REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree	Year	Name of Author
PhD	2006	DAVID, A.L.M.

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Development of ultrasound-guided gene therapy to the sheep fetus

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July 2005

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Abstract

Fetal gene therapy may treat genetic diseases before significant organ damage, target stem cell populations and avoid immune sensitisation. Candidate diseases include cystic fibrosis, haemophilia and lysosomal storage disorders. This thesis developed ultrasound-guided delivery of viral vectors to the sheep fetus for treatment of these diseases.

For haemophilia B treatment we delivered adenovirus vectors containing the β-galactosidase reporter gene (adlacZ) or the human factor IX gene (adhFIX) by ultrasound guidance to the early gestation sheep fetus, when it is considered to be pre-immune. Intraperitoneal injection allowed the earliest time point for gene delivery, achieved the highest hFIX levels and the most localised β-galactosidase expression. Therapeutic hFIX levels were detected after intramuscular and intra-amniotic delivery suggesting that these are potentially alternative sites for therapeutic gene expression. For each route examined, no humoral immune response was observed to the transgene, although antibodies to the adenovirus vector were identified. We achieved intravascular delivery via umbilical vein injection; therapeutic hFIX levels were detected.

We developed ultrasound-guided transthoracic injection of the mid-gestation fetal trachea for cystic fibrosis treatment. β-galactosidase expression, measured by ELISA, was low after delivery of adlacZ vector alone, but increased 10 fold when the vector was complexed with DEAE dextran. Pretreatment of the fetal airways with sodium caprate increased expression by 90 fold; the effect of the two agents was synergistic. Perflubron instillation following vector injection redistributed transgene expression from the large to the small airways. We developed ultrasound-guided fetal intragastric injection and achieved widespread transgene expression throughout the gastrointestinal epithelia after adlacZ vector delivery.

For brain manifestation of lysosomal storage disorders we injected adlacZ vectors to the fetal ventricles under ultrasound guidance. Transduction of the choroid plexus was seen. Future application of integrating vectors such as lentivirus may allow for long term therapeutic correction and induce immune tolerance to the transgene.

Acknowledgements

I am indebted to Charles Rodeck for his encouragement and advice during the course of this thesis and for his continuing support in my academic career. I would also like to thank Charles Coutelle for his positive energy, ideas and good counsel when things were tough. I must also thank Donald Peebles for his valued support in the day-to-day running of the project and for his help in performing, developing and directing me through the ultrasound guided fetal procedures. In addition Maznu Miah, Dany Perocheau and Ali Ruthe provided invaluable technical support on the project. Thank you also to Boaz Weisz for his assistance in the later stages of the work.

This thesis is the result of a fantastic and fruitful collaboration between the Department of Obstetrics and Gynaecology at University College London and the Gene Therapy Research Group at Imperial College, London. I am extremely grateful to all the members of this group especially Simon Waddington, Suzy Buckley, Lisa Gregory and Megha Nivsarkar for allowing me to work alongside them in their labs and for being so patient. Particular thanks go to Mike Themis for supporting much of the laboratory work in this thesis and for his knowledge and expertise in the field of gene therapy. At Hammersmith Hospital my thanks go to Terry Cook who was always so helpful with histological tissue analysis.

My thanks must also go to the staff of the Biological Services Units at UCL and the Royal Veterinary College. In particular it was a pleasure to work with Alistair Wallis and David Manners and I thank them for their time and skills in animal care. I would like to thank Douglas Stewart and Kathleen Tennant at the RVC for their advice concerning large animal pathology and Jill Maddox, University of Melbourne, Australia for supplying primers to endogenous sheep sequences.

Finally I would not have been able to achieve any of this without the wonderful support of my family. To my mother who babysat at the drop of a hat, to my son who gave mummy time to write "her book", to my daughter who happily played on the floor while I wrote much of it on the computer, and to my husband whose love and encouragement and many cups of tea saw me through the production of this work, thank you.

Glossary of Terms

AC	abdominal circumference
adlacZ	adenovirus vector containing the β-galactosidase transgene
adhFIX	adenovirus vector containing the human factor IX transgene
adhCFTR	adenovirus vector containing the human CFTR transgene
β-gal	β-galactosidase
BAL	bronchoalveolar lavage
BPD	biparietal diameter
BrDU	5-bromo2'-deoxy-uridine
BSA	bovine serum albumin
CsCl	caesium chloride
CFTR	cystic fibrosis transmembrane regulator
CMV	cytomegalovirus
D-Mem	Dulbecco's Modified eagle media
DMSO	dimethylsulfoxide
DOGS	dioctadecylamidoglycyl spermine
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EIAV	equine immune anaemia virus
FCS	fetal calf serum
FL	femur length
HE	intrahepatic
HIV	human immune deficiency virus
hFIX	human factor IX clotting factor
IA	intra-amniotic
IC	intracardiac
IM	intramuscular
IP	intraperitoneal
IT	intratracheal
LMP	low melting point
MLV	Moloney Leukemia Virus
NaCl	sodium chloride
Na caprate or C10	sodium caprate
OSL	occipito-snout length

p	particles
PBS	physiological buffered saline
pen/strep	penicillin/streptomycin
pfu	particle forming units
RSV	Rous sarcoma virus
SD	standard deviation
SDS	sodium dodecyl sulphate
Sendai lacZ	Sendai vector containing the β-galactosidase transgene
TE buffer	tris-EDTA buffer
Tris	tris (hydroxymethyl) methylamine
UA	umbilical artery
UV	umbilical vein
VSV-G	Varicella zoster virus protein G
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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A. Introduction

A 1 The rationale for prenatal gene therapy

Fetal somatic gene therapy has the potential to treat inherited genetic diseases *in utero* before significant organ damage has occurred. Rapidly expanding stem cell populations may be targeted and the introduction of transgenes to the fetus during development of the immune system could result in immune tolerance and facilitate repeat treatment postnatally. Finally, fetal gene therapy would give a third option to parents following prenatal diagnosis of inherited disease, where currently termination of pregnancy or acceptance of an affected child have been the only choices. Genetic diseases such as cystic fibrosis (CF) and haemophilia B which are life threatening, and for which there are no currently acceptable treatments available are suggested targets for this therapy.

A 2 Candidate diseases for in utero gene therapy

A 2.1 Cystic fibrosis, a common systemic genetic disease

A 2.1.1 Cystic fibrosis pathology and genetics

Cystic fibrosis appears to be an ideal candidate for treatment with *in utero* gene transfer. The Cystic Fibrosis Transmembrane Regulator (CFTR) gene encoding the CFTR protein is mutated in this disease resulting in abnormal electrolyte transport in airway, sweat ductal, intestinal and pancreatic ductal epithelia. CFTR is an integral membrane component of the apical portion of many epithelial cells (Crawford I et al., 1991). It functions as a transmembrane chloride channel regulated by cyclic adenosine 3',5'-monophosphate (cAMP) and controls other ion channels such as the epithelial Na+ channel (Stutts MJ et al., 1995). Compared with other secretory epithelia, CFTR is expressed only in very small amounts in normal or CF airway epithelia (Trapnell BC et al., 1991). *In vitro* studies where normal and CF airway cells were mixed, suggest that only 6-10% of cells expressing normal CFTR are required to correct the ion transport defect in all cells of an epithelial monolayer (Johnson LG et al., 1992).
The predominant site of CFTR expression in the non-CF human bronchus is the submucosal glands, in particular the ducts and serous tubules (**Figure A 1**) In CF lung there is a similar distribution of mutated CFTR expression as in the non-CF lung, with increased expression in the collecting ducts (Engelhardt JF et al., 1992). This is important for attempts to target gene transfer to the most suitable site in the airways for

treatment of CF.

In the human fetal lung CFTR distribution follows a cephalocaudal pattern of maturation and differentiation (Gaillard D et al., 1994) being present in the trachea at 7 weeks gestation, in glandular buds and ducts of the trachea and lower epithelial airways between 12-15 weeks gestation, and in the apical domain of tracheal and bronchial ciliated cells from 25 weeks gestation onwards. CFTR is also expressed in other tissues throughout normal human fetal development, in the yolk sac during embryogenesis, and in the epithelia of the pancreatic ducts, the small intestine, the genital ducts and particularly the airways during fetal life (Trezise AE et al., 1993).

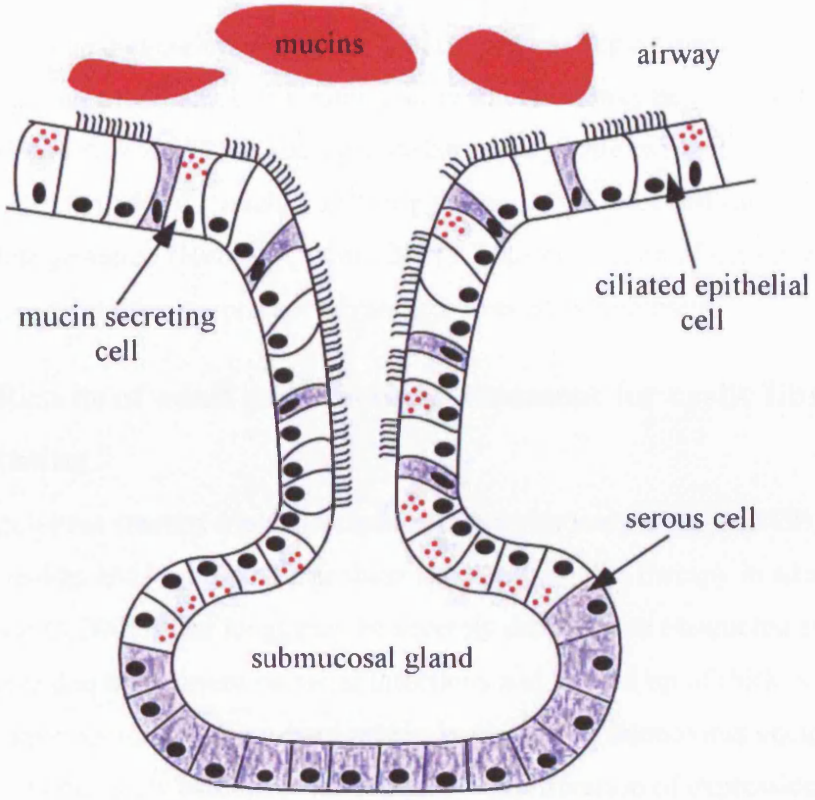


Figure A 1: The variety of CFTR-expressing cellular targets for gene therapy of the CF airway.

Submucosal glands are found beneath the airway and express abundant CFTR protein in serous cells in the base of the glands. CFTR is also highly expressed in a non-ciliated cell in gland ducts and surface airway epithelia. Ciliated cells of the surface airway epithelia express low levels of CFTR. Cell types that express the highest levels of CFTR are shaded in purple. Adapted from (Driskell RA and Engelhardt J, 2003).

The expression profile of CFTR in the fetal lung suggests a crucial role for this protein in lung epithelial maturation. In the fetal sheep lung, high CFTR expression is observed during the pseudoglandular phase when the bronchial tree and acini form, but this falls

during the later canalicular phase (Broackes-Carter FC et al., 2002). One study delivering an adenovirus vector containing the CFTR gene to the amniotic fluid of normal or CFTR knockout fetal mice suggested that the CFTR gene may have an important physiological role in normal fetal development (Larson JE et al., 2000a). Survival of the homozygote normal mice was much lower than expected, and histological analysis of the lungs showed accelerated secretory cell proliferation and differentiation. Whether this is due to overexpression of the CFTR gene during lung development however, is unclear.

A 2.1.2 Cystic fibrosis pathology begins in utero

There is evidence that the cystic fibrosis disease process begins during fetal development since by the mid-trimester, pancreatic ducts may be blocked by mucin secretion (Boué A et al., 1986) and a pro-inflammatory state exists in fetal CF airways with infiltration of fetal CF trachea and lung by numerous mast cells and macrophages in mid to late gestation (Hubeau C et al., 2001). Thus correction of the underlying CF defect during fetal life may present advantages over adult treatment.

A 2.1.3 Results of adult gene therapy treatment for cystic fibrosis are disappointing

Phase 1 adult gene therapy trials directed towards pulmonary disease in CF show equivocal results and highlight the problems of applying this therapy in adults (Bigger B and Coutelle C, 2001). The lungs may be severely damaged or obstructed even in young adult patients due to recurrent bacterial infections and a build up of thick, sticky mucus. Immune responses to the vector, particularly in the case of adenovirus vectors, limit the dose that may be safely administered, and reduce the duration of expression. There is also a lack of targeting to the epithelial stem cells. Thus most groups working on fetal gene delivery in animal models have suggested research towards treatment of this disease.

The major difficulties with the adult gene therapy trials have been to measure the efficiency of gene transfer and to quantify transgene expression using an appropriate clinical endpoint. Measurement of the potential difference (PD) across the epithelial surface has been used to demonstrate functional correction in clinical trials although measurements *in vivo* in the lower airways are difficult to perform. The research has therefore been targeted to the nose although it is unclear how far results in the nasal epithelia can be extrapolated to the pulmonary epithelia.

Most trials have used adenovirus vectors. When applied to the nasal epithelium, transient correction of the chloride transport defect was seen in some studies (Zabner J et al., 1993,Hay JG et al., 1995) although others have failed to show any such correction (Knowles MR et al., 1995). Even when less immunogenic second and third generation vectors were used, the patients mounted a local and systemic immune response. Instillation to the airway epithelium had similar results (Zuckerman JB et al., 1999). Adeno-associated virus has been used as an alternative vector. Application to the maxillary sinus gave transient correction to the PD and resulted in a 25% reduction in the incidence of recurrent sinusitis, a common complaint of CF patients (Wagner JA et al., 1999). These encouraging results unfortunately could not be repeated in the lungs after nebulized delivery (Flotte TR et al., 2003). Non-viral gene delivery systems have also been studied. Application of DNA/liposome formulations partially and transiently corrected the PD in the nasal epithlium. In contrast to findings from adenovirus trials, minimal immune responses were elicited and repeat administration was both safe and successful (Hyde SC et al., 2000).

A 2.2 Haemophilia B, a model for many inborn errors of metabolism

A 2.2.1 Haemophilia B pathology and genetics

Haemophilia B is another disease that would be particularly suitable for gene therapy *in utero*. This X-linked hereditary haemorrhagic disorder occurs in 1in 25000 males and is caused by the absence or dysfunction of factor IX (FIX) clotting factor which is involved in the intrinsic pathway of the coagulation cascade (**Figure A 2**) (Furie B et al., 1994). This results in recurrent haemorrhage into muscles and joints with subsequent arthiritis, and cerebrovascular accidents.

Current treatment of haemophilia B uses replacement therapy with human Factor IX (hFIX). Extensive experience with protein concentrates over the last 25 years has shown that maintenance of factor IX levels above 1% of normal adult human levels (50 ng/ml) in patients with severe haemophilia significantly reduces the frequency of spontaneous bleeding into joints and soft tissues, and reduces the risk of fatal intracranial bleeding (Lofqvist T et al., 1997).

However, a number of patients develop antibodies to therapy leading to ineffective treatment and occasional anaphylaxis, and induction of immune tolerance to the replacement hFIX is only partly successful (Lusher JM, 2000). Indeed, the complications of haemophilia treatment have in some cases been far worse than the

disease itself, increasing the morbidity and mortality of patients (Soucie JM et al., 2000). Restoration of the functional hFIX gene by *in utero* gene transfer could provide long-term treatment using a single injection, avoid immune-sensitisation and also prevent any haemorrhagic complications that could occur at the time of delivery.

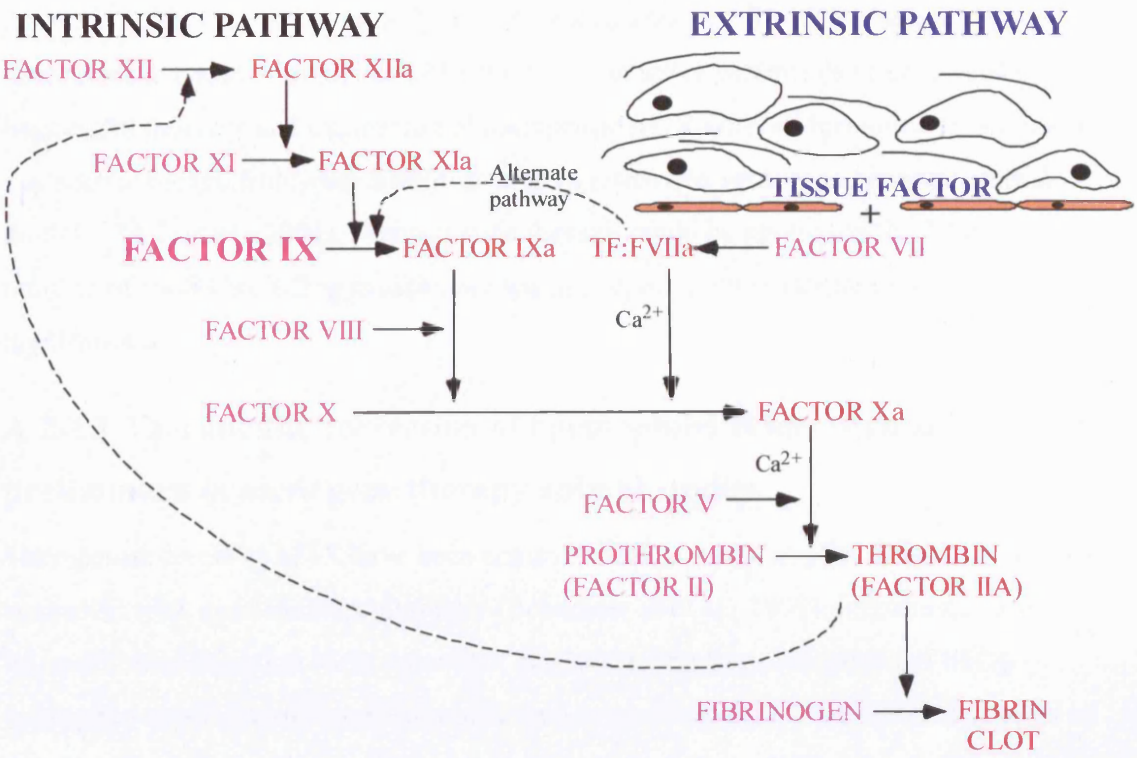


Figure A 2: The coagulation cascade.

Coagulation is initiated via the extrinsic pathway. Broken endothelium in a blood vessel allows factor VII present in the blood to recognize and bind to tissue factor (TF, factor III) that is present on the surface of fibroblasts and smooth muscle cells of the vessel wall. This produces the factor VII:tissue factor activated complex (TF:VIIa) which activates factor IX. A cascade quickly develops, culminating in the production of a fibrin clot. Small amounts of thrombin generated by the extrinsic pathway amplify the coagulation process by activating the intrinsic pathway via factors XI and VIII, which generates large amounts of thrombin.

A 2.2.2 Adult gene therapy studies in animal models of haemophilia show promising results

Since the hFIX coagulation factor is required in the blood and can be secreted functionally from a variety of tissues, the actual site of production is not so important as long as therapeutic plasma levels are realized. Adult gene therapy strategies have therefore concentrated on application to the muscle or the liver.

Successful delivery and expression of FIX has been achieved in adult animal models of haemophilia B following portal intravascular administration of adenovirus (Kay MA et al., 1994) and retrovirus vectors (Kay MA et al., 1993). Sustained FIX expression was

also observed after intramuscular injection of adult haemophiliac dogs with adeno-associated viral (AAV) vectors expressing canine FIX (Chao H et al., 1999,Herzog RW et al., 1999,Herzog RW et al., 1999,Chao H et al., 1999) and after intravascular injection of adult haemophiliac mice with AAV vectors expressing hFIX (Snyder RO et al., 1999). These results culminated in the first clinical trial in humans that resulted in low level hFIX expression (Kay MA et al., 2000) although it was prematurely terminated because of evidence of liver toxicity in some patients (Kaiser J, 2004). Successful delivery and expression of therapeutic hFIX without formation of antibodies has been achieved following administration of retrovirus vectors in neonatal animal models (Xu L et al., 2003). Prenatal gene therapy could be applied to the fetus via a number of routes including muscle, peritoneal, hepatic, intravascular or skin application.

A 2.2.3 Therapeutic correction of haemophilia B has been achieved in preliminary *in utero* gene therapy animal studies

Therapeutic levels of hFIX have been achieved after *in utero* application of adenovirus vectors to fetal mice via intra-amniotic (Schneider H et al., 1999), intramuscular or intraperitoneal injection (Schneider H et al., 2002). The transient nature of hFIX expression observed after intra-amniotic delivery was thought to be due to a number of factors including shedding of the transduced keratinocytes and the humoral immune response to the transgene and/or vector. Intravascular delivery of adenovirus hFIX vectors to fetal mice has resulted in long-term tolerance of hFIX into adulthood on repeated applications of purified proteins (Waddington SN et al., 2003b). Recently our group has demonstrated that *in utero* application can provide long-term postnatal correction of the haemophiliac phenotype in FIX deficient mice (Waddington SN et al., 2004b). This provides proof of principle that gene therapy applicaton *in utero* may allow induction of immune tolerance.

A 2.3 Mucopolysaccharidosis type VII, a congenital lysosomal storage disorder affecting the central nervous system

A 2.3.1 Mucopolysaccharidosis type VII pathology and genetics

The lysosomal storage disorders (LSDs) are a group of congenital deficiencies of one or more lysosomal enzymes. In mucopolysaccharidosis type VII (MPS type VII), a deficiency of β-glucuronidase activity leads to accumulation of undegraded

glycosaminoglycans in lysosomes. Clinically, patients develop hepatosplenomegaly, mental and growth retardation, hearing and vision defects, skeletal deformities and die of cardiac failure. Many of the LSDs present already during fetal life with hydrops fetalis and prenatal diagnosis can be performed by detection of β-glucuronidase deficiency in chorionic villi or fetal blood (Geipel A et al., 2002). Although individually rare, as a group the LSDs occur in approximately 1 in 7500 live births and are one of the more prevalent groups of inherited diseases in humans (Wraith JE, 2002).

Treatments are currently limited to enzyme replacement therapy and bone marrow transplantation. For non-neuropathic forms of LSDs such as type 1 Gaucher disease, enzyme replacement therapy has been shown to be effective (Weinreb NJ et al., 2002). However, the short half-life of lysosomal enzymes in the circulation means that patients need biweekly parenteral administration that increases the risk of an immune response to the infused enzyme, particular in patients with null mutations. In addition, systemically administered enzyme is unable to cross the blood-brain barrier and is therefore ineffective for those LSDs that have central nervous system disease manifestation such as MPS type VII. *In utero* haematopoietic stem cell transplantation has been attempted but was not effective at preventing neurological deterioration. This might be due to poor engraftment of donor cells, but more probably, is because of a failure of cross-correction in some LSDs, whereby the corrective enzyme produced by the transplanted cells fails to reach the lysosomes of deficient cells (Martino S et al., 2002).

The LSDs are considered to be good candidates for gene therapy since they are well-characterized single gene disorders. Localised gene transfer to an organ such as the liver may provide therapeutic levels of enzymes in the circulation for correction of non-neurological manifestations, since a proportion of newly synthesized lysosomal enzymes are secreted into the systemic circulation and are recaptured by distant cells. Based on the observed enzyme levels in patients with mild late-onset disease, the amount of enzyme needed to correct the deficiency may only be 1-10% of normal levels (Cheng SH and Smith AE, 2003). As discussed previously however, direct gene delivery to affected neural cells is thought to be necessary to treat central nervous system manifestations and this will require a strategy that can bypass the blood-brain barrier and deliver sufficient enzyme to the brain.

A 2.3.2 Studies of gene transfer to the central nervous system show promising results.

Several studies have shown encouraging results, particularly in neonatal animal models of LSDs. The cells of the CNS are relatively quiescent, even in the fetus, and this precludes the use of vectors that require cell division for transduction. The age of the animal and the site of injection determine the efficacy of treatment. Intravenous injection of adenovirus vectors encoding β- glucuronidase into neonatal MPS type VII mice is more efficacious at preventing CNS manifestations than treatment of adult mice (Kamata Y et al., 2003). This is probably due to the relative immaturity of the blood brain barrier which is not fully intact until 10 – 14 days of life in rodents (Stewart PH and Hayakawa EM, 1987). Gene transfer was low level however, even when AAV vectors were applied (Daly TM et al., 1999).

Injection of the brain parenchyma with recombinant AAV2 (Frisella WA et al., 2001) or feline immunodeficiency virus vectors (Brooks AI et al., 2002) encoding β-glucuronidase improved cognitive function in neonatal and adult MPS type VII mice. Enzyme expression was concentrated at the sites of injection but cellular pathology was reduced in all areas of the brain, suggesting that enzymes secreted from transduced cells were taken up by uninfected cells. Delivery of AAV2 vector into the lateral ventricles of normal or MPS type VII mice resulted in more widespread but low level gene transfer to the central nervous system (Passini MA and Wolfe JH, 2001) although this could be improved by application of AAV1 vectors (Passini MA et al., 2003).

Translating these results to a large animal model has been successful for some extraneurological manifestations of LSDs. Intravenous administration of retrovirus vectors containing canine β- glucuronidase to neonatal MPS type VII dogs reduced the bone and joint abnormalities and heart valve defects that commonly occur in this animal model (Ponder KP et al., 2002) but enzyme activity in the brain was low, suggesting that neonatal systemic application may not prevent neurological pathology.

A 3 Development of the fetal immune system

A 3.1 The human fetal immune system develops throughout gestation

The principle that fetal gene therapy may avoid the development of immune reactions to the vector or transgene product depends critically on the time of *in utero* gene delivery

at which fetal tolerance might be induced. The human immune system develops progressively through the first trimester and is not fully functional until 1-2 years after birth (Riley RL, 1998). Definitive haematopoiesis is now believed to be established from an intraembryonic source of haematopoietic progenitors associated with the ventral endothelium of the aorta in the 4 - 5 week old human embryo and not from the yolk sac as previously thought (Tavian M et al., 1996,Tavian M et al., 1999). Haematopoietic stem cells then migrate to the liver from 5 weeks of gestation and pre-B and B-cells are identifiable in fetal liver from 12 weeks of gestation (Kamps WA and Cooper MD, 1984). B cells subsequently migrate to the bone marrow from 16 weeks of gestation. T cell precursors increase in number in the fetal liver from 7 weeks of gestation and migrate to the fetal thymus between 8 and 9 weeks of gestation where they mature (Haynes BF et al., 1988). T lymphocytes are found in the circulation and the fetal liver from 13 weeks of gestation (Pahal GS et al., 2000) but are not capable of producing a definitive cytotoxic response until the end of the first trimester (Mackenzie IZ and Maclean DA, 1980). Natural killer cells are present in the human fetal liver and spleen from 9 and 15 weeks of gestation respectively. Even at this early gestation these cells can mount a cytotoxic reaction to selected target cells such as tumours and virus-infected cells without the presence of MHC class I or II antigens to recognize their targets, although their lytic activity increases with gestational age (Phillips et al., 1992).

A 3.2 The development of the fetal sheep immune system parallels that in humans

The immune system of the sheep develops similarly to humans making the sheep model particularly useful for investigating immune reactions to vector and transgene product. Haematopoiesis is thought to begin in the yolk sac and fetal liver from 17 days of gestation (term = 145 days) although an intraembryonic source of haematopoietic progenitors associated with the aorta has not yet been investigated in the fetal sheep. The liver is the major haematopoietic organ from 20 days of gestation. There is evidence of a pre-thymic population of immature lymphoid cells that is no longer recognizable by 40 days of gestation (Miyasaka M and Morris B, 1988). It is probable these lymphoid cell precursors populate the primitive thymus and initiate lymphopoiesis that is observed from 35 days of gestation (Mackay CR et al., 1986). Lymphocyte migration within the thymus occurs at 45 days of gestation and 5 days later the first T-cells are observed in the fetal circulation. By 60 days of gestation the thymus has a distinctive cortex and medulla and by 100 days it has a mature complement of T-cells

(Morris B and Miyasaka M, 1985). Lymphocytes are seen in the spleen from 55 days, the lymph nodes from 60 days, the bone marrow from 75 days and the Peyer's patches from 80 days of gestation (Alsalami MT and Filippich LJ, 1999). Immunoglobulin G and M first appear in fetal plasma at 56 and 77 days of gestation respectively and levels increase with gestational age (Sawyer M et al., 1978). Mature neutrophils however, are observed only from 123 days of gestation and enzymes involved in degradation are not present until term.

A 3.3 The development of the fetal immune response in sheep has been extensively investigated.

Much of the early work on the ontogeny of the fetal immune response was performed in the fetal sheep because of the easy surgical manipulation of the fetus, and the synepitheliochorial placenta in the sheep which does not permit passage of gammaglobulin from mother to fetus (Morris B, 1986). This ensures that all antibodies in the fetal serum are derived from the fetus and not from the mother and provides an ideal opportunity to test how a deliberate experimental exposure of the fetus to antigen will affect the development of immune competence.

The idea that the immune system 'learns' what is 'self' was first suggested following studies of dizygotic twins in cattle that were found to be born with and retain red blood cells of dizygotic origin (Owen RD, 1945). These naturally occurring chimeras arise because blood vessel anastomoses in the placenta allow reciprocal transfer of red blood cells between the twins. The concept that tolerance is induced during fetal life was thus proposed. A comprehensive set of experiments tested this hypothesis of 'actively acquired tolerance' in the fetal mouse (Billingham RE et al., 1953,Billingham RE et al., 1956). Cells from the spleen, testis or kidney of one strain of mouse were injected into the fetus of another strain at 16-17 days of gestation. The treated mice as adults did not reject skin grafts taken from the donor strain but could reject grafts from a third mouse strain. Injection of neonatal mice elicited neither tolerance or immunity and a null period of immunological reactivity was proposed. Later experiments showed that either tolerance or immunity could be induced in the neonatal mouse depending on the dose of innoculum (Howard JG and Michie D, 1962).

Attempts to induce tolerance in the fetal sheep using the conditions adequate for tolerance induction in the fetal mouse have failed to achieve comparable results. This is most likely because of differences in the maturation of the fetal immune response between the two species. Transplantation experiments in the fetal sheep show that skin

allografts are uniformly rejected as early as 77 and 80 days of gestation (Schinkel PG and Ferguson KA, 1953,Silverstein AM et al., 1964) and renal allografts are similarly rejected when placed at 70 days of gestation (Neiderhuber JE et al., 1971). Skin grafts at 65 days of gestation however, were accepted for at least 21 days with no evidence of cellular infiltration, and permanent survival of skin grafts beyond term has been achieved in fetuses transplanted with adult skin allografts at 55 days of gestation (McCullagh P, 1988).

In addition to the time of grafting, the nature of the antigen is also important in determining whether tolerance is induced. For example fetal lambs at 55 days of gestation uniformly reject skin allografts from fetal sheep aged up to 85 days but retain skin allografts from fetal sheep at 95 days of gestation and adult sheep (McCullagh P, 1989). Transplantation of other tissues such as blood products or splenic allografts into fetal sheep even at 50 and 60 days of gestation has proved unsuccessful in inducing tolerance when the fetuses were challenged as adults with donor blood cells (Moore NW and Rowson LEA, 1961,Mitchell RM, 1959).

The fetal sheep immune response to intrauterine infection also shows a different modality depending on the antigen. Exposure to Border disease virus as a fetus results in a state of immunological tolerance as an adult and persistent shedding of virus particles (Nettleton PF and Entrican G, 1995). In contrast, exposure of the fetus to Akabane virus infection in early gestation produces a viraemia that persists until 75 days of gestation when neutralizing antibodies appear and eliminate the virus (McClure S et al., 1988).

Silverstein and his colleagues performed a series of investigations to determine the gestational age at which the fetal lamb is able to respond to a specific antigenic challenge (Silverstein AM et al., 1963a,Silverstein AM et al., 1963b). A hierarchy of response among the antigens was observed with the earliest antibody produced to bacteriophage φX from 60 days, to ferritin from 96 days and to albumin from 120 days of gestation. Responses to diphtheria toxoid, Salmonella typhosa organisms and BCG could not be elicited in the sheep until after birth. They could not correlate the appearance of a particular immune capability with a stage of lymphopoiesis. The development of the humoral immune response over time has also been studied by Fahey and colleagues using serial fetal blood sampling via vascular catheters implanted into fetuses challenged with various antigens (Fahey KJ and Morris B, 1978,Fahey KJ and Morris B, 1974). Most fetuses acquired a capacity to respond to several antigens between 60 and 75 days of gestation. For all antigens tested, there was a higher

antibody titre, a longer duration of response and a wider range of antibody production in older fetuses, showing that the humoral immune response matures through gestation. The immune response to antigens again appeared in a sequential fashion as described by Silverstein and colleagues. The hierarchy however, was not particularly precise and there was also a wide variation amongst individual fetuses.

Many have proposed that a 'window of opportunity' exists in the first third to half of pregnancy during which time introduction of foreign genetic material may not elicit an immune response but may allow development of tolerance. All these data however, would suggest that in the sheep fetus, the nature of the antigen itself is of prime importance in determining if an immune response is mounted and whether tolerance is induced.

A 4 Gene delivery vectors

A 4.1 Adenovirus vectors

Adenovirus vectors are attractive candidates for gene therapy since they have continually achieved highly efficient gene transfer *in vivo*. Adenovirus coding sequences necessary for viral reproduction are deleted, rendering them replication-incompetent. They are stable, have a relatively high capacity for transgene insertion and can be obtained at high titre, making systemic administration in humans and large animal models feasible. The adenovirus genome replicates in an extra-chromosome state, which circumvents the risk of insertional mutagenesis but results in only transient transgene expression. Another advantage is their tropism for most cells of the human body, including the respiratory epithelium and their efficient transduction of both quiescent and actively dividing cells. Adenoviruses are thus a popular choice as a gene delivery vehicle and are currently being used in approximately one-quarter of all gene therapy clinical trials, second only to retroviruses (Journal of Genetic Medicine website www.wiley.co.uk/genmed/clinical). For these studies we required a 'pathfinder' vector, suitable for exploring different technical approaches to fetal vector delivery, and adenoviruses were therefore chosen as the optimum vector.

A 4.1.1 The adenovirus virion

The adenovirus virion is a nonenveloped icosahedral particle about 70-90 nm in size with an outer protein shell surrounding an inner nucleoprotein core (**Figure A 3**).

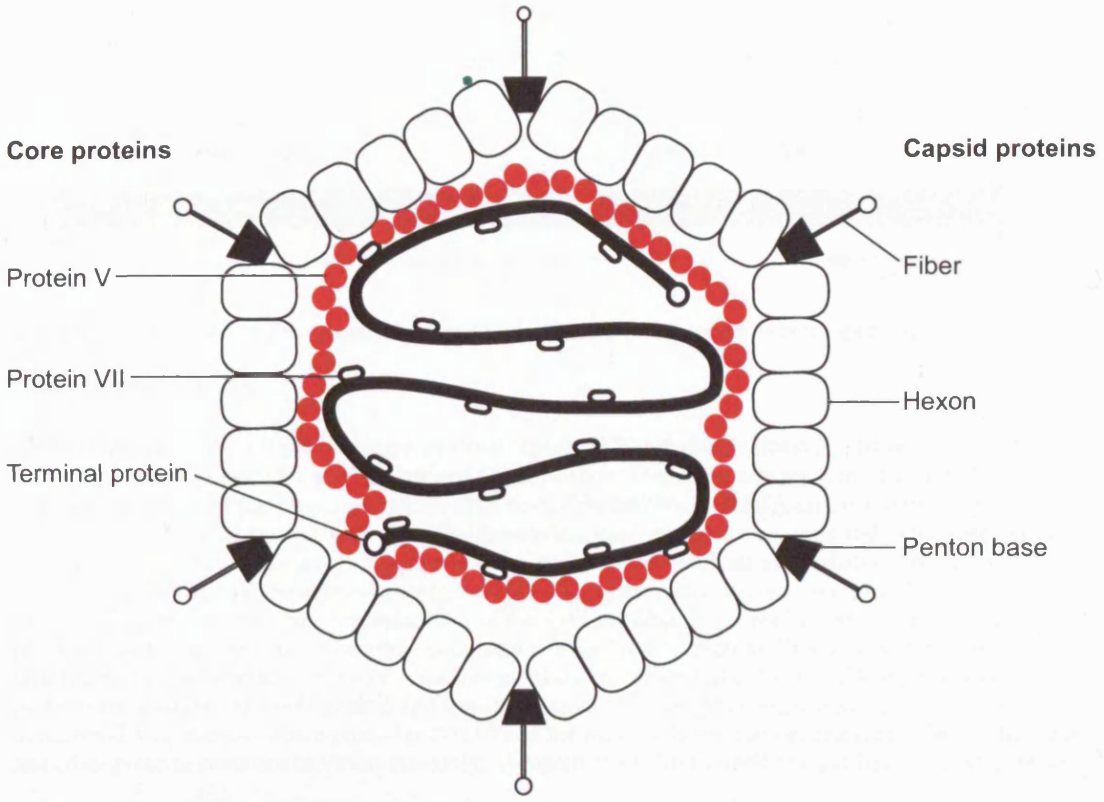


Figure A 3: An adenovirus particle.

The outer protein shell contains trimers of the hexon protein. A penton base forms the capsid vertices and anchors the fiber protein, the part responsible for primary attachment of virions to the cell surface. The core contains proteins and the linear double-stranded DNA that is approximately 36 kb long. Adapted from (McConnell MJ and Imperiale MJ, 2004).

A map of the double stranded DNA of the adenovirus genome and transcription units is shown in **Figure A 4**. Initial attachment of virion particles to the cell surface occurs by binding of the fiber knob to the coxsackievirus B and adenovirus receptor (CAR). CAR is a transmembrane protein of the immunoglobulin family present in many tissues including the heart, lung, liver and brain and it normally functions as a cell-to-cell adhesion molecule on the basolateral surface of epithelial cells (Honda T et al., 2000). After initial attachment to the cell surface, an exposed RGD motif on the penton base interacts with members of the αv integrin family to trigger virus internalization by clathrin-dependent receptor-mediated endocytosis (Wickham TJ et al., 1993). Viral particles are captured within the endosome from which they escape and are trafficked in the cytoplasm along microtubules towards the nucleus. They dock with the nuclear pore complex, the capsid disassembles allowing importation of the viral genome and commencement of DNA replication (McConnell MJ and Imperiale MJ, 2004).

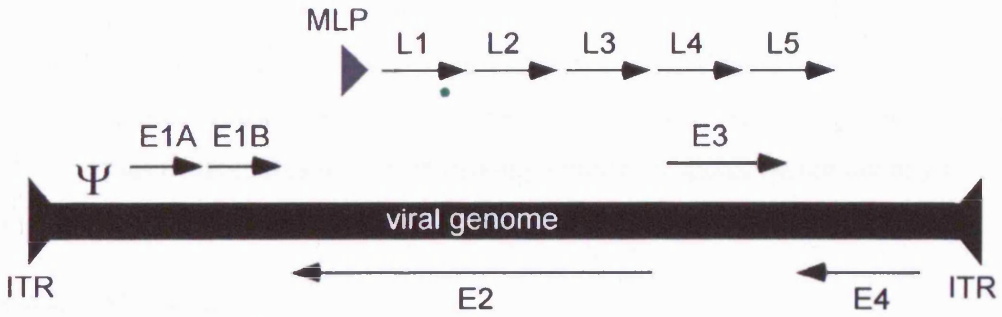


Figure A 4: Map of the double stranded DNA of the adenovirus genome and transcription units.

Each end of the genome has an inverted terminal repeat (ITR) covalently linked to the terminal protein. The packaging sequence (Ψ) is at the left end of the genome. Genes encode on both strands of DNA in overlapping units and are transcribed in the direction of the arrows. The E1A unit is the first to be expressed. Its proteins transactivate the other adenovirus early transcription units and induce the cell to enter S phase in order to create an environment optimal for virus replication. Products of the E1B unit inhibit programmed cell death thus keeping the cell alive so as to maximize viral yields. The E2 region encodes the proteins necessary for replication of the viral genome: DNA polymerase, a preterminal protein to maintain integrity of the DNA ends, and a DNA-binding protein. Products of the E3 region undermine the host immune response and allow persistence of infected cells. The E4 region encodes proteins involved in cell cycle control and transformation. The late genes expressed from L1-L5 are transcribed from the major late promoter (MLP) and the transcripts encode structural proteins of the virus and other proteins involved in virion assembly. Adapted from (McConnell MJ and Imperiale MJ, 2004).

For virion assembly to occur, hexon trimerization takes place first in the cytoplasm. The trimers are then translocated to the nucleus where they associate with penton and other capsid components to form the protein shell around the genome core proteins. The structural proteins mature to form fully infectious virions that cause cell lysis and are released approximately 30 hours after infection.

For the studies described here we used first generation replication incompetent adenovirus vectors that are created by replacing the E1 region with a transgene of interest. To produce the viral vector, the E1 gene that is critical for viral replication is provided *in trans* by the 293 cell line, a human embryonic kidney-derived line that has been transformed by the adenovirus E1 region (Graham FL et al., 1977). Recombination between the E1 region sequences and recombinant virus can give rise to replication competent viral progeny that have functional E1 genes, and therefore all vector stocks are assayed to exclude the presence of replication-competent viruses.

Problems with adenovirus vectors include their transient expression, significant toxicity and immunogenicity *in vivo*. Even fetal administration has been associated with an immune response (McCray PB et al., 1995) particularly after repeat exposure to the vector (Iwamoto HS et al., 1999). Attempts have been made to reduce the immunogenicity and toxicity of adenovirus vectors by deleting parts of the adenovirus

genome which has led to the generation of the so called 'gutless vectors' in which essentially all adenovirus coding sequences have been eliminated (Chen HH et al., 1997). However the use of an immunogenic vector in these exploratory studies is advantageous because it is important that any immune response to the therapy is detected and evaluated.

A 4.2 Retrovirus

Vectors that are able to integrate into the host genome such as retroviruses and lentiviruses offer the possibility of permanent gene delivery. Retrovirus vectors have single stranded RNA genomes and have been developed based on the Moloney murine leukaemia virus (MLV) whereby all structural genes are removed to render them fully replication-defective (**Figure A 5**).

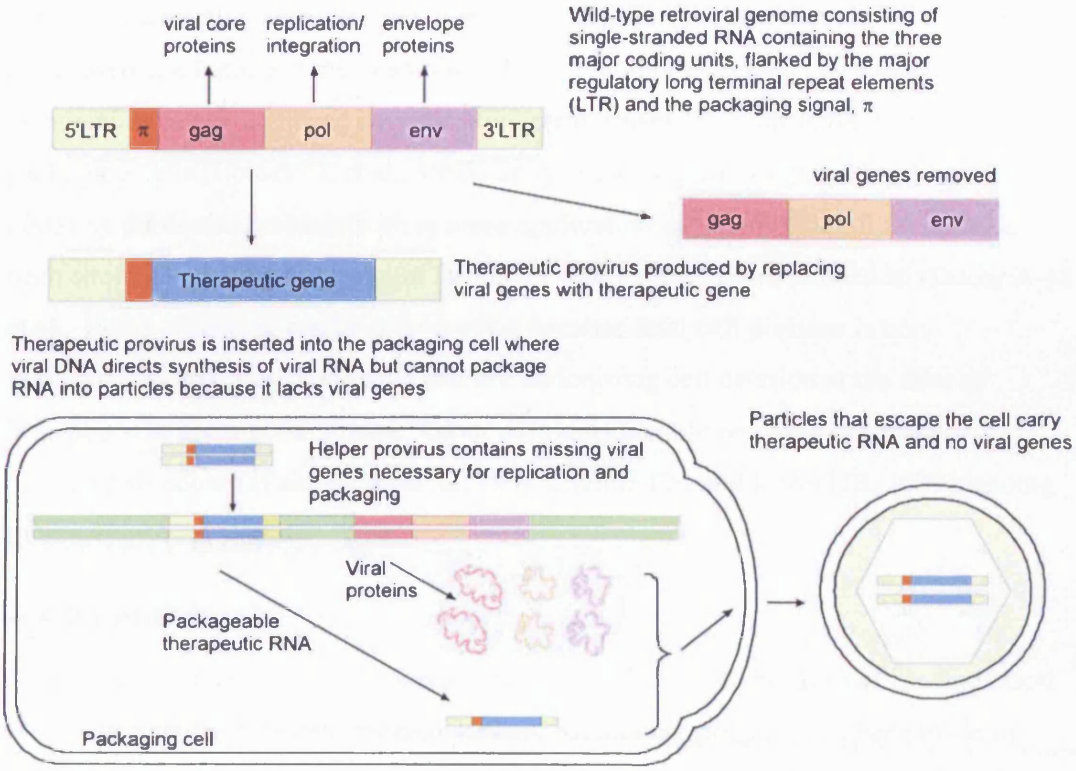


Figure A 5: Propagation of replication defective retrovirus vectors from wild type retrovirus.

Although only fairly low virus titres can be produced, virus gene transfer may be improved by complexing vectors with cationic agents (Themis M et al., 1998), or by the administration of retrovirus producer cells *in vivo* to allow localised gene delivery close to the site of cell transfer (Douar A-M et al., 1997,Russel DW et al., 1995).

Pseudotyping of the vector, by replacing the natural envelope of the retrovirus with a heterologous envelope such as the G protein of Vesicular Stomatitis Virus (VSV-G), results in improved mechanical stability allowing concentration of the vector by ultracentrifugation to increase viral titre (Burns JC et al., 1993).Retroviruses require dividing cells for gene transfer (Miller DG et al., 1990). They may be better suited therefore, for use in fetal rather than adult tissues where cells are rapidly dividing and are not fully differentiated. This is reflected by the relatively greater efficiency of retrovirus-mediated gene transfer to fetal hematopoietic progenitors when compared with adult cells from sheep and humans (Ekhterae D et al., 1990). They are also much less immunogenic than adenoviruses which is important for long term gene transfer and expression (McCormack et al., 1997).

The main problem with retrovirus vectors is their instability *in vivo*. Human serum can almost completely inactivate some retrovirus particles (Welsh RJ et al., 1975) which limits their use for intravenous administration although increased resistance to serum inactivation can be achieved by generating retroviruses from particular human packaging cells (Cosset FL et al., 1995) or by pseudotyping (Engelstädter M et al., 2001). A particular problem with *in utero* application is that amniotic fluid has also been shown *in vitro* to have a mild inhibitory effect on retrovirus infection (Douar A-M et al., 1996). This may hinder transduction because fetal cell division is non-synchronized and only those cells that are undergoing cell division at the time of infection will become integrated. Other problems include reports of premature promoter shutdown (Palmer TD et al., 1991,Challita PM and Kohn DB, 1994) leading to transcriptional shutoff.

A 4.3 Lentivirus

Alternative vectors such as lentiviruses have been developed because of the limitation of infection to dividing cells by retroviruses. Significant progress has been made in recent years in the development of lentivirus vectors, a retrovirus sub-group based on the Human Immunodeficiency Virus (HIV) (Trono D, 2000). To reduce the risk of pathogenesis, third generation HIV vectors are now available that use only a fractional set of HIV genes and a conditional packaging system to protect against generating productive recombinants (Dull T et al., 1998). Self-inactivating vectors in which viral enhancer and promoter sequences have been deleted are also now available (Miyoshi H et al., 1998). HIV vectors are capable of transferring genes into nondividing cells such as neurons (Naldini L et al., 1996) and quiescent haematopoietic progenitor cells, (Case

SS et al., 1999) which will be particularly useful for these tissue targets. Lentiviruses can be made more stable by pseudotyping that allows virus titres to be improved by ultracentrifugation. This offers the opportunity of infecting a greater number of cells *in vivo* and different envelopes allow targeted gene transfer to specific tissues, for example to the nervous system (Mazarakis ND et al., 2001) and airways (Kobinger GP et al., 2001). Both the EIAV vector, a vector derived from non-primate animal lentiviruses, (Mitrophanous K et al., 1999) and Feline Immunodeficiency Virus (FIV) (Wang G et al., 1999) have been developed in an attempt to create vectors for use in human treatment that are not associated with any human pathology. Recent work in our lab has shown that high level sustained transgene expression can be achieved in a variety of tissues using a VSV-G pseudotyped EAIV vector in fetal mice after intravascular administration (Waddington SN et al., 2003c). A potential problem for lentivirus vectors is that they integrate into the genome randomly and may therefore be able to cause insertional mutagenesis.

A 4.4 Sendai virus

The negative strand RNA cytoplasmically replicating Sendai virus, a member of the paramyxovirus family, has recently been developed as a gene transfer vector. Early vectors still capable of self-propagation were found to provide very high levels of marker gene expression in a wide range of tissues including bronchial epithelium (Yonemitsu Y et al., 2000), skeletal muscle (Shiotani A et al., 2001) and vascular endothelium (Masaki I et al., 2001). Second generation vectors, although still capable of intra-cytoplasmic replication of the RNA genome, are incapable of intercellular propagation. In these vectors, genes encoding surface glycoproteins including the haemaglutinin-neuraminidase (HN) protein or the fusion (F) protein, which are responsible for cell binding and infection, have been deleted from the viral genome (Inoue M et al., 2003). Injection of F-deficient Sendai virus vector into the fetal mouse via various routes including intra-vascular, intra-amniotic, intra-muscular, intra-peritoneal and intra-spinal resulted in expression of marker gene in gut wall, lung, muscle, peritoneal mesothelia and dorsal route ganglia respectively (Waddington SN et al., 2004a). Further optimisation will be needed to develop these first generation constructs into clinically applicable vectors.

A 4.5 Reporter genes can be used as indicators of successful transduction

There is as yet no sheep model of cystic fibrosis or haemophilia B in which correction of the disease process using gene therapy can be tested. Instead reporter genes such as β-galactosidase and human Factor IX (hFIX) can be used as indicators of successful transduction and to monitor for their expression. β-galactosidase allows tracking of vector gene transfer and expression in tissues at set time points, and can be detected in sacrificed tissues directly by a blue staining assay (X gal) or indirectly using immunohistochemistry. The level of hFIX, detectable in the plasma by ELISA analysis can be used to follow gene expression after birth without the need for sacrifice. Later readministration of hFIX or the vector to born animals injected *in utero* can be used to examine whether immune tolerance has been achieved. PCR and RT-PCR can be used for both vectors to follow vector spread and expression on the DNA and RNA levels respectively.

A 5 Fetal gene therapy studies

In utero gene therapy has so far been investigated in a broad range of small animals using a variety of techniques. Studies in large animals have mainly used sheep, since primates are more costly and difficult to maintain.

A 5.1 Intravascular injection

Delivery of vectors to the systemic fetal circulation appears to be a highly effective route for targeting gene therapy to a range of fetal tissues, and particularly to the liver (Senoo M et al., 2000). This can be accomplished either by injection via the umbilical vein (Themis M et al., 1999,Yang EY et al., 1999,Senoo M et al., 2000), by intracardiac injection (Wang G et al., 1998), or by injection into the yolk sac vessels (Schachtner SK et al., 1996). In all small animals and in two sheep studies these procedures have been performed at laparotomy. The ductus arteriosus was transfected using a liposome/DNA plasmid after surgical exteriorization of the ductus in fetal sheep at 90 days of gestation (Mason CA et al., 1999). Adenovirus vector injection into the umbilical vein at 60 days gestation in sheep has been achieved after externalization of a loop of umbilical cord at laparotomy and hysterotomy (Yang EY et al., 1999).

Two studies have performed intravascular delivery via ultrasound guidance. In the late gestation fetal rabbit, ultrasound-guided intracardiac delivery of adenovirus vectors was achieved (Wang G et al., 1998). Transgene expression was observed in up to 40% of

fetal hepatocytes, with less in the fetal renal cortex and glomeruli. As expected transgene expression was transient, declining to <1% by 21 days after injection, although this was not associated with a fetal immune response to the vector or transgene. The procedure had a 25-40% mortality rate.

Our group performed ultrasound-guided percutaneous injection into the umbilical vein to deliver adenovirus vectors carrying the β-galactosidase marker gene or the human factor IX gene to fetal sheep from 102 days gestation (Themis M et al., 1999). Similarly, 30% of fetal hepatocytes were β-galactosidase positive and the fetal adrenal cortex also showed less but still significant transgene expression. Although vector DNA was found by PCR analysis in the gonads, extensive further investigation by RT-PCR could not detect any gene expression. The duration of transgene expression was studied using the levels of human factor IX in fetal and neonatal plasma, that reached therapeutic levels within a week of delivery in two animals and subsequently declined. Maximum adenovirus expression is expected 48-72 hours after introduction of the vector. Several other factors that determine the kinetics of transgene expression include vector elimination, promoter shutdown, the half-life of the protein and immune reactions. Antibodies against the vector and transgene were observed in some animals with low plasma human factor IX levels.

A 5.2 Other routes for targeting the fetal circulation and liver

Gene delivery to the liver has also been observed after vector application via other routes. Intramuscular injection is mainly used to target the muscle for treatment of muscular dystrophies but this route may be used for ectopic production of proteins such as hFIX in the treatment of haemophilias. Intramuscular injection of adenovirus vectors into fetal mice resulted in gene transfer to the muscle, liver and lung albeit transiently (Yang et al., 1999b). Hepatocytes were also targeted by intrahepatic injection of fetal mice (Lipshutz GS et al., 1999a,Lipshutz GS et al., 1999b,Lipshutz GS et al., 2000) and rats (Hatzoglou M et al., 1995). Intraperitoneal injection has been used for successful gene transfer to multiple tissues including the liver in fetal mice (Lipshutz GS et al., 1999b) and sheep (Porada CD et al., 1998,Tran ND et al., 2000). Retrovirus vectors were injected into the peritoneal cavity of fetal sheep at 57-65 days of gestation and long-term transduction of hematopoietic stem cells (HSCs) in the bone marrow and blood could be demonstrated 5 years later. Since this indicated haematogenic spread, gene delivery to the germline was also investigated, but was not detected by extensive PCR analysis of the sperm derived from rams injected *in utero*.

A 5.3 Intra-amniotic application

Gene delivery to the amniotic cavity is achieved by laparotomy in small animals such as rodents and guinea pigs (Papaioannou VE, 1990) and by ultrasound guided intra-amniotic injection in larger animals such as the sheep and primate. Sheep studies have so far used catheters implanted at laparotomy to deliver the vectors to the amniotic cavity (Iwamoto HS et al., 1999,Holzinger A et al., 1995). One group has performed ultrasound-guided intra-amniotic injection of mid-trimester rhesus macaque fetuses (Larson JE et al., 2000b). Transgene expression was seen after vector delivery to the amniotic cavity in the amniotic membranes, the fetal skin, the gut and the mucosae in all investigated species (Douar A-M et al., 1997,Larson JE et al., 1997,Holzinger A et al., 1995,Iwamoto HS et al., 1999,Sekhon HS and Larson JE, 1995). Indeed, therapeutic plasma concentrations of human coagulation factor IX were achieved in fetal mice after intra-amniotic injection of adenovirus vectors carrying the human factor IX gene (Schneider H et al., 1999) and the transgenic protein remained detectable after birth. This suggests that transduction of keratinocytes *in utero* may be able to deliver proteins to the circulation as well as to treat hereditary skin disease. Unfortunately the efficiency of gene transfer particularly to the pulmonary epithelium is low (Boyle MP et al., 2001) which could be due to dilution of the vector by the relatively larger volume of the amniotic fluid. Fetal airway smooth muscle spontaneously contracts rhythmically from early gestation in humans and other mammals (McCray PB, 1993,Schittny JC et al., 2000). Fetal breathing movements are observed in the sheep (Cooke IRC and Berger PJ, 1990) from 50 days of gestation and in the human from 10 weeks of gestation (Baconnais S et al., 1999). These increase in incidence as gestation proceeds and lead to an intake of amniotic fluid to the lungs against the continuous outflow of tracheal fluid (Badalian SS et al., 1993). It may be possible to enhance these breathing movements using agents such as theophylline (Moss IR and Scarpelli EM, 1981) to carry the vector further into the fetal airways.

A 5.4 Vector delivery to the fetal airways

Direct injection of the lung parenchyma has been attempted to access the fetal airways but with poor results. Our group delivered an adenovirus vector containing the β-galactosidase gene by ultrasound guidance at laparotomy through the intact uterine wall into the lung parenchyma of a fetus at 98 days of gestation. Gene transfer was restricted to the site of injection and predominantly in alveolar macrophages and no spread within the airways could be detected (Prof T Kiserud, personal communication). In mid-

gestation fetal primates, ultrasound guided delivery of lentivirus vectors to the lung resulted in low level transgene expression in the fetal airways (Tarantal AF et al., 2001a).

Direct instillation of vector into the trachea has been more successful but this has so far only been attempted in fetal sheep using highly invasive techniques at laparotomy that carry a significant morbidity and mortality. Three groups instilled retrovirus (Pitt BR et al., 1995) or adenovirus (McCray PB et al., 1995,Vincent MC et al., 1995) vectors via catheters placed in the trachea under direct vision and demonstrated low level gene expression in the proximal airways. Another group used fetoscopic guidance to place catheters in the trachea for adenovirus vector delivery (Sylvester KG et al., 1997,Yang EY et al., 1999). This led predominantly to alveolar infection but the efficiency of gene transfer of the entire airway was improved by the use of a balloon catheter to occlude the trachea. As expected transgene expression was transient in many animals and may have been limited by the induced inflammatory response that was occasionally seen. Delivery to the fetal airways using ultrasound-guided embryofetoscopy should be possible but further work is needed on improving gene transfer to these tissues.

A 5.5 Agents to enhance epithelial cell transduction

The epithelium of the upper airways is notably resistant to adenovirus transfection (Grubb BR et al., 1994). Complexing adenovirus vectors with various polycations such as DEAE dextran, significantly increases gene transfer to the mouse lung (Kaplan JM et al., 1998). The polycations are believed to improve binding of complexed adenovirus to the target cell surface via neutralisation of the adverse charge interaction between negatively charged adenovirus particles and the negative charge of the cell glycocalyx and abundant sialic acid residues (Arcasoy SM et al., 1997b). Exposure of epithelial cell monolayers to polycations may also transiently increase paracellular permeability (McEwan GT et al., 1993) allowing the virus to reach the basolateral surface where CAR and integrins are expressed.

Sodium caprate, the sodium salt of a medium chain fatty acid improves adenovirus gene transfer to primary human airway epithelial cells *in vitro* (Coyne CB et al., 2000) and *in vivo* application to the mouse airways enhances adenovirus-mediated gene transfer (Gregory LG et al., 2002). The effect is believed to be mediated via the tight junction that is the strongest binding among cell junctions and creates a regulated barrier in the apical junctional complex. The tight junction consists of a continuous ring of associated proteins that include the occludins and claudins (Denker BM and Nigam SK, 1998). A

reversible redistribution of the cytoskeleton and structural dilations in the tight junction occur when sodium caprate is applied to epithelial cells (**Figure A 6**). Sodium caprate enhances transmucosal absorption in the gastrointestinal epithelia *in vitro* (Lindmark T et al., 1998) and is used as an absorption enhancing agent to promote intestinal transmucosal hydrophilic drug absorption (Anderberg EK et al., 1993).
Fluorocarbon liquids such as perflubron improve distribution of adenovirus vectors to adult airways epithelia and enhance gene expression in normal and diseased lungs (Weiss DJ et al., 2001). Perflubron is used clinically in liquid ventilation of neonates (Shaffer TH et al., 1992) and for adult resuscitation following shock (Younger JG et al., 1997). It is believed to enhance gene transfer by reducing the surface tension at the air-liquid interface in the airways and recent evidence has suggested that it may also increase the permeability of epithelial tight junctions (Weiss DJ et al., 2003).

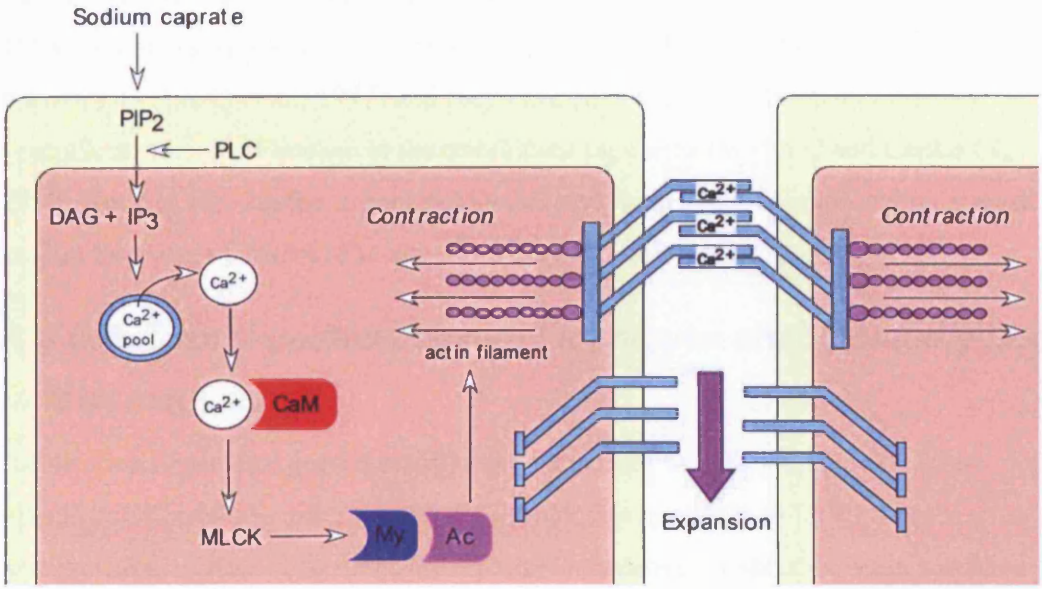


Figure A 6: The proposed mechanism of action of sodium caprate on epithelial tight junctions.

Sodium caprate increases the intracellular calcium levels through interaction with phospholipase C in the membrane. Calcium released in a chain of reactions activates calmodulin-dependent contraction of the actin microfilament which opens the tight junction. PIP_2: phosphatidylinositol-4,5-diphosphate; PLC: phospholipase C; DAG: diacylglycerol; IP_3: insitol-1,4,5-triphosphate; CaM: calmodulin; MLCK: myosin light chain kinase; My: myosin; Ac: actin. Adapted from (Hayashi M et al., 1999).

A 5.6 Gastrointestinal delivery

Intrapharyngeal delivery has been attempted in fetal rabbits at laparotomy to target the fetal gastrointestinal system as a model for the treatment of meconium ileus due to cystic fibrosis (Wu Y et al., 1999). Gene transfer to the small bowel enterocytes was

achieved but there was significant maternal and fetal loss related to anaesthesia and the invasive surgery used. Ultrasound-guided injection of barium into the fetal stomach of rabbits has been performed successfully (Brandt et al., 1997c) and this technique could be extended to deliver gene therapy to the fetal gut. We have observed gene delivery to the gut of fetal mice after intra-amniotic vector application most likely as a result of fetal swallowing (Douar A-M et al., 1997).

A 5.7 Delivery to the nervous system

Most of the work on gene transfer to the fetal brain has involved engraftment of transduced fetal neural progenitors into adult small animals, for potential treatment of neurodegenerative disorders such as Parkinson's disease. Adenovirus vectors efficiently infect and transfer genes to fetal rat neurons (van Esseveldt KE et al., 1997) and long term expression up to 7 months after infection was observed (van Esseveldt KE et al., 1998). Gene transfer to fetal human neurons is also achievable by recombinant retroviruses (Lin Q et al., 1997) and they have been applied to the fetal rat lateral ventricle to study cell lineage in the developing rat cortex (Walsh C and Cepko CL, 1988). Such *in vivo* studies are techically demanding in small animals and no similar studies have been performed in any large animal fetal models.

A 6 Ultrasound-guided delivery techniques are commonly used in fetal medicine

Application of prenatal gene therapy in the fetus must be safe, reliable and cost-effective. Ultrasound-guided percutaneous injection may help to fulfill these prerequisites because procedures are minimally-invasive, of short duration and have already been developed for diagnostic and treatment purposes in clinical practice. There are a range of ultrasound-guided techniques available to target the human fetus from early in gestation, many of which might be adapted to deliver gene therapy (**Figure A 7**). For some organ systems, such as the brain and gut, there was no suitable ultrasound-guided injection technique available.

Coelocentesis allows access to the extraembryonic coelom in the early first trimester with a success rate of >95% at 6-11 weeks of gestation (Wilson JM and Wivel NA, 1999). It may be of little use, however for *in utero* gene therapy because of the limited transfer from the extraembryonic coelom via the amniotic membrane to the amniotic cavity (Jauniaux E and Gulbis B, 2000). Also, the risk of miscarriage in ongoing pregnancies is approximately 25% (Ross J et al., 1997).

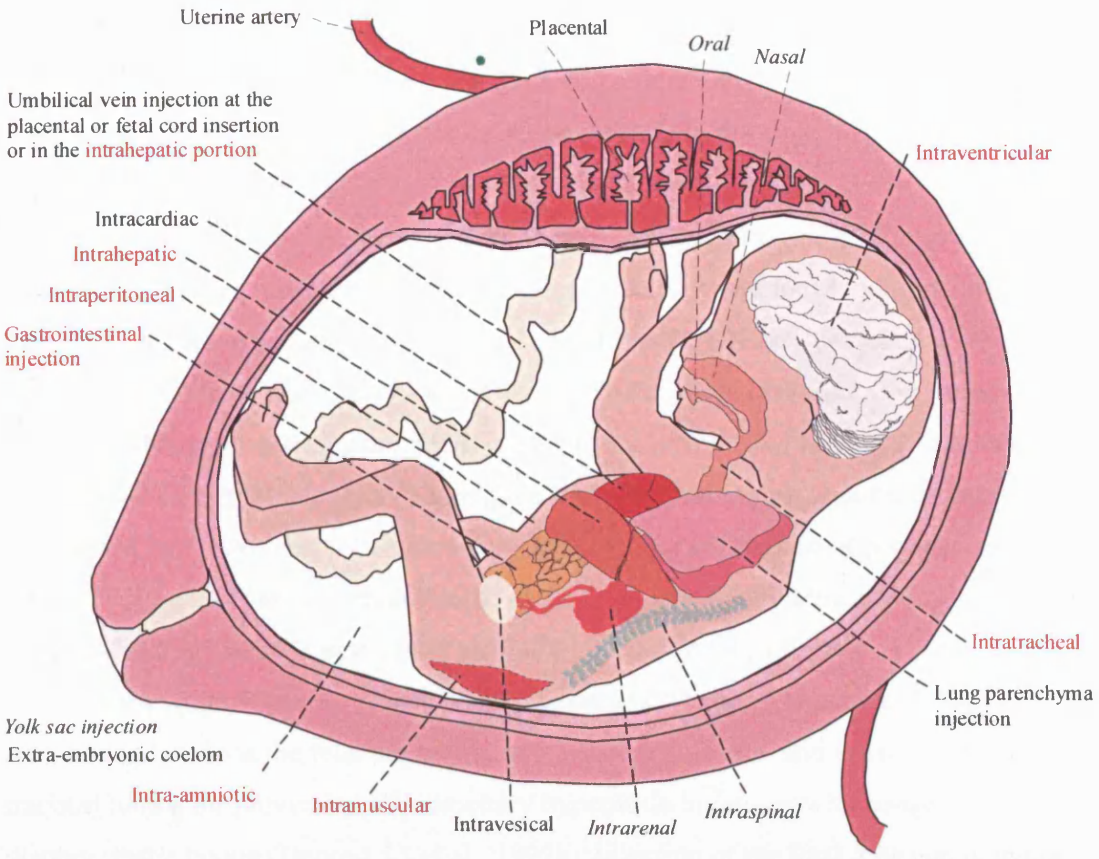


Figure A 7: Ultrasound-guided injection techniques.

Routes in red were developed and applied in this thesis to the fetal sheep animal model. Routes in italics have not yet been applied to the fetal sheep by ultrasound guided injection.

Amniocentesis may be performed from 8 weeks, although it is more commonly used from 15 weeks of gestation onwards because of the risk of fetal limb defects. However as we have seen, intra-amniotic application may be of limited use in fetal gene therapy and accessing the systemic circulation has greater potential.

Fetal blood can be sampled in the second trimester under ultrasound-guidance either from the placental cord insertion or from the intrahepatic vein (Nicolaides KH et al., 1986,Nicolaidis P et al., 1991). The procedure has a good success rate, is low risk and is used commonly for rapid karyotyping or fetal blood transfusion (Nicolini U et al., 1990).

From 12 weeks of gestation ultrasound-guided intracardiac puncture for fetal blood sampling has been performed on patients undergoing surgical termination of pregnancy (Jauniaux E et al., 1999a). Similarly, radiolabelled fetal liver cells were successfully injected into the heart of fetuses aged 13 weeks of gestation under ultrasound-guidance

(Westgren M et al., 1997) prior to prostaglandin termination of pregnancy. No fetal heart rate abnormalities were detected and all fetuses were alive at least 6 hours after the procedure. In clinical practice however, intracardiac puncture is usually only reserved for delivery of potassium chloride for selective fetocide in multiple pregnancy (Golbus MS et al., 1988a).

Intraperitoneal injection has been used for *in utero* stem cell transplantation from 14 weeks of gestation (Touraine, 1999) and is an alternative route for blood transfusion prior to 18 weeks of gestation (Rodeck CH and Deans A, 1999). Ultrasound guided transabdominal liver biopsy has been performed in the second trimester for prenatal diagnosis of some congenital metabolic syndromes from enzyme activity (Vaughan JI and Rodeck CH, 2001). The procedure has a low complication rate but has been mostly superseded by DNA analysis. Corticosteroid therapy for maturation of preterm infant lungs and vitamin K have been delivered to the fetus *in utero* by ultrasound guided intramuscular injection (Larsen JF et al., 1978,Ljubic A et al., 1999).

Access to the fetal trachea is possible using fetoscopy. Detachable occlusive balloons have been placed via the fetal larynx under fetoscopic guidance and inflated within the tracheal lumen for prevention of pulmonary hypoplasia in fetuses with congenital diaphragmatic hernia (Deprest JA et al., 1997b). Injection of the fetal lung parenchyma has been used to decompress large unilocular macrocystic congenital cystic adenomatoid malformations of the lung (Nugent CE et al., 1989).

A 7 The fetal sheep animal model is particularly suitable for investigating fetal gene therapy

The sheep fetus is well established as an animal model relevant to human fetal physiology, has a good tolerance to *in utero* manipulations and a consistent gestation period of 145 days, which is approximately half that of the human. As in humans, the gestational age of the fetus can be accurately determined by ultrasound using fetal measurements similar to those used in clinical practice (Barbera A et al., 1995) and ultrasound is routinely used in farming practice to check for multiple pregnancy early in gestation (Aiumlamai S et al., 1992). Ultrasound-guided injection techniques in sheep such as fetal blood sampling (Newnham JP et al., 1994), intra-amniotic delivery of endotoxin (Moss TJM et al., 2002), fetal intramuscular steroid injection (Jobe AH et al., 1996) and fetal intraperitoneal injection of a DNA synthesis label (Greenwood PL et al., 1999) have also been performed. More recently, ultrasound has been used to examine the fetal sheep trachea (Kalache KD et al., 2001). Unfortunately there are no sheep and

few other large animal models of human genetic disease available for testing of gene therapy. It is for this reason that small animals such as mice are commonly used, although their size precludes development of minimally invasive techniques of application.

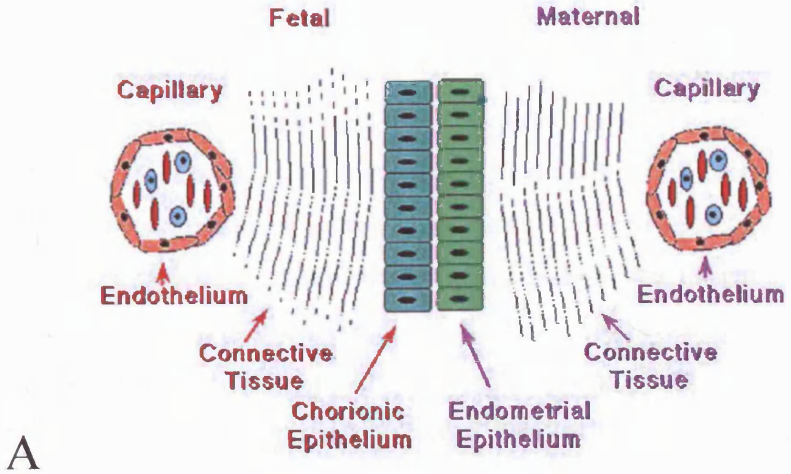
There are some differences between ovine and human biology particularly in the growth of the fetus that must be considered (Newnham JP and Kelly RW, 1993). The fetal growth rate in late gestation sheep is over double that in humans (36 vs 15 g/kg fetal wet weight per day) although the percentage body fat at term is a quarter of that in humans (2% vs 16%) (Fowden AL, 1995). These differences are responsible for the close relationship between maternal nutrition and lamb survival and the thermal disadvantage of the neonatal lamb from its high surface area to weight ratio.

There are also differences in the structural development of the human and sheep fetus. In particular the placenta, the stomach and lungs are considered in more detail below since they are relevant to the research described in this thesis.

A 7.1 The sheep placenta and membranes

The discoid human placenta provides only a single disk-like zone of intimate maternofetal contact that can generally be avoided during ultrasound-guided fetal injection. The sheep placenta however, consists of spot like regions of maternofetal interdigitations (placentomes) containing a tuft of fetal villi called a cotyledon attached to 60-150 endometrial thickenings called caruncles. The placentomes are spread throughout the uterine cavity and may be difficult to avoid during ultrasound-guided uterine interventions. Evidence from ultrasound-guided amniocentesis in the fetal goat that has a similar placental structure indicates that placentome puncture is an abortion risk (Lovell KL et al., 1995).

The maternofetal barrier in sheep is the most complete placental barrier possible, separating the maternal and fetal blood by six tissue layers, three derived from the mother (maternal capillary endothelium, maternal endometrial connective tissue, maternal endometrial epithelium) and three derived from the fetus (trophoblast, chorionic connective tissue, fetal endothelium) (Benirschke K and Kaufmann P, 1990) (**Figure A 8 A**). Syncytia are formed by fusion of fetal chorionic binucleate cells and maternal endometrial epithelial cells producing an epitheliochorial placenta (Wooding FB, 1992).



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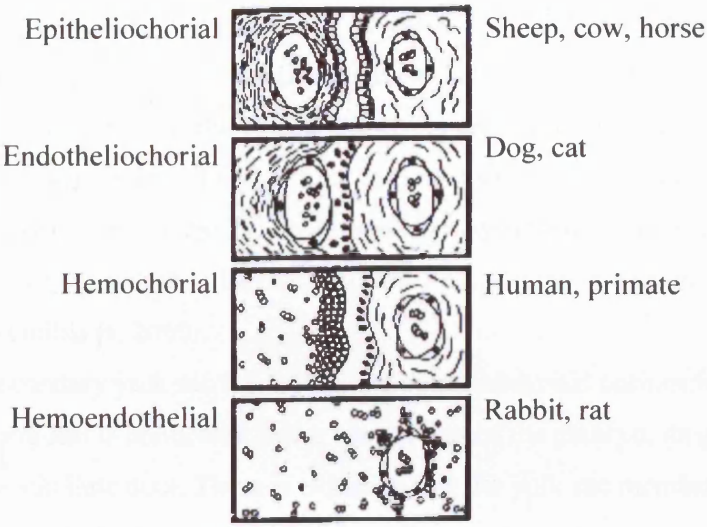


B

Figure A 8: Potential barriers between fetal and maternal circulations.

(A) The six layers of the epitheliochorial placenta in the sheep are illustrated. (B) Comparative placentation showing the cellular barriers between maternal and fetal blood for several species.

In humans however, there is extensive invasion of the endometrium by the trophoblast that removes the three maternal tissue barriers and results in a hemomonochorial placenta at term (**Figure A 8 B**). Probably as a result of these structural differences, gammaglobulin does not pass from the mother to the fetus in the sheep, but is able to cross the placenta in humans.

The placental weight in sheep increases to a maximum at about 90 days of gestation and then declines (Barcroft J and Barron DH, 1946) while in humans placental weight increases to a maximum at 34-36 weeks and then remains static. Placental transport capacity keeps pace with fetal growth however, with similar increases in uterine blood

flow in late gestation sheep and humans (Reynolds LP and Redmer DA, 20021).
The exocoelomic cavity or extraembryonic coelom surrounds the embryo and fetus during most of the first trimester. Although not directly in contact with the embryo, the coelomic fluid is only separated from it by the thin amniotic membrane and thus might provide an alternative route to access the embryo and early fetus. The sheep has a substantial allantoic compartment that increases in size through gestation reaching a volume of 750ml at term. The allantoic fluid is largely derived from the fetal urine via the urachus which drains up to 50% of the urine in the fetal bladder, whilst the remaining urine drains via the urethra to the amniotic compartment (Wlodek ME et al., 1988).
In the human fetus the allantois is relatively rudimentary, arising from the roof of the yolk sac as a posteriorly directed diverticulum after the formation of the tail fold. It eventually opens into the cloaca, the proximal end forming the apical part of the bladder and the distal end becoming a fibrous cord connected to the umbilicus (Sadler TW, 1990). The amniotic cavity develops and grows inside the extraembryonic coelom before fusing with the placental chorionic plate at the end of the first trimester (Jauniaux E and Gulbis B, 2000).
The secondary yolk sac lies within the extraembryonic coelom from the 6^{th} week of gestation and is connected to the ventral part of the embryo, its gut and main circulation via the vitelline duct. There is evidence that the yolk sac membrane may transfer molecules from the extraembryonic coelom to the embryonic compartments and into the gut and circulation (Gulbis B et al., 1998). In addition, haemopoietic stem cells of myeloid potential are generated within the yolk sac although the multipotent, lympho-myeloid stem cells are now thought to emerge within the ventral wall of the aorta, within the embryo itself (Peault B and Tavian M, 2003). As in the human, the yolk sac in the sheep is rudimentary.

A 7.2 The sheep lung

Lung development is divided into stages based on the events occurring primarily in the gas-exchange area. The sheep and human lung undergo similar stages of development but the timing of these stages in gestation is different. Human lung development can be divided into three chronological periods, the embryonic, fetal and postnatal periods. In the early embryonic period the lung bud appears towards the end of the fourth week as a ventral bud of the prospective oesophagus that grows into the surrounding mesenchyme

by successive dichotomous divisions. By the end of seven weeks the lung resembles a small tubulo-acinar gland termed the pseudoglandular phase of development.
The embryo enters the fetal period during which there are three morphologically defined phases to reach a functionally mature organ at birth. Development occurs from proximal to distal tissues, and there is considerable overlap between the phases and from segment to segment within the lung. In the pseudoglandular stage up to 17 weeks of gestation, all the prospective conductive airways form and the most distal tubules representing the future acini appear (**Figure A 9 A**). In the canalicular phase there is widening of the tubules and further branching within the acini to produce canaliculi. Thinning of the epithelium occurs and the respiratory surface becomes vascularized (**Figure A 9 B**). The cuboidal epithelium differentiates into type I and type II pneumocytes towards the end of this phase. The saccular phase from 24 weeks up to birth, is marked by completion of respiratory surface vascularization. There is a marked decrease in interstitial tissue and elastin is deposited underneath the epithelium (**Figure A 9 C**). This initiates the formation of alveoli in the alveolar phase (**Figure A 9 D**) that extends from 36 weeks through to the postnatal period.
In humans, with an extended period of gestation, the pseudoglandular stage is virtually complete by 50% of gestation and the alveolar stage is clearly established by 90% gestation although many areas of the lung are still in the saccular stage beyond term (**Figure A 10**).
In the fetal sheep the pseudoglandular stage extends further to 85 – 90 days (60%) gestation, the canalicular and saccular stages are foreshortened but the alveolar stage is clearly established by 125 days of gestation (86%, **Figure A 10**) (Pringle KC, 1986). Mature type II surfactant producing pneumocytes are abundant by 120 days of gestation, although lambs delivered before 140 days of gestation seldom survive unless they receive exogenous surfactant

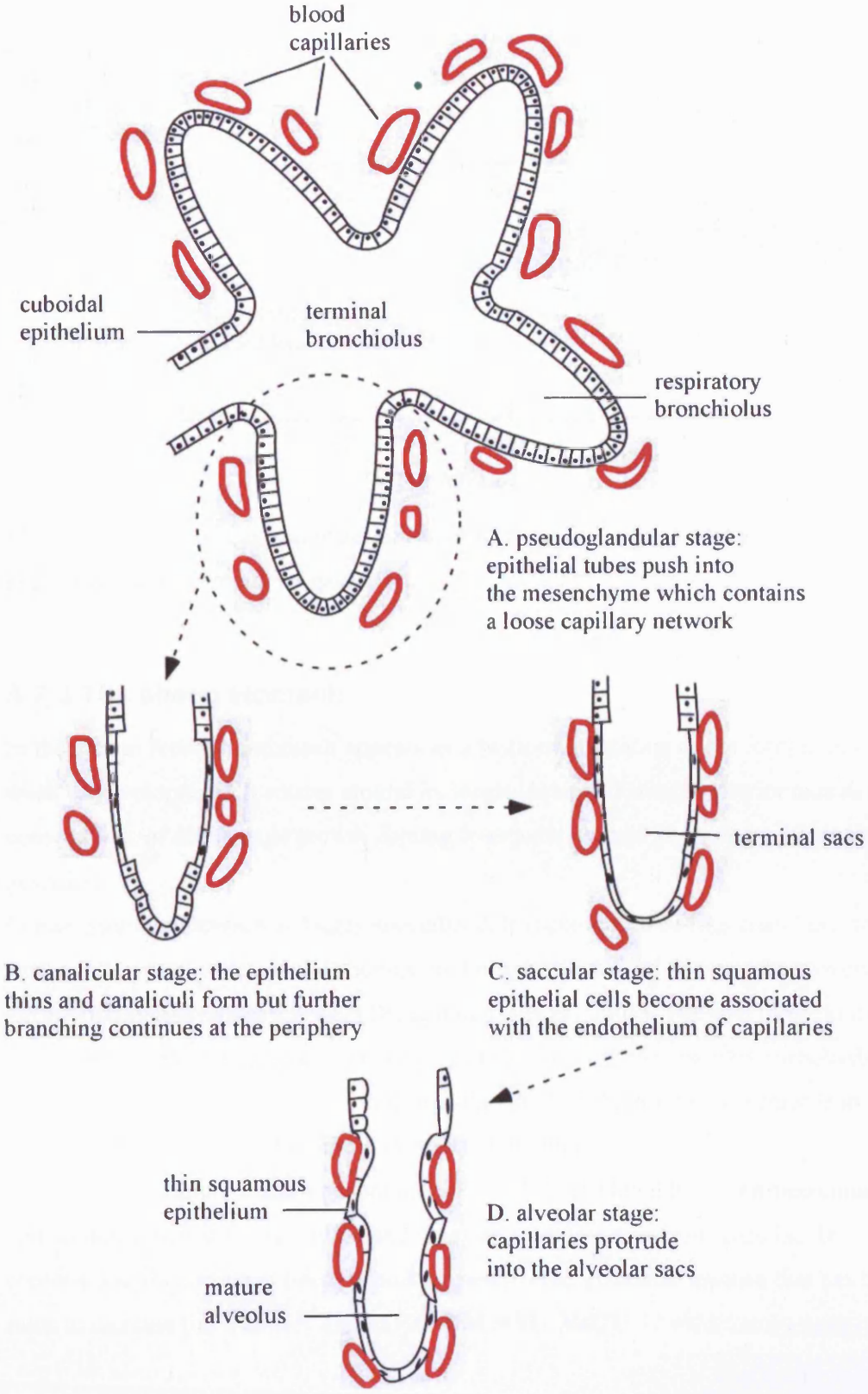


Figure A 9: The histological and functional development of the lung.

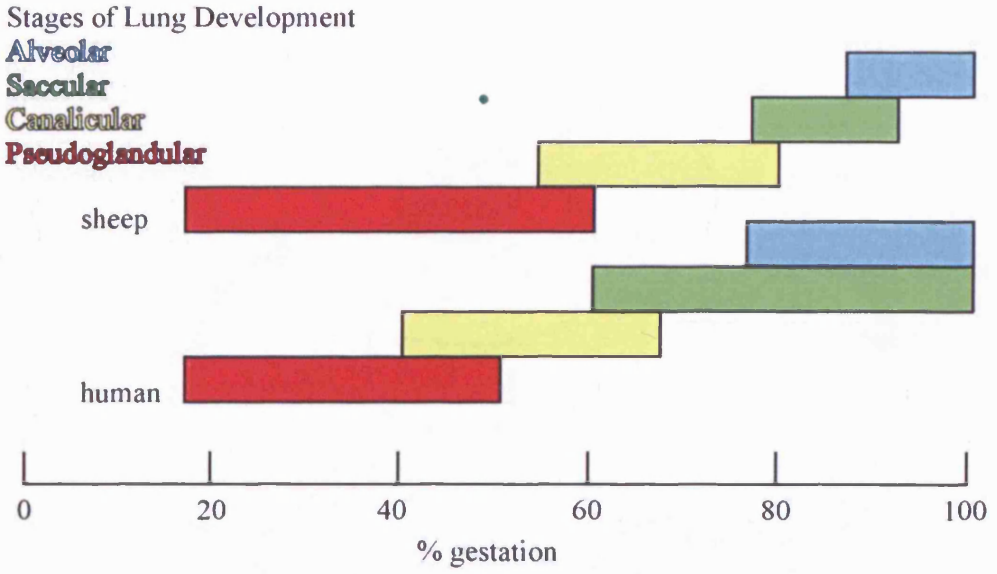


Figure A 10: A comparison of fetal lung development in the human and sheep according to the percent of gestation.

A 7.3 The sheep stomach

In the human fetus, the stomach appears as a fusiform dilatation of the foregut in the 4th week of development. It rotates around its longitudinal and anteroposterior axis as a consequence of differential growth coming to assume its final position by 12 weeks of gestation.

In ruminants the stomach is highly specialized. It is composed of four chambers, the rumen, reticulum, omasum and abomasum through which food passes successively during rumination (**Figure A 11**) (Thyagarajan B et al., 2001). The first three, known collectively as the forestomach, are developed to cope with the complex carbohydrates that form a large part of ruminant diet, and only the last chamber is comparable in structure and function to the simple stomach of the human.

The rumen, reticululum and omasum are thin walled, and lined by a stratified cutaneous epithelium, which in the reticulum and omasum, contains numerous papillae. In contrast, the abomasum is lined by pink slime-covered glandular mucosa that has large folds to increase the secretory area (Dyce KM et al., 2002).

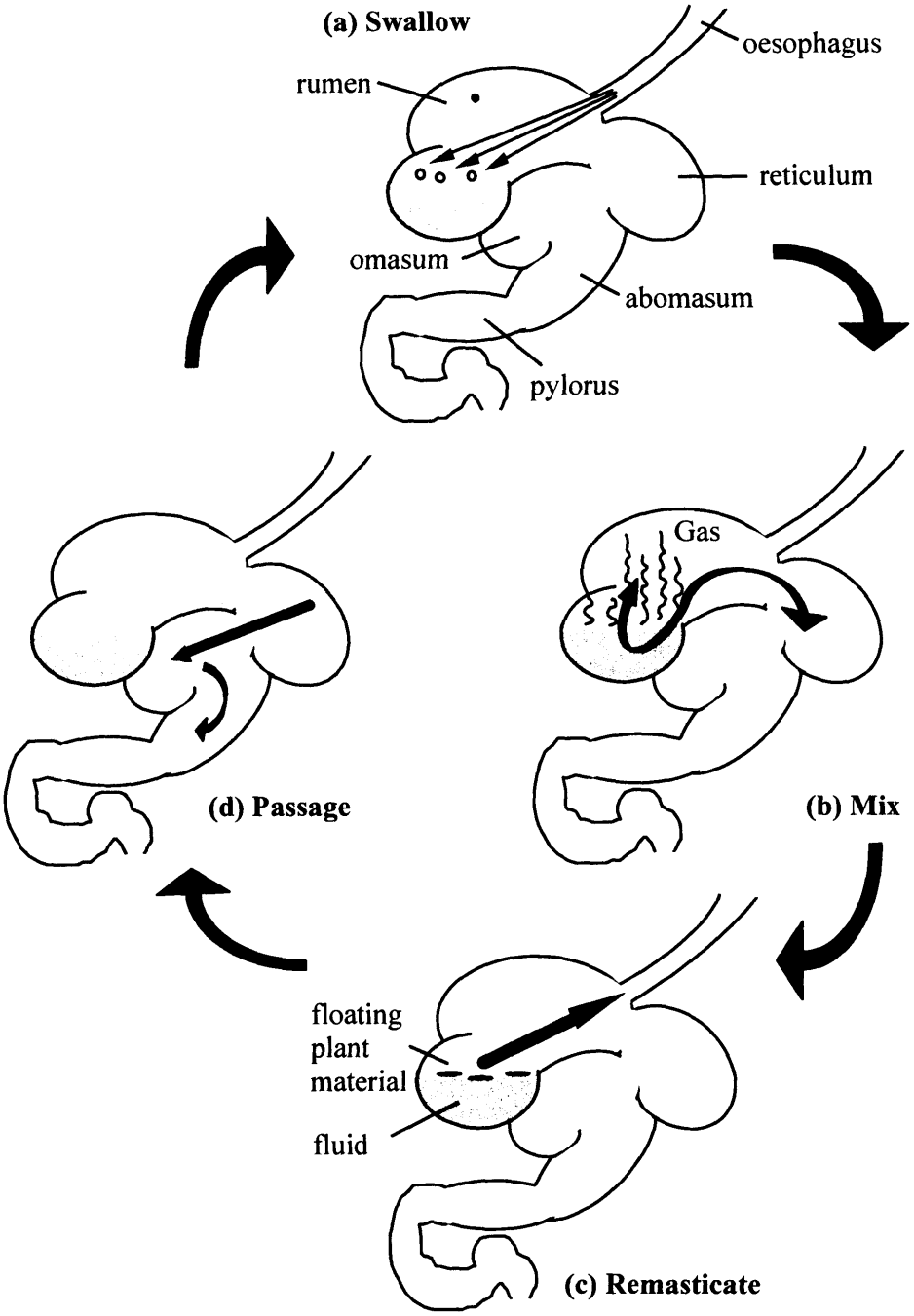


Figure A 11: Rumination in the ovine stomach.

(a) The large rumen, which is about 75% of the stomach volume, receives the food after it is swallowed and serves as a large holding and fermentation vat. The smaller reticulum represents about 8% of the stomach volume. (b) During rumination, rhythmic contractions pass through the rumen and reticulum to circulate the food round the two compartments. Small food particles sink and pass into the reticulum while large undigested plant fibers float on the surface of the ruminal fluid and (c) are regurgitated for remastication in the mouth. The omasum, which occupies 4% of the stomach volume, absorbs fatty acids and water, and (d) is responsible for transferring food from the reticulum to the abomasum. The abomasum, representing about 13% of the stomach volume, is the true stomach in which enzymatic and acidic hydrolysis take place.

The primitive stomach is identifiable as an expansion of the foregut spindle in the early sheep embryo from 18 days of gestation. The spindle rotates 90° to the left by 23 days and the four chambers are discernible by 26 days of gestation (Latshaw WK, 1987). By 34 days of gestation the rumen has developed two cranially directed growths, the dorsal and ventral sacs, and the reticulum is clearly separated from it. As the rumen grows it comes to lie on the left side of the abdominal cavity and the dorsal and ventral sacs become directed caudally. Rapid expansion of the rumen pushes the omasum and abomasum to the right of the abdominal cavity and by the end of the embryonic period the position and relative sizes of the compartments of the ruminant stomach are the same as in the adult. The growth of the rumen slows while the abomasum expands, so that at birth the abomasum accounts for more than half the stomach volume. This is because in the suckling neonate, fermentation is unnecessary, and the milk bypasses the rumen through a reticular groove entering the abomasum for digestion. Ruminal growth catches up after birth on weaning and the relative sizes of compartments reach that of the adult by 1 year of age.

B. Aims

Fetal gene therapy has been extensively investigated in small animal models. There is a need however, to translate this research into a large animal model using clinically applicable delivery methods before application in the human fetus.

The aims of this study are:

B.1 To establish a large animal model to test minimally invasive delivery methods in preparation for future application of *in utero* gene therapy in humans.

B.2 To target gene therapy to the fetus for treatment of haemophilia, cystic fibrosis in the airways and small bowel, and mucopolysaccharidosis type VII using ultrasound guided delivery methods.

B.3 To investigate reporter gene spread, expression, the fetal and maternal immune response, and the morbidity and mortality using adenovirus as a pathfinder vector.

C. Methods

C 1. General chemicals and reagents

The manufacturers of chemicals and reagents, and the details of buffers and solutions are listed in **Appendix 1 and 2.**

C 1. Care of sheep and ultrasound guided procedures

Sheep of Romney breed were used. Each ewe was ear tagged twice with an identification number and the sheep flock was vaccinated against Toxoplasma, Chlamydia and Clostridium annually. All procedures on animals were conducted in accordance with UK Home Office regulations and the Guidance for the Operation of Animals (Scientific Procedures) Act (1986).

C 1.1 Timed mating of ewes

Tupping of sheep was begun in early autumn during the sheep breeding season and approximately 50 sheep were tupped each year. To enable timed mating of sheep, Chronogest® sponges (Flugestone acetate) containing 30mg progesterone were placed in the vagina of ewes for 2 weeks to induce ovulation. Two days after removal of the progesterone sponges, ewes were put in a pen with the ram overnight. The ram was marked on its belly with Ram Raddle®, a coloured powder mixed with liquid paraffin which is transferred and marks the back of the ewe once she has been served. Marked ewes were presumed to have been tupped. Ewes that were unmarked or not marked clearly were put back for a 'return service' with the ram 2 weeks later. In some cases ewes were tupped but were not pregnant when scanned. These 'empty' ewes were not used in experiments. When the sheep breeding season was advanced (March onwards) superovulation was occasionally required. Chronogest® sponges were placed vaginally for 12 days followed by an intramuscular injection of 1000 IU Folligon® (Pregnant Mares Serum Gonadotrophin). The ewe was placed in with the ram 2 days later.

C 1.2 Confirmation of pregnancy using ultrasound

Following tupping, sheep on the farm were scanned every month by a commercial sheep sonographer using a wide sector ultrasonic scanner (Oviscan 3, BCF Technology Limited, Livingston, Scotland). This operates on an 85° or 170° sector at varying depths of field that allows the sonographer to image the entire uterus and its contents by

placing the transducer on the naturally bare areas in front of the udder. The pregnancy and fetal number were confirmed and entered onto a spreadsheet (Excel 7.0, **Figure C 1**) to manage the details of tupping.

Ewe No.	Sponging Date	Tupping Date	USS	Week beginning Monday 14-Jan-02	21-Jan-02	28-Jan-02	04-Feb-02	11-Feb-02	18-Feb-02
2451	27-Sep-01	11-Oct-01	S	95	102	**109**	**IT8**		
2479	27-Sep-01	11-Oct-01	S	95	**102**	**IT7**			
2471	05-Oct-01	19-Oct-01	S	87	94	101	108	**115**	**IT10**
2450	05-Oct-01	19-Oct-01	S	87	94	101	**108**	**IT9**	
2181	15-Nov-01	29-Nov-01	S	46	**53**	**60**	**UV5**		
2486	15-Nov-01	29-Nov-01	S	46	**53**	**IC1**			
2469	22-Nov-01	06-Dec-01	S	39	46	53	**60**	**UV6**	
2540	22-Nov-01	06-Dec-01	S	39	**46**	**53**	**IC2**		
2546	29-Nov-01	13-Dec-01	T	32	39	46	53	**60**	**UV 7 + UV8**
2523	29-Nov-01	13-Dec-01	R	returned 31/12/02					
2544	06-Dec-01	20-Dec-01	T	25	32	39	46	**53**	**HE 6 + HE7**
2533	06-Dec-01	20-Dec-01	E	empty					

Figure C 1: Example of a sheep tupping list.

Ewes were put in overnight with the ram for tupping 2 weeks after placement of intravaginal progesterone sponges. Ewes not tupped were put back in with the ram 2 weeks later (returned). Sheep were delivered to the sheep holding facility one week before surgery and designated an experimental code (eg **IT8**) at surgery. USS: ultrasound scan; S: singleton; T:twin; E: empty; R: returned

C 1.3 Sheep diet

Ewes were housed on the farm out on grass or in sheds during the winter. Pregnant ewes were moved to the sheep holding facility in a lorry one week before use to allow acclimatization and condition scored on arrival. They were housed together in pairs in pens on straw at ambient temperature. They had free access to running water and hay and were given a daily portion of Super Ewe & Lamb UFAS compound feed according to their gestational age (**Table C 1**).

Table C 1: Daily feeding of sheep according to gestational age.

Gestational age	Ewe carrying twins		Ewe carrying singleton	
	Low condition score (2 – 3)	High condition score (4 – 5)	Low condition score (2 – 3)	High condition score (4 – 5)
Up to 91 days	200g	100g	100g	50g
92 – 105 days	400g	200g	200g	100g
106 – 119 days	600g	400g	400g	200g
120 – 133 days	800g	600g	500g	350g
134 – birth	1000g	800g	700g	500g

The feed is a concentrated source of oil, protein and vitamins and the amount given to sheep was increased as gestation progressed. This ensures adequate nutrition for the ewe

and reduces the risk of toxaemia caused by the large late gestation fetus compressing the rumen and reducing the ability of the ewe to digest enough hay for nutrition.

C 1.4 Confirmation of gestational age using ultrasound

All pregnant sheep were scanned the day after arrival at the sheep holding facility to confirm pregnancy, fetal number and gestation age. Ewes were caught, turned up and held in a sitting position for scanning using a Vingmed Sonotron CFM 800 ultrasound scanner (Horten, Norway) as shown in **Figure C 2**. The sonographer applied ultrasound coupling medium to the lower abdomen and scanned the uterus using a 5.0 MHz probe (TN100119A) held just above the udders. Gestational age was checked from measurements of fetal abdominal circumference, occipito-snout length and biparitetal diameter according to standard tables (Barbera A et al., 1995,Kelly RW and Newnham JP, 1989).

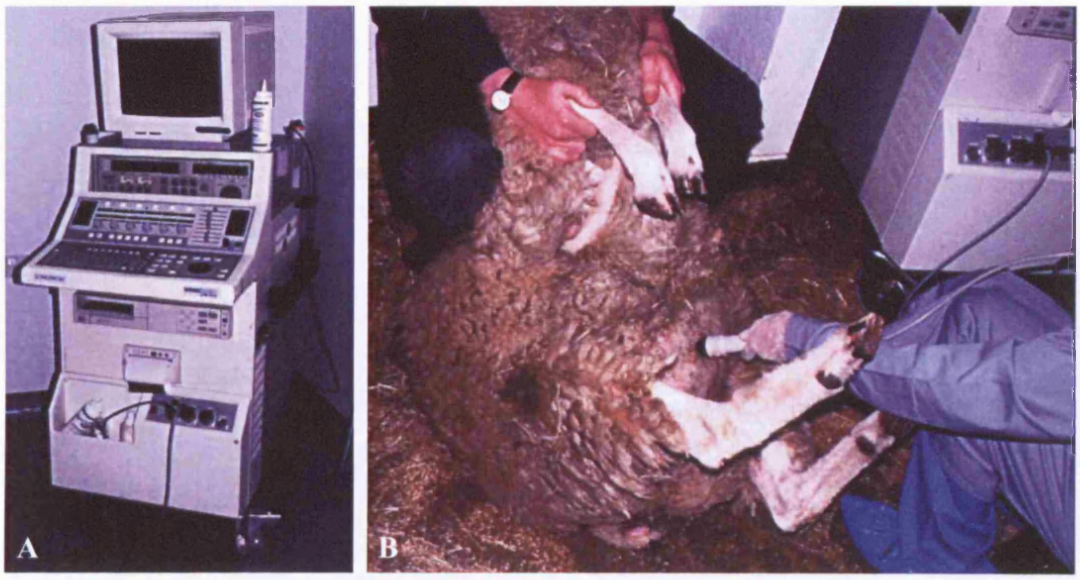


Figure C 2: Confirmation of gestational age in the sheep fetus by ultrasound.

(A) A Vingmed Sonotron 800 CFM ultrasound scanner being used to check the gestational age in a pregnant ewe that has been turned up (B).

C 1.5 Sheep anaesthesia

Before surgery ewes were starved for twelve hours with free access to water and bedded on wood chips. This is to prevent bloating which can occur when sheep under anaesthesia are unable to belch (eructate) normally and release methane gas which is a by-product of fermentation of their food (Wolfensohn S and Lloyd M, 1998).

On the morning of surgery sheep were moved to theatre restrained in a cart. The wool was clipped from their necks, the jugular vein was cannulated using a 19 Gauge butterfly winged perfusion set (Terumo Europe NV, Leuven, Belgium) and 15 ml of sodium thiopentone (10% w/v), was given intravenously over 1 minute for induction. Once asleep the ewes were rolled onto the operating table and intubated supine with a 9.0 mm cuffed endotracheal tube (Portex, UK) using a laryngoscope (Penlon, UK) to visualize the vocal cords. A cuff was inflated on the endotracheal tube to prevent inhalation of regurgitated ruminal contents and the head was lowered to allow drainage of saliva and ruminal fluid (Taylor PM, 1991). Because access to the whole abdomen was needed for procedures the animals were kept in dorsal recumbency. Over long periods the rumen can compress the lungs causing dyspnoea and the vena cava reducing venous return to the heart. This was not felt to be a problem in our procedures because they were of sufficiently short duration. Anaesthesia was maintained on 1.5-2 litres/minute halothane and 4 litres/minute oxygen using a Magill circuit (Medishield ventilator, Manley Serovent, **Figure C 3**).

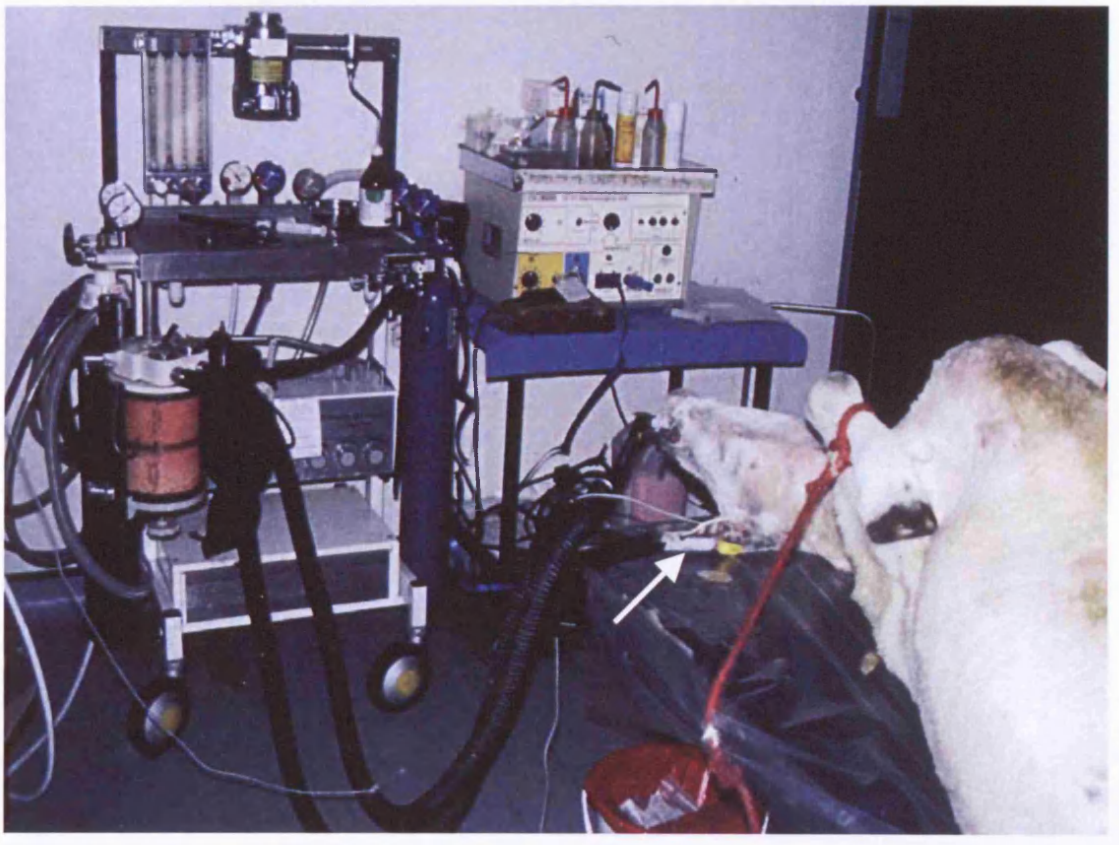


Figure C 3: General anaesthesia in the sheep.

The pregnant ewes were intubated and maintained on halothane and oxygen. The pulse oximeter is arrowed.

A pulse oximeter (5250 RGM, Ohmeda) placed on the ewe's ear was used to monitor oxygen saturation and pulse rate (**Figure C 3**) and this data was documented every 15 minutes together with respiration rate, halothane concentration and level of anaesthesia. Maternal blood was taken for pre-operative analysis of antibodies to the vector and transgene and for human factor IX levels. Blood was collected into BD Vacutainer tubes (BD Vacutainer systems, Plymouth, UK) containing 0.105 M sodium citrate for plasma (9NC, blue topped bottle) and into plain tubes for serum (SST, brown topped bottle).

Following surgery sheep were recovered while placed on their sternum in a cart and extubated once strong swallowing reflexes returned. They were then returned to their pen.

C 1.6 Ultrasound guided procedures on sheep

Once anaesthetized, the wool was clipped from the ewe's abdomen and a detailed ultrasound examination of the uterus and its contents was performed using a 3.3 MHz probe (TK100105A) on the Vingmed ultrasound scanner (**Figure C 4**).

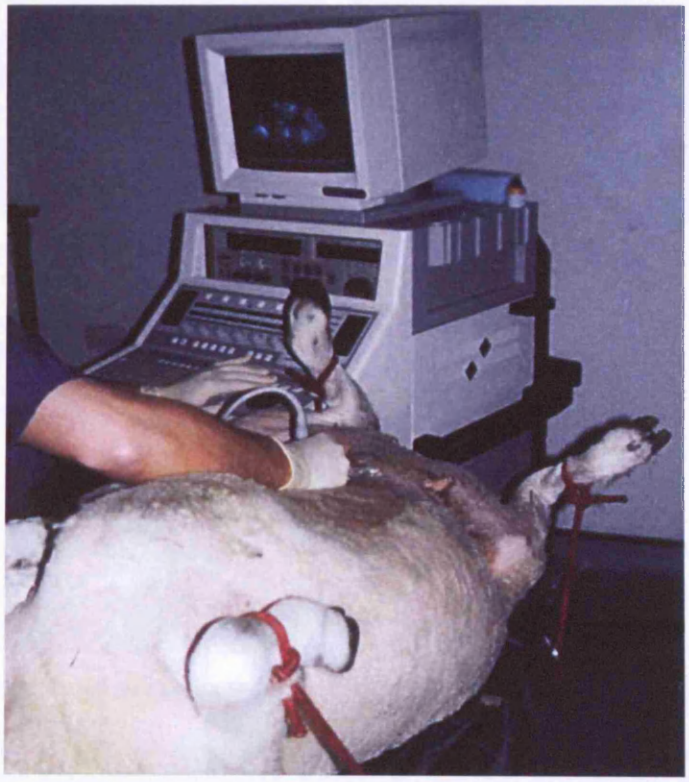


Figure C 4: Ultrasound examination of the fetus prior to fetal injection.

Ultrasound coupling medium was used during scans and small aliquots were sterilized by irradiation for use during operative procedures. The following fetal measurements

were taken of each fetus where fetal size allowed: crown rump length, femur length, abdominal circumference, biparietal diameter and occipito-snout length (**Figure C 5**)

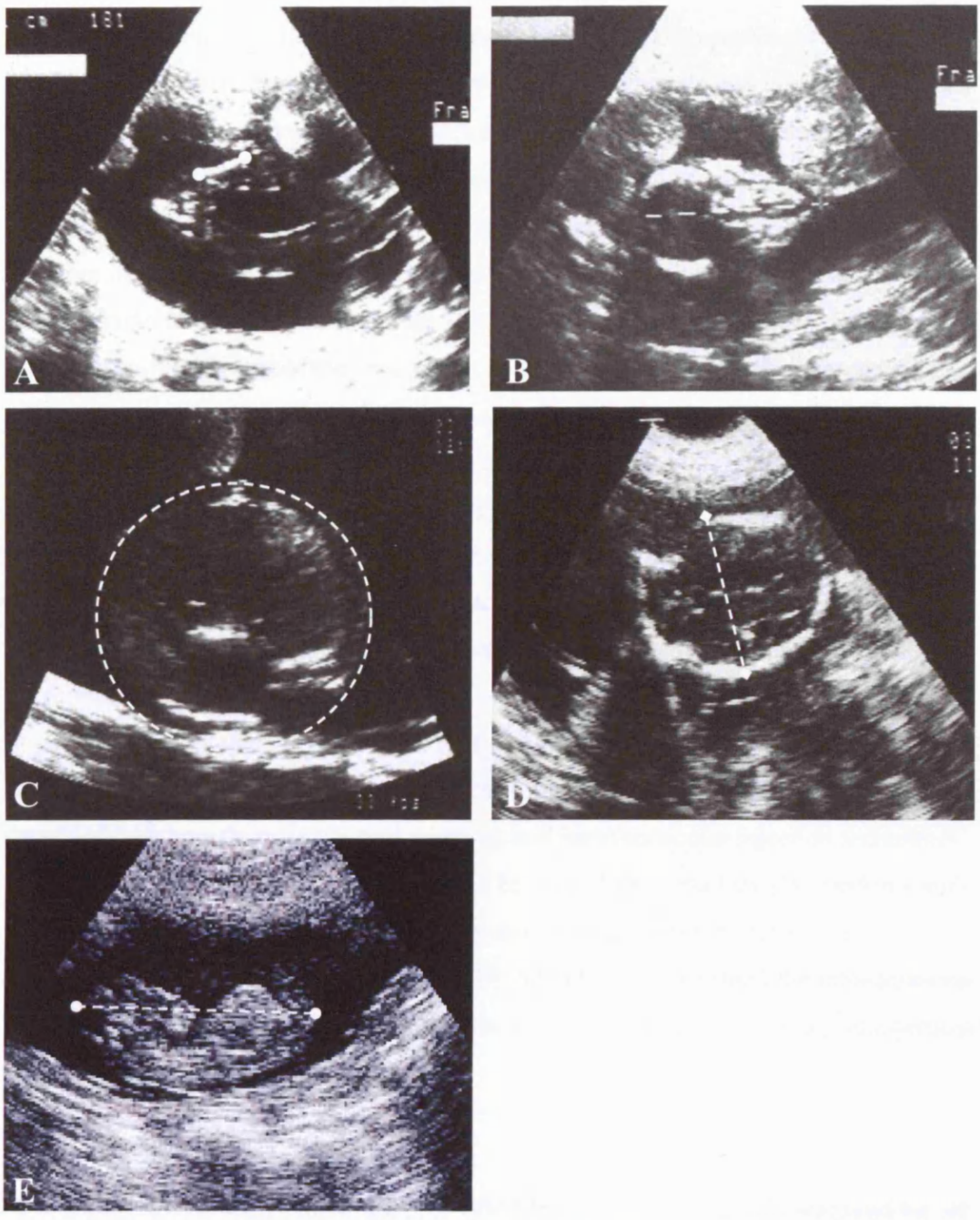


Figure C 5: Ultrasound measurements of fetal sheep.

(A) FL (10.1mm) and (B) OSL (40.2mm) at 60 days of gestation (UV5). (C) AC (162mm) at 83 days of gestation (UV14). (D) BPD (22mm) at 60 days of gestation (B2). (E) CRL (27.4mm) at 36 days of gestation (A7).

The abdomen was then scrubbed with povidone iodine antiseptic solution prior to injection. Analgesia was not considered necessary since all procedures involved a single

injection of the needle and these procedures are routinely carried out in humans without the need for analgesia.

Under ultrasound guidance the needle was inserted through the maternal skin, the uterus and into the amniotic cavity avoiding passage through the placentomes if possible. For each injection route a freehand technique was used. The needle was positioned and the viral vector or colloidal carbon was injected followed by 40μl 0.9% saline to flush the vector from the dead space of the spinal needle, the volume of which was confirmed by the manufacturer. This was important because in many of these experiments, very small volumes of virus were applied. The time to successful injection was measured from the first insertion of the needle through the fetal skin to removal of the needle from the fetus after successful delivery of the viral vector and/or other agents into the appropriate part of the fetus. A failed attempt at injection was defined as insertion of the needle through the fetal skin and then failure to access the appropriate part of the fetus that was being targeted. All ultrasound examinations and procedures were videotaped so that intraoperative events could be reviewed after surgery. As fluid was being delivered to fluid filled areas of the fetus such as the trachea, stomach, peritoneal and amniotic cavity, umbilical vein and lateral cerebral ventricles, turbulence was observed, which we termed "microbubbles".

Dr Donald Peebles, Senior Lecturer in Fetal Medicine at University College London performed the intravascular injection procedures in early to mid gestation fetal sheep, developed the transthoracic tracheal injection and intraventricular injection techniques. During the course of the work for this thesis he trained Dr Anna David to perform mid-gestation intravascular injection procedures and early gestation intramuscular, intraperitoneal and intrahepatic injections. Dr Anna David performed the intra-amniotic and intragastric injection procedures and was also responsible for pre and post-operative ultrasound surveillance of the fetal sheep.

C 1.6.1 Intravascular injections

A 22 Gauge Quincke type point spinal needle (Becton Dickinson, UK) was used for all procedures up to 84 days of gestation. A size 20 Gauge Quincke type point spinal needle (Becton Dickinson, UK) was used for experiments at later gestational ages. The inner-to-inner diameter of the umbilical vein was measured at the intrahepatic portion and at the placental cord insertion before injection. For umbilical vein injections, the needle was placed directly into the umbilical vein and a small volume of blood withdrawn to confirm correct needle placement. If a large enough blood sample was

obtained, fetal serum and plasma was kept for pre-operative analysis of antibodies to the vector and transgene and for human factor IX levels. On injection of the vector, microbubbles were viewed passing along the vessel. The inner-to-inner diameter of the umbilical vein was measured after injection at the injection site.
Intracardiac injections involved placement of the needle through the anterior chest wall directly into the left ventricle and blood was again withdrawn to confirm correct needle placement. On injection of the vector, microbubbles were viewed in the heart chambers.

C 1.6.2 Intra-amniotic injections

A 22 Gauge Quincke type point spinal needle (Becton Dickinson, UK) was used for all procedures. For intra-amniotic injections, 1ml amniotic fluid was first withdrawn and frozen at –80°C for later analysis. Correct placement of the needle was confirmed by observing microbubbles moving around the fetus in the amniotic sac after injection of 100µl 0.9% saline fluid. The viral vector was then injected.

C 1.6.3 Tracheal injections

Larger Echotip spinal needles (Cook, UK) were used during these procedures varying from 17-22 Gauge. The trachea was injected either in the fetal neck or via the fetal thorax. At each procedure the needle was inserted into the trachea under ultrasound guidance and tracheal fluid was withdrawn using a syringe to check correct needle placement. The viral vector and other solutions were instilled down the needle or down catheters inserted through the needle in some experiments. Microbubbles could be seen flowing down the trachea and distal airways on ultrasound. The inner-to-inner diameter of the trachea was measured before and after injection in the distal third of the fetal neck and just superior to the level of the pulmonary artery in the fetal chest.

C 1.6.4 Placement of detachable balloons in the trachea

Detachable goldvalve embolisation balloons (CathNet-Science, Paris, France) were placed in the trachea under ultrasound guidance. These balloons are used in clinical practice during endovascular interventions to therapeutically occlude aneurysms, arterio-venous fistulae and great vessels. They have also been used in the treatment of congenital diaphragmatic herniae in the fetal sheep model and in humans (personal communication, Prof Jan Deprest, Center for Surgical Technologies, Faculteit Geneeskunde, Leuven, Belgium).

The catheter on which the detachable balloon is mounted was first primed with 0.9% normal saline solution. The "Y" connector attached to the proximal end of the catheter was similarly primed. The wire mandrel was passed through the catheter until only 1mm extended beyond the distal end of the catheter. The integrity of the balloon was tested prior to the experiment. A blunt needle (22 Gauge) was primed with 0.9% normal saline solution and the balloon was placed on the end so that the tip was inserted to the top of the valve. The balloon was inflated with a volume of 0.9% normal saline fluid according to manufacturer's instructions (**Figure C 6 A**), and detached from the needle. The solution used to inflate the balloon must be the same osmolality as the lung fluid to prevent rapid deflation or inflation of the balloon by movement of water across the latex.

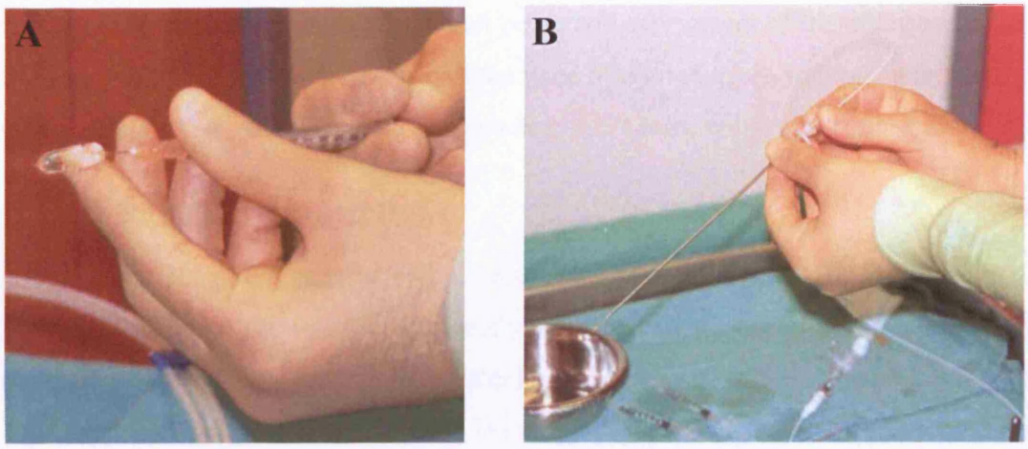


Figure C 6: Preparation of the balloon and catheter system before injection into the fetal trachea.

Testing the integrity of the balloon by inflation with 0.9% normal saline fluid down a 22 Gauge blunt needle (A). The deflated balloon is mounted on the end of the support catheter and passed down the injection needle to check for smooth passage (B).

The inflated balloon was placed on the wire mandrel and inserted onto the distal end of the support catheter. The wire mandrel was withdrawn 1cm to avoid damaging the balloon and the distal end of the support catheter was inserted to the top of the balloon valve. The wire mandrel was withdrawn a further 4cm to allow the balloon to deflate completely. The wire mandrel was then reinserted up to the balloon but not beyond, to provide support during balloon placement.

The needle used for the injection procedure was prelubricated with 0.9% normal saline solution and the fit of the balloon and catheter system tested down the needle (**Figure C 6 B**). It was then placed in the trachea under ultrasound guidance, the cannula withdrawn and tracheal fluid aspirated to confirm correct needle placement. The

detachable balloon mounted on the catheter was passed down the needle until it was in the trachea just proximal to the carina. The wire mandrel was withdrawn and at the same time 0.9% normal saline was injected via the "Y" connector to compensate for the dead space. The balloon was inflated by injection of 0.9% normal saline down the catheter to its maximal dimensions that were visualised on ultrasound. The needle was then withdrawn from the fetal skin so as to avoid damaging the catheter and the catheter was detached from the balloon by a firm pull leaving the inflated balloon in place. The dimensions of the inflated balloon and the inner-to-inner diameter of the trachea above and below the balloon were measured. The chest dimensions including the anteroposterior, transverse and longitudinal diameters were measured by ultrasound at surgery. The ewe was recovered and the fetuses were scanned the day after surgery and weekly thereafter for viability, fetal well being and assessment of the diameter of the balloon and trachea. The chest dimensions were measured again immediately prior to post mortem examination when the ewe was given a terminal general anaesthetic.

C 1.6.5 Intragastric injections

A 22 Gauge Quincke type point spinal needle (Becton Dickinson, UK) was used in all procedures. The needle was placed directly into the fetal stomach viewed in a transverse plane either through the anterior abdominal wall or occasionally via the abdominal ribs. The position of the needle was viewed in two planes to confirm correct placement and 100μl gastric fluid was withdrawn. On injection of the vector, microbubbles could be seen flowing inside the fetal stomach. The inner-to-inner diameter of the stomach and its cavities was measured before and after injection in the anteroposterior, transverse and longitudinal planes.

C 1.6.6 Intraperitoneal or intrahepatic injections

A 22 Gauge Quincke type point spinal needle (Becton Dickinson, UK) was used for all procedures. The needle entered the anterior abdominal wall superior to the intrahepatic portion of the umbilical vein for intrahepatic injections or below the level of the cord insertion just superior to the fetal bladder for intraperitoneal injections. The position of the needle was viewed in 2 planes to confirm correct placement before delivery of the viral vector. Microbubbles could be seen flowing in the peritoneal cavity confirming intraperitoneal injection. On intrahepatic vector administration, an echodense area developed within the hepatic parenchyma at the injection site.

C 1.6.7 Intramuscular injections

For intramuscular delivery a 22 Gauge Quincke type point spinal needle (Becton Dickinson, UK) was inserted along the length of the femur and/or the buttock. On intramuscular vector administration, an echodense area could be seen along the length of the muscle.

C 1.6.8 Intraventricular injections

A 22 Gauge Quincke type point spinal needle (Becton Dickinson, UK) was inserted through the coronal suture or skull in a posterior direction into the posterior horn of the lateral ventricle. The position of the needle was viewed in 2 planes to confirm correct placement before delivery of the viral vector. Microbubbles could be seen within the lateral ventricles on vector instillation.

C 1.7 Post-operative care

The day after a procedure, the wellbeing of the ewe was assessed and fetal survival was determined on ultrasound scan. Ewes and fetuses were subject to post mortem analysis at various time points following procedures and some animals were allowed to come to birth. This was to allow examination of fetal transgene expression in blood at time intervals following injection and to assess the humoral immune response to the vector and transgene. Ultrasound examination of fetal wellbeing and growth was performed weekly on those animals surviving longer than 2 days after operation.

C 1.8 Post mortem examination

Ewes were euthanized using an overdose (40ml) of intravenous 20% pentobarbitone. Maternal and fetal serum and plasma was collected for analysis of antibodies and human factor IX levels. The ewes were subjected to a limited post-mortem examination and samples of liver, spleen, lung, heart, thymus, adrenal, kidney, brain and ovary were taken into 10% formaldehyde for histology and snap frozen in liquid nitrogen for PCR analysis. A full post-mortem examination was performed on the fetal sheep with widespread sampling of tissues from all organ systems according to the post mortem sheet (**Appendix 3**). Multiple tissue blocks were taken from the sites of injection. The peritoneum was stripped from the small and large bowel prior to fixing or freezing. The lung and trachea were removed *en bloc* in tracheal injection experiments before sectioning.

For tracheal occlusion experiments using an inflated balloon, the trachea was cannulated with a 4mm paediatric endotracheal tube (Portex) and the lungs were inflated with 6% formalin and fixed at 25cm H_2O using a manometer for 72 hours, before sectioning. Tissues from ewes or fetal sheep that died following procedures were subject to microbiological examination at the Royal Veterinary College, Hawkshead, Potters Bar, Herts to help determine the cause of death. All post mortem procedures were performed by Dr Anna David.

C 1.9 Care and investigation of lambs born following in utero procedures

Ewes that were allowed to come to birth were vaccinated with Heptavac-P plus® one month before their delivery date to prevent Clostidium and Pasteurella infection in the lambs. A further 2 doses were given to the lambs at 10 weeks and 16 weeks of age. Blood was taken from lambs as soon as possible after delivery for analysis of antibodies and human factor IX levels and then six weekly thereafter. Analysis of blood count, biochemistry, liver function and bile acids was also performed at birth and 3 monthly thereafter at the Royal Veterinary College pathology department. After discussion with Dr Kathleen Tennant, Veterinary Pathologist at Royal Veterinary College, Hawkshead, Potters Bar, an optimum data set for blood analysis was drawn up (**Table C 2**).

Table C 2: Blood analysis of lambs born after ultrasound-guided *in utero* injection.

Conc.: concentration

Haematological analysis	Serum biochemistry	Liver function tests
White blood cell count	Total protein conc.	Glutamate dehydrogenase
Neutrophil count and %	Albumin conc.	Aspartate transaminase
Lymphocyte count and %	Globulin conc.	Gamma-glutamyltransferase
Monocyte count and %	Sodium conc.	Creatine kinase
Eosinophil count and %	Potassium conc.	Total bilirubin conc.
Basophil count and %	Chloride conc.	Bile acids
Red blood cell count	Bicarbonate conc.	
Haemoglobin conc.	Anion gap	
Haematocrit	Calcium conc.	
Mean red cell volume and haemoglobin conc.	Inorganic phosphorus conc.	
Platelet count	Urea conc.	
	Creatinine conc.	

For blood count determination, 4.5ml blood was collected into a BD Vacutainer (BD Vacutainer systems, Plymouth, UK) containing 0.054ml potassium EDTA (K3E, purple

topped bottle). Results of blood tests from animals older than 1 year were compared with normal ranges from adult sheep. There are no normal ranges available for lambs however, and therefore results of blood tests from lambs were compared with those from non-experimental lambs of the same age and breed that were also on the farm. All analyses were performed by the Department of Pathology, Royal Veterinary College, Hawkshead, Potters Bar.

Lambs were kept with their mothers until weaning at approximately 16-20 weeks of age. Ewes were then subject to post mortem examination, serum and plasma collection and tissue sampling for histology and PCR analysis. Tail docking was performed routinely on lambs using rubber rings before they were one week old.

C 1.10 Liver biopsy of lambs born following *in utero* procedures

Liver biopsy was performed with lambs standing up without sedation by a qualified veterinary surgeon (Dr John Fishwick, Royal Veterinary College, Hawkshead, Potters Bar). The right flank was clipped and the site of the biopsy identified as the 11^{th} intercostal space at a level just anterior to the tuber coxae, the most cranial part of the pelvis (**Figure C 7**). The area was scanned with ultrasound to confirm the liver was adjacent to the body wall at this point and to identify any liver pathology. The diameter of the bile duct was measured.

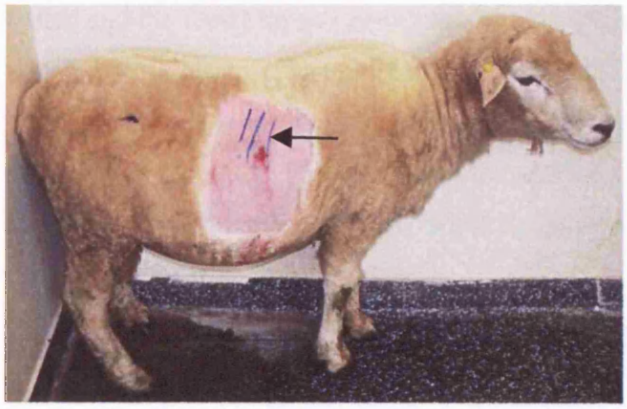


Figure C 7: Liver biopsy in an adult sheep.

The unsedated standing sheep has local anaesthetic placed into the skin just over the 11^{th} rib (arrowed). The landmarks of the 11^{th}, 12^{th} and 13^{th} ribs have been marked on the shaved skin. The biopsy is performed in the 11^{th} intercostal space.

The skin was cleaned with povidone iodine antiseptic solution and infiltrated with 2.5ml of 5% procaine hydrochloride for local anaesthesia. A 9cm long 14 Gauge veterinary

Quick-Core Biopsy Needle (Cook, Australia) was inserted approximately 4cm into the liver to obtain a liver core. Four liver cores were taken, placed in 70% formalin for 24 hours and then processed as for other pathology specimens. To close the wound, two nylon skin sutures were placed in the skin. An intramuscular injection of 750mg amoxycillin trihydrate was given for infection prophylaxis.

C 2. Production of viral vectors

C 2.1 Cell culture

The following cell lines were used for investigations in this thesis: 293 cells, NIH 3T3 cells, HeLa cells, HT1080 cells and HBE cells. Cell lines were taken either from existing cell culture plates or from liquid nitrogen frozen stocks. These were defrosted quickly to 37°C in a waterbath, and washed to remove residual dimethylsulfoxide in the storage media by diluting 10x in D-Mem containing 10% FCS and 5% pen/strep, and pelleting by centrifugation for 5 minutes at 100 x g (Centurion 4000 Series). Cells were then resuspended in fresh media and cultured at 37°C, 5% CO_2 in Nunclon™ 75 cm^2 triple flasks for adenovirus production or 6 well culture cluster plates for transfection experiments.

When cells grown in triple flasks were 50% confluent, the growth media was removed and the cells washed with 25ml PBS. To remove the cells, 25ml 1 x trypsin-EDTA was added and the flasks tapped gently for 30 – 45 seconds before 25ml growth medium was added to neutralize the trypsin. Cells were pelleted by centrifugation for 5 minutes at 100 x g and the supernatant discarded before resuspension in 10ml new growth medium by vigorous pipetting. This was then diluted 200x in new growth medium, distributed between 4 new Nunclon™ 75 cm^2 triple flasks equally and cultured at 37°C, 5% CO_2. Cells could be split every 2 – 3 days.

C 2.2 Adenovirus vector production

Replication deficient first generation adenovirus constructs deleted for the E1 and E3 regions and containing the lacZ reporter gene (Stratford-Perricaudet LD et al., 1992) and the human Factor IX gene (unpublished construct) were originally supplied by Transgene, Strasbourg, France. Replication first generation adenovirus constructs deleted for the E1 and E3 regions and containing the hCFTR gene (AV1CF2) were originally supplied by B. Trapnell, Cincinatti, Ohio, USA. Dr Anna David was responsible for production, purification and titration of adenovirus vectors for the first

year of the research under the supervision of Dr Michael Themis, Lecturer, Gene Therapy Research Group, Imperial College, London. Production of adenovirus vectors for further work was performed by other members of the Gene Therapy Research Group.

C 2.2.1 Adenovirus vector preparation

The human embryonic kidney 293 cell line was used to produce adenovirus vectors. These provide the E1A gene that has been deleted from the adenovirus vector construct in *trans*. 293 cells were grown to 70% confluence in twelve $75cm^3$ triple flasks in 125ml D-Mem, 10% FCS and 5% pen/strep at 37°C and 5% CO_2. Cells were infected with new media containing D-Mem, 5% FCS, 5% pen/strep and 5 x 10^8 plaque forming units adenovirus and incubated at 37°C and 5% CO_2 for two to three days until cells began to lift off the plate. Cells were completely dislodged from the plate by gentle tapping of the flask and the bathing medium was then centrifuged at 4,000 x g for 10 minutes. The supernatant was discarded and the cells were resuspended in 10ml of new 10% medium and freeze/thawed three times to lyse the cells and release the virus. This was then centrifuged at 2000 xg for 10 minutes to remove cell debris.

The supernatant was subjected to two rounds of ultra-centrifugation in a caesium gradient to purify the adenovirus particles. The three caesium chloride solutions were prepared and filtered using a 0.20μm syringe filter (Sartorius AG, Goettingen, Germany) into a sterile bottle. The first caesium gradient was prepared by placing 12ml of 1.4g/ml CsCl (heavy solution) underneath 12ml of 1.25g/ml CsCl (light solution) in two 35ml Polyallomer centrifuge tubes (Beckman, Palo Alto, CA, USA). The supernatant was split into two, added to the top with a covering of mineral oil and the tubes balanced. Ultra-centrifugation was at 28,000 rpm for 2 hours at 4°C (Sorvall Ultra-Pro 80 centrifuge). The bottom band containing the adenovirus particles was retrieved with a sterile 21G needle (BD Microlance, Becton Dickinson, UK), care being taken not to remove genomic DNA in the top band. The virus was split into two samples and each sample was added on top of 5ml of 1.34 g/ml CsCl (medium solution) in a 15ml Polyallomer centrifuge tube (Beckman, Palo Alto, CA, USA) to form a new gradient. Mineral oil was added on top, the tubes balanced and ultracentrifuged at 35,000 rpm for 20 hours at 4°C (Sorvall Ultra-Pro 80 centrifuge). The bottom band containing adenovirus was removed with a sterile 21G needle (BD Microlance, Becton Dickinson, UK) and then purified to remove all traces of cytotoxic CsCl. The virus solution was injected into a 3-50μl dialysis cassette (Pierce, Rockford, USA) containing

an equal volume of 2 x sucrose. This was then dialysed overnight in three washes of 1x 5M sucrose solution and the cleaned adenovirus solution was kept at 4°C prior to titration.

C 2.2.2 Titration of adenovirus vectors

The titre of adenovirus vectors was determined using a physical method of measurement at a range of optical densities (OD). This method is based on the correlation between protein concentration of adenovirus preparations and absorbance at 260nm: one absorbance unit is equivalent to 1.1 x 10^{12} adenovirus particles (Maizel JV et al., 1968).

Vector titration using biological methods such as plaque assay is imprecise because they depend on factors such as the cell type used and the diffusion rate of the viral vector. Results can vary by as much as 30% within a day. Physical methods however are much less variable with interday precision values of less than 5% (Hutchins B et al., 2000) and have been recommended by the Food and Drug Administration in their Guidance for Industry (Food and Drug Administration, 1998). OD measurement of viral titre also has drawbacks. For example the purity and formulation of the vector preparation influence the absorbance of the sample, and physical measurement of viral titre does not give an indication of the infectious titre which makes analysis of toxicity effects difficult.

For OD measurement of viral titre the dialysed virus was diluted with 10mM Tris-HCl, pH 8.0 and the absorbance at 220, 260, 280, 320 and 340 nm determined using a spectrophotometer (Lucy 1, Anthos).

Quality of vector: The virus is dead if $OD_{320nm}/OD_{260nm} > OD_{220nm}$

The virus is aggregated if $OD_{340nm}/OD_{260nm} > OD_{220nm}$

Titre of vector: Titre (particles/ml) = OD_{260nm} x 1.1 x 10^{12} particles/ml.

C 2.2.3 Testing for replication competent adenovirus

Virus batches were tested for the absence of replication competent adenovirus (RCA). A rapid method used primers to amplify the E1A region of adenovirus (Genbank AY147066) which is deleted in the replication incompetent adenovirus vector. A PCR product from amplification of the E1A region will arise from replication competent virus particles or by contamination of the virus with genomic DNA from the human embryonic 293 kidney producer cell line. Samples of virus batches were subjected to 40 cycles of 1st round amplification followed by 30 cycles of nested amplification (**Table**

C 3). The detection limit of the PCR reaction was one replication competent adenovirus in 1 x 10^9 replication competent particles.

Table C 3: PCR conditions for amplification of the E1A adenovirus gene.

1st round product size	795bp
1st round forward primer	GGACCAGCTGATCGAAGAGG
1st round reverse primer	CCACCGGGTGTATCTCAGG
Annealing temperature	60°C
$MgCl_2$ concentration in reaction	1.3 mM
Nested product size	580bp
Nested forward primer	GTAATGTTGGCGGTGCAGGA
Nested reverse primer	CTCTAGACACAGGTGATGTCG
Annealing temperature	58 °C
$MgCl_2$ concentration in reaction	1.5 mM

AdRSVβgal contains a Rous Sarcoma Virus (RSV) promoter driving the expression of the β-galactosidase reporter gene. The human factor IX gene on AdTG9397 is driven by the Cytomegalovirus (CMV) promoter and contains the adenovirus tripartite leader sequence and the simian virus 40 polyadenylation site. The human CFTR gene on AV1CF2 is driven by the Rous Sarcoma Virus (RSV) promoter.

Viruses were prepared freshly in PBS prior to injection into fetal sheep. In this study we used fetal sheep from a wide range of gestational ages that were growing rapidly. The dose of vector applied to each fetus was therefore standardized to a dose (particles) per kg fetal weight using estimated fetal weight according to the gestational age at injection. There was no published data on fetal weight according to fetal measurements or fetal weight in sheep of Romney Marsh breed. We initially used data on fetal weight at gestational age from studies in Merino sheep (Cloete JHL, 1939) and unspecified breeds (Joubert DM, 1956)(**Figure C 8**). As our study progressed, we were able to use data from fetal weight at post mortem examination to estimate the fetal weight in our sheep. The dose per kg was then confirmed using the fetal weight at post mortem 2 days after injection. This was because about 20% of our fetuses were from twin gestations and were slightly smaller than singletons of the same gestational age. For fetuses that were

euthanased longer than 2 days after injection, we reported the fetal weight for the gestational age at injection.

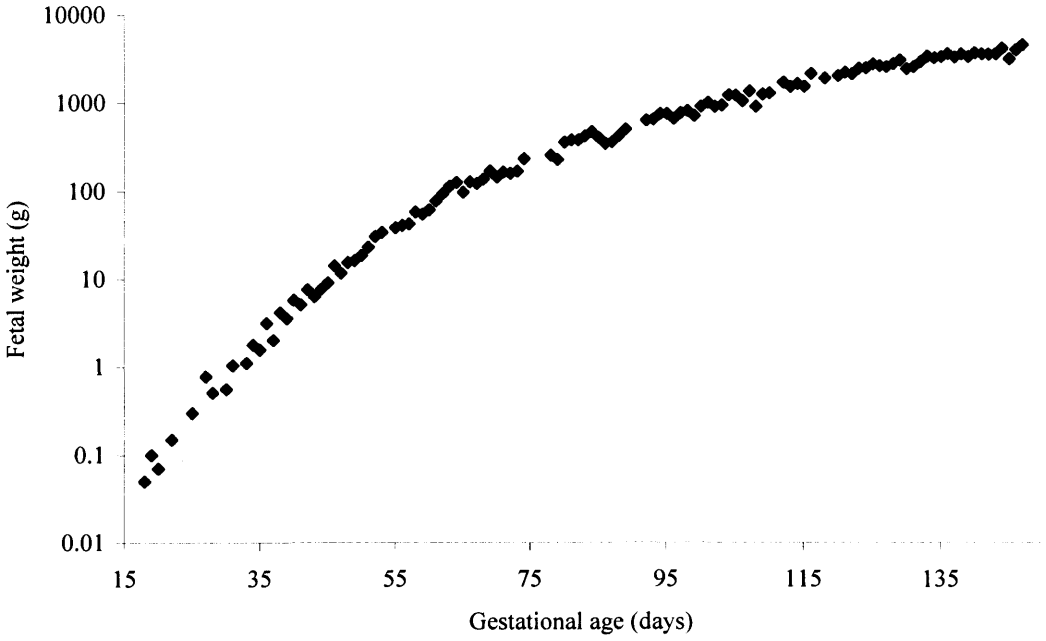


Figure C 8: Weight of singleton male sheep fetuses according to gestational age.

Taken from (Joubert DM, 1956).

C 2.3 Alternative vectors

C 2.3.1 Retrovirus vectors

Retrovirus vectors (Moloney leukemia virus, MLV) containing the lacZ reporter gene under the control of the LTR were provided by Francois Cosset, University of Lyon, France.

C 2.3.2 Lentivirus vectors

EIAV vectors pseudotyped with the VSV-G or the Mokola envelope and containing the lacZ reporter gene or hFIX gene were provided by Oxford BioMedica, Oxford, UK.
HIV vectors pseudotyped with the VSV-G or Mokola envelope and containing the lacZ reporter gene were provided by Professor Adrian Thrasher, Institute of Child Health, London, UK.

C 2.3.3 Sendai vectors

Second generation Sendai vectors deleted for the fusion (F) protein, a gene that encodes a surface glycoprotein, and containing the lacZ reporter gene were provided by Dr Michael Bitzer, Tuebingen, Germany.

C 2.4 *Ex vivo* transfections

Tissue from the small bowel, trachea and liver of fetal sheep at various gestational ages were transfected *ex vivo* to determine adenovirus mediated gene transfer. The effects of the transduction enhancing agents sodium caprate and DEAE dextran were also evaluated in the fetal small bowel and trachea *ex vivo*. Experiments were performed by Dr Anna David, Dr Lisa Gregory and Dr Michael Themis, Gene Therapy Research Group, Imperial College, London.

C 2.4.1 *Ex vivo* transfection of the fetal liver

Thin sections of fetal liver taken at post mortem analysis were cultured in a 100mm x 20mm cell culture dish with D-Mem containing 10% FCS and 5% pen/strep in an incubator at 37°C and 5% CO_2. Adenovirus vectors ($5x10^8$ particles) containing the β-galactosidase reporter gene with or without complexing with DEAE dextran (5μg/ml) were applied to the medium for 24 hours. The medium was replaced with fresh medium for a further 24 hours and then the tissues were stained with X-gal solution for assessment of β-galactosidase transgene expression.

C 2.4.2 *Ex vivo* transfection of the fetal trachea

A 4cm long piece of trachea from a mid-gestation fetal sheep was opened and divided into 4 sections. These were cultured in a liquid-air interface on a filter paper platform soaked in D-Mem culture medium containing 10% FCS, 10% NCTC-135 medium, 2mM L-glutamine, 100U/ml pen/strep, 1μg/ml insulin and 30ng/ml dexamethasone in a 100mm x 20mm cell culture dish. PBS or sodium caprate was applied to the epithelial surface. The cell culture dishes were sealed within a pressure jar (Oxoid) filled with 95% CO_2/5%O_2 to 1.5 bar (BOC) and the jar and contents were incubated at 37°C for 10 min. Following pre-treatment with sodium caprate or PBS, adenovirus vectors containing the β-galactosidase reporter gene ($5x10^8$ particles) were applied to each section of trachea for 30 min, with or without DEAE dextran complexation (5μg/ml). Tracheae were washed with PBS and incubated for 28 h in the same gas mixture which

was replaced after 4 h and 15 h. Tissues were homogenised in lysis buffer and levels of transduction were determined by ELISA. Results of gene transfer were grouped according to treatment and analysed statistically.

C 2.4.3 *Ex vivo* tranfection of the fetal gut

This was performed in a similar way as *ex vivo* transfection of the fetal liver. The small bowel was opened and cultured in a 100mm x 20mm cell culture dish with D-Mem containing 10% FCS and 5% pen/strep in an incubator at 37°C and 5% CO_2. Adenovirus vectors containing the β-galactosidase reporter gene ($5x10^8$ particles) were applied to the medium together with sodium caprate and/or DEAE dextran in the same combinations and incubated for 24 hours. The medium was replaced with fresh medium for a further 24 hours and a section of the small bowel was stained with X gal solution to determine qualitatively the areas of the small bowel that had been transfected. The remainder of each section was taken for assessment of gene transfer by β-galactosidase ELISA analysis and results were grouped according to treatment and analysed statistically.

C 3. Histological analysis

C 3.1 Tissue samples

Tissue samples were fixed in 10% formaldehyde overnight, transferred to 70% ethanol and processed into paraffin in cassettes. Paraffin sections were cut at 2-5μm thickness, floated out on warm water and mounted and air-dried onto glass slides. These were immersed in hematoxylin solution made up in PBS pH 7.2 for 5 minutes, washed 3 times in PBS before being immersed in 1% eosin counterstain solution made up in PBS pH 7.2 for 5 seconds and washed 3 times in PBS. Sections were processed through alcohol into xylene and then mounted in DPX mountant medium and a coverslip placed over the section and allowed to dry. Slides were analysed under normal light microscopy with the assistance of Professor Terry Cook, Department of Pathology, Hammersmith Hospital, Imperial College, London.

C 3.2 Fixation of brains

At post mortem examination 2 days following ultrasound guided intraventricular injection, the fetal abdomen and chest were opened and a sample of fetal liver and gonad was snap frozen in liquid nitrogen for PCR analysis. The thoracic aorta was

cannulated with an 18 Gauge intravenous cannula (Becton Dickinson, Franklin Lakes, NJ, USA) which was held in place by a 3.0 silk tie and the left atrium opened to allow perfusion to occur. The fetal brain was initially perfused with 100ml PBS (pH 7.4) followed by 100ml 4% paraformaldehyde buffered with H_3PO_4 at pH 7.4 for 20 minutes. The skull bone was removed and the brain, brain stem and upper thoracic spinal cord removed in one piece and placed in 1% paraformaldehyde at 4°C with rotation for 2 hours. The brain was then placed overnight in 30% sucrose in 10mM PBS for cryoprotection, and then snap frozen. The tissue was mounted on a Leica CM 1900 cryostat (Leica microsystems, Nuslock GMBH, Germany) at –20°C. Approximately 250 sections were cut alternating at 20μm or 50μm thickness and placed onto gelatinized slides (BDH twin frost Super Premium microscope slides 1.0-1.2mm thick, dipped in 1g gelatin dissolved in 200ml water and left to set overnight) and kept at –80°C.
Frozen sections were hydrated with distilled water and spread on the slide, which was then air dried at room temperature for 10 minutes. Sections were fixed in 4% formaldehyde in PBS for 5 minutes, rinsed with PBS and then stained accordingly. For X-gal staining the slides were placed in X-gal solution overnight in the dark, rinsed with PBS and counterstained with neutral red. Haematoxylin & eosin staining for histology and β-galactosidase immunohistochemistry was performed as previously described. All procedures were performed by Dr Anna David.

C 4. Evaluating the effect of viral vectors on the composition fetal fluids

All analyses were performed by Dr Anna David.

C 4.1 Amniotic fluid

The osmolality and electolyte content of the amniotic and allantoic fluids differ greatly. The results from fluid samples taken prior to vector injection were compared to those from samples of allantoic fluid taken at post mortem. This was used to confirm that the vector had been correctly delivered into the amniotic cavity.
Amniotic fluid was analysed at postmortem analysis to determine if the adenovirus vector had affected the biochemical composition and osmolality. Because amniotic fluid was snap frozen immediately after removal at post mortem, it was not possible to analyse for an inflammatory response to the vector.

C 4.1.1 Determination of electrolyte content and osmolality of amniotic fluid

An osmometer was used to determine the osmolality or concentration of solution by means of freezing-point measurement (The Advanced™ Micro-Osmometer Model 3MO, Advanced Instruments, Mass, USA). After calibration with known osmolality standards, 20µl of amniotic fluid was presented to a thermistor probe and was rapidly cooled to below its freezing point. A mechanical pulse induced the sample to freeze and the heat of fusion then released caused the sample temperature to rise to the equilibrium temperature where ice and water are maintained, which is the freezing point of the solution. The osmometer routinely determined differences of 2 mOsm/kg H_2O and each sample was tested twice.

C 4.2 Tracheal fluid

C 4.2.1 Inflammatory response to vector injection

Tracheal fluid (500µl,TF) was removed from the fetal trachea into a syringe at post mortem using a needle inserted through the tracheal wall once the lungs and trachea were exposed by dissection. Broncho-alveolar lavage (BAL) was performed after removal of the lungs and trachea from the fetus by flushing 2ml PBS down the trachea and recollecting the fluid. Cells and debris in the TF and BAL fluid was pelleted by centrifugation at 2000 rpm for 10 minutes and the pellet was resuspended in 500 µl 0.1% BSA in PBS. Cytospin preparations were prepared by centrifugation of 100µl cell suspension onto glass slides at 350 rpm for 5 minutes (Cytospin 3, Shandon). The slide was air-dried and fixed in methanol for 5 minutes. Following Wright–Giemsa staining, cells were identified, differentially counted and the number expressed per high power field and compared to results from tracheal fluid taken just before vector injection.

C 4.2.2 Electrolyte changes in response to vector injection

The osmolality and electolyte content of tracheal fluid taken prior to vector injection and at post mortem examination were compared as previously described to assess the fetal response to the vector.

C 4.3 Gastric fluid

C 4.3.1 Inflammatory response to vector injection

Gastric fluid taken before and after vector injection was analysed for an inflammatory response to the vector in a similar way to tracheal fluid.

C 4.3.2 Electrolyte changes in response to vector injection

The osmolality and electolyte content of gastric fluid taken prior to vector injection and at post mortem examination as described previously were compared to assess the fetal response to the vector.

C 5. Evaluating the effect of fetal fluids on adenovirus transfection in vitro

All experiments were performed by Dr Anna David.

C 5.1 Effect of fetal tracheal fluid on adenovirus transfection *in vitro*

Human bronchial epithelial cells (HBEs) were grown from existing cell cultures in the laboratory. Dead cells and old culture medium was removed from HBE cells at 70% confluence grown in 75 cm^2 cell culture flasks. The flasks were washed with 10ml PBS, incubated with 3ml 1 x trypsin-EDTA at 37°C for 2 minutes and the flasks gently tapped to remove the cells. The cells were washed in 7ml culture medium, D-Mem containing 10% FCS and 5% pen/strep and pelleted by centrifugation for 5 minutes at 100 x g (Centurion 4000 Series). The supernatant was discarded before resuspension in 10ml new growth medium by gentle pipetting. To determine the number of cells per ml culture medium, the cells were counted using a haemocytometer (Superior, Marienfeld, Germany). Four 24-well culture plates were plated with 7 x 10^4 cells in 1 ml new culture medium per well and cultured at 37°C, 5% CO_2 for 24-48 hours until cells were 30% confluent. The culture medium was removed from the 24-well plates and replaced with 500µl fresh medium.

Adenovirus vector (10^{11} particles in 10µl, AdlacZ) was incubated with fetal tracheal fluid (100µl) at various gestational ages for 30 minutes at room temperature. Serial dilutions of the vector in tracheal fluid were then made with PBS to achieve the following concentrations: 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 adenovirus particles in 10µl fluid to give a multiplicity of infection (MOI) of 10,000, 1,000, 100, 10, 1 and 0.1 respectively. The serial dilutions of adenovirus vector were added to the 24-well plates

in quadruplicate to give one plate for tracheal fluid at each gestational age and a further plate for virus that had not been preincubated with tracheal fluid. Plates were incubated at 37°C, 5% CO_2 for 24 hours and the medium was then refreshed for a further 24 hours. After removal of the medium, the cells were fixed by adding 1ml of fix solution to each well for 20 minutes and washing three times with PBS. The cells were incubated with X gal solution (1ml per well) overnight in the dark and the number of blue cells per well were counted. The experiment was repeated in triplicate using adenovirus concentrations at which it was possible to count the number of blue cells per well.

C 5.2 Effect of fetal gastric fluid on adenovirus transfection *in vitro*

Using the same method as for fetal tracheal fluid experiments, adenovirus vector was incubated with gastric fluid from fetal sheep at three different gestational ages and then applied to human bronchial epithelial cells cultured in 24-well culture plates. The number of blue cells per well were counted and the experiment repeated in triplicate using appropriate adenovirus concentrations.

C 6. Detection and quantitification of β-galactosidase transgene expression

C 6.1 X-gal histochemistry

Tissue samples were placed in 100% ethanol at post-mortem and fixed overnight before washing with PBS. Samples were then incubated in X-gal staining solution containing 40 mM potassium ferrocyanide, 40mM potassium ferricyanide, 1mM $MgCl_2$ in PBS and X-gal dissolved in DMSO at 40mg/ml overnight in a rotary shaker protected from the light. For the intra-tracheal injection experiments, X-gal staining solution was injected down the main bronchioles to ensure distal airways were reached. Samples were washed five times in PBS, and stored in 10% formaldehyde. X-gal blue staining was visualised by light microscopy. After washing with PBS, tissue samples were fixed in 100% methanol prior to clearing with benzyl benzoate: benzylalcohol (2:1). Cleared samples were then visualised by light microscopy. Stained tissue was fixed in paraffin blocks and 3 μm sections cut. Sections were counterstained with eosin and nuclear localisation of the lac Z stain was observed within the airway epithelia. Analysis was done by Dr Anna David, Dr Lisa Gregory and Mr Dany Perocheau, Gene Therapy Research Group, Imperial College, London.

C 6.2 X gal ELISA

A commercially available kit (Boehringer Mannheim, Mannheim, Germany) was used to quantify the levels of X gal expression according to manufacturer's instructions. Tissues were cut into 5mm cubes, washed with PBS, freeze thawed in 1ml lysis buffer and then homogenized using a Polytron PT 1200 CL homogenizer (Kinematica PG). Homogenized tissues containing soluble components of the cells including the β-galactosidase enzyme were centrifuged at 13000xg for 10 minutes to remove cellular debris and tested immediately or frozen at –60°C.

The protein concentration in the tissue samples was determined by a colorimetric method using the bicinchoninic acid (BCA) protein assay kit. This uses the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium and the chelation of two molecules of BCA with Cu^{+} to form a purple-coloured reaction product (Smith PK et al., 1985). Protein standards were prepared using BSA (fraction V) diluted in distilled water to 2, 1, 0.5, 0.25, 0.125 and 0.063 mg/ml protein. The standard or sample (10µl per well) was incubated with BCA reagent (200µl per well) in a 96 well plate at 37°C for 20 minutes. The plate was cooled and the absorbance was read on a spectrophotometer (Lucy 1, Anthos) at 562nm. Every serial dilution or tissue sample was tested on two wells and the final result taken as the average. The absorbance values for the calibration dilutions were plotted against the standard concentrations of BSA to produce a linear calibration curve and the protein concentration of the tissue samples was determined. Tissue samples were diluted so that the absorbance of the sample fell within the linear range of the β-galactosidase assay, usually at a protein level of less than 1000pg/ml.

The β-galactosidase assay consisted of a microtiter plate containing 12 x 8 wells precoated with a monoclonal mouse antibody to β-galactosidase. The β-galactosidase enzyme standard dilutions were prepared to obtain a calibration curve. The sensitivity of the assay is to a level of 30pg/ml. To each well was added 200µl of the calibration dilutions or tissue extracts containing 200µg protein and the plate was incubated for 1 hour at 37°C. The plate was washed three times with washing solution and then incubated with digoxigenin-labelled antibody to β-galactosidase (200µl per well) for 1 hour at 37°C. Wells were washed three times with washing solution and then incubated with a digoxigenin antibody conjugated with peroxidase (200µl per well) for 1 hour at 37°C. Wells were washed three times with washing solution and the bound peroxidase revealed by incubation with the peroxidase substrate ABTS for 15-40 minutes at room temperature yielding a green coloured reaction product. The absorbance of samples was

measured at 405nm using a spectrophotometer (Lucy 1, Anthos). Every serial dilution or sample was tested on two wells and the final result taken as the average. The absorbance values for the calibration dilutions were plotted against the standard concentrations of β-galactosidase to produce a nearly linear calibration curve and the β-galactosidase concentration of tissue samples was determined. Analysis was done by Dr Anna David, Dr Lisa Gregory and Mr Dany Perocheau, Gene Therapy Research Group, Imperial College, London.

C 6.3 β-galactosidase immunohistochemistry

β-galactosidase was detected immunohistochemically in tissues using a mouse monoclonal and a rabbit polyclonal antibody, respectively, followed by a standard avidin-biotin peroxidase method (Polak JM and Van Noorden S, 1997). Paraffin sections on slides were dewaxed in 3 changes of xylene, and brought to water through 3 changes of 100% ethanol and one change of 70% ethanol. Endogenous peroxidase from red blood cells was blocked by immersion of slides in 0.3% hydrogen peroxide in water for 30 minutes. Slides were washed in water and then subjected to trypsin digestion to unmask antigen sites that may have become cross-linked during fixation. A fresh solution of 0.1% trypsin was added to 0.1% calcium chloride in 0.0005M Tris buffered saline at 37°C and brought to pH 7.8 with 0.1M sodium hydroxide. Slides were immersed for 10 minutes, rinsed in water, placed in PBS and then onto the Sequenza (Shandon, UK), a modified rack system in which each well has a low volume (100μl) for easy processing of large numbers of slides. Non-specific antibody binding to proteins in the tissue preparations was prevented by applying 5% non-immune goat serum to each slide for 10 minutes, the serum being from the species which provided the second layer antibody. The slide was drained off, avidin applied for 15 minutes, rinsed with PBS and biotin applied for 15 minutes followed by a final rinse with PBS. For β-galactosidase immunohistochemistry, slides were then incubated overnight at 4°C with anti-βgalactosidase primary antibody raised in mouse diluted to 1/1000 in 1% PBS. They were then rinsed for 5 minutes with PBS before application of biotinylated secondary goat anti-mouse antibody at 1/500 dilution in 1% PBS for 30 minutes at room temperature. Slides were rinsed for 5 minutes with PBS and peroxidase-labelled streptavidin at 1/500 dilution in 1% PBS added for 30 minutes at room temperature. A final 5 minute rinse of 1% PBS was done before the peroxidase was developed by immersion of the slides in 50mg/ml diaminobenzidine tetrahydrochloride in 1% PBS

and 0.01% hydrogen peroxide for 10 minutes. Slides were counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene and allowed to dry. Positive staining was seen as a dark brown reaction product when viewed with a light microscope. A positive control and negative control were used throughout. The positive was a section of fetal liver with strong staining from a previous experiment; the negative was a test section which was not exposed to the primary antibody but immersed in 1% PBS. Immunohistochemistry analysis was performed at Department of Pathology, Hammersmith Hospital, Imperial College under the supervision of Professor Terry Cook.

C 7. Detection and quantitification of human factor IX transgene expression

C 7.1 Determination of human factor IX concentration in plasma

C 7.1.1 Optimisation of human factor IX ELISA

The ELISA kit for hFIX measurement was designed for use on human plasma and had been evaluated for use with late gestation sheep plasma. To check that the assay was suitable for testing early gestation sheep plasma we spiked plasma from a non-injected fetal sheep with serial dilutions of hFIX. This was analysed, together with a sample of normal human plasma to act as a control and with a serial dilution of hFIX in buffer. The resulting calibration curve showed that the assay was able to detect hFIX in early gestation sheep plasma to the same level of detection as in human plasma.
Expression of hFIX in fetal plasma following injection in early gestation was much lower than expression after late gestation intravascular injection. The lowest accurate level of detection of the ELISA kit was not known although the standard calibration curve went only to a hFIX level of 25ng/ml. To test the lowest detection level of hFIX in our experiments we serially diluted early gestation fetal sheep plasma with hFIX to a level of 0.21% of normal hFIX levels (10.5ng/ml). Similarly we analyzed the standard calibration series of hFIX in dilution buffer to this level. **Figure C 9** shows that that the concentration of hFIX in the fetal sheep plasma could be accurately determined to this level.
Initial optimisation of the hFIX ELISA and analysis of samples from the first year of work was done by Dr Anna David. Further analysis by Dr Megha Nivsarkar, Gene Therapy Research Group, Imperial College, London has taken the lower limit of

detection of the hFIX ELISA to 0.11% (5ng/ml) and this is the lowest limit of detection that was used for analysis of fetal sheep plasma samples in this thesis.

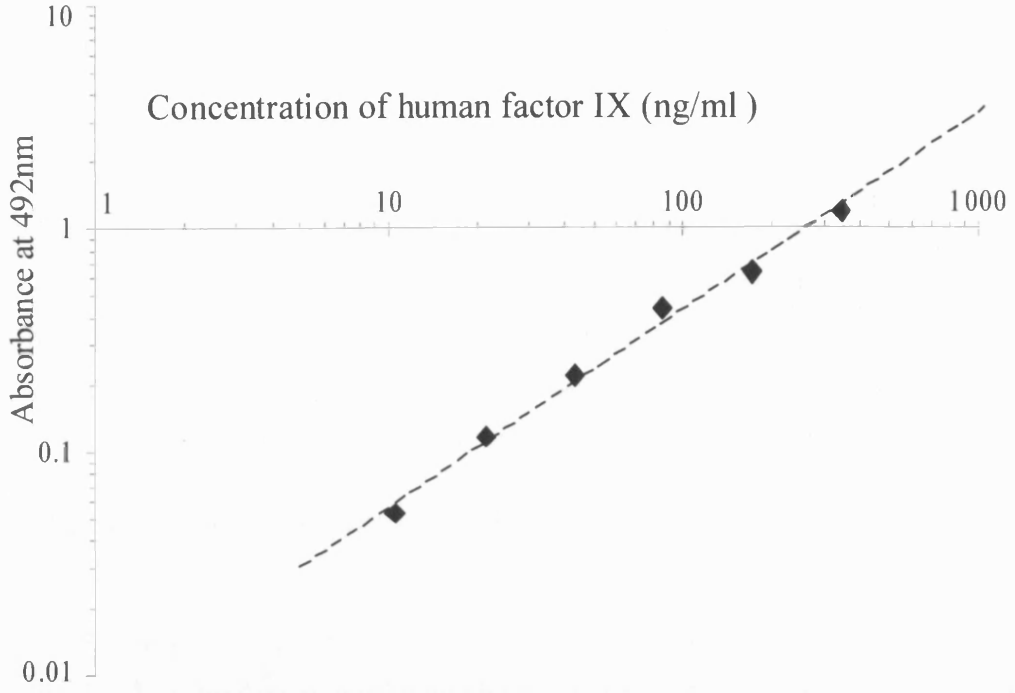


Figure C 9: Determining the lower limit of detection of hFIX in early gestation fetal sheep plasma by ELISA analysis.

Early gestation fetal sheep plasma was spiked with reducing concentrations of hFIX and the absorbance at OD 492 nm plotted on a double logarithmic graph.

C 7.1.2 Human factor IX ELISA

Whole blood (4.5ml) was collected into tubes containing 0.109 M trisodium citrate (0.5ml). Plasma was obtained after centrifugation at 2500 x g for 10 minutes and kept frozen at –30°C until analysis. A calibration series of six serial dilutions was prepared by dissolving a human factor IX antigen standard, t, in dilution buffer from t/16 to t/512. The standard, t, corresponded to a concentration of 105% of adult human factor IX levels or 5 µg/ml, according to the kit manufacturer. Two blanks were also tested, a buffer blank containing only dilution buffer and a sheep blank containing a 1+50 dilution of fetal serum from a non-experimental animal of the same gestational age in dilution buffer. Serum samples were diluted 1+50 with dilution buffer.

The assay consisted of a plate of sixteen wells coated with specific rabbit anti-human factor IX primary antibody that captures the human factor IX to be measured. Each well was incubated for 2 hours at room temperature with 200 µl of serial dilution, blank or

sample. Wells were washed five times with washing solution and then incubated with 200 µl of rabbit anti-factor IX secondary antibody coupled with peroxidase for a further 2 hours at room temperature. Wells were washed five times with washing solution again and the bound peroxidase enzyme in the wells was revealed as a yellow colour by incubation with 200 µl ortho-phenylenediamine in hydrogen peroxide for exactly 3 minutes at room temperature. The reaction was stopped by adding 200 µl of 3 M sulphuric acid and the absorbance at 492nm was measured ten minutes later on a spectrophotometer (Lucy 1, Anthos). Every serial dilution, blank or sample was tested on two wells and the final result taken as the average. The absorbance from samples and serial dilutions were adjusted for the sheep blank by subtraction and the calibration results plotted on log-log graph paper. The calibration curve was drawn and the human factor IX levels for samples were derived by reading directly from the curve.

C 7.2 Immunohistochemistry for human factor IX

The method was essentially the same as for β-galactosidase immunohistochemistry but used a primary rabbit anti-human factor IX antibody and a secondary swine anti-rabbit antibody. The standard avidin-biotin peroxidase method was then followed (Polak JM and Van Noorden S, 1997). Non-specific antibody binding to proteins in the tissue preparations was prevented by applying 5% non-immune swine serum to each slide for 10 minutes, the serum being from the species which provided the second layer antibody. The positive was a section of fetal liver with strong staining from a previous experiment; the negative was a test section that was not exposed to the primary antibody but immersed in rabbit serum at 1/4000 dilution. Immunohistochemistry analysis was performed at Department of Pathology, Hammersmith Hospital, Imperial College under the supervision of Professor Terry Cook.

C 8. Assessment of cell division by BrdU immunohistochemistry

Tissues for BrdU staining were fixed in carnoys solution (60ml methanol, 30ml chloroform and 10ml glacial acetic acid). Immunohistochemistry for BrdU proliferation was performed using a similar technique to that for β-galactosidase staining. The slides were dewaxed and endogenous peroxide was blocked as above. After rinsing in PBS the slides were immersed in 1M HCl preheated to 60°C for 5 minutes to dissociate the histones and partially denature the DNA. After a further rinse in PBS, non-specific

antibody binding to proteins in the tissue preparations was prevented by applying 20% non-immune rabbit serum to each slide for 30 minutes at room temperature. The serum was tapped off and the slides then incubated with a 1/50 dilution of mouse anti-BrdU 1° antibody with 5% normal sheep serum overnight at 4°C. Slides were rinsed twice with PBS and then incubated with a 1/200 dilution of rabbit anti-mouse biotinylated 2° antibody and 5% normal sheep serum for 45 minutes at room temperature. This was followed by a standard avidin-biotin peroxidase method (Polak JM and Van Noorden S, 1997) and slides were counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene and allowed to dry. Each tissue had a test negative that was not exposed to the primary antibody but immersed in 20% non-immune rabbit serum. Immunohistochemistry analysis was performed by Dr Anna David at Department of Pathology, Hammersmith Hospital, Imperial College under the supervision of Professor Terry Cook.

C 9. PCR analysis of vector spread in tissues

Preparation of genomic DNA from tissue samples and PCR analysis for the first half of the work in this thesis was performed by Dr Anna David. Subsequent analysis was done by Mr Maznu Miah and Mr Dany Perocheau, Gene Therapy Research Group, Imperial College, London. Preparation of genomic DNA from sheep semen samples, and optimisation of PCR amplification of the hFIX transgene and endogenous sheep sequences was performed by Dr Anna David.

C 9.1 Preparation of genomic DNA from tissue samples

Tissues were chopped with a sterile blade on a sterile petri dish and ground well with a sterile pellet mixer in a 1.5ml Eppendorf tube to which was added 700µl proteinase K buffer and 17.5µl of proteinase K (10 mg/ml) solution. After incubation at 55°C overnight, RNA was digested by incubation with 5µl of Rnase (500 µg/ml) at 37°C for 4 hours. Insoluble material was removed by centrifugation at 4000 x g for 10 minutes at room temperature and the supernatant transferred into a new tube. To this was added 750µl equilibrated phenol at pH 7.9 and the contents were mixed vigorously on a vortex. After centrifugation at 14,000 x g for 10 minutes at 4°C, the supernatant containing the phenol and genomic DNA was transferred into a new tube. To remove any remaining protein and traces of phenol, 500µl chloroform was added and the contents mixed vigorously on a vortex. Another centrifugation at 14,000 x g for 10 minutes at 4°C was performed and the supernatant transferred to a new tube. To aid

DNA precipitation, 10% by volume sodium acetate (pH5.0, 3M) was added followed by 2-3 x volume of cold isopropanol and the DNA was seen to precipitate on inversion. The DNA was pelleted by centrifugation at 14,000 x g for 10 minutes at 4°C and the supernatant carefully removed and discarded. The pellet was washed with 500µl cold 70% ethanol, centrifuged at 14,000 x g for 10 minutes at 4°C and the supernatant was discarded. The DNA was carefully air-dried and resuspended in 200µl TE buffer pH 8.0 overnight. To determine the correct concentration for PCR analysis, serial dilutions of the DNA solution were electrophoresed along with a 1Kb DNA ladder on a 0.3% agarose gel (electrophoresis grade) containing ethidium bromide (10mg/ml) at 50kv for 1 hour. The gel was visualized under an ultra-violet transilluminator (Uvitec STX.20.M). The dilution that was just visible under transillumination was used for PCR analysis, equivalent to 100ng DNA.

C 9.2 Preparation of genomic DNA from sheep semen samples

Attempts to extract genomic DNA from lamb semen samples using the standard DNA extraction protocol were unsuccessful. Sperm nuclei are impervous to lysis with SDS/proteinase K because they are ramified with cross-linked thiol rich proteins (Calvin HL and Bedford JM, 1971). A protocol for DNA extraction from human sperm was provided by the Home Office Forensic Science Service (Gill P et al., 1985) and tested on sheep semen samples as described below.

Fresh sheep semen samples were incubated overnight in a 1.5ml Eppendorf tube at 37°C in 0.01 M Tris-HCl, 0.01 M EDTA, 0.1 M NaCl (pH8.0) containing 2% SDS, 20 µg/ml proteinase K and 0.039 M DTT. The DNA was purified by two phenol/chloroform extractions as described above and precipitated by addition of 0.1 volume 2M sodium acetate and 2.5 volumes of absolute ethanol. The DNA was pelleted by centrifugation at 15,000g for 5 minutes at 4°C, washed with 70% ethanol, repelleted and resuspended in 200µl TE buffer pH 8.0 overnight. Samples were diluted and electrophoresed on a 0.3% agarose gel (electrophoresis grade) containing ethidium bromide (10mg/ml) as described above and visualized under an ultra-violet transilluminator (Uvitec STX.20.M). To confirm the presence of genomic sheep DNA, samples were amplified in a PCR using primers designed to endogenous sheep sequences (TATA box binding protein, TBP, see later).

C 9.3 PCR analysis

Spread of the adlacZ or adhFIX vector after the different routes of fetal injection was tested by PCR analysis for the presence of adenovirus β-galactosidase or hFIX cDNA respectively in a range of fetal and maternal tissues. The specificity and sensitivity of the tissue analysis are both important factors in the polymerase chain reaction and these aims were addressed by performing first round followed by nested PCR analysis. The first round primers amplify DNA from part of the target gene and the products accumulate exponentially. Generally 30 cycles are performed before one of the reaction components becomes limiting. To improve the sensitivity a further amplification step is required. Nested PCR analysis also allows improvement in the specificity of the reaction by using primers that amplify a shorter part of the DNA from the target gene, within the sequence targeted by the first round primers.

The primers and conditions optimal for PCR analysis of adlacZ DNA were already determined in the laboratory. Primers designed to amplify part of the hFIX gene and endogenous sheep sequences were tested and the conditions for amplification of DNA optimized. In all cases a Biometra T3 Thermocycler was used and the PCR final reaction volume was 20µl. Each reaction contained 1x Mg free reaction buffer, 0.5 U Taq polymerase, 200 µM dNTPs, 2.5µM forward and reverse primers and $MgCl_2$ at different concentration according to the primers used (**Table C 4**). The PCR cycle was 94°C for 1 minute, annealing temperature of Tm – 4oC for 1 minute (**Table C 4**), 72°C for 1 minute for 35 cycles followed by a final extension step of 72°C for 10 minutes. PCR amplified products were subjected to electrophoresis alongside a 100bp DNA ladder on a 2% agarose gel containing ethidium bromide (10mg/ml) at 100kv for 1 hour. The gel was visualized with an ultra-violet transilluminator (Uvitec STX.20.M). Samples that were negative for first round product were subjected to 20 cycles of nested PCR using 2 µl of first round product applying the above conditions. Nested PCR amplified products were also subjected to gel electrophoresis and visualized as above. For each PCR amplification of transgene, DNA samples from non-injected fetuses of similar gestational ages and a water blank were used as negative controls, and the viral vector used as a positive control. PCR amplification of endogenous sheep sequences used the viral vector and water blank as a negative control and DNA samples from non-injected fetuses of similar gestational ages as a positive control. Each tissue was subjected to PCR analysis twice.

C 9.3.1 PCR amplification of adenovirus β-galactosidase vector

First round primer sequences were designed to amplify DNA sequences from position 4105-4879 of the AdRSVβgal virus from a range of sheep tissues. This region contains a part of the β-galactosidase reporter gene and adenovirus E1B sequences 3' to this region. Nested primer sequences were designed to amplify AdRSVβgal virus DNA between positions 4271-4779.

Table C 4: PCR conditions for amplification of the AdRSVβgal vector.

1st round product size	774bp
1st round forward primer	GTTCAACATCAGCCGCTACAG
1st round reverse primer	TATGTTTACCGCCACACTCGC
Annealing temperature	56°C
$MgCl_2$ concentration in reaction	1.5mM
Nested product size	508bp
Nested forward primer	GTCGCTACCATTACCAGTTGG
Nested reverse primer	TTATCGATACCGTCGACCTCG
Annealing temperature	60 °C
$MgCl_2$ concentration in reaction	1.5mM

C 9.3.2 Optimization of PCR amplification of human factor IX transgene

The conditions and primers for amplification of hFIX DNA were not known in the laboratory and therefore first round and nested primers specific to the human factor IX gene were designed and the conditions needed for amplification and their specificity were investigated. First round primers and nested primers were designed to amplify DNA sequences from position 997-1502, and from position 1047-1444 respectively of the human factor IX gene.

The optimal magnesium chloride ($MgCl_2$) concentration in the final reaction mix for amplification of hFIX DNA was determined for the first round reaction by performing the same PCR reaction at a range of $MgCl_2$ concentrations from 4.0mM to 1.0mM. At each $MgCl_2$ concentration there were four PCR reactions which amplified DNA extracted from (1) adhFIX vector, (2) the liver of a fetal sheep that had received an intraperitoneal injeciton of adhFIX, (3) the liver of a non-injected sheep fetus as a sheep

blank and (4) distilled water as a second blank. The optimal $MgCl_2$ concentration was found to be 1.5mM (data not shown).

To confirm the same optimal $MgCl_2$ concentration for nested PCR analysis the same four PCR reactions were repeated using the nested hFIX primers at the range of $MgCl_2$ concentrations. **Figure C 10** shows the product of the nested PCR amplification of DNA from the liver of a fetus that received an intraperitoneal injection of adhFIX at the range of $MgCl_2$ concentrations from 4.0mM to 1.0mM. Again the optimal $MgCl_2$ concentration was found to be 1.5mM. Annealing temperatures between 55°C and 70°C were also tested to determine the optimal temperature for the first round and nested PCR reaction (data not shown).

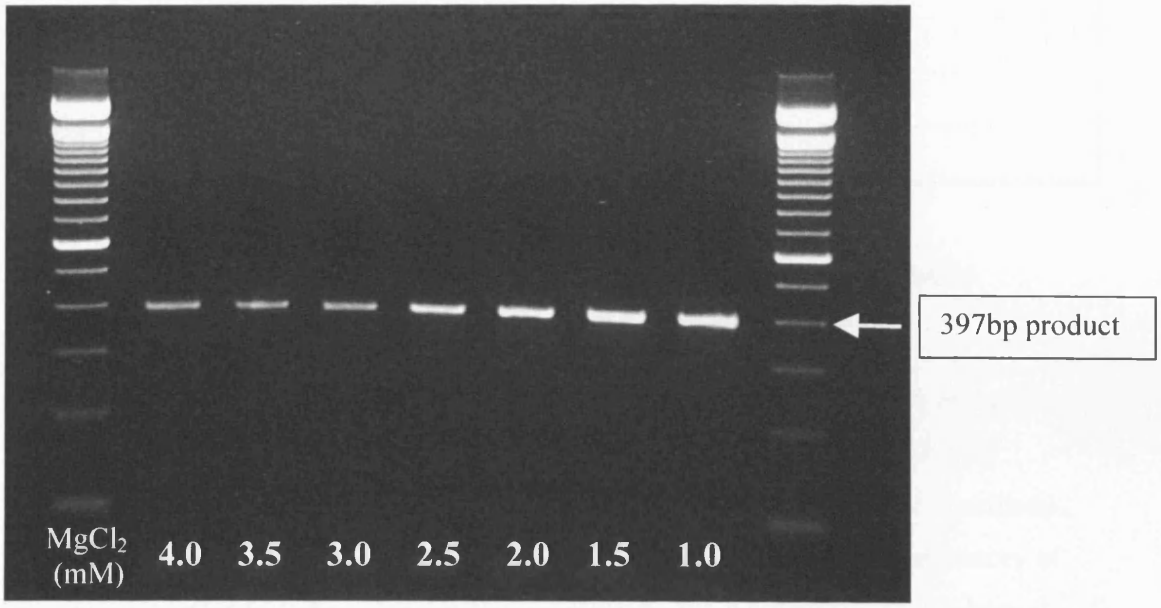


Figure C 10: Optimization of nested PCR analysis of hFIX for magnesium chloride ($MgCl_2$) concentration in the reaction mix.

DNA was amplified for 30 cycles using 1st round primers designed to amplify the hFIX gene. 5µl of the 1st round product was then amplified for a further 20 cycles using nested hFIX primers at reducing $MgCl_2$ concentrations in the nested PCR reaction mix from 4.0mM to 1.0mM. The gel shows the product of amplification of DNA from the liver of a fetal sheep that received an intraperitoneal injection of adhFIX, at reducing concentration of $MgCl_2$ from 1.0mM to 4.0mM.

To determine the specificity of the hFIX primers for hFIX DNA they were also tested on fetal sheep DNA, and DNA extracted from two cell lines, mouse fibroblast NIH 3T3 cells and human fibrosarcoma HT 1080 cells, that had been transfected with adlacZ or adhFIX vectors, as used to test the specificity of the endogenous sheep sequence primers. The hFIX primers only amplified DNA from cell lines that had been transfected with the adhFIX vector (data not shown)

C 9.3.3 PCR amplification of human factor IX transgene

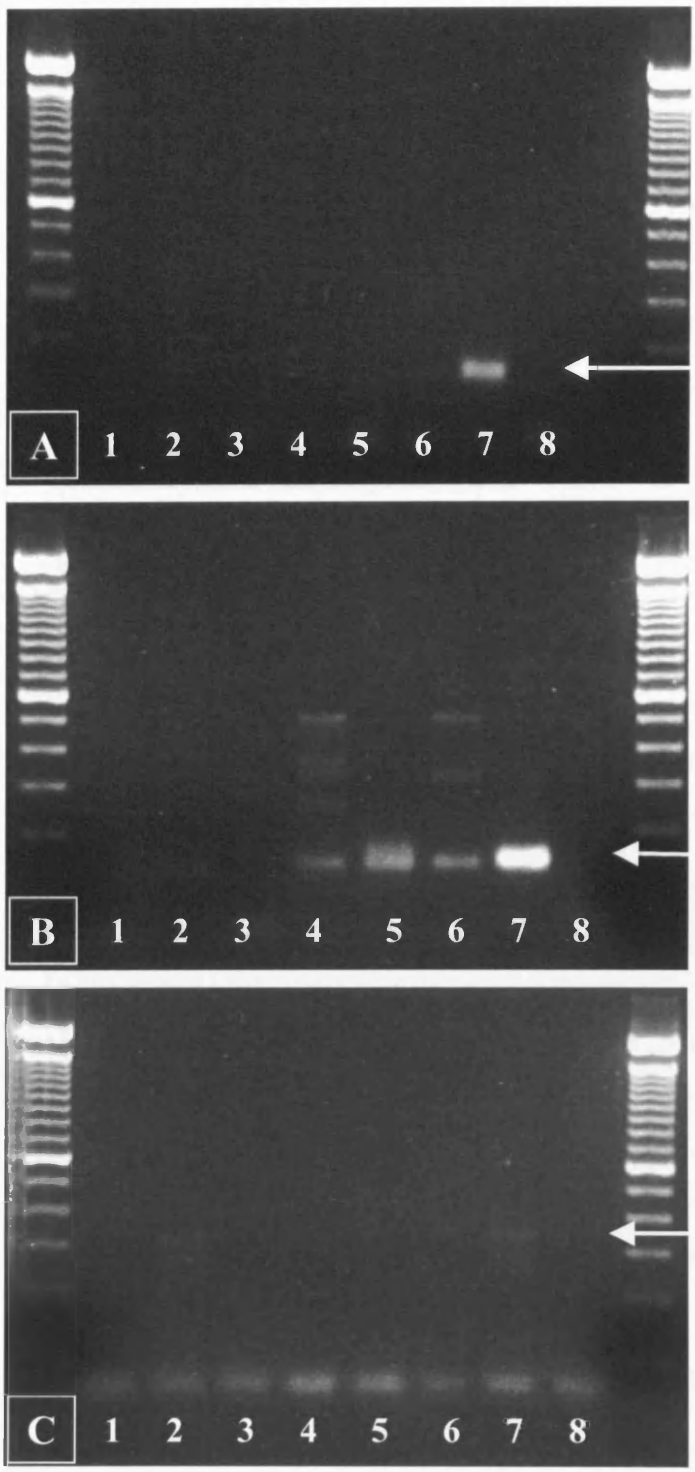
The final reaction conditions are listed below (**Table C5**).

Table C 5: PCR conditions for amplification of the human FIX transgene.

1st round product size	505 bp
1st round forward primer	TGGAAGCAGTATGTTGAT
1st round reverse primer	GCATTCTGTGGAGGCTCTAT
Annealing temperature	60°C
$MgCl_2$ concentration in reaction	1.5mM
Nested product size	397 bp
Nested forward primer	TGGCGGCAGTTGCAAGGATGAC
Nested reverse primer	GGAGAAGATGCCAAACCAGGTGA
Annealing temperature	67°C
$MgCl_2$ concentration in reaction	1.5mM

C 9.3.4 Optimization of PCR amplification of endogenous sheep sequences

As a positive control to show the presence of sheep genomic DNA in DNA extractions from tissue samples we obtained primers to endogenous sheep sequences (kindly provided by Dr Jill Maddox, University of Melbourne) and tested them for specificity. These primers were designed from cattle sequences and amplified sheep sequences of transcription factor COUP-TF1 (COUPTF1, GenBank ref AJ249440), TATA box binding protein (TBP, GenBank ref L47974) and scavenger receptor class B type 1 (SRB1, GenBank ref AF019384). Primers were first tested on fetal sheep DNA, and DNA extracted from two human cell lines, mouse fibroblast NIH 3T3 cells and human fibrosarcoma HT 1080 cells, that had been transfected with adlacZ or adhFIX vectors. **Figure C 11** shows three gels on which products of PCR amplification using the three sheep primers were run. The COUP-TF1 primer amplified DNA from fetal sheep (**Figure C 11 C, lane 7**) and NIH3T3 cells transfected with adlacZ vector (**Figure C 11 C, lane 2**) and both bands were weak. The SRB1 primer amplified DNA from almost all samples tested (**Figure C 11 B**) and therefore was non-specific for sheep DNA. The TBP primer amplified DNA from fetal sheep only (**Figure C 11 A**) and did not amplify DNA from two cell lines transfected with the adlacZ or adhFIX vector.



Gel lanes:
1: NIH3T3 cDNA
2: NIH3T3 adlacZ cDNA
3: NIH3T3 adhFIX cDNA
4: HT1080 cDNA
5: HT1080 adlacZ cDNA
6: HT1080 adhFIX cDNA
7: sheep cDNA
8: dH_2O blank

127bp product, TBP

156bp product, SRB1

345bp product, COUP-TF1

Figure C 11: Testing the specificity of oligonucleotide primers to endogenous sheep sequences by PCR analysis.

DNA samples extracted from 2 human cell lines (NIH3T3 cells and HT1080 cells) transfected with adlacZ or adhFIX vector were amplified for 30 cycles using primers designed to amplify endogenous sheep sequences. The three gels show amplification with primers to (A) TATA box binding protein (TBP), (B) 3'UT of scavenger receptor class B type 1 (SRB1) and (C) 3'UT of transcription factor COUP-TF1 (COUPTF1).

The TBP primer was therefore the most specific for fetal sheep and it was used in future investigations as a positive control for sheep DNA.

C 9.3.5 PCR analysis of endogenous sheep sequences

The final reaction conditions are listed below (**Table C 6**).

Table C 6: PCR conditions for amplification of the TBP gene.

1st round product size	127 bp
1st round forward primer	TCTGTCTATTCTGGAGGAGCAGCAAC
1st round reverse primer	TGCCTGCTGGGACGTCGACT
Annealing temperature	65°C
$MgCl_2$ concentration in reaction	1.5mM

C 10. Determination of fetal and maternal antibody responses to transgene and vector

Antibody analyses were performed by Dr Megha Nivsarkar, Gene Therapy Research Group, Imperial College, London. Sera were taken from each treated fetus at post-mortem or after birth of the treated lambs at six-weekly intervals. Maternal serum samples were taken prior to operation as a negative control and at post mortem analysis. Because of the small size of early gestation fetuses no pre-treated fetal serum was obtainable and therefore serum from non-treated fetuses at similar gestational ages were used for comparison as a sheep blank.

Whole blood (10ml) was collected into a serum separation tube, allowed to clot before centrifugation at 2500 x g for 10 minutes and the serum was stored at –30°C.

C 10.1 Detection of antibodies to adenovirus by ELISA

A 96 well plate was incubated overnight at 4°C with stock adlacZ vector at $1x10^9$ particles/ml in 200 µl dilution buffer from the human factor IX ELISA kit followed by five washes with PBS. A calibration series was prepared by diluting a cross-reactive goat anti-adenovirus antibody in dilution buffer and deriving a standard curve (**Figure C 12**). The lower limits of detection of the assay are shown in **Table C 7**.

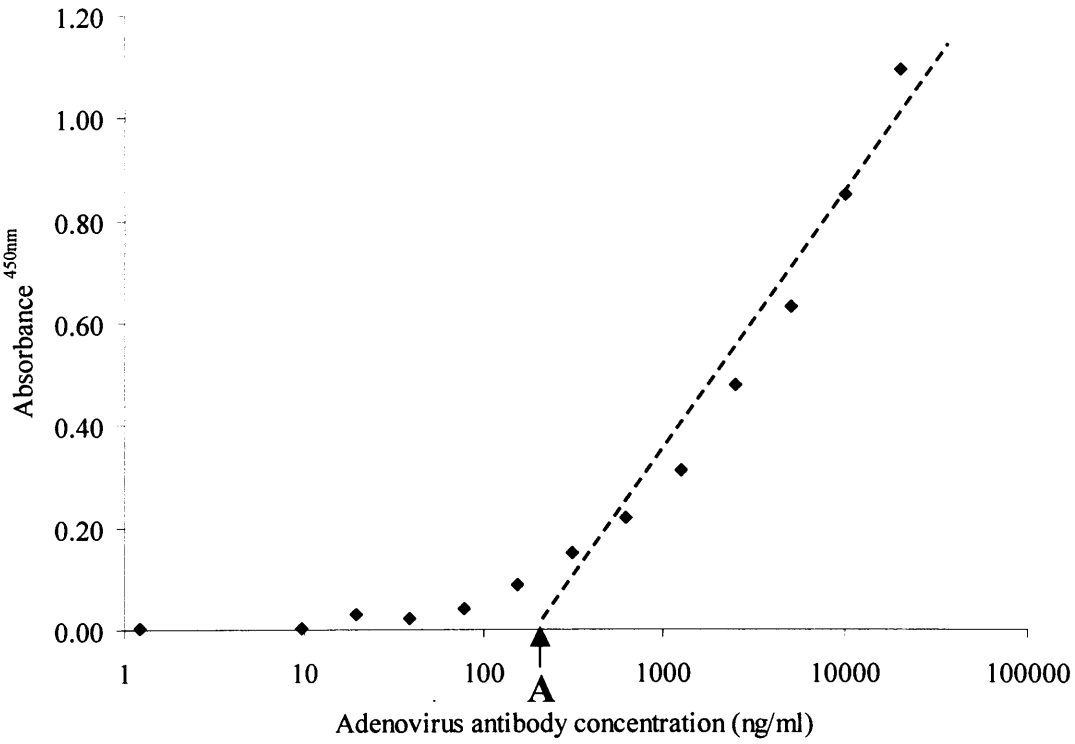


Figure C 12: Example of a standard curve to calibrate the anti-adenovirus antibody ELISA.

The lower limit of reliable estimation of antibody concentration is the point at which the dotted line crosses the x axis (**A**).

Table C 7: The lower limits of detection of the antibody ELISA assays on fetal sheep serum.

ELISA antibody assay	Lower limit of detection of antibodies (ng/ml)	Lower limit of reliable estimation of antibody concentration (ng/ml)
Anti-adenovirus	40	300
Anti-hFIX	20	200
Anti-β-galactosidase	70	580

Samples and sheep blanks (serum from non-treated fetuses of similar gestational ages) were diluted 1+50 with dilution buffer and 200 µl incubated in each well at room temperature for 4-12 hours. After washing six times with washing solution, a horseradish peroxidase labelled rabbit anti-sheep IgG was applied in 200 µl dilution buffer and incubated for 4 hours. Wells were washed six times with washing solution and the bound peroxidase was allowed to react with 200 µl ortho-phenylenediamine in hydrogen peroxide for exactly 3 minutes at room temperature. This produces a characteristic yellow colour, the intensity of which is proportional to the antibody level. The reaction was stopped with 200 µl of 3 M sulphuric acid and the absorbance at

450nm was measured ten minutes later on a spectrophotometer (Lucy 1, Anthos). Every serial dilution, blank or sample was tested on two wells and the final result taken as the average. A standard calibration curve was drawn (**Figure C 12**) and the concentration of antibody to adenovirus determined.

C 10.2 Detection of antibodies to human factor IX transgene by ELISA

The presence of human factor IX antibodies was detected similarly on 96-well plates coated with human factor IX at a standard dilution of 1 ng/ml. A cross-reactive goat anti-human factor IX antibody was used as a positive control to derive the calibration series and the lower limits of detection of the assay are shown in **Table C 7**. The concentration of antibody to human factor IX in serum samples was determined by reading the absorbance at 450nm from the standard calibration curve.

C 10.3 Detection of antibodies to β-galactosidase transgene by ELISA

The presence of antibodies to the β-galactosidase transgene were detected similarly on 96-well plates coated with β-gal protein at a standard dilution of 0.25 μg/ml. A cross-reactive sheep anti-β-gal antibody was used as a positive control to derive the calibration series. The lower limits of detection of the assay are shown in **Table C 7**. The concentration of antibody to β-gal in serum samples was determined by reading the absorbance at 450nm from the standard calibration curve.

D Prenatal gene therapy of haemophilia B

For treatment of Haemophilia B the primary target organ would be the liver and this is therefore also a model for many diseases caused by mutations in proteins synthesized in the liver. The clotting factor protein that is deficient in haemophilia B however, is secreted into and required in the general circulation. Thus any other organ that is able to secrete the functional protein efficiently may serve as a producer of therapeutic protein. We therefore first investigated intravascular delivery and then moved on to alternative routes of injection.

D 1. Targeting the liver and circulation for treatment of haemophilia B

D 1.1 Adenovirus efficiently transduces early gestation fetal sheep liver *ex vivo*

Previous experiments showed that the adlacZ vector was able to transduce late gestation fetal sheep liver with high efficiency after umbilical vein administration (Themis M et al., 1999). Before applying our vector to fetal sheep *in vivo* at earlier gestations, we tested whether adenovirus vectors were able to transfer genes to the early gestation fetal liver *ex vivo*. The livers of two non-injected animals (55 and 60 days of gestation) were sectioned at post mortem examination and infected with 1 x 10^8 particles adlacZ vector with or without DEAE dextran complexation (**Figure D 1**).

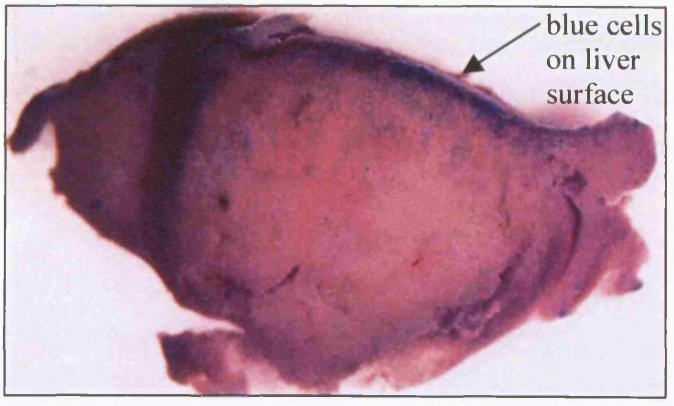


Figure D 1: β-galactosidase transgene expression in the early gestation fetal sheep liver after *ex vivo* application.

AdlacZ vector complexed with DEAE dextran (5μg/ml) was applied to the liver of a fetal sheep aged 55 days of gestation *ex vivo*. Blue dots are seen all over the surface of the liver to which the vector has been applied, indicating positive X-gal staining.

X-gal staining 48 hours after application showed moderate β-galactosidase expression was improved as judged by subjective analysis, with DEAE dextran complexation.

D 1.2 Evaluation of the umbilical vein for ultrasound guided injection

Direct intravascular administration to the umbilical vein is the most effective route for gene application to the whole fetus with predominant delivery to the liver, which is also the natural site of production of hFIX (Themis M et al., 1999). Ultrasound-guided injection of the umbilical vein (UV) can be performed at three sites, the placental cord insertion, the fetal cord insertion as the UV enters the liver, termed intrahepatic UV or in the free umbilical cord. Clinically the UV is accessed either at the placental cord insertion or intrahepatically because the free cord is too mobile to inject. There were no studies on ultrasound examination of the fetal sheep UV and so before commencing experiments to inject the UV we examined it using ultrasound from early to mid-gestation. Fetal sheep undergoing other injection procedures were examined and ewes were also scanned prior to surgery to determine fetal number in order to confirm viability and gestational age. The ability to visualise the UV was assessed and its inner-to-inner diameter was measured. The intrahepatic UV was seen in all but one of the fetuses examined (n = 67) and was measured in the transverse plane as it passed into the anterior third of the liver. We found a virtually linear increase in diameter with age between 51 and 105 days of gestation from 1.2mm up to 5.4mm (**Figure D 2**).
The placental cord insertion was visualised in only 27 out of 67 fetuses examined and was less likely to be seen at the earlier gestational ages (**Figure D 3**). The difficulty visualising the placental cord insertion was due to the placentomes that line the whole uterine cavity. These tended to obscure the placental cord insertion, particularly in early gestation when the cord is narrow and the blood flow velocity was so low that it was not possible to use Doppler examination to visualise flow in the umbilical vessels.

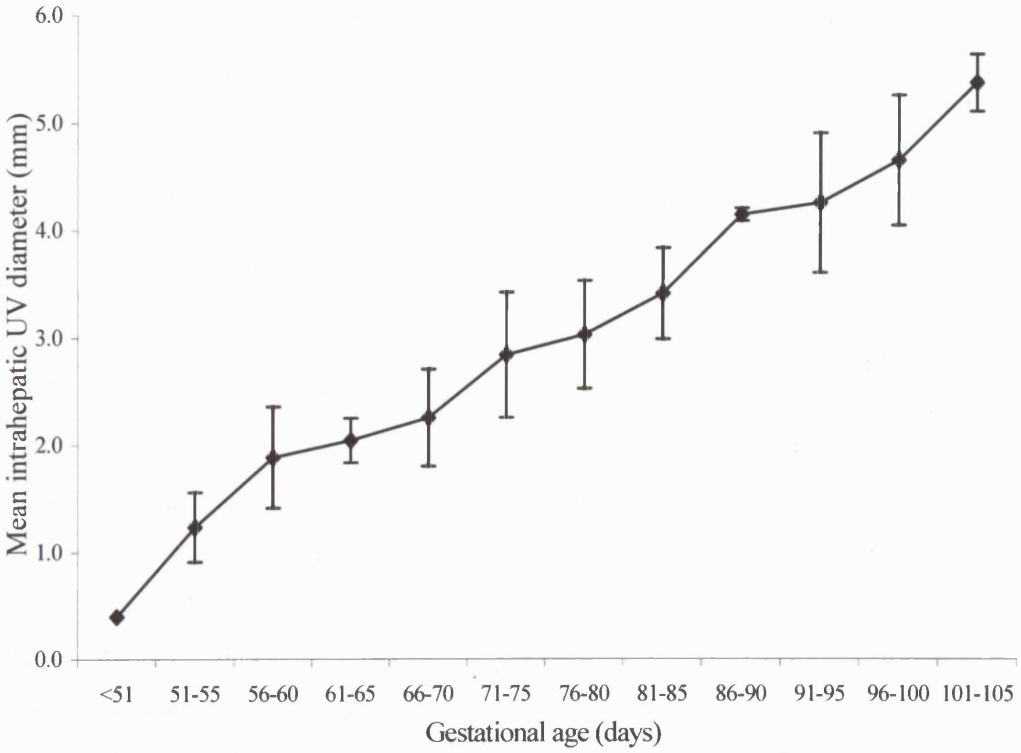


Figure D 2: The diameter of the intrahepatic UV in the sheep fetus.

The mean diameter is shown ± SD. Refer to **Figure D 3** for number per age group.

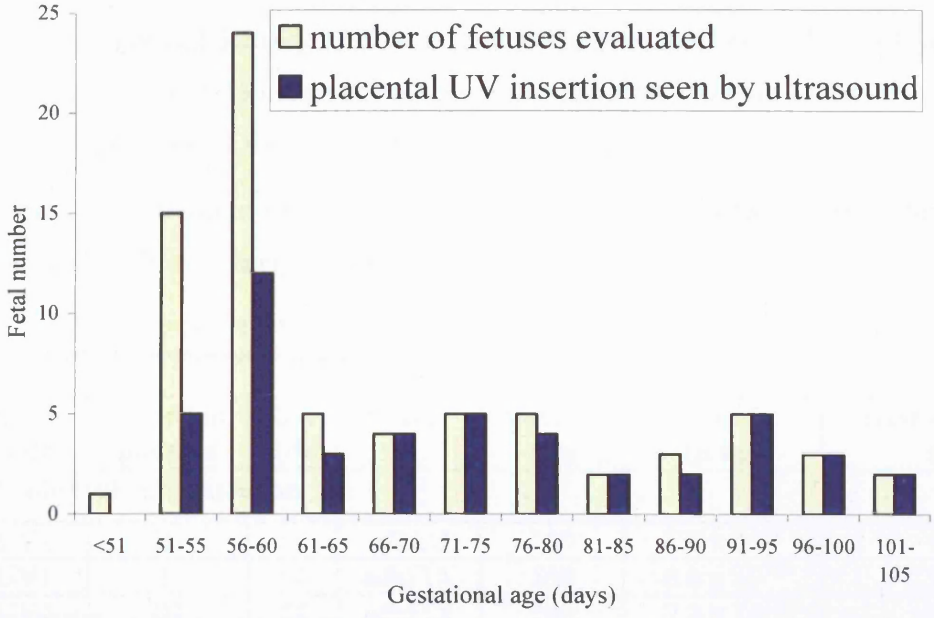


Figure D 3: Ultrasound visualisation of the UV at the placental cord insertion in the sheep fetus.

D 1.3 Ultrasound guided injection of the umbilical vein of early gestation fetal sheep

We began our experiments in early gestation because we believed that the fetus would be large enough to tolerate vector delivery but may not yet have developed immune competence. We wanted to determine the earliest gestational age at which we could confidently and safely inject the fetal circulation using ultrasound-guidance.
We first studied ultrasound guided injection of the umbilical vein between 53-54 days of gestation (0.37 term), which is equivalent to 14 weeks of gestation in humans.
The tolerance dose of adenovirus in early gestation fetal sheep was unknown but our previous study in late gestation fetal sheep had shown that a dose of 4.8×10^{11} pfu was tolerated at intrahepatic UV injection (Themis M et al., 1999). From measurement of the weight of late gestation fetuses we therefore calculated the dose per kg tolerated in late gestation to be $1\text{-}2 \times 10^{11}$ pfu/kg which is approximately equivalent to $1\text{-}2 \times 10^{12}$ p/kg. The tolerance dose of early gestation sheep was unknown to us and we anticipated fetuses may be more sensitive to adenovirus toxicity in early gestation, so we started with a dose of 1×10^{11} p/kg.

D 1.3.1 Ultrasound guided injection of the umbilical vein at 53 days gestation results in procedure-related fetal death

We first attempted delivery of the vectors into the umbilical vein (UV, n = 4) at 54 days of gestation by ultrasound guided percutaneous injection applying $1.0 \times 10^{11} - 2.2 \times 10^{13}$ p/kg adlacZ or adhFIX vector (**Table D 1**).

Table D 1: Ultrasound-guided intravascular injection of adenovirus vectors or colloidal carbon to early gestation fetal sheep.

GA: gestational age; F: injection at the fetal cord insertion; IH: injection at the intrahepatic UV; P: injection at the placental cord insertion.

Sheep code	Injection position	GA (d)	Vector	Volume (µl)	Dose (p/kg)	Time of death (h)
Umbilical vein injection						
UV2	IH	54	adlacZ	100	1.0×10^{11}	24
UV1	F	54	adhFIX	500	6.6×10^{12}	24
UV4	IH	54	adhFIX	200	2.2×10^{13}	20
UV3	P	54	carbon	200	-	24
Intracardiac injection						
IC1	-	53	adlacZ	100	2.8×10^{10}	20
IC2	-	53	carbon	100	-	24

The intrahepatic UV measured between 1.4-1.9mm and the UV at the placental cord insertion measured between 1-1.3mm in diameter and was very difficult to detect. Insertion of the 22 Gauge needle near or within the UV resulted in vessel narrowing probably caused by fluid extravasation compressing it.

Administration of vector to the UV at its insertion into the fetus (n = 1) or within the fetal liver parenchyma (n = 2) resulted in death within 24 hrs of the procedure. Post mortem analysis showed extensive intraperitoneal haemorrhage in both fetuses injected at the intrahepatic UV probably due to bleeding from the site of UV injection. There was autolysis with bacterial colonisation of the fetal lung and liver on histological analysis of one fetus but not in the other; bacterial culture of the vector preparation was negative.

In clinical practice, fetal blood sampling is more commonly performed in the UV at the placental cord insertion rather then the fetal cord insertion or intrahepatic UV (Nicolaides and Rodeck, 1987). However the placental cord insertion was difficult to visualise at this gestational age because placentomes obscured the view. To determine whether fetal death was as a result of virus toxicity or due to the injection procedure, we injected colloidal carbon marker dye into the placental cord insertion (n = 1). In this fetus of a twin pregnancy fetal positioning prevented visualisation of the intrahepatic UV although the placental cord insertion was clearly visible. Fetal death was again observed within 24 hours of the procedure and post mortem findings were similar to those observed before, suggesting that the intravascular injection procedure rather than adenovirus toxicity was the cause of death.

We considered using a finer needle such as a 25 Gauge that would cause less trauma, and that has been used successfully for fetal blood sampling from the placental cord insertion at an equivalent gestational age in humans (Orlandi F et al., 1990). On testing the 25 Gauge needle at post mortem examination, we found it to be too pliable for easy use and withdrawal of blood or injection of fluid was very slow. We concluded that injection of the UV at this gestational age in the fetal sheep was not technically possible.

D 1.3.2 Ultrasound guided intracardiac injection at 53 days of gestation results in procedure related fetal death

Having failed to achieve umbilical vein injection at 53 days of gestation, we attempted intracardiac injection (IC, n = 2) as an alternative route into the circulation at the same early gestational age, applying 2.8 x 10^{10} p/kg adlacZ vector or colloidal carbon (**Table**

D 1). Technically this procedure was relatively straightforward compared with umbilical vein injection at this gestational age. Fetal bradycardia however, was evident immediately after insertion of the needle into the left ventricle. The vector or colloidal carbon was injected and following removal of the needle a pericardial effusion was clearly seen suggestive of haemorrhage into the pericardial cavity (**Figure D 4A and B**) and fetal death occurred within 24 hours.

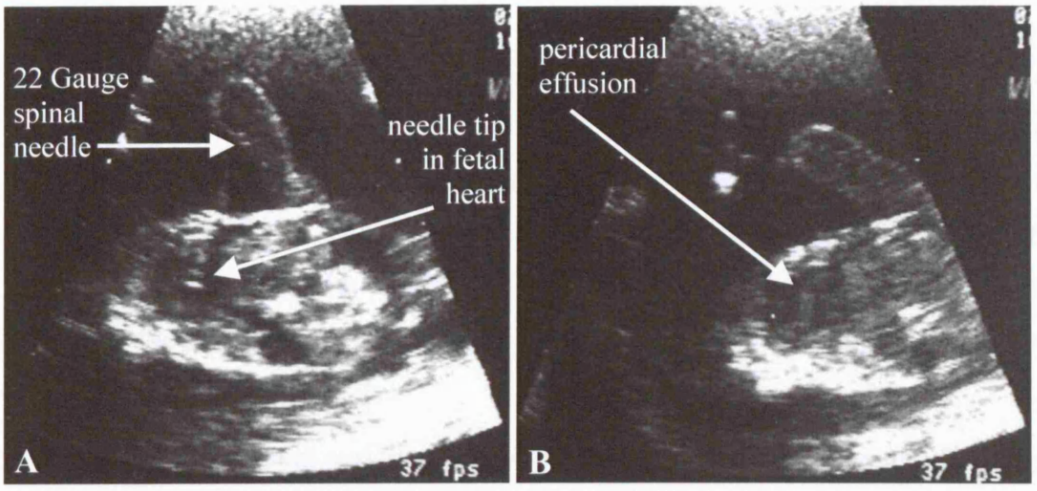


Figure D 4: Ultrasound guided intracardiac injection in an early gestation fetal sheep.

The ultrasonograms show a fetus (IC1) in longitudinal section at 53 days of gestation during intracardiac delivery of adlacZ vector. (A) shows a 22 Gauge spinal needle entering the left ventricle of the fetal heart. Following injection fetal bradycardia occurred and (B) a pericardial effusion was visible suggesting haemorrhage from the left ventricle into the pericardial sac.

Postmortem analysis of one fetus 24 hours after the operation showed a large pericardial blood clot suggesting haemorrhage and tamponade as the cause of death. No evidence of bacterial infection was found on culture of fetal tissues. The other fetus sacrificed 48 hours after operation showed signs of autolysis, and a heavy growth of Bacillus licheniformis, a fleece commensal, was found on culture of the fetal tissues and placenta. It is likely this bacterial infection was a postmortem event.

D 1.4 Ultrasound guided injection of the umbilical vein of fetal sheep at 60 days of gestation

Our results had so far suggested that the intravascular procedure was the cause of fetal death at 53 days of gestation, probably due to the size disparity between the UV at this gestation and the 22 Gauge needle. We therefore investigated whether fetuses that were one week older (60 days of gestation) would tolerate the UV injection procedure better.

This is equivalent to 16 weeks gestation (0.4 gestation) in humans, at an age when a 22 Gauge needle has been successfully used clinically for fetal blood sampling (Orlandi F et al., 1990)

D 1.4.1 The umbilical vein can be injected at 60 days of gestation but not always reliably

At 60 days of gestation it was still not possible to see the placental cord insertion reliably and the umbilical vein at the fetal cord insertion was fragile. We injected the umbilical vein therefore within the hepatic parenchyma (n = 11) and delivered 1.0-1.4 x 10^{11} p/kg adlacZ or adhFIX vectors (**Table D 2**). The mean intrahepatic UV diameter in these animals before injection was 2.3 mm. A view of the fetal abdomen in transverse section at the level of the fetal cord insertion was obtained during injection (**Figure D 5 A**) and the needle passed through the fetal liver into the UV (**Figure D 5 B**). Fetal blood (20µl) was withdrawn at each attempt to confirm correct positioning of the needle before the vector was injected.

Table D 2: Post mortem and histological findings following intrahepatic UV injection of fetal sheep at 60 days of gestation.

PM: post mortem; h: hours; d: days; m: months; Vol: volume of vector injected; Microbubbles: visualisation of microbubbles during vector injection; IP: intraperitoneal;

Sheep	PM sampling	vector	Dose (p/kg)	Vol (µl)	Micro-bubbles	PM findings	Liver histology
UV13	died 24 h	adhFIX	1.2 x 10^{11}	100	no	IP blood clot	tissue autolysis
UV9	died 24 h	adhFIX	1.17 x 10^{11}	100	no	IP blood clot	tissue autolysis
UV5	2 d	adlacZ	1 x 10^{11}	100	no	friable liver	infarcts & haemorrhage
UV16	2 d	adlacZ	1.4 x 10^{11}	200	no	normal	normal
UV6	2 d	adhFIX	1.1 x 10^{11}	100	yes	normal	infarcts & haemorrhage
UV10	2 d	adhFIX	1.15 x 10^{11}	100	no	IP blood clot	infarcts
UV7	9 d	adhFIX	1 x 10^{11}	100	no	pale mottled area distal liver	infarcts
UV8	9 d	adhFIX	1 x 10^{11}	100	yes	pale mottled area distal liver	infarcts
UV11	29 d	adhFIX	1 x 10^{11}	100	no	normal	normal
UV12	78 m	adhFIX	1 x 10^{11}	100	yes	normal	normal
UV15	70 m	adhFIX	1 x 10^{11}	100	yes	normal	normal

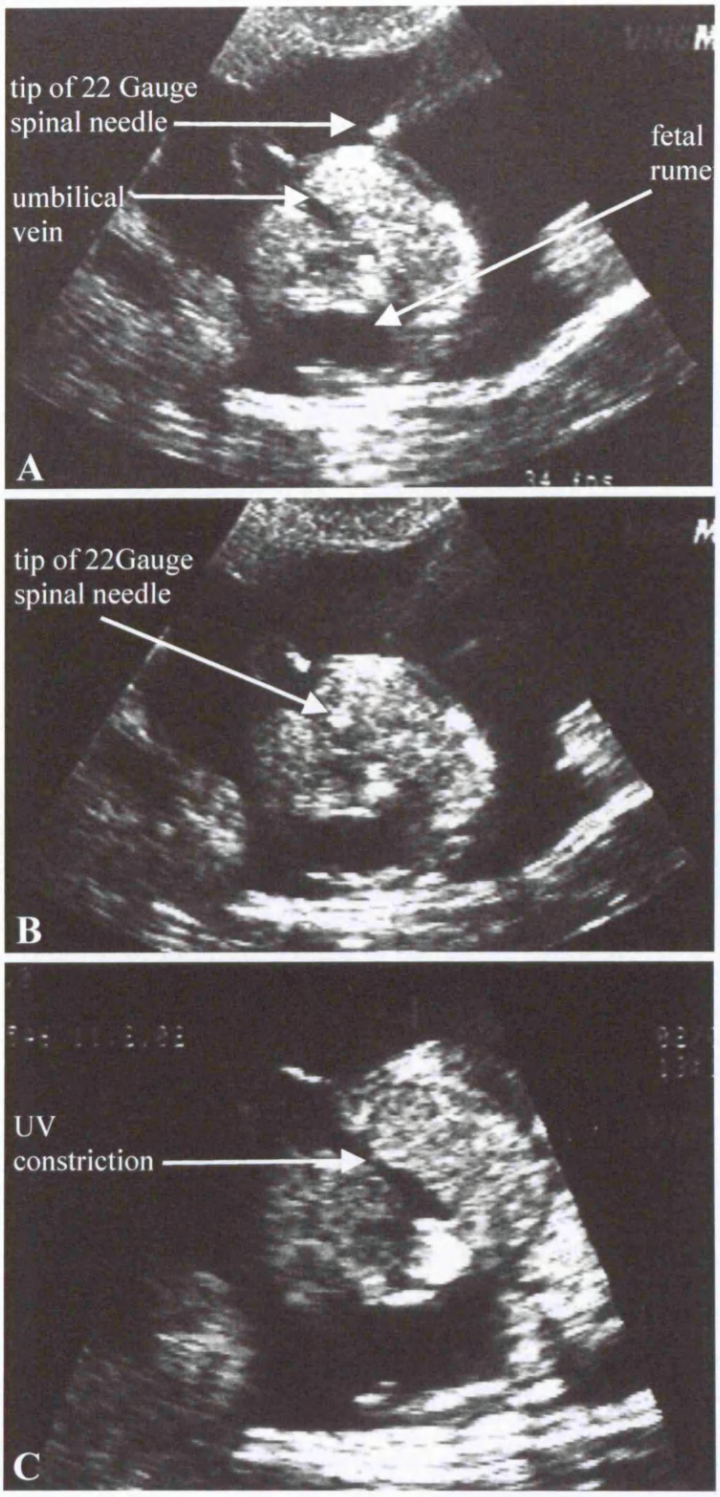


Figure D 5: Ultrasound-guided injection of the intrahepatic UV in an early gestation sheep fetus.

A sheep fetus (60 days of gestation) is shown in cross-section during UV delivery of adhFIX vector. (A) 22 Gauge spinal needle approaches the fetus from the right side, passes through the hepatic parenchyma and (B) the tip can be seen within the UV as it traverses the fetal liver. (C) UV narrowing post op at the site of injection.

The presence of microbubbles in the UV during vector injection was recorded at the time of injection and rechecked on the videotape recording. We observed the liver for echogenicity surrounding the UV that would suggest injection of the hepatic parenchyma rather than intravascular delivery. Such extravascular injection occurred in one case (UV9), and attempts to reposition the needle were unsuccessful. This case was deemed to be a procedure failure (**Figure D 6**).

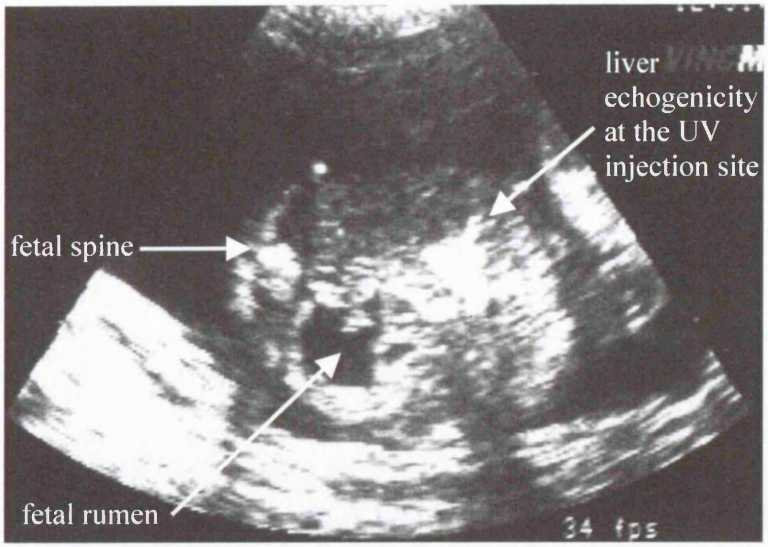


Figure D 6: Failed injection of the intrahepatic UV.

Ultrasonogram of a sheep fetus in cross-section at 60 days of gestation (UV9) after attempted umbilical vein injection of adhFIX vector. There is echogenicity of the liver parenchyma surrounding the umbilical vein after inadvertant extravascular injection of the vector.

Blood was successfully withdrawn prior to vector injection in all fetuses, at the first attempt in 7 fetuses, at the second attempt in 4 fetuses and requiring a third attempt in 1 fetus. The reason for repeated attempts at UV injection was narrowing of the UV that occurred after the majority of injection attempts, successful or unsuccessful (**Figure D 5 C**). This obscured the view of the UV and lengthened the procedure time. Excluding the procedure failure, the mean UV diameter reduced from 2.3mm to 1.8mm and this change was statistically significant (paired Students *t*test < 0.002, **Table D 3**).
The mean time to successful injection was 12 min 58 sec (SD 11 min 23 sec, range 3 min 43 sec – 41 min 33 sec). Microbubbles were only seen in 4 of the 11 cases (**Table D 2**), on two occasions passing along the UV, through the chambers of the heart and down the aorta. Although there was no echogenicity of the hepatic parenchyma seen in the remaining cases, microbubbles were not observed, even with the UV in clear view.

On this basis therefore, we can only be confident that the vector had been successfully delivered into the circulation in 4 of 11 cases (36%).

Table D 3: The change in the intrahepatic UV diameter with ultrasound-guided UV injection in fetal sheep aged 60 days of gestation.

There was no data available from UV12 and data from UV9 was excluded because the vector injection was extravascular.

Sheep	UV diameter (mm)		change in UV diameter
	before	after	
UV5	2.5	2.0	-20%
UV6	2.4	2.4	0
UV7	2.3	2.1	-9%
UV8	2.3	1.6	-30%
UV10	2.6	1.6	-38%
UV11	2.3	1.9	-17%
UV13	2.1	1.9	-10%
UV15	2.1	1.4	-33%
UV16	1.9	1.7	-11%

D 1.4.2 Umbilical vein injection at 60 days of gestation has low morbidity and mortality

Fetal survival using this technique was 82% (**Table D 2**). Two fetuses died within 24 hours of injection of adhFIX injection. In both animals, the umbilical vein became severely narrowed after an unsuccessful first attempt at injection and fetal bradycardia was observed. After allowing recovery of the fetal heart rate, a second injection attempt was made. In one fetus the vector appeared to be delivered into the umbilical vein (UV13) although no microbubbles were seen in the UV. In the other (UV9) an echogenic area appeared within the liver parenchyma suggesting extravascular intrahepatic injection. Post mortem analysis of both fetuses the following day showed extensive intraperitoneal haemorrhage suggesting this as the cause of death. Culture of fetal tissues demonstrated the presence of Aeromonas hydrophila, a common fleece commensal in one animal and Escherichia coli, a common gut commensal in the other, and these were most probably post mortem infections. Culture of the adenovirus vector showed no evidence of bacterial infection.

Analysis of four fetuses at post mortem 2 days after operation showed no obvious site of injection although there was a small blood clot on the lower left surface of the liver in one animal (UV10). There was an obvious needle track in the liver of two animals

(UV5 & UV6) with haemorrhage and necrosis surrounding the UV confirming that bleeding had occurred from the UV injection site (**Figure D7 A**).

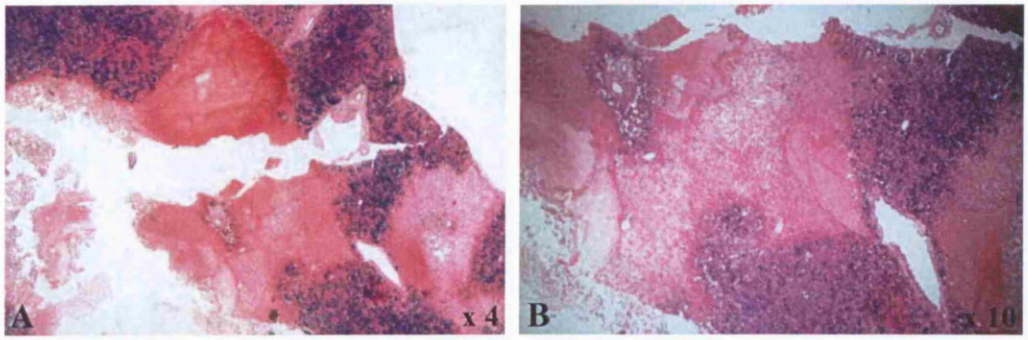


Figure D 7: Histological analysis of the fetal liver after intrahepatic UV injection of adhFIX vector

Light microscopy (H & E stain) 2 days after injection of 1.1 x 10^{11} p/kg adhFIX vector at 60 days of gestation shows (A) infarction, haemorrhage and necrosis of the central liver and (B) infarction and necrosis in the peripheral left lobe of the liver of one fetus (UV6) 2 days after UV injection. Original magnifications are as indicated.

In three cases histological analysis showed small areas of infarction, particularly subcapsular in the left or right lobes of the liver in all fetuses (**Figure D 7 B**). Analysis of two fetuses sampled 9 days after injection of adhFIX showed only pale areas on the periphery of the liver at post mortem (**Figure D 8 A & B**) which were confirmed to be subcapsular infarcts on histological analysis (**Figure D 8 C & D**). We postulated that the infarcts could be due to narrowing of the UV following the vector injection that temporarily reduced the blood supply to the peripheral liver. We did not believe that adenovirus toxicity would have caused the necrosis since injection of the vector into the UV is unlikely to have preferentially entered the peripheral liver. There were no abnormal findings on analysis of one fetus 29 days after injection of adhFIX vector. Two sheep were allowed to survive postnatally and studied for 18 months. Serum levels of liver enzymes and bile acids were initially raised but then returned to within normal limits and the data is presented in **Section D 5**. Post mortem and histological examination of the animals at 18 months of age showed mild chronic portal inflammation that was commonly seen in ewes, but no other abnormality.

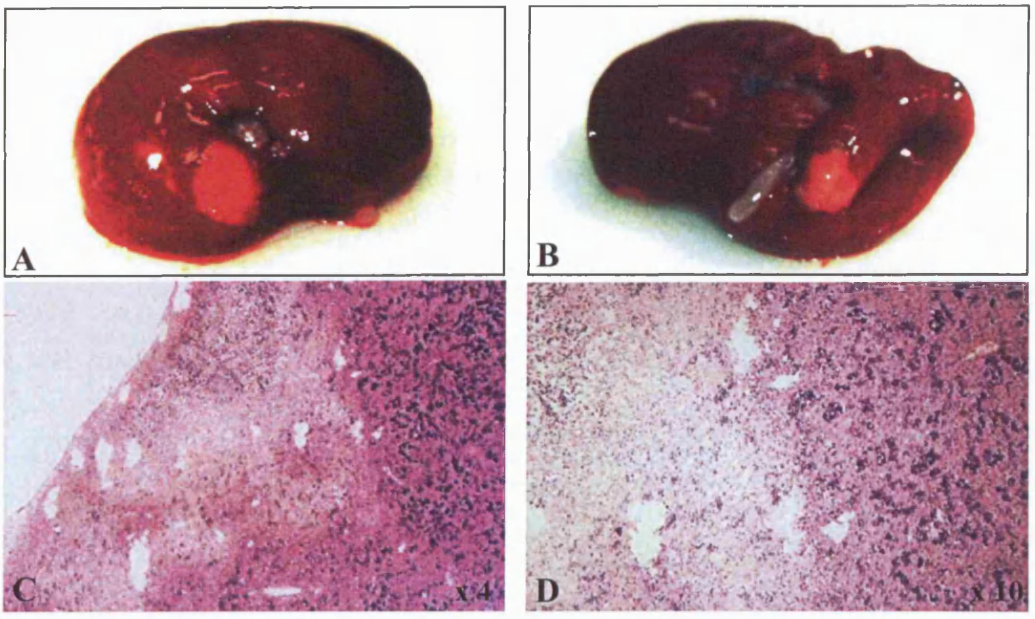


Figure D 8: Post mortem and histological analysis of the fetal liver 9 days after intrahepatic UV injection of adhFIX vector.

After intrahepatic injection of 1 x 10^{11} p/kg adhFIX vector there are pale areas on the periphery of the fetal liver at post mortem analysis (A and B) that were confirmed to be subcapsular infarcts on light microscopy (H & E stain, C and D). Original magnifications are as indicated.

D 1.4.3 Transgene expression is observed following umbilical vein injection at 60 days of gestation

X-gal staining and immunohistochemistry for expression of β-galactosidase was negative in both fetuses sampled 2 days after UV injection, at a time when we would expect to see maximum transgene expression (**Table D 4**).

For hFIX expression, fetal blood was sampled at post mortem examination rather than by ultrasound guided injection. At that stage of the project we were not confident of our ability to retrieve blood from the UV safely or reliably in mid-gestation and we were only able to anaesthetise each animal once. We have now amended the Home Office project licence to permit two anaesthetic procedures on one animal, allowing blood to be sampled from the fetus once under anaesthesia at a later gestational age.

Measurement of hFIX levels 2, 9 and 29 days after injection and at birth were 582 and 0ng/ml (UV6 and UV10), 199.5 and 145ng/ml (UV 7 and UV8), 0ng/ml (UV 11) and 0ng/ml (UV12 and UV15) respectively (**Table D 4** and **Figure D 9**).

Table D 4: Transgene expression after ultrasound-guided intraheptatic UV injection in early gestation.

Adenovirus vectors (1 – 1.4 x 10^{11} p/kg) were delivered to fetal sheep at 60 days of gestation. h: hours; d: days; Microbubbles: microbubbles visualised during vector injection; nt: not tested

Sheep	Sampling	Vector	Micro-bubbles	X gal or FIX expression (ng/ml)	β-gal immuno or FIX immuno
UV13	died 24 h	adhFIX	no	nt	nt
UV9	died 24 h	adhFIX	no	nt	nt
UV5	2 d	adlacZ	no	negative	negative
UV16	2 d	adlacZ	no	negative	negative
UV6	2 d	adhFIX	yes	582	positive cells in central liver round haemorrhage
UV10	2 d	adhFIX	no	0	nt
UV7	9 d	adhFIX	no	199.5	negative
UV8	9 d	adhFIX	yes	145	positive cells in central & left liver, no haemorrhage
UV11	29 d	adhFIX	no	0	nt
UV12	birth	adhFIX	yes	0	nt
UV15	birth	adhFIX	yes	0	nt

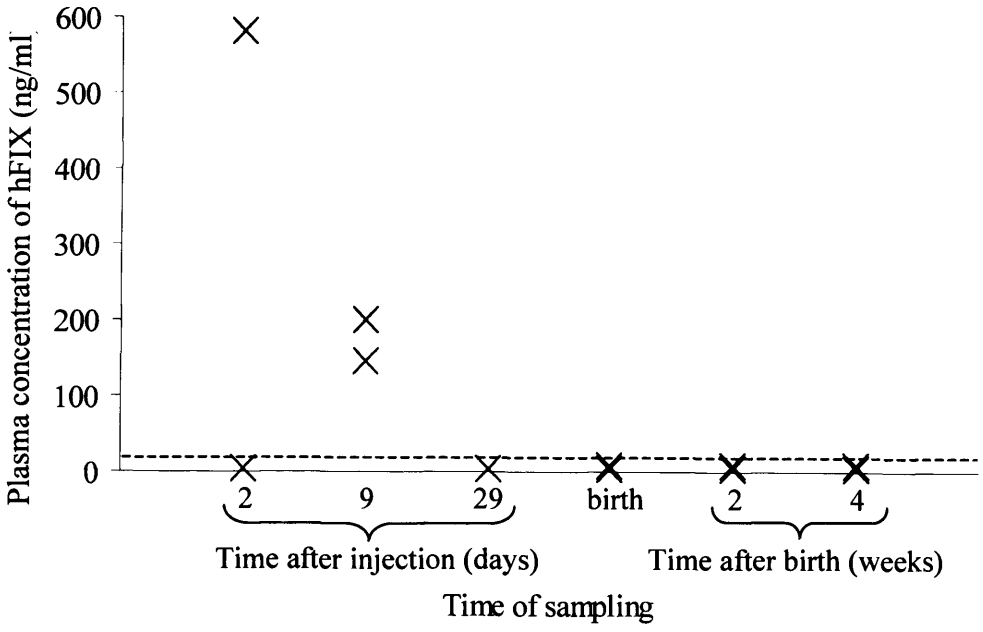


Figure D 9: Expression of hFIX transgene after ultrasound-guided UV injection of adhFIX vector.

After injection of adhFIX vector (1 – 1.4 x 10^{11} p/kg) into fetal sheep at 60 days of gestation, fetal blood was sampled at post mortem analysis. The concentration of hFIX in fetal or lamb plasma was determined by ELISA. The dotted line represents the lowest level of detection (5ng/ml) in the assay.

Immunohistochemistry for hFIX expression was positive in two fetuses, sampled at 2 and 9 days respectively after intrahepatic UV injection (UV6 and UV8, **Figure D 10**). These fetuses were those in which microbubbles were observed passing along the intrahepatic UV and in which fetal plasma hFIX levels of 582 and 145ng/ml respectively, had been found. Therapeutic levels of hFIX (199.5ng/ml) were also observed in the plasma of the injected cotwin of one of these animals (UV7). Microbubbles were not observed within the UV at the time of injection and immunohistochemistry for hFIX expression was negative. This may be due to sampling of the liver in this particular fetus since each liver was divided into three and then subdivided into two further samples for PCR or histological/immunohistochemical analysis.

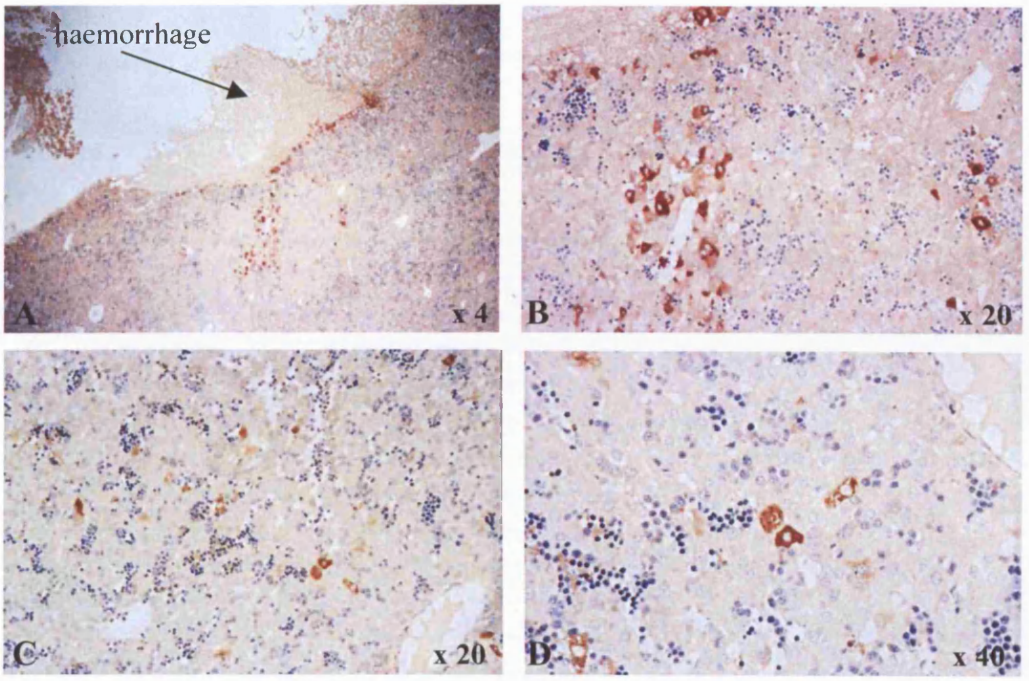


Figure D 10: Immunohistochemistry for hFIX expression after ultrasound-guided intrahepatic UV injection of adhFIX vectors.

Fetuses were sampled 2 days and 9 days after intrahepatic UV injection of adhFIX vector ($1 - 1.1 \times 10^{11}$ p/kg) at 60 days of gestation. Two days after injection (UV6) there is positive staining of hepatocytes adjacent to an area of haemorrhage in the central portion of the liver (A and B) and staining of scattered hepatocytes around the area of haemorrhage (C). Nine days after injection (UV8) there are scattered positively stained hepatocytes in the left lobe of the liver (D).

D 1.4.4 Haematogenic vector spread is observed after umbilical vein injection at 60 days of gestation

We investigated the biodistribution of vector in two animals sampled two days after intrahepatic UV injection of adlacZ and adhFIX vector respectively. PCR of tissues two days after adlacZ injection (UV16) showed no evidence of vector on the 1st round or more sensitive nested PCR analysis in fetal or maternal tissues. PCR of tissues two days after adhFIX (UV6), showed the presence of transgenic hFIX cDNA in the fetal adrenal gland and spleen on 1st round analysis. Low level spread was detectable in a wide range of tissues including the placenta, fetal liver, umbilical cord and heart by nested PCR analysis (**Table D 5**).

Table D 5: Vector spread after intrahepatic UV injection.

AdhFIX vector (1.1×10^{11} p/kg) was delivered into the UV of a fetal sheep at 60 days of gestation (UV6) and was detected in fetal and maternal tissues by PCR analysis 48 hours after injection. 1st: 1st round PCR analysis; 2nd: nested PCR analysis; nt: not tested, m denotes maternal tissues.

Tissue		1st	2nd
Liver	Right lobe	–	+
		–^{m}	–^{m}
	Left lobe	–	+
		nt^{m}	nt^{m}
Gonad		–	–
		–^{m}	–^{m}
Lung		–	+
		–^{m}	–^{m}
Placenta		–	+
Umbilical cord		–	+
Thymus		–	–
Heart		–	+
Spleen		+	+
Small bowel		–	+
Adrenal		+	+
Kidney		–	+
Skin		–	+
CNS cortex		–	+

We concluded that ultrasound-guided injection of the umbilical vein at 60 days of gestation gave therapeutic levels of hFIX expression and haematogenic vector spread. The injection technique was difficult to reproduce reliably however,and was associated with significant morbidity.

D 1.5 Ultrasound-guided umbilical vein injection is technically easier and is reliable at or after 67 days of gestation in the sheep fetus

Because of the technical difficulties in performing umbilical vein injections at 60 days of gestation, we investigated this application route at later gestations to determine the earliest gestational age at which umbilical vein injection could be performed reliably. We performed experiments from 67 up to 95 days of gestation (n = 13). We used the adlacZ vector (n = 11) and adhFIX vector (n = 1, UV14) and sampled fetal tissues 2 days after injection (**Table D 6**). We injected the adlacZ vector rather than the adhFIX vector for most of these experiments because we wanted to locate transgene expression within the tissues, especially the liver. To define at which gestational age UV injection could be performed reliably, we studied fetuses at increasing age from 60 days of gestation.

We also performed an intrahepatic UV injection of retrovirus (MLV) containing the lacZ gene complexed with DOGS (n = 1, UV24). This fetus was sampled 7 days later and the data on the technique and post mortem findings are presented here. Results of transgene expression for this fetus are presented in **Section H**.

We did not perform experiments later than 95 days of gestation since a previous study had already established that ultrasound-guided injection of the intrahepatic UV was possible from 102 days of gestation (Themis M et al., 1999). These experiments had shown therapeutic levels of hFIX expression (221ng/ml) in the fetal blood 24 hours after injection and widespread β-galactosidase expression was observed immuno-histochemically 3 days after injection.

To confirm intravascular delivery in the earlier gestation injections, colloidal carbon was delivered with the vector in two cases. In four cases the adlacZ vector was complexed with DEAE dextran (5μg/ml) that we had shown to improve adenovirus transfection of fetal liver *ex vivo*. We were unsure however, of the effect of intravascular DEAE dextran on fetal wellbeing and so it was not used in every experiment.

D 1.5.1 Visualisation of the UV at the placental insertion is not adequate enough for ultrasound-guided injection at or after 67 days of gestation in the sheep fetus

In all cases during initial ultrasound examination of the fetus we examined the UV at the placental or fetal cord insertion or during its intrahepatic course. A decision was

made on the most suitable position to inject the UV on the basis of visualisation. Initially we planned to inject the UV at the placental cord insertion since this is the route of choice in clinical practice (Vaughan JI and Rodeck CH, 2001). Cordocentesis at the fetal intrahepatic UV is usually used when difficulties arise either with access or due to sampling failure at the placental UV insertion. There were however, some problems with the placental insertion injection route in the sheep fetus. The placental UV insertion was only visualised adequately in 9 out of 13 cases. To distinguish between the two UVs and two UAs in the sheep fetus, the vessels were insonated with Doppler (**Figure D 11**). It is important to identify the UV since fluids injected into the UA would be directed first towards the placenta and not towards the fetus. In contrast the normal human fetus has only one UV that is easy to identify.

Blood flow in the UV vessels was always detected by Doppler examination from 71 days of gestation onwards. At earlier gestational ages however, UV blood flow was detected in only one of the three fetuses in which the placental insertion was adequately visualised. This was most likely due to the low flow rate in the vessels at earlier gestations and a more advanced ultrasound machine may have been able to detect such flow.

At all gestational ages examined the placental insertion of the UV ran along the inner surface of the uterus making it difficult to determine the optimum injection site. The umbilical cord was short so that frequently the fetal abdomen was positioned close to its placental insertion and the UV appeared to run within the membranes along the uterine wall and over the placentomes. The diameter of the vessel became narrower the more distal it was from the umbilical cord. Postmortem examination of a placenta at 74 days of gestation (**Figure D 12**) revealed that the umbilical cord approached the uterine wall, entered the chorion where the two UAs and UVs divided, one of each distributed to each uterine horn. Within the chorionic mesoderm the UA gave off branches to the placentomes, the more proximal branches being the largest and the diameter of the UA reduced the further the distance from the umbilical cord.

The fetal cord insertion was examined as another possible site of UV injection. In clinical practice this route is rarely used because of the passive movements of the fetus on the needle and this was also a problem in the sheep fetus. The UV was clearly visible in free loops of cord although detection of blood flow using Doppler was not usually possible. Early gestation injection of the UV in a free loop of cord can lead to traumatic haemorrhage because of the paucity of Wharton's jelly, an embryonic connective tissue that surrounds and supports the umbilical vessels. In the human fetus, the Wharton's

jelly area in the umbilical cord increases with gestational age (Ghezzi F et al., 2001). Histologically we noted an increase in the amount of Wharton's jelly in the fetal sheep umbilical cord as gestation advanced.

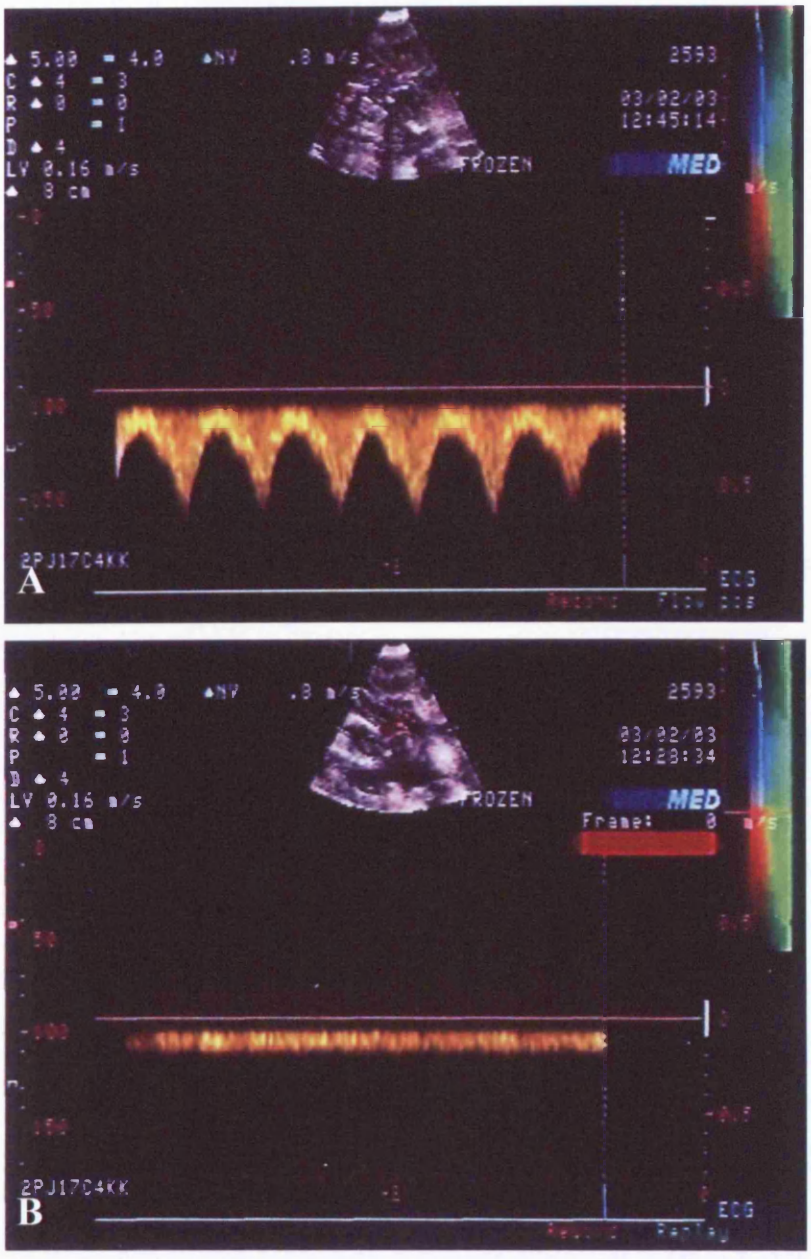


Figure D 11: Doppler measurement of blood flow in the umbilical vein and artery of fetal sheep.

Ultrasonograms show (A) the umbilical artery and (B) umbilical vein at the placental cord insertion of a fetal sheep aged 71 days of gestation (UV19). The top part of each sonogram shows the vessel being imaged with color-flow. A sample volume is placed on the vessel of interest and a Doppler sonogram is produced in the bottom part of each sonogram showing the Doppler frequencies measured. The Doppler frequency is a measure of velocity in the direction of the ultrasound beam from which the color-flow and Doppler spectrum displays are produced. In sonogram (A) the waveform is pulsatile indicating this is the umbilical artery, whereas in sonogram (B) the waveform is non-pulsatile indicating this is the umbilical vein. Both waveforms are inverted indicating blood flow away from the probe.

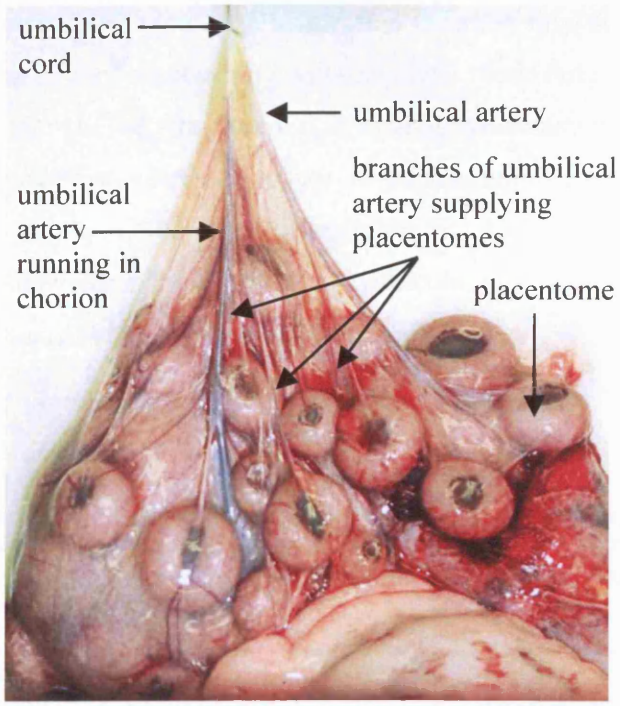


Figure D 12: The umbilical cord and placenta in a sheep fetus aged 74 days of gestation.

The intrahepatic UV was the most easily visualised in all fetuses. An advantage of this route is that there is no need to insonate the vessel with Doppler to determine its origin. This was therefore the injection route of choice in the sheep fetus.

D 1.5.2 Ultrasound-guided injection of the intrahepatic UV is achieved at or after 67 days of gestation in the sheep fetus with low morbidity and mortality

Injection procedures were straightforward in 10 out of 13 fetuses (**Table D 6**). The intrahepatic UV was injected in 9 cases and the placental UV insertion was injected in 1 case (**Figure D 13**). Injection of the UV was achieved at the first attempt in 10 cases and at the second attempt in 1 case; microbubbles were visible in all these cases. The mean time to successful injection in these cases was 10 min 8 secs (± 7 min 5 secs, range 1 min 44 secs – 20 min 7 secs).

In two cases there were multiple attempts to inject the UV at various positions but no microbubbles were visualised during vector injection. In the first fetus (UV19), the placental UV insertion was clearly visible and blood flow was detected within the UA and UV on Doppler examination. A 22 Gauge spinal needle was inserted into the UV in two separate attempts but no blood was withdrawn. The intrahepatic UV was obscured

by the fetal spine and an attempt at UV injection failed. A final attempt to access the UV in a free loop of cord successfully withdrew fetal blood but no microbubbles were seen during injection of the viral vector. Ultrasound assessment of the fetus 16 hours after injection showed no heartbeat. At post mortem examination there was haemorrhage at the placental cord insertion, in the free loop of cord and a small blood clot posterior to the liver at the UV insertion. Microbiological culture of fetal tissues was sterile and the cause of death was haemorrhage.

Table D 6: Post mortem and histological findings following UV injection of fetal sheep from 67 days of gestation.

* denotes twin fetus; GA: gestational age; d: days; h: hours; IH: injection of the intrahepatic UV; cord: injection of the UV in the umbilical cord; P: injection of the placental cord insertion; C: colloidal carbon; D: DEAE dextran (5μg/ml); Vol: volume of vector injected; Microbubbles: visualisation of microbubbles during vector injection; IP: intraperitoneal;

Sheep	sampling	route	GA (d)	Dose (p/kg)	C	D	Vol (μl)	Micro-bubbles	PM findings	Histological findings
UV20	2 d	IH	67	4.4×10^{11}	+	–	300	yes	normal	normal
UV17	2 d	IH	68	8.2×10^{10}	–	–	150	yes	IP blood clot	normal
UV18	2 d	P	68	9.7×10^{10}	–	–	150	yes	normal	normal
UV19	died 16 h	cord; IH + P x 2 failed	71	1.5×10^{11}	+	–	300	no	cord + placental haemorrhage	cord haemorrhage
UV21*	2 d	IH	71	1.7×10^{11}	–	+	350	yes	normal	normal
UV22*	2 d	IH	71	1.7×10^{11}	–	+	350	yes	injection site	normal
UV23	2 d	IH; P x 2 failed	74	4.3×10^{10}	–	+	500	no	injection site blood clot	normal
UV24	7d	IH	76	3.2×10^{9}	–	–	4000	yes	IP adhesion	normal
UV14	2 d	IH	83	2.5×10^{10}	–	–	100	yes	normal	normal
UV28	2 d	IH	87	1.1×10^{11}	–	+	500	yes	normal	normal
UV25*	died 24 h	IH	95	5×10^{12}	–	–	1000	yes	injection site	autolysed liver
UV26*	2 d	IH	95	2.7×10^{12}	–	–	1000	yes	injection site blood clot	normal
UV27	2 d	IH	95	8.5×10^{10}	–	–	500	yes	injection site blood clot	liver haemorrhage

In the second fetus (UV23) the intrahepatic UV was accessed and fetal blood was withdrawn. We were unsure however, that the needle tip was correctly placed within the UV and injection of PBS (100μl) went into the hepatic parenchyma. The extravascular

PBS and resulting narrowing of the intrahepatic UV obscured the view. At the placental insertion blood flow was detectable within the UV by Doppler examination. As the needle tip was advanced into the vessel, it slipped away and despite attempts at three different positions along the vessel, no blood was withdrawn. At a final injection attempt into the intrahepatic UV blood was withdrawn but no microbubbles were seen during injection of the viral vector.

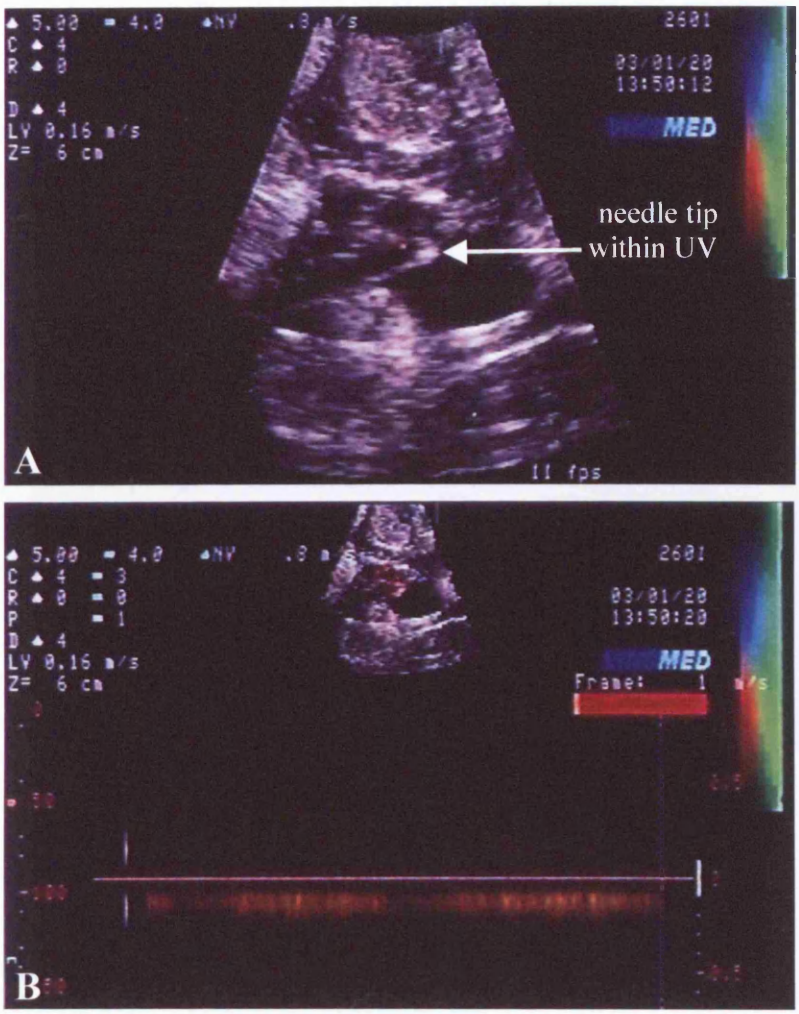


Figure D 13: Injection of the UV at the placental cord insertion in a sheep fetus.

In a fetus aged 68 days of gestation (UV18) the sonograms show (A) the tip of a 22 Gauge spinal needle within the UV and (B) Doppler examination of the injected vessel showing non-pulsatile blood flow, confirming this is the UV prior to injection of the adenovirus vector.

There were 2 fetal deaths (mortality rate 15%), one as previously described. The other was of a twin fetus (UV25) in which no heartbeat was detected 24 hours after straightforward intrahepatic UV injection and no analysis for transgene expression was performed. Its cotwin was alive (UV26) and therefore post mortem examination was

deferred until 48 hours after injection to allow for maximal transgene expression. There was no haemorrhage or injection site visible at post mortem examination and microbiological culture of fetal tissues was negative. Because of the delay in post mortem examination the liver was autolysed and this prevented useful histological analysis. The cause of death was thought to be the inadvertently high dose of vector (5 x 10^{12} p/kg) that this fetus received because of its unexpected small size.

Post mortem examination of the remaining fetuses showed minor abnormal findings (**Table D 6**). The injection site was visible on the fetal liver surface in 5 fetuses (**Figure D 14 A**) and a small intraperitoneal blood clot was seen at the site of liver injection in 3 fetuses (**Figure D 14 B**). Histological examination of the central liver lobe of one fetus (UV27) 2 days after injection showed haemorrhage (**Figure D 14 C**).

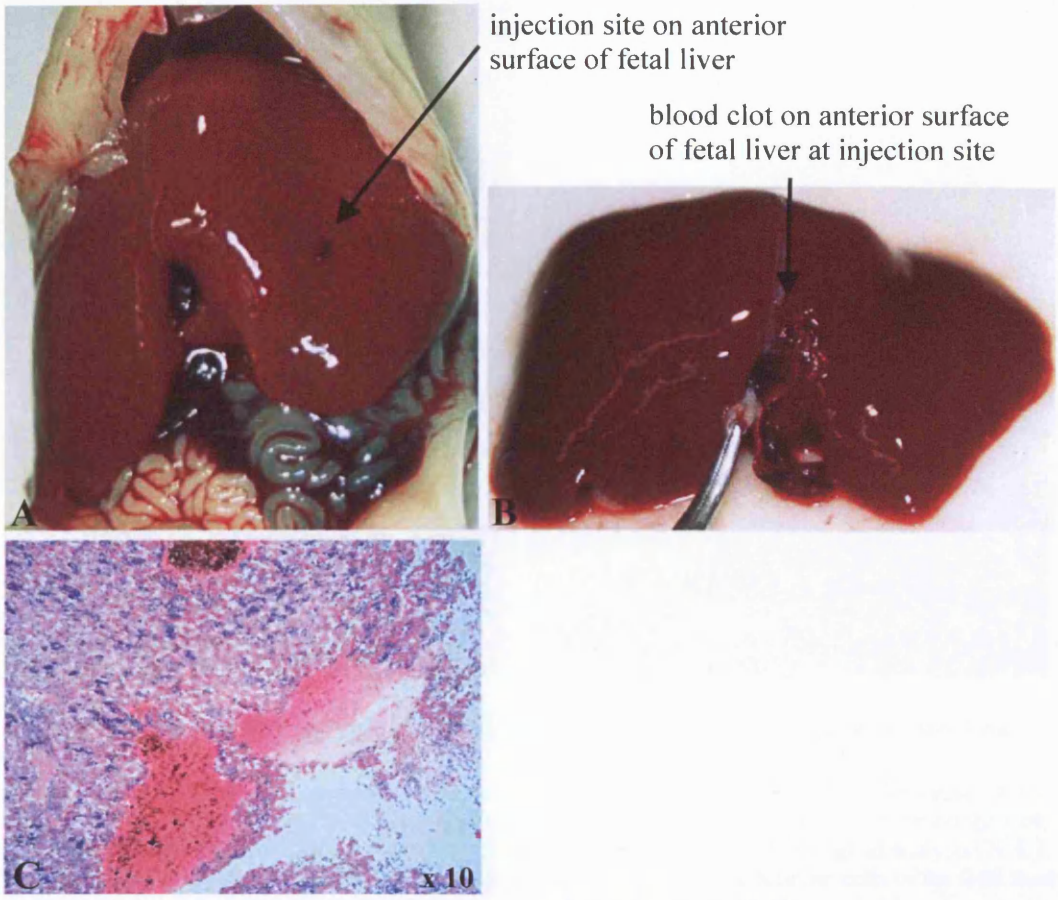


Figure D 14: Post mortem and histological findings after intrahepatic UV injection.

Two days after receiving intrahepatic UV injection of adhFIX vector (8.5 x 10^{10} – 2.7 x 10^{12} p/kg), on the anterior surface of the fetal liver (A) the injection site is visible after injection at 71 days of gestation (UV22) and (B) a small intraperitoneal blood clot is adherent to the injection site after injection at 95 days of gestation (UV26). (C) Histological examination (H & E stain) shows haemorrhage in the central liver lobe 2 days after intrahepatic UV injection at 95 days of gestation (UV27).

Histology of the remaining fetal tissues 2 days after injection of adenovirus was normal. A small peritoneal adhesion was present in the fetus examined 7 days after intrahepatic UV injection of retrovirus vector (UV24).

Post mortem analysis of the fetus that received intrahepatic UV injection of colloidal carbon (UV20) revealed carbon deposits in the liver (**Figure D 15 A**), peritoneal cavity, thoracic duct and inferior vena cava. Histological section showed carbon was present in the Kupffer cells of the liver (**Figure D 15 B**) and in a lymph node adjacent to the thymus on histological examination (**Figure D 15 C**). This demonstrates that there was broad haematogenic spread of colloidal carbon following UV injection.

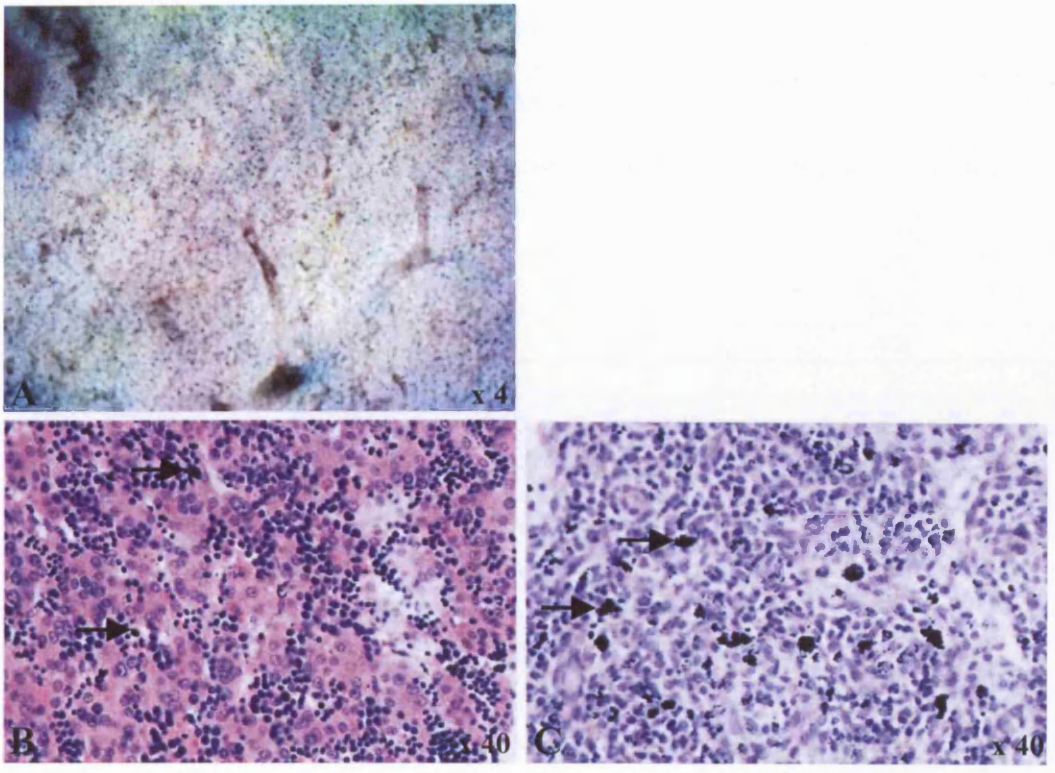


Figure D 15: Systemic spread of colloidal carbon after umbilical vein injection.

Post mortem and histological analysis of fetal tissues 2 days after UV injection of adhFIX vector (4.4 x 10^{11} p/kg) and colloidal carbon in a sheep fetus aged 67 days of gestation. Colloidal carbon can be seen (A) throughout the fetal liver parenchyma on post mortem analysis, and on histological analysis (H & E stain) black deposits of colloidal carbon (arrowed) are seen (B) within the Kupffer cells of the fetal liver and (C) within a thymic lymph node. Original magnifications are as indicated.

D 1.5.3 Ultrasound guided umbilical vein injection at or after 67 days of gestation does not result in significant narrowing

The UV diameter at the site of injection was measured before and after injection (**Table D 7**). The change in the UV diameter ranged from a slight increase (n = 3), no change (n = 5) to a reduction (n = 4) and was not statistically significant (one tailed, paired students *t* test, p = 0.09). The different effects of injection on the UV diameter at 60 days and from 67 days of gestation is probably due to the larger fetal size and the UV being less fragile that would reduce the impact of bleeding and fluid extravasation.

Table D 7: Change in the UV diameter after ultrasound-guided injection in fetal sheep from 67 days of gestation.

* denotes twin fetus; GA: gestational age; d: days; IH: injection of the intrahepatic UV; cord: injection of the UV in the umbilical cord; P: injection of the placental cord insertion.

Sheep	GA (d)	Injection route	UV diameter (mm)		change in UV diameter
			before	after	
UV20	67	IH	2.3	2.4	+ 4%
UV17	68	IH	2.6	2.2	-15%
UV18	68	P	2.9	2.5	-14%
UV19	71	cord	2.8	2.7	-4%
UV21*	71	IH	2.0	2.0	0
UV22*	71	IH	2.7	2.7	0
UV23	74	IH	2.7	2.7	0
UV24	76	IH	3.1	3.2	+3%
UV28	87	IH	4.2	4.3	+2%
UV25*	95	IH	4.7	4.7	0
UV26*	95	IH	4.1	4.1	0
UV27	95	IH	5.1	4.1	-20%

D 1.5.4 Transgene expression after umbilical vein injection of adenovirus vectors at or after 67 days of gestation

Fetal and maternal tissues were tested for expression of the lacZ transgene 48 hours after UV injection of adlacZ vector. Very little positive staining was detectable (**Table D 8**), particularly at earlier gestations. Two preparations of adlacZ vector were used in these experiments. The first preparation (UV17-UV22) was simultaneously used in fetal sheep gastric injection experiments in which strong lacZ transgene expression was detectable. In two cases (UV17 and UV18), a diluted aliquot of the vector was made to reach the correct concentration for injection. Following UV injection, titration of this diluted aliquot by OD_{260nm}, was found to be negative, although the OD_{260nm} of the

undiluted vector preparation following manufacture had measured 1 x 10^{13} p/ml. These fetuses received a slightly lower vector dose (8.2 –9.7 x 10^{10}p/kg) than we had planned (1 x 10^{11} p/kg) because their weight was greater than we had estimated by ultrasound biometric examination, although the difference is small. Two further fetuses received this vector (UV21 and UV22) but a fresh dilution of the original vector preparation was used. In all four fetuses, no transgene expression was detectable and we were concerned that the vector preparation might be degrading with age.

A second fresh adlacZ vector preparation of lower titre (2 x 10^{11} p/ml) was used for experiment UV23 (4.3 x 10^{10} p/kg complexed with DEAE dextran 5μg/ml)) because of concerns with the first preparation but no transgene expression was detectable. It became apparent from simultaneous adult mouse experiments that this fresh preparation was not functional *in vivo* despite showing activity *in vitro*. Subsequent testing of the first adlacZ vector preparation showed it still to have good activity *in vitro* and in *in vivo* adult mouse experiments, and it was used therefore for experiments UV25 - UV28.

Table D 8: Transgene expression 2 days after ultrasound-guided UV injection of adenovirus vectors.

* denotes twin fetus; GA: gestational age; d: days; good: vector shown to be active *in vivo* in adult mice or fetal sheep gut; *poor*: vector inactive *in vivo* or in experimental aliquot; C: colloidal carbon; D: DEAE dextran (5μg/ml); + to (+) indicates degree of transduction observed after X gal staining or β-galactosidase immunohistochemistry. (+) indicates single positive cells or sparse staining; – indicates no staining; nt: not tested

Sheep	sampling	GA (d)	Dose (p/kg)	Vector quality	C	D	X gal or hFIX expression	β-gal immuno
UV20	2 d	67	4.4 x 10^{11}	good	+	–	(+) UV in cord	–
UV17	2 d	68	8.2 x 10^{10}	*poor*	–	–	–	–
UV18	2 d	68	9.7 x 10^{10}	*poor*	–	–	–	–
UV21*	2 d	71	1.7 x 10^{11}	good	–	+	–	–
UV22*	2 d	71	1.7 x 10^{11}	good	–	+	–	–
UV23	2 d	74	4.3 x 10^{10}	*poor*	–	+	–	–
UV14	2 d	83	2.5 x 10^{10}	good	–	–	0% hFIX	nt
UV28	2 d	87	1.1 x 10^{11}	good	–	+	–	–
UV26*	2 d	95	2.7 x 10^{12}	good	–	–	+ small bowel, duodenum & caecum	(+) right liver
UV27	2 d	95	8.5 x 10^{10}	good	–	–	+ bladder, surface of gonad, thymus, adrenal, brain	–

Injection of the intrahepatic UV at 67 days of gestation with adlacZ (UV20, 4.4 x 10^{11} p/kg) following sodium caprate pretreatment, resulted in positive β-galactosidase expression limited to the UV in the cord (**Figure D 16 A**) despite post mortem and histological evidence of widespread distribution of colloidal carbon via this injection route. Immunohistochemical analysis for β-galactosidase expression in the UV was negative; this could have been due to the effect of different sampling sites for X gal staining and β-galactosidase immunohistochemistry tissue analysis. The vector quality was good since it was the same batch as that used in gastric injection experiments. Biologically active vector was also used in experiments UV21, UV22 and UV28 (1.1 – 1.7 x 10^{11} p/kg), in which the vector was complexed with DEAE dextran (5μg/ml) but there was no transgene expression detectable.

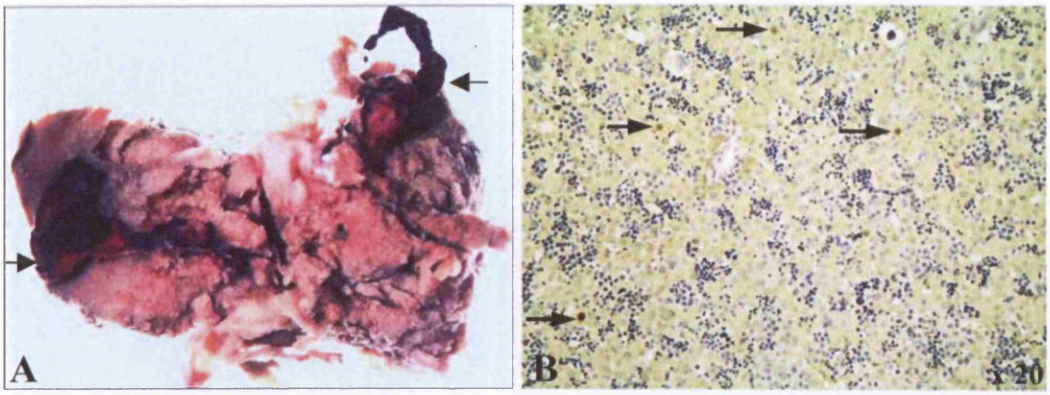


Figure D 16: β-galactosidase transgene expression 2 days after umbilical vein injection of adlacZ vector.

After injection of adlacZ vector (4.4 x 10^{11} and 2.7 x 10^{12} p/kg) there is (A) positive X gal staining (arrowed) of the umbilical vein within the umbilical cord of a fetus aged 67 days of gestation (UV20). (B) Scanty positive expression (arrowed) is seen in the hepatocytes on β-galactosidase immunohistochemical analysis of the right lobe of the liver of a fetus aged 95 days of gestation (UV26).

The two fetuses injected at the latest gestational age (95 days) showed low level β-galactosidase expression. In one fetus (UV26, 2.7 x 10^{12} p/kg) immunohistochemical analysis of the right lobe of the liver showed occasional positive β-galactosidase expression in cells (**Figure D 16 B**). There were occasional positively stained villi in the fetal small bowel following X-gal staining which was not confirmed on β-galactosidase immunohistochemistry (data not shown). Positively X-gal stained cells were also detected within the bladder, thymus, brain, adrenal and on the surface of the fetal gonad

(UV27, 8.5 x 10^{10} p/kg, **Figure D 17**) although immunohistochemical analysis of these tissues was negative.

In conclusion we observed variable and only low level transgene expression after umbilical vein injection of adlacZ vectors. The experiments were complicated by the difficulties we experienced with the vector efficacy. It is of note that X gal staining was seen in the two fetuses that received the highest adlacZ vector doses, one of which also received sodium caprate pretreatment.

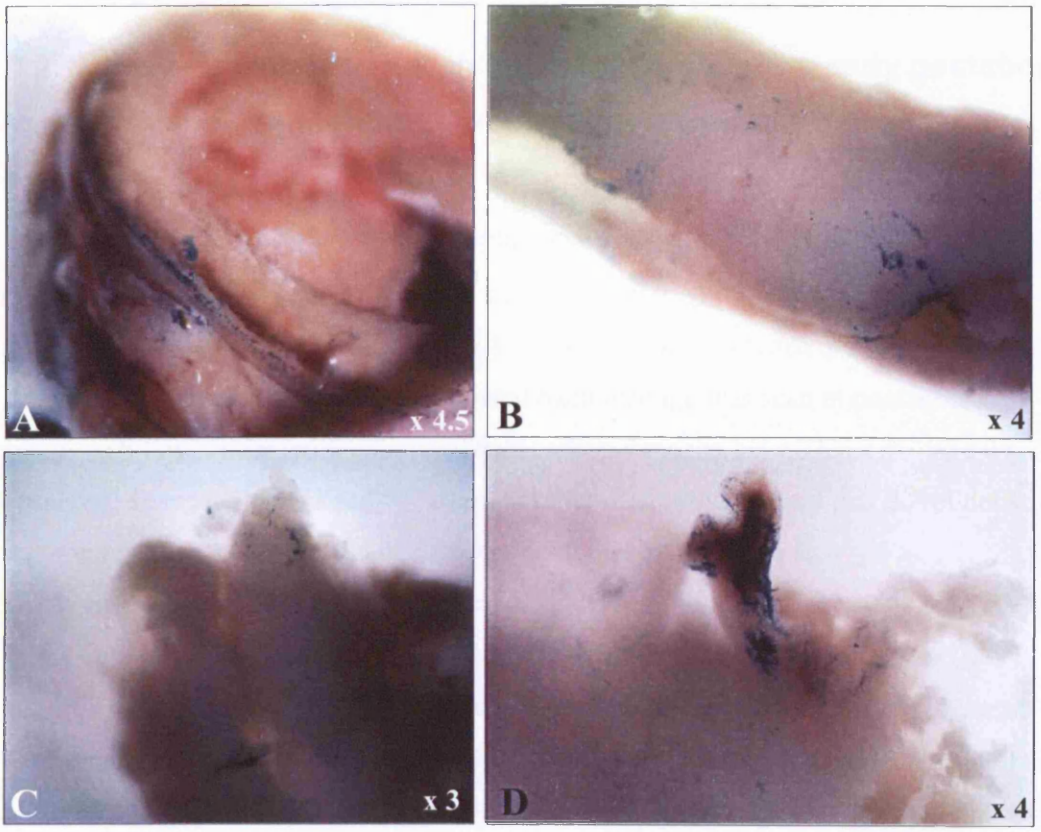


Figure D 17: X gal staining of fetal tissues after injection of adlacZ vector into the umbilical vein.

After UV injection of adlacZ vector (8.5 x 10^{10} p/kg) of a fetal sheep aged 95 days of gestation (UV27) there is scanty positive staining in the fetal adrenal (A), bladder lumen (B), thymus (C) and the serosal surface of the gonad (D). Original magnifications are as shown.

D 2 Other routes of vector administration in early gestation: intrahepatic injection

Our limited success in delivering adenovirus vectors directly to the fetal circulation of early gestation sheep fetuses prompted us to explore alternative routes of vector administration. Hepatocytes have been successfully targeted in fetal mice by direct intrahepatic injection (Lipshutz GS et al., 1999b,Lipshutz GS et al., 1999a) and some spread to other organs and tissues was observed.

D 2.1 Ultrasound guided intrahepatic injection in the early gestation sheep fetus is a straightforward procedure with low morbidity and mortality

We began to investigate this route of application by injecting colloidal carbon marker dye (200µl) into the fetal liver at 54 days of gestation (n = 1, **Table D 9**) to test the procedure related morbidity and mortality and to look for evidence of systemic spread from this injection route. A small peritoneal haemorrhage was seen at post mortem analysis and light microscopy showed local carbon deposits but no liver damage was observed. There was no spread of carbon to other organs suggesting that direct delivery into the liver at this gestational age did not achieve systemic spread.

A low and a high dose (HE_L, 2.9 – 8.6 x 10^{10} p/kg and HE_H, 1.8- 3.2 x 10^{13} p/kg, **Table D 9**) of adenovirus vector were then used in this set of experiments on fetuses (n = 11) at 52-57 days of gestation to test whether gene transfer could be achieved. The mean time for injection was 7 min 34 sec (± SD 2 min 24 sec, range 4 min to 12 min) and all injections were successful at the first attempt. We observed echogenicity of the hepatic parenchyma confirming correct injection placement (**Figure D 18**).

Fetal survival was 81%. Two fetal deaths were in twin fetuses injected with low dose adhFIX vector that aborted 48 hours after injection. This was most likely due to a procedure related extensive bacterial infection that was detected by histological analysis of fetal and placental tissues and confirmed as Escherichia coli by culture.

Post mortem examination of fetuses 2 days after HE_L adenovirus vector injection was normal (**Table D 9**). Injection of HE_L adlacZ (n = 2) resulted in central necrosis at the site of vector administration observed by light microscopy of the fetal liver in one fetus (**Figure D 19 A**).

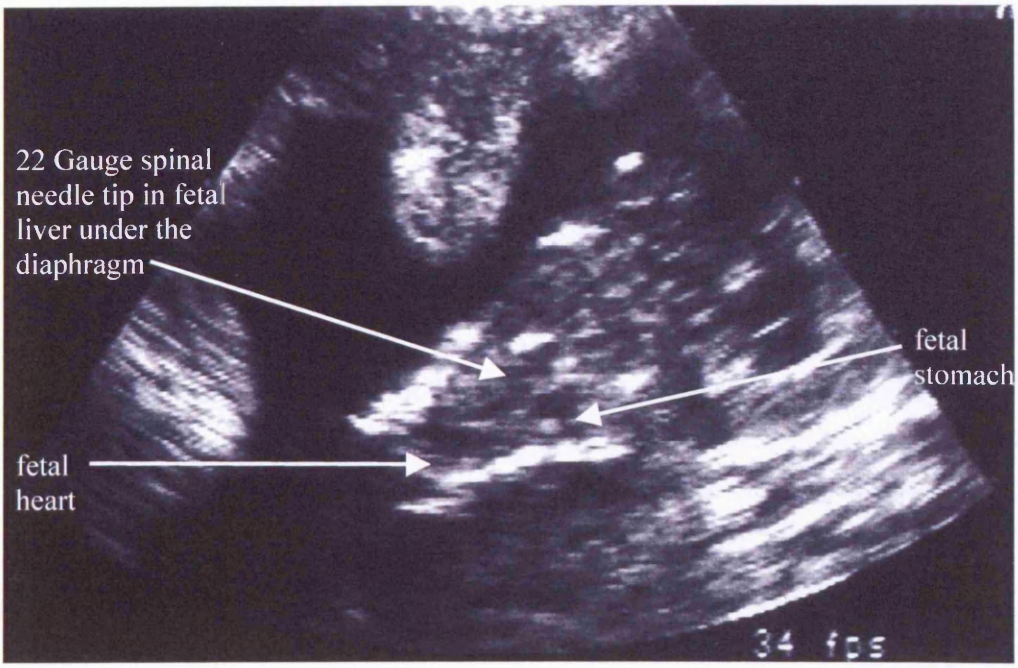


Figure D 18: Ultrasound guided intrahepatic injection.

The ultrasonogram shows intrahepatic injection of adhFIX to a sheep fetus at 53 days of gestation. The fetus is in longitudinal section and the needle passes up into the liver parenchyma on the right side of the fetal abdomen under the diaphragm.

Table D 9: Post mortem and histological findings after ultrasound-guided intrahepatic injection of adenovirus vectors or colloidal carbon to early gestation sheep.

d: days; h: hours; m: months; Vol: volume of vector, *: twin fetuses; PM: postmortem; IP: intraperitoneal.

Sheep	sampling	GA (d)	Vector	Vol (µl)	Dose (p/kg)	PM findings	Histological findings
HE12	2d	54	carbon	200	–	IP clot	normal
Low dose vector (HE_L)							
HE11	2d	52	adlacZ	100	3.5×10^{10}	normal	liver necrosis
HE8	2d	53	adlacZ	100	7.0×10^{10}	normal	normal
HE6*	died 24 h	54	adhFIX	50	5.7×10^{10}	aborted	bacteria
HE7*	died 24 h	54	adhFIX	100	2.9×10^{10}	aborted	bacteria
HE9*	2d	53	adhFIX	100	8.6×10^{10}	normal	normal
HE10*	2d	53	adhFIX	100	8.0×10^{10}	normal	normal
High dose vector (HE_H)							
HE2	2d	54	adlacZ	200	1.8×10^{13}	liver bruising	liver haemorrhage + necrosis
HE3	2d	52	adhFIX	200	3.2×10^{13}	liver bruising	normal
HE4	9d	57	adhFIX	200	2.0×10^{13}	normal	normal
HE5	40 m	57	adhFIX	200	2.0×10^{13}	normal	mild portal chronic inflammation

We had observed that an adenovirus dose of 10^{13} p/kg was tolerated when injected into the fetal muscle and therefore next applied this high dose to the fetal liver (n = 4). To achieve delivery of a higher dose of adenovirus vector, we increased the volume injected from 100 to 200 µl but we did not observe any associated complications. At post mortem analysis there was bruising of the anterior abdominal wall and liver in two animals sampled 2 days after injection but no evidence of the injection site was detectable in the animal sampled 9 days after injection. Histological analysis of the central liver showed necrosis around an area of haemorrhage at the site of vector administration in one animal (**Figure 19 B,** HE2). Neither macroscopy nor histology of the two fetuses injected with high dose adhFIX vector showed evidence of hepatic trauma, necrosis or inflammation when analysed 2 days and 9 days after injection although this was possibly due to not sampling exactly at the site of injection.

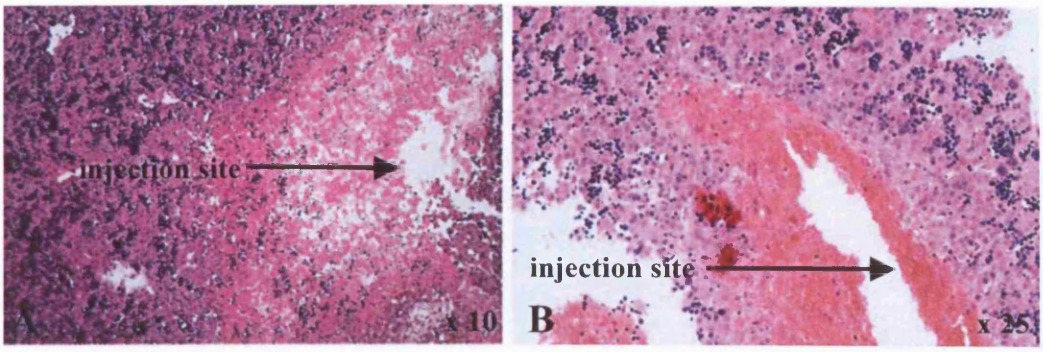


Figure D 19: Histological analysis of the liver after intrahepatic adlacZ vector injection.

Two days after ultrasound-guided intrahepatic delivery of adlacZ vector to two fetal sheep at 52 days of gestation, light microscopy (H & E stain) shows necrosis around an area of haemorrhage at the site of vector administration. The dose of vector delivered was (A) low (3.5 x 10^{10}p/kg, HE11) and (B) high (1.8 x 10^{13} p/kg, HE2). Original magnifications are as indicated.

Post mortem and histological examination of the animal that came to birth following early gestation intrahepatic injection of adhFIX (HE5) was performed at 40 months of age. This showed mild chronic portal inflammation that was a common finding in the liver of many of the ewes.

To conclude, intrahepatic injection was technically much easier than intravascular injection because of the larger target, and it had a low mortality rate. Adenovirus is known to be hepatotoxic and therefore the morbidity we observed could have been related to delivery of the adenovirus vector itself rather than the injection technique.

D 2.2 Intrahepatic delivery of adenovirus vectors in early gestation fetal sheep does not result in good transgene expression

Transgene expression was only detectable in one fetus receiving the low dose of adenovirus vector (**Table D 10**) where immunohistochemical analysis for β-galactosidase 2 days after adlacZ vector injection showed positively stained hepatocytes around an area of necrosis (**Figure D 21 A**). X gal staining was negative and there was no detectable transgene expression in the remaining fetal tissues.

Table D 10: Transgene expression after ultrasound-guided intrahepatic injection of adenovirus vectors in early gestation fetal sheep.

* denotes twin fetus; GA: gestational age; d: days; ++ to (+) indicates degree of transduction observed after X gal staining or β-galactosidase immunohistochemistry. (+) indicates single positive cells or sparse staining; – indicates no staining; nt: not tested

Sheep	Vector	Sampling (d)	Dose (p/kg)	X gal staining or hFIX plasma level	β-gal or hFIX immuno-histochemistry
Low dose vector (HE_L)					
HE11	adlacZ	2	3.5×10^{10}	–	++ central liver
HE8	adlacZ	2	7.0×10^{10}	–	–
HE9*	adhFIX	2	8.6×10^{10}	0%	nt
HE10*	adhFIX	2	8.0×10^{10}	0%	nt
High dose vector (HE_H)					
HE2	adlacZ	2	1.8×10^{13}	–	(+)
HE3	adhFIX	2	3.2×10^{13}	30 ng/ml (0.6%)	–
HE4	adhFIX	9	2.0×10^{13}	0%	nt
HE5	adhFIX	birth	2.0×10^{13}	7 ng/ml (0.14 %)	nt

D 2.3 Increasing the dose of adenovirus vector does not improve transgene expression following intrahepatic injection.

We increased the dose of adenovirus vector delivered in an attempt to increase transgene expression (**Table D 10**). The animal sampled 2 days after injection of high dose adhFIX showed only low levels of hFIX at 30ng/ml (0.6%) and no hFIX was detectable in the animal sampled 9 days after injection (**Figure D 20**). The lamb born after *in utero* therapy expressed very low levels of hFIX at the limit of reliable detection of the assay (7ng/ml, (0.14%) at birth, 6.5ng/ml (0.13%) at 8 weeks and 12ng/ml (0.24%) at 16 weeks postnatal. No hFIX was found by 6 months of age (**Figure D 20**).

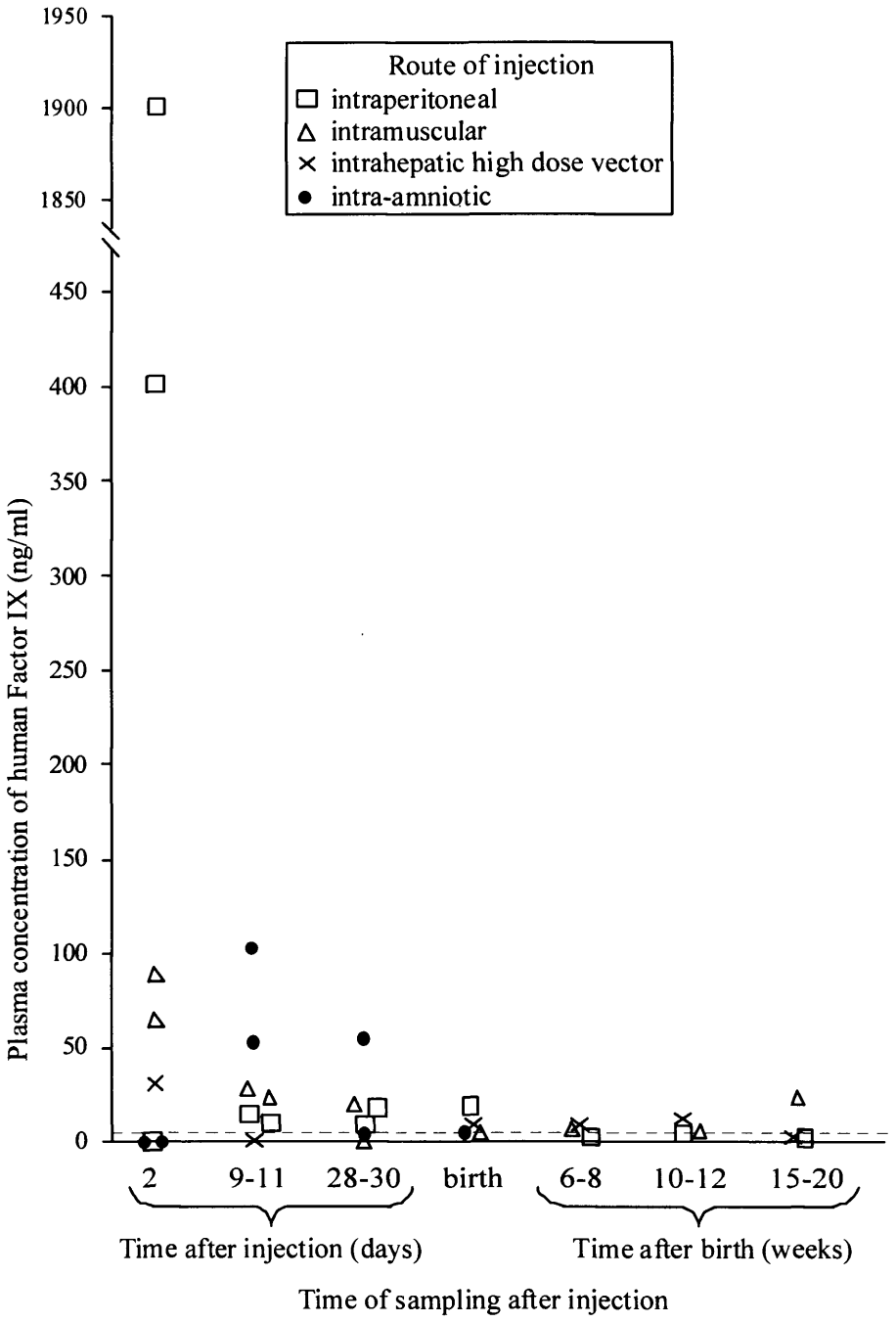


Figure D 20: Expression of hFIX transgene after ultrasound guided delivery of adhFIX vector to early gestation fetal sheep.

Sheep fetuses aged 33-60 days of gestation were delivered adhFIX vector via intrahepatic ($1.8 - 3.2 \times 10^{13}$ p/kg), intraperitoneal ($8.2 \times 10^{11} - 5.3 \times 10^{12}$ p/kg), intramuscular ($6.8 \times 10^{11} - 3 \times 10^{13}$ p/kg) or intra-amniotic ($1 - 4.5 \times 10^{13}$ p/kg) injection. Concentrations of human factor IX in fetal or lamb plasma were determined by ELISA analysis. Fetal samples were collected at postmortem. The dotted line represents the limit of reliable detection by the assay (5 ng/ml).

A single β-galactosidase expressing hepatocyte was detected surrounding an area of necrosis on immunohistochemical analysis 2 days after high dose adlacZ injection (**Figure D 21 B**) although X gal staining was negative.

In conclusion we found very low levels of gene transfer after intrahepatic injection of low or high dose adenovirus vectors suggesting this route of injection cannot be used as an alternative to intravascular delivery to achieve systemic gene transfer in early gestation fetal sheep.

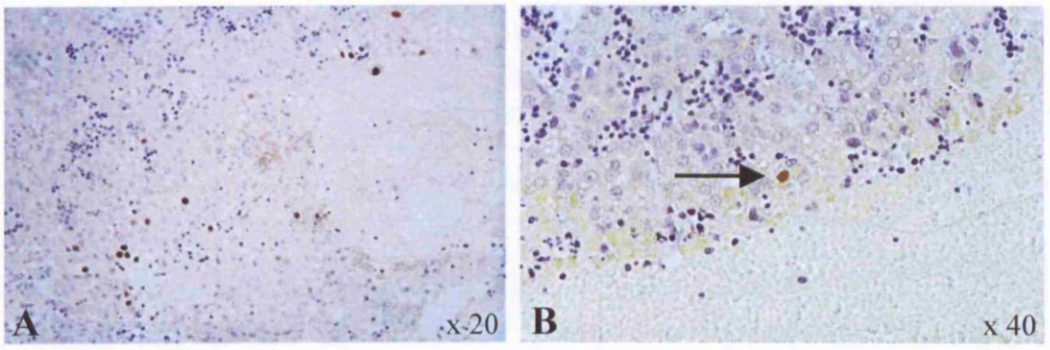


Figure D 21: β-galactosidase transgene expression after ultrasound-guided intrahepatic injection.

Two days after ultrasound guided intrahepatic delivery of adlacZ vector to two fetal sheep at 52 days of gestation, β-galactosidase immunohistochemical analysis shows (A) a few β-galactosidase expressing hepatocytes following low dose adlacZ injection (3.5×10^{10}p/kg, HE11) and (B) a single β-galactosidase expressing hepatocyte after high dose adlacZ injection (1.8×10^{13} p/kg, HE2) surrounding an area of central necrosis. Original magnifications are as indicated.

D 2.4 Intrahepatic injection of adenovirus vectors did not achieve significant vector spread to other fetal tissues

To test if the injury to the liver parenchyma would have inhibited or promoted the escape of vector into the general circulation via the hepatic sinus we studied its biodistribution in two animals by sensitive PCR analysis of various fetal tissues. We investigated the presence of adlacZ vector in the fetus that had received high dose vector (HE2) and in which a single positive β-galactosidase expressing hepatocyte was observed. AdlacZ vector was not detected in any fetal or maternal tissues investigated 2 days after injection (**Table D 11**, gel electrophoresis not shown).

Table D 11: Vector spread after delivery of adenovirus vectors to fetal sheep in early gestation.

Transgenic hFIX (Table A) or adenovirus lacZ vector (adlacZ, Table B) was detected in fetal and maternal tissues (n = 7) by PCR analysis after intrahepatic (HE), intraperitoneal (IP) or intramuscular (IM) injection of 7 fetal sheep sacrificed 48 hours after injection. Tissues that tested positive on 1st round PCR analysis (1st) were not generally subjected to nested PCR analysis (2nd). n: not available; m denotes maternal tissues; *indicates tissue from experiment IM1.

A: hFIX	HE (HE3)		IP (IP9)		IM (IM 1* or IM2)	
Tissue	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**
Liver	+	+	+		+	
	$+^{m}$		$-^{m}$	$+^{m}$	$+^{m}$	
Gonad	–	–	+		–	–
	$-^{m}$	$-^{m}$	–	$+^{m}$	$-^{m*}$	$-^{m*}$
Lung	–	–	+		+	
	$-^{m}$	$-^{m}$	$+^{m}$		$-^{m}$	$-^{m}$
Placenta	–	–	–	+	–	+
Umbilical cord	–	–	+		n	
Thymus	–	–	+		+	
Heart	+	+	+		+	
Spleen	–	–	+		+	
Small bowel	–	–	+		n	
Adrenal	–	–	+		$+^{*}$	
Kidney	–	–	+		–	–
Skin	n		+		+	
Muscle	n		n		+	
CNS cortex	–	–	–	–	$-^{*}$	$-^{*}$

B: adlacZ	HE (HE2)		IP (IP3)		IM (IM 5)	
Tissue	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**
Liver	–	–	+		+	
	$-^{m}$	$-^{m}$	$-^{m}$	$+^{m}$	–	$-^{m}$
Gonad	–	–	–	+	–	+
	$-^{m}$	$-^{m}$	–	$+^{m}$	$-^{m}$	$-^{m}$
Lung	–	–	+	+	–	+
	$-^{m}$	$-^{m}$	$-^{m}$	$-^{m}$	$-^{m}$	$-^{m}$
Placenta	–	–	–	+	–	+
Umbilical cord	–	–	+	+	n	
Thymus	–	–	n		–	+
Heart	–	–	–	+	–	+
Spleen	–	–	n		+	+
Small bowel	–	–	+	+	n	
Adrenal	–	–	–	–	–	+
Kidney	–	–	–	+	–	+
Skin	n		–	+	+	+
Muscle	n		n		+	+
CNS cortex	–	–	–	+	–	–

It is possible that the liver tissue used in the PCR analysis was not taken from the injection site since each tissue was split into three sections at post mortem for X gal staining, immunohistochemistry and PCR analysis. We therefore investigated spread of the adhFIX vector in the animal that showed low level expression of hFIX 2 days after injection of high dose adhFIX (HE3). The hFIX transgene was detected in the fetal liver and heart, and in the maternal liver by 1st round PCR analysis. Nested PCR analysis revealed no further spread to any other fetal or maternal organs (**Table D 11**, gel electrophoresis not shown). The finding of vector in the maternal liver was surprising in view of the lack of vector spread to the majority of fetal tissues. It indicates that haematogenic spread occurred, perhaps at the time of injection via the placentome or uterus, although lacZ expression was not detected in any maternal tissues including the liver, heart, lung or gonad. We concluded that the high adenovirus dose of 10^{13} p/kg injected into the hepatic parenchyma might have caused the hepatic necrosis observed in one fetus and did not result in significant vector spread to other fetal tissues.

D 3 Other routes of vector administration in early gestation: intraperitoneal injection

Intraperitoneal injection has been used successfully as an indirect route to the circulation and the liver for the delivery of fetal haematopoietic cells (Touraine, 1999) and for transduction of hematopoietic precursor cells (Porada CD et al., 1998). Gene therapy studies have shown that this route gives access to the liver in fetal mice (Hatzoglou M et al., 1995,Lipshutz GS et al., 1999b) and fetal sheep (Tran ND et al., 2000), probably via the lymphatic system. We therefore tested this route in the fetal sheep as an alternative to intravascular delivery in early gestation.

D 3.1 Ultrasound guided intraperitoneal injection is a straightforward procedure in the early gestation sheep fetus

We first applied colloidal carbon marker dye (200µl) to the peritoneal cavity of a fetal sheep at 52 days of gestation to test the technical aspects of the procedure (**Table D12**). Light microscopy 2 days after injection showed a small blood clot in the peritoneal cavity and widespread carbon deposits in the glomeruli of the kidney, the liver sinusoids and spleen (**Figure D 22**).

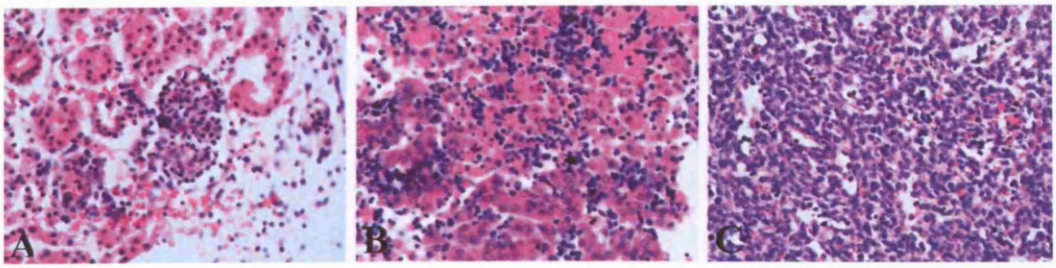


Figure D 22: Widespread deposition of carbon after ultrasound-guided intraperitoneal injection of colloidal carbon in early gestation.

Light microscopy (H & E stain) of a fetal sheep 2 days after intraperitoneal injection at 52 days of gestation shows carbon deposits in (A) the glomeruli of the fetal kidney, (B) the liver sinusoids and (C) the spleen. Original magnification was x 40 in all cases.

We then applied both lacZ and hFIX encoding adenoviruses at doses between 4.8 x 10^{11} and 1.1 x 10^{13} p/kg into the peritoneal cavity of sheep fetuses (n = 15) at 40-56 days of gestation (**Table D 12, Figure D23**).

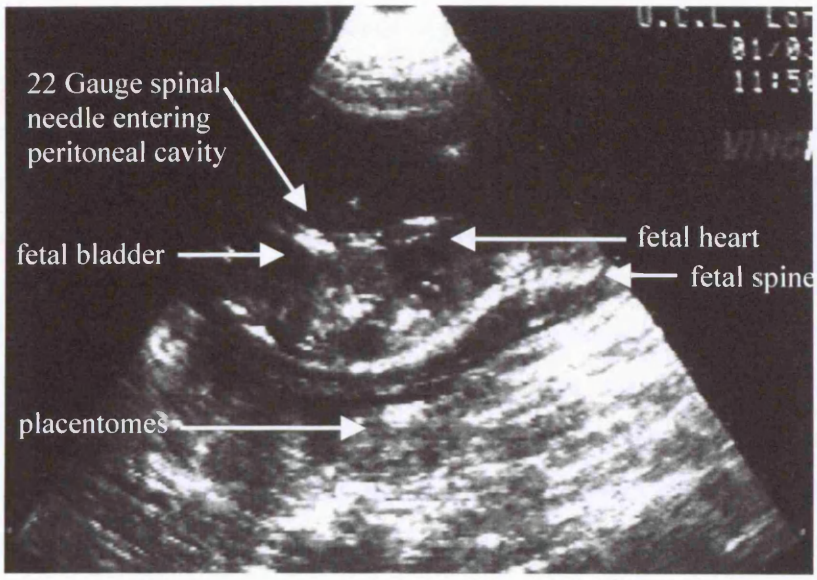


Figure D 23: Ultrasonogram showing intraperitoneal delivery of adhFIX to an early gestation sheep fetus.

The fetus (53 days of gestation) is in longitudinal section, with the 22 Gauge needle entering the peritoneal cavity just superior to the fetal bladder.

Three fetuses in this series of experiments were found to be unexpectedly 2 weeks younger in their gestational age at operation due to an error in tupping dates (40 days instead of 54 days of gestation). Fetal injection was still performed, although in one fetus, review of the videotape recording after the intervention showed the needle

passing beyond the fetal peritoneal cavity and the vector was actually delivered into the amniotic cavity. Results from this fetus (IP1) and its twin that received a poor stock of adhFIX vector (IP2) are not reported further. After these initial difficulties, the technique was as simple to perform as intrahepatic injection and the procedure was successful at the first attempt. The needle position could be confirmed by the observation of microbubbles in the injected fluid moving within the peritoneal cavity. In one case the procedure took over 20 minutes due to difficult fetal lie and inexperience of the operator. Excluding this case, the mean time to successful injection was 3 min (± 1 min 39 sec, range 45 sec to 5 min 9 sec).

D 3.2 Ultrasound guided intraperitoneal injection has a low morbidity and mortality in the early gestation sheep fetus

Fetal survival was 80% (**Table D 12**). In two of the three fetuses that died 24 hours after injection (IP11 + IP14), post mortem analysis showed severe bleeding into the abdominal cavity and histological evidence of placental inflammation and bacterial colonization. Neither culture of the vector nor of the fetal tissues showed any infectious agent. We concluded that these fetal losses were a result of the procedure itself rather than vector related. The other fetal death (IP16) was most likely due to bacterial infection introduced at the time of injection, since there was no evidence of haemorrhage on post mortem or histological analysis and the culture of fetal tissues confirmed Bacillus licheniformis, a common fleece commensal.

Of those fetuses surviving the procedure, four (27%) showed evidence of peritoneal inflammation at post mortem or histological analysis. At two days after injection, one fetus (IP9) had focal fibrosis over the small bowel with moderate chronic inflammation that may have been associated with an intraperitoneal bleed. In one fetus analysed 29 days after injection (IP7) there were dense peritoneal adhesions at post mortem examination and fibrosis over the surface of the bowel, testes and heart on light microscopy. In its cotwin (IP6), the small bowel serosa was oedematous.

One of two sheep born after intraperitoneal injection of adhFIX had a marked kyphoscoliosis. Clinical examination and X-ray of the skeleton did not reveal any other abnormalities. By 6 months of age however, it was noted that the lamb had bilateral undescended testes. Kyphoscoliosis and undescended testis are both relatively common unrelated abnormalities in lambs. The rate of cryptorchidism is around 1% with a possible autosomal recessive basis (Jubb KVF et al., 1993) and kyphoscoliosis occurs in

approximately 0.5% of lambs (Dennis SM, 1993). It is possible that inflammation in the peritoneal cavity following intraperitoneal injection of adhFIX vector may have prevented the normal movement of the testis into the scrotal sac in late gestation, resulting in undescended testis. All other lambs have been normal.

Table D 12: Post mortem and histological findings following ultrasound-guided intraperitoneal injection of adenovirus vectors or colloidal carbon to early gestation sheep.

d: days; h: hours; m: months; Vol: volume of vector injected; *: twin fetuses; PM: postmortem; IP: intraperitoneal;

sheep	sampling	GA (d)	Vector	Vol (µl)	Dose (p/kg)	PM findings	Histological findings
IP18	2d	52	carbon	200	–	IP clot	carbon deposits
IP10	2d	40	adlacZ	100	1.1×10^{13}	normal	normal
IP3*	2d	52	adlacZ	500	1.5×10^{12}	bruised abdominal wall	normal
IP4*	2d	52	adlacZ	500	1.5×10^{12}	bruised abdominal wall	normal
IP11	died 24 h	55	adhFIX	100	1.5×10^{12}	IP haemorrhage	cocci in fetal tissues + placenta
IP14	died 24 h	54	adhFIX	100	2.7×10^{12}	IP haemorrhage	bacterial colonization of tissues
IP16	died 24 h	53	adhFIX	100	4.8×10^{11}	normal	bacilli in fetal tissues
IP9	2d	56	adhFIX	100	1.6×10^{12}	IP clot	focal fibrosis small bowel
IP17	2d	53	adhFIX	100	1.8×10^{12}	normal	normal
IP15	2d	53	adhFIX	100	8.2×10^{11}	normal	normal
IP12*	9d	55	adhFIX	100	1.0×10^{12}	normal	normal
IP13*	9d	55	adhFIX	100	1.0×10^{12}	normal	normal
IP6*	29d	54	adhFIX	100	1.0×10^{12}	normal	oedematous small bowel serosa
IP7*	29d	54	adhFIX	100	1.0×10^{12}	dense IP adhesions	mild fibrosis over heart, bowel + heart
IP5	33 m	54	adhFIX	250	5.3×10^{12}	dense peritoneal + moderate pleural adhesions	portal tract inflammation + lung fibrosis
IP8	33 m	54	adhFIX	100	1.0×10^{12}	mild perihepatic + pleural adhesions	liver fibrosis

The two sheep that received intraperitoneal injection of adhFIX were examined at post mortem nearly 3 years after birth. In one animal (IP5), dense peritoneal adhesions involving the omentum, bowel and liver made it difficult to identify the abdominal contents; in addition there were moderate pleural adhesions. Histological analysis showed mild chronic inflammation in the portal tract of the liver, mild lung fibrosis, enlarged para-splenic lymph nodes and fibrous thickening of the liver capsule which contained histologically foreign material that appeared to be of plant origin (**Figure D 24 A – E**). There are two possible sources of this material. It may have been introduced during postnatal life from a bowel perforation, however during postnatal life the lamb was well and showed no signs of peritonitis. Alternatively it may have been introduced into the fetal peritoneal cavity from the ewe's abdomen during vector injection.

Post mortem examination of the other sheep that came to birth (IP8) showed moderate perihepatic and pleural adhesions and there was a chronic infiltrate in the portal tracts of the liver on histological analysis. No plant material was identified in the fibrous tissue, although this may be due to sampling at the time of post mortem. A liver biopsy, taken from this lamb at 2 years of age to investigate hepatocellular dysfunction that had been diagnosed on abnormal serum liver function tests and bile acid levels (see **section D 5**), showed a chronic infiltrate in the portal tracts (**Figure D 24 F**). It is unlikely that the liver biopsy caused the peritoneal adhesions since it was straightforward and the animal made an uneventful recovery.

Mild chronic portal tract inflammation was a common finding on histological analysis of the liver of ewes. It should be noted that intraperitoneal adhesions have never been seen in any of the ewes at post mortem examination, and pleural adhesions were observed in only one ewe that developed pneumonia following general anaesthesia.

In the fetuses that were examined 2 or 9 days after injection, a small intraperitoneal blood clot or bruising of the anterior abdominal wall was found at post mortem analysis in four cases (27%). In conclusion intraperitoneal injection of adlacZ vectors was associated with some peritoneal pathology but this may be related to the particular vector applied rather than the route of injection itself.

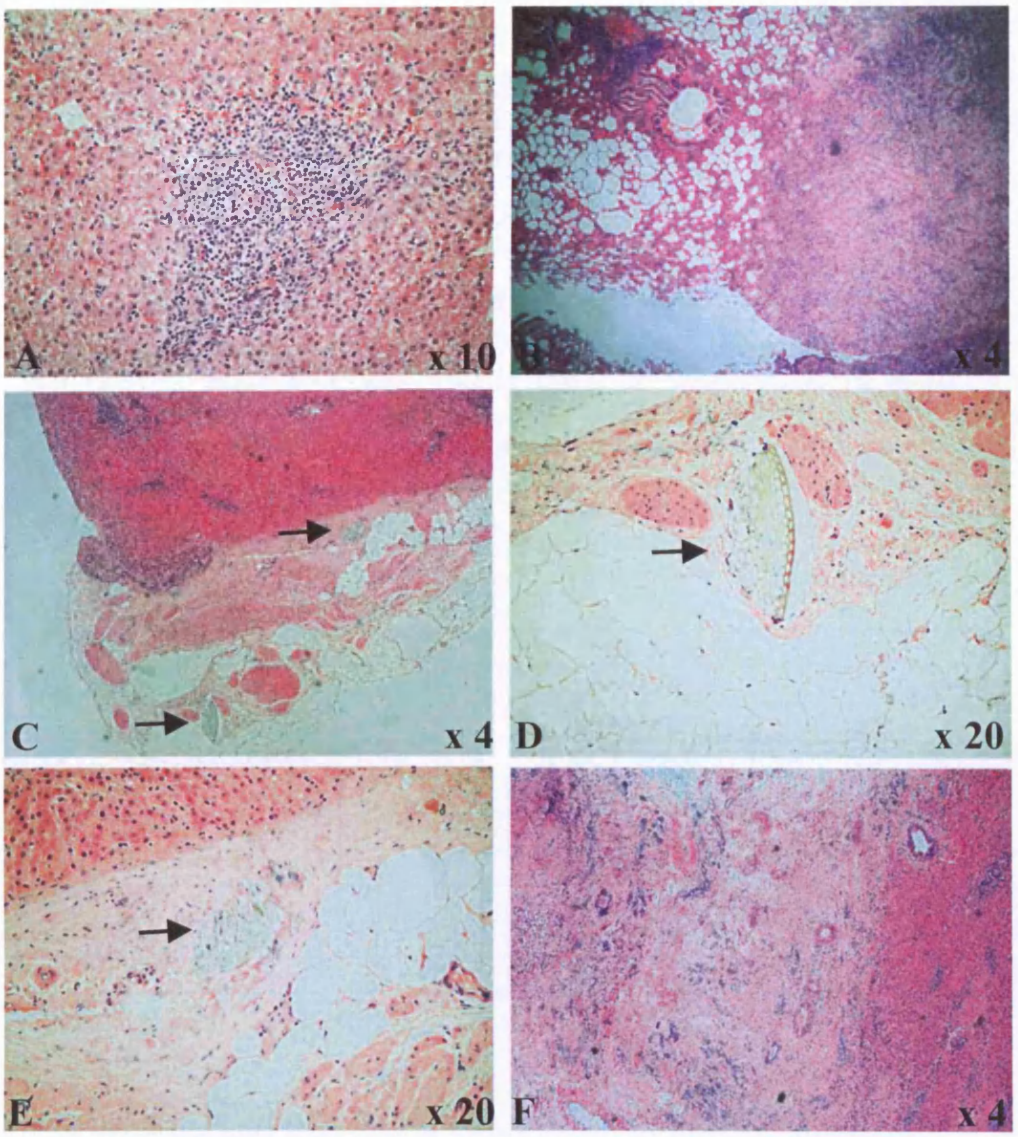


Figure D 24: Histological findings in adult sheep after early gestation intraperitoneal injection of adhFIX vector.

After intraperitoneal injection of adhFIX vector as fetuses, histological analysis (H & E stain) showed in one sheep (IP5) there was (A) chronic mild inflammation in the portal tract of the liver, (B) lung fibrosis surrounding a chronic abscess and (C) fibrous thickening of the liver capsule which contains foreign material (arrowed) that is of plant origin and shown enlarged in (D) and (E). (F) Liver biopsy from the other sheep (IP8) showed a mild chronic inflammatory infiltrate in the portal tracts. Original magnifications are as indicated.

D 3.3 Therapeutic levels of hFIX are observed after early gestation ultrasound-guided intraperitoneal injection

Therapeutic levels of hFIX were detected in the plasma of two fetuses sampled 2 days after injection (IP9 and IP17, 400 and 1900 ng/ml respectively, **Table D 13, Figure D 20**). By 9 and 29 days after injection hFIX levels were still detectable (8.5 – 18 ng/ml)

but below the therapeutic range (50 ng/ml). One animal showed a hFIX level of 16.5 ng/ml at birth (**Figure D 20**) but this fell to undetectable levels from 5 months of age. In a second animal, allowed to deliver at term, no hFIX was observed 5 months after birth (the samples from birth until this time point were lost).

Table D 13: Transgene expression after ultrasound-guided intraperitoneal injection of adenovirus vectors in early gestation fetal sheep.

* denotes twin fetus; GA: gestational age; d: days; ++ to +++ indicates degree of transduction observed after X gal staining or β-galactosidase immunohistochemistry; – indicates no staining; nt: not tested

sheep	PM	GA (d)	Vector	Dose (p/kg)	X gal staining or hFIX plasma level ng/ml (%)	β-gal or hFIX immuno-histochemistry
IP10	2d	40	adlacZ	1.1×10^{13}	nt	+++
IP3*	2d	52	adlacZ	1.5×10^{12}	nt	++
IP4*	2d	52	adlacZ	1.5×10^{12}	nt	–
IP9	2d	56	adhFIX	1.6×10^{12}	400 (8%)	nt
IP17	2d	53	adhFIX	1.8×10^{12}	1900 (38%)	nt
IP15	2d	53	adhFIX	8.2×10^{11}	0	nt
IP12*	9d	55	adhFIX	1.0×10^{12}	9.5 (0.19%)	nt
IP13*	9d	55	adhFIX	1.0×10^{12}	14.5 (0.29%)	nt
IP6*	29d	54	adhFIX	1.0×10^{12}	18 (0.36%)	nt
IP7*	29d	54	adhFIX	1.0×10^{12}	8.5 (0.17%)	nt
IP5	33 m	54	adhFIX	5.3×10^{12}	16.5 (0.33%)	nt
IP8	33 m	54	adhFIX	1.0×10^{12}	nt	nt

D 3.4 Widespread transgene expression in serosa walls and adjacent tissue is observed after ultrasound guided intraperitoneal injection in early gestation

Immunohistochemistry for β-galactosidase 2 days after injection of the adlacZ vector at 52 days of gestation showed widespread expression in the subcapsular hepatocytes, the small bowel serosa and the surface of the umbilical cord (**Figure D 25**).

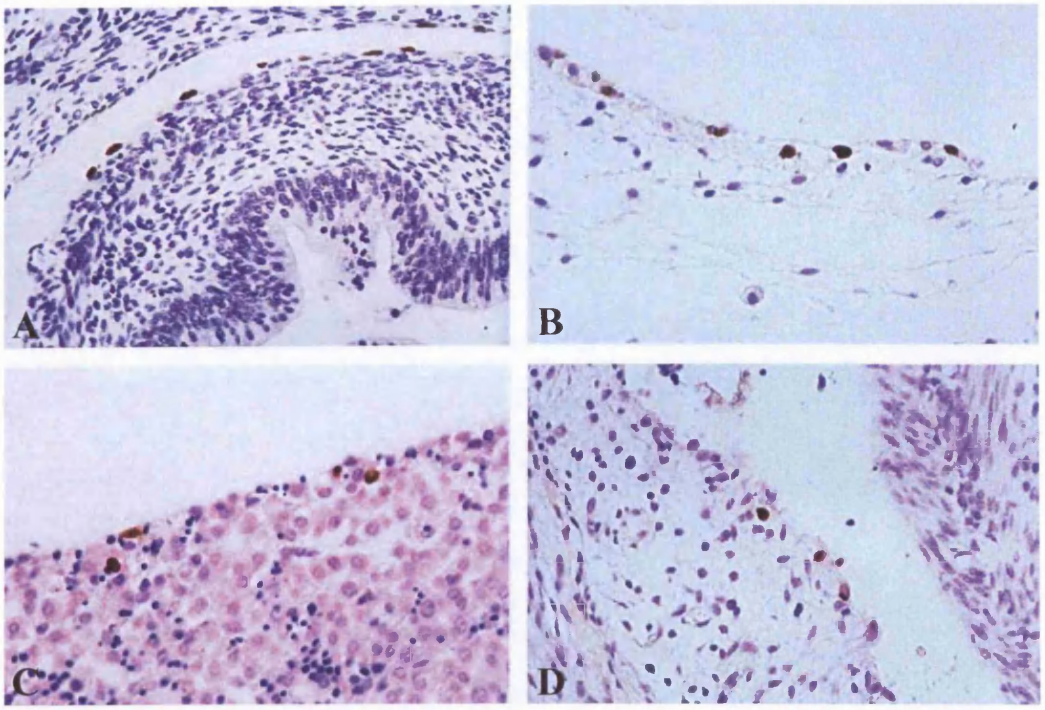


Figure D 25: β-galactosidase transgene expression after ultrasound-guided intraperitoneal delivery of adlacZ vector in early gestation.

Immunohistochemical analysis (haematoxylin counterstain) 2 days after IP injection of a fetal sheep (52 days of gestation, IP3, 1.5 x 10^{12} p/kg), positive staining is seen in the (A) fetal small bowel serosa, (B) surface of the umbilical cord, (C) fetal liver and (D) bowel mesentery. Original magnifications are x 40.

Positive lacZ expression was also observed after intraperitoneal injection at 40 days of gestation with widespread expression in the subcapsular hepatocytes, the pleura and within the chest and abdominal wall (**Figure D 26**). During the injection procedures vector delivery was clearly seen by microbubble movement in the peritoneal cavity and the needle tip was visualized at all times, reducing the possibility that the needle penetrated the pleural cavity. The pleuroperitoneal canals that connect the peritoneal with the pleural cavity close with the formation of the pleuroperitoneal membranes at the end of the embryonic period. In humans this occurs at approximately 7 weeks of gestation (Noden DM and De Lahunta A, 1985) which approximates in sheep to 25 days of gestation, well before the time of our experiment and so it is unlikely that the vector entered the pleural cavity via these channels.

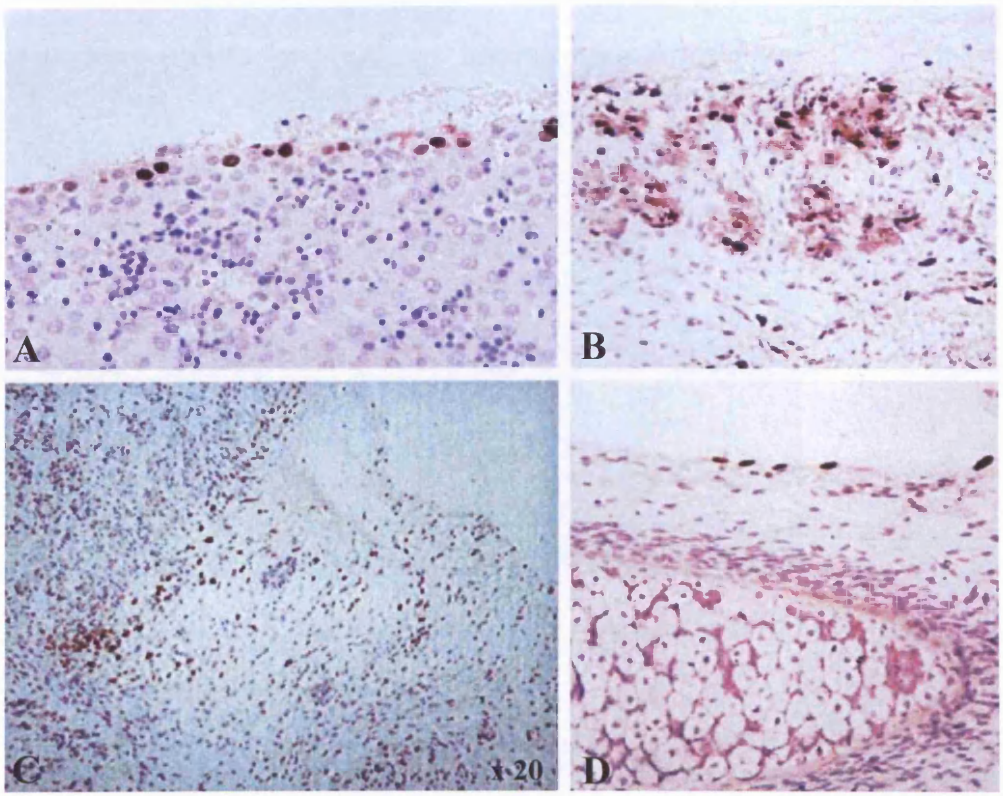


Figure D 26: β-galactosidase transgene expression after ultrasound-guided intraperitoneal delivery of adlacZ vector to a fetal sheep at 40 days of gestation.

Immunohistochemical analysis (haematoxylin counterstain) 2 days after IP injection of adlacZ (IP10, 1.1 x 10^{13} p/kg), shows positive staining in the (A) fetal liver, myocytes in (B) fetal chest wall and (C) anterior abdominal wall, and (D) fetal pleural epithelial cells. Original magnification x 50 except where indicated.

Another possibility is that the vector passed into the chest via lymphatic stomata connecting the pleural and peritoneal cavities. These appear early in gestation in the mammalian embryo (Shao XJ et al., 1998) and drain fluid from the peritoneal cavity to the lymphatic system (Nakatani T et al., 1996). Adenovirus particles (70nm in diameter) may pass through these stomata, enter the lymph glands and gain access to the systemic circulation. However this still would not explain the presence of vector in the chest wall and we must assume that the needle inadvertently entered the chest cavity probably because of the small size of this fetus.

D 3.5 Broad haematogenic vector spread is observed after intraperitoneal injection in early gestation

We investigated the biodistribution of adenovirus vector after early gestation intraperitoneal injection in two fetuses, one receiving adlacZ vector at 54 days (IP3) and the other that received adhFIX vector at 56 days of gestation (IP9, **Figure D 27**).

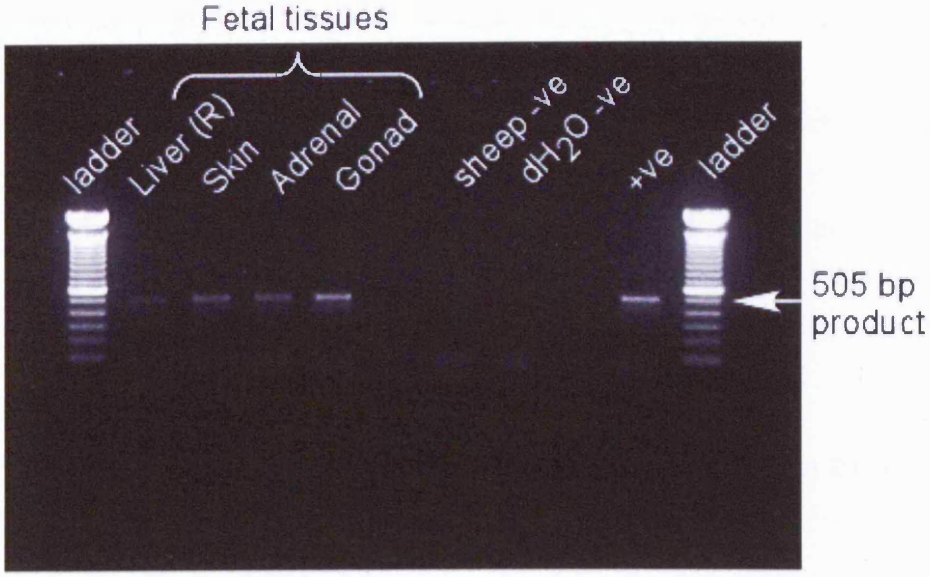


Figure D 27: AdhFIX vector spread after ultrasound-guided intraperitoneal injection in early gestation.

1st round PCR analysis in some fetal tissues 2 days after IP injection at 56 days of gestation (IP9). In this 2% agarose gel transgenic hFIX cDNA was detected in genomic DNA extracted from the fetal liver, skin, adrenal and gonad after 1st round PCR. Positive adhFIX virus (+ve) and negative blank (dH_20 -ve) and genomic DNA from a non-injected sheep fetus (sheep -ve) were used as controls. ladder: 100bp DNA ladder.

Widespread distribution of adlacZ vector to virtually all fetal tissues including the fetal gonads was detected by PCR analysis (**Table D 11**). This suggested that haematogenic vector spread occurred via this route of injection. Low level spread of vector to the maternal liver and ovaries, but not to the maternal lung was detected by nested PCR. In conclusion, ultrasound guided intraperitoneal delivery of adenovirus vectors had a low morbidity and mortality and gave widespread and high levels of transgene expression that were therapeutic in the case of the hFIX transgene.

D 4 Other routes of vector administration in early gestation: intramuscular injection

Intramuscular injection is an established route for vector administration by which *in vivo* expression of hFIX after injection of adenovirus and adeno-associated virus (AAV) hFIX vectors has been achieved in adult and fetal mice (Schneider H et al., 2002,Mitchell M et al., 2000). We investigated this route of administration in early gestation fetal sheep to determine if striated muscle could be used as an alternative site of transgene expression. To maximize our chances of achieving therapeutic hFIX

expression in the fetal circulation we delivered a high adenovirus vector dose of up to 3 x 10^{13} p/kg.

We did not specifically investigate adenovirus mediated transfection of fetal sheep muscle *in vitro*. Gene transfer to fetal sheep muscle *in vitro* is achievable using retrovirus vectors (John HA, 1994) and comprehensive studies in the fetal mouse have shown adenovirus mediated transduction to be an efficient process, dependent on binding between the adenovirus fiber knob and the Coxsackie Adenovirus Receptor (Bilbao R et al., 2003b).

D 4.1 Ultrasound guided intramuscular injection can be achieved in early gestation

Both adlac Z and adhFIX vectors were administered to the quadriceps and hamstring thigh muscles and gluteal buttock muscles of sheep fetuses (n = 11, **Figure D 28**). The vector was delivered in a total volume of 150-450µl by 1-4 injections depending on the adenovirus titre (**Table D 14**).

Table D 14: Ultrasound-guided intramuscular injection of adenovirus vectors in early gestation fetal sheep.

GA: gestational age; d: days; Vol: volume of vector injected; Injection no.: number of injections.

Sheep	GA (d)	sampling	Vector	Vol (µl)	Dose (p/kg)	Injection no.	Injection position
IM5	54	2d	adlacZ	280	2.7 x 10^{13}	4	R buttock
IM1	50	2d	adhFIX	150	9.9 x 10^{12}	1	L buttock
IM2	55	2d	adhFIX	450	3.0 x 10^{13}	4	L buttock + thigh
IM3	51	2d	adhFIX	350	1.6 x 10^{13}	4	L + R thigh
IM8	52	died 3d	adhFIX	400	6.8 x 10^{11}	2	R buttock + thigh
IM4	61	9d	adhFIX	400	2.7 x 10^{13}	4	L buttock + thigh
IM11	54	9d	adhFIX	400	6.8 x 10^{11}	2	R buttock + thigh
IM7	52	28d	adhFIX	400	2.7 x 10^{13}	4	L buttock + thigh
IM10	52	30d	adhFIX	400	6.8 x 10^{11}	3	L buttock + thigh
IM6	54	birth	adhFIX	320	2.1 x 10^{13}	4	R buttock + thigh
IM9	52	birth	adhFIX	400	6.8 x 10^{11}	2	L buttock + thigh

Four fetuses received adenovirus vector doses of 6.8 x 10^{11} p/kg rather than the higher dose of 1 x 10^{13} p/kg because of low adenovirus titre in that vector preparation. The procedure at this early age was technically more difficult than intrahepatic or intraperitoneal injections. We chose to inject the fetal thigh and buttock muscle because this is the largest accessible muscle group at this gestational age in the sheep fetus (Joubert DM, 1956).

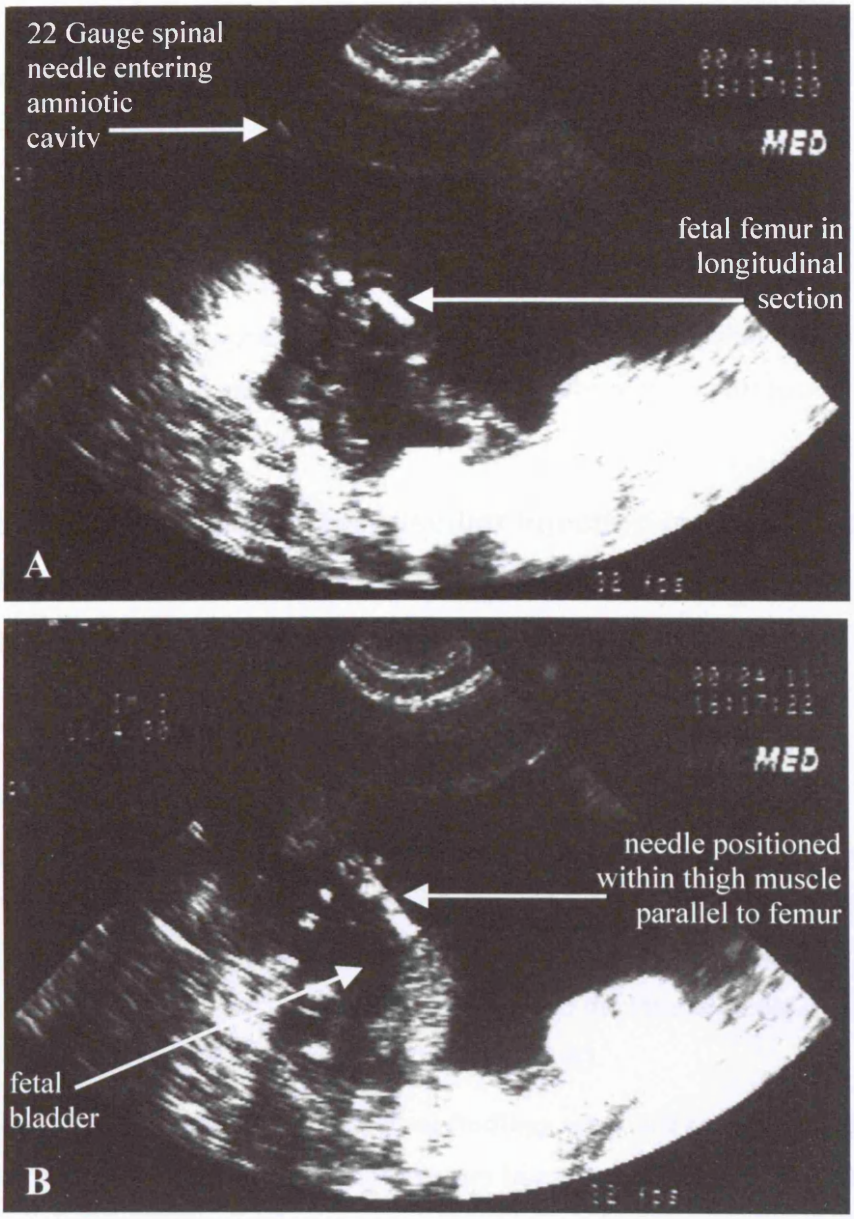


Figure D 28: Ultrasound-guided intramuscular injection in early gestation fetal sheep.

The ultrasonograms show injection of the thigh muscle of a sheep fetus aged 51 days of gestation. (A) A 22 Gauge spinal needle enters the amniotic cavity and lines up with the fetal femur in longitudinal section prior to injection. (B) The needle has now slid along the lateral side of the femur and is positioned within the thigh muscle parallel to the femur.

The cross-sectional diameter of the fetal thigh or buttock muscle on ultrasound measured only 2 – 3 mm while the bevel of the needle measured 2mm. Thus insertion of the needle tip into the muscle in a transverse section would have resulted in immediate loss of the vector into the amniotic fluid from the needle tip. To avoid this and to ensure a longer needle track, we placed the needle into the muscle parallel to the bone (**Figure**

D 28 B) and injected the thigh and/or buttock at a number of sites. Echogenic foci could be observed within the muscle parenchyma confirming vector placement. Despite these precautions, it is likely that only a proportion of the administered dose remained *in situ* and we therefore applied a slightly higher dose initially.

The mean time to successful injection was 18 min 49 sec (± SD 5min 4 sec, range 14 min – 25 min 40 sec). The reason for the long procedure time was the need to perform a number of intramuscular injections on the same fetus to achieve a high enough vector dose. This occasionally required the fetus to be repositioned to be able to access the buttock as well as the thigh muscle.

D 4.2 Ultrasound guided intramuscular injection in early gestation has a low morbidity and mortality

Fetal survival was 91%; one ewe and fetus died as a result of overwhelming sepsis 3 days after injection secondary to chorioamnionitis and fetal abortion. Death was probably due to bacterial infection at the time of injection since culture of the viral vector was negative.

During post mortem examination of the remaining fetuses, slight bruising was observed at the site of intramuscular injection in 3 fetuses (30%) and a 1cm haematoma was noted at the site of buttock injection 30 days after surgery (IM10, **Table D 15**). Some focal haemorrhage (2 fetuses, 7%) and inflammation in the muscle tissue (3 fetuses, 10%) was detected by light microscopy (**Figure D 30 A**).

Table D 15: Post mortem and histological findings after ultrasound-guided intramuscular injection of adenovirus vectors in early gestation fetal sheep.

d: days; m: months; PM: post mortem.

Sheep	sampling	Vector	Dose (p/kg)	PM findings	Histological findings
IM5	2d	adlacZ	2.7×10^{13}	bruising	normal
IM1	2d	adhFIX	9.9×10^{12}	normal	muscle haemorrhage
IM2	2d	adhFIX	3.0×10^{13}	bruising	muscle inflammation
IM3	2d	adhFIX	1.6×10^{13}	normal	muscle haemorrhage
IM8	died 3d	adhFIX	6.8×10^{11}	aborted	bacteria
IM4	9d	adhFIX	2.7×10^{13}	bruising	muscle inflammation
IM11	9d	adhFIX	6.8×10^{11}	normal	muscle inflammation
IM7	28d	adhFIX	2.7×10^{13}	normal	normal
IM10	30d	adhFIX	6.8×10^{11}	haematoma	normal
IM6	41 m	adhFIX	2.1×10^{13}	perihepatic peritoneal adhesions	mild portal tract inflammation
IM9	35 m	adhFIX	6.8×10^{11}	normal	mild portal tract inflammation

One of two sheep born after intramuscular injection of adhFIX had a misaligned jaw (IM9). This did not interfere with feeding, is a common finding in lambs (Dennis SM, 1993) and is unlikely to have been a result of the gene therapy treatment. Animals born following *in utero* intramuscular injection were examined 3 years after birth. Dense perihepatic adhesions involving the diaphragm and omentum were observed in one animal (IM6) and histological analysis of the liver showed mild portal tract inflammation but no obvious cholestasis. Examination of the other animal (IM9) similarly only found mild chronic portal inflammation that was also observed in the liver of many of the ewes.

D 4.3 Therapeutic levels of hFIX are observed after early gestation ultrasound-guided intramuscular injection

Therapeutic levels of hFIX were detected in the plasma of two fetuses sampled 2 days after injection (IM1 and IM2, 83 and 65.5 ng/ml respectively) and one fetus sampled 9 days after injection (IM 4, 87.5 ng/ml, **Table D 16, Figure D 20**). Transgenic hFIX protein was detected in a further two fetuses at 9 and 30 days after injection although the levels were sub-therapeutic (IM11 and IM10, 23.5ng/ml and 19.5ng/ml respectively). Of the two animals that were born, only one gave a measurable hFIX level of 5ng/ml that became undetectable after 20 weeks postnatally (**Figure D 20**).

Table D 16: Transgene expression after ultrasound-guided intramuscular injection of adenovirus vectors in early gestation fetal sheep.

* denotes twin fetus; GA: gestational age; d: days; ++ to +++ indicates degree of transduction observed after X gal staining or β-galactosidase immunohistochemistry; – indicates no staining; nt: not tested

Sheep	sampling	Vector	Dose (p/kg)	X gal staining or hFIX plasma level ng/ml (%)	β-gal or hFIX immuno-histochemistry
IM5	2d	adlacZ	2.7×10^{13}	nt	+++
IM1	2d	adhFIX	9.9×10^{12}	83 (1.67%)	++
IM2	2d	adhFIX	3.0×10^{13}	65.5 (1.31%)	++
IM3	2d	adhFIX	1.6×10^{13}	0	++
IM4	9d	adhFIX	2.7×10^{13}	87.5 (1.75)	++
IM11	9d	adhFIX	6.8×10^{11}	23.5 (0.47%)	nt
IM7	28d	adhFIX	2.7×10^{13}	0	nt
IM10	30d	adhFIX	6.8×10^{11}	19.5 (0.39%)	nt
IM6	41 m	adhFIX	2.1×10^{13}	5 (0.10%)	nt
IM9	35 m	adhFIX	6.8×10^{11}	0	nt

Positively stained myocytes were seen by immunohistochemical analysis for hFIX in four fetuses at 2 and 9 days after injection of the adhFIX vector (**Table D 16, Figure D 29 B and C**). No positive staining was observed in any other fetal or maternal tissues.

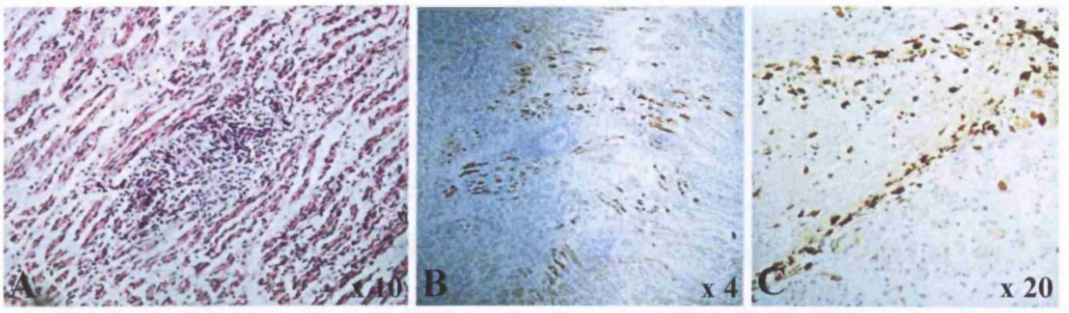


Figure D 29: Histological analysis and hFIX transgene expression after ultrasound-guided intramuscular injection.

Nine days after injection at 61 days of gestation (IM4) there is (A) focal haemorrhage and inflammation in the fetal quadriceps hamstring muscle tissue detected by light microscopy (H & E stain). Positively stained myocytes are seen on immunohistochemistry analysis for hFIX expression (haematoxylin counterstain) (B) and (C) in a fetus 2 days after injection of adhFIX vector at 51 days of gestation (IM3). Fetuses received 1.6 - 2.7 x 10^{13} p/kg adhFIX vector. Original magnifications are as indicated.

D 4.4 β-galactosidase transgene expression is observed in the fetal muscle and liver after intramuscular injection

Immunohistochemical analysis of fetal tissues 2 days after injection of adlacZ vector showed the presence of nuclear localised ß-galactosidase expression in myocytes and the overlying skin with some spread to the liver as seen by staining of a few hepatocytes (**Table D 16, Figure D 30**).

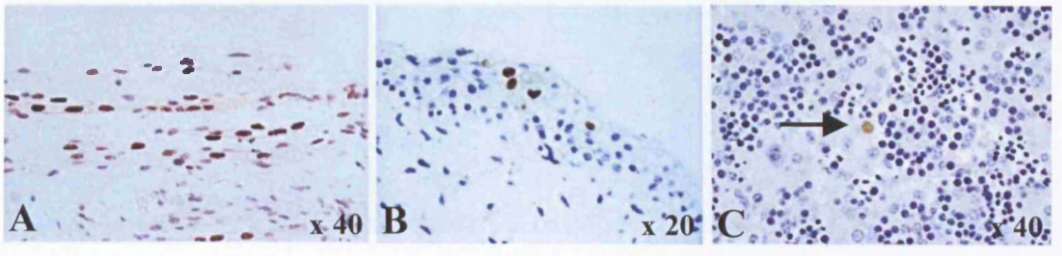


Figure D 30: β-galactosidase transgene expression after ultrasound-guided intramuscular injection of adlacZ vector.

Immunohistochemistry (haematoxylin counterstain) two days after IM injection of adlacZ vector at 54 days of gestation (IM5, 2.7 x 10^{13} p/kg) shows nuclear localised ß-galactosidase expression (A) in myocytes in the fetal gluteus muscle and (B) in the overlying skin with (C) spread to the liver as seen by staining of a single stained hepatocyte. Original magnifications are as indicated.

D 4.5 Broad haematogenic vector spread is observed after intramuscular injection in early gestation

Widespread distribution of the adlacZ vector or hFIX transgene was found by PCR in almost all investigated fetal tissues albeit by the more sensitive nested PCR analysis (**Table D 11, Figure D 31**). First round PCR product was detected in the fetal muscle and liver in agreement with the results of β-galactosidase immunohistochemical analysis. This suggests that low level haematogenic spread occurred following intramuscular injection. A representative gel showing nested PCR analysis for the presence of the adlacZ vector in fetal and maternal sheep tissues is shown in **Figure D 31.** Low level spread to the fetal gonad was only detectable in the fetus injected with adlacZ vector.

In conclusion, we have shown that ultrasound guided intramuscular delivery of adenovirus vectors could be achieved in the early gestation sheep fetus with minimal morbidity or mortality, achieving haematogenous vector spread and therapeutic levels of transgene expression. This supports the concept that the muscle may be useful as an ectopic source of potentially therapeutic proteins in some systemic congenital disorders.

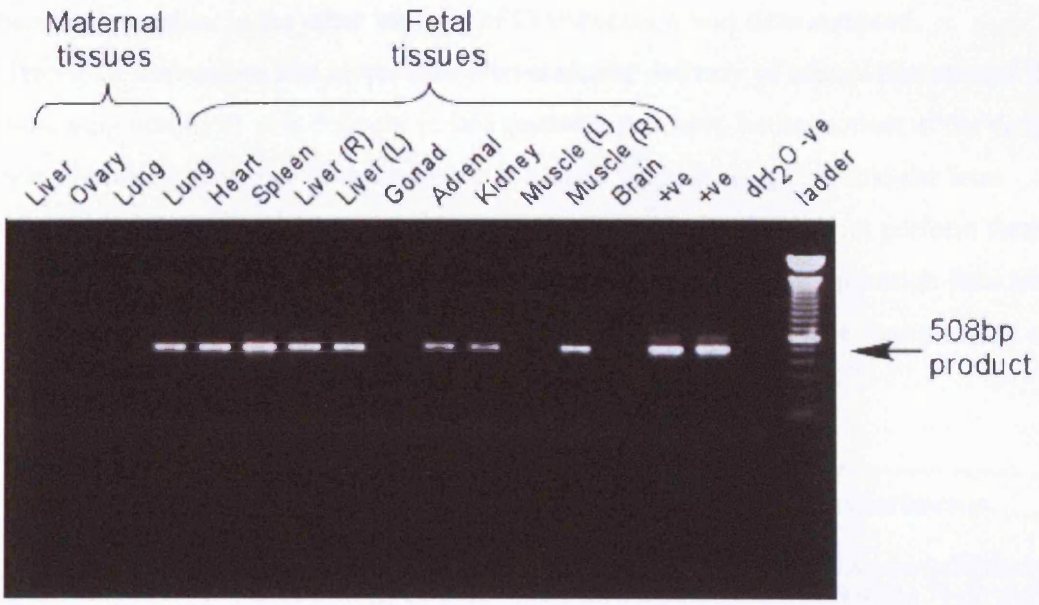


Figure D 31: Vector spread after ultrasound-guided intramuscular injection of adlacZ vector.

Nested PCR analysis in fetal and maternal tissues two days after IM injection of adlacZ vector at 54 days of gestation (IM5). In this 2% agarose gel transgenic adlacZ cDNA was detected in genomic DNA extracted from the fetal liver, ovary, lung, heart, spleen, adrenal, kidney, and right buttock muscle (injected side) after nested PCR. Positive adhFIX virus (+ve) and negative blank (dH_20 -ve) were used as controls. ladder: 100bp DNA ladder.

D 4.6 Ultrasound-guided intramuscular injection of adenovirus vectors in late gestation gives only low level tissue transduction

We had previously shown that intravascular delivery of adenovirus vectors in late gestation fetal sheep resulted in widespread tissue transduction and therapeutic transgene expression (Themis M et al., 1999). Initially one of our aims was to perform a series of intramuscular injections in late gestation fetal sheep using the same adenovirus vector as a comparison with results from umbilical vein delivery at the same age (Themis M et al., 1999). However because of time and resource constraints we decided to limit these experiments to 2 experiments in 4 twin fetuses.

Sheep fetuses at 125-137 days of gestation (n = 4, **Table D 17**) received an injection of high or low dose adhFIX vector into the quadriceps and hamstring muscle. The procedure was straightforward because of the much larger muscle bulk available, and in two fetuses up to 1ml of vector was injected with ease. Post mortem and histological analysis 2 days after injection in 2 fetuses showed no abnormality.

Levels of hFIX analysed by ELISA measured 23.5 and 16ng/ml 2 days after injection in one twin pair. One lamb born after high dose *in utero* treatment had detectable hFIX levels of 7ng/ml at birth, falling to 5.5ng/ml at one week of age and subsequently hFIX was undetectable; in the other lamb no hFIX expression was demonstrated.

Transgene expression was lower after intramuscular delivery of adenovirus vectors than following umbilical vein delivery in late gestation probably because most of the vector was confined to the muscle mass and only a small proportion spread into the fetal circulation. Because of the low level gene transfer observed we did not perform further intramuscular injection experiments using the adlacZ vector in late gestation fetal sheep. Nevertheless these results show that it is possible to achieve low level transduction of the fetal musculature at this late gestation.

Table D 17: Overview of late gestation intramuscular injection experiments.

Sheep fetuses were injected with adenovirus vectors containing the human factor IX gene (adhFIX) by ultrasound guided intramuscular injection. d: days, *: twin fetus pairs, p/kg: particles/kg.

Sheep	GA (d)	sampling	Vector	Vol (µl)	Dose (p/kg)
IM12*	125	2d	adhFIX	1000	2.3×10^{14}
IM13*	125	2d	adhFIX	100	2.3×10^{13}
IM14*	137	birth	adhFIX	1000	1.0×10^{14}
IM15*	137	birth	adhFIX	100	2.7×10^{13}

D 5 Health of lambs born after early gestation adenovirus vector administration

All lambs born following early gestation *in utero* injection of adhFIX were examined at delivery and during postnatal development. Blood was analysed to investigate the effect of fetal injection of adenovirus vectors on the long term health of lambs. Blood was taken as soon after birth as possible and at regular intervals. Results from blood samples taken for blood count, serum biochemistry, liver function tests (LFTs) and bile acids were compared to international standards for adult sheep (International Sheep Information System, ISIS). Because the standard reference ranges were available only for adult sheep of Mouflon species we also examined blood from three normal Romney lambs aged 3 months old present on the farm for comparison. Serum from 10 adult non-pregnant Romney ewes that had been on the farm for 6 months was also tested to provide an adult reference range for LFTs and bile acids.

D 5.1 Haematological analysis and serum biochemistry of sheep is normal

An increased platelet count was found in the blood from both injected and normal lambs that returned to the ISIS normal range by 6 months of age. All other haematological values and serum biochemistry were within the normal range from birth.

D 5.2 Analysis of liver function tests suggests hepatocellullar injury in some sheep born after early gestation *in utero* adhFIX injection

The serum levels of liver enzymes can indicate whether hepatic disease is present and assess its functional impact. The diagnostic usefulness of particular liver enzymes depends on the domestic animal species investigated and their age, and this must be considered when interpreting results (Meyer DJ, Harvey JW, 1998).

The serum level of aspartate transaminase (AST) was above the upper limit of normal (195 U/l) in one sheep at 6 and 7 months of age (IM6, 242 and 297 U/l) and another at 18 months of age (IP8, 203 U/l). In both animals the level of serum creatine kinase was normal, thus excluding muscular damage as the cause of the raised AST.

While abnormal levels of AST provide only a general indicator of hepatic injury in ruminants, the serum concentration of the liver enzyme glutamate dehydrogenase (GLDH) is the most specific for hepatocellular injury. In ruminants, GLDH is raised in hepatic necrosis and bile-duct obstruction (Kaneko JJ et al., 1997). The level of GLDH

in 2 non-injected control lambs at 2-3 months of age (50 U/l) was higher than the normal range quoted for adult sheep (up to 20 U/l, **Figure D 32**) suggesting that the normal adult range cannot be applied to young lambs.

We observed similar levels of GLDH initially in the serum of almost all the sheep that had received early gestation *in utero* adenovirus injection. From 6 months of age however, very high levels of GLDH were detected in one of the sheep injected via the intramuscular route (IM6) that subsequently settled to within or just above the normal range by 18 months of age. One of the two sheep that received intraperitoneal injection (IP8) had continuously raised levels of serum GLDH significantly above the upper limit of normal (33U/l). The sheep injected via the umbilical vein (UV12, UV15) and the sheep that received high dose intrahepatic adenovirus (HE5) had raised levels of GLDH at 6 months that then normalised by 1 year of age. Thus by a year after birth, serum GLDH levels were only significantly raised in one animal (IP8).

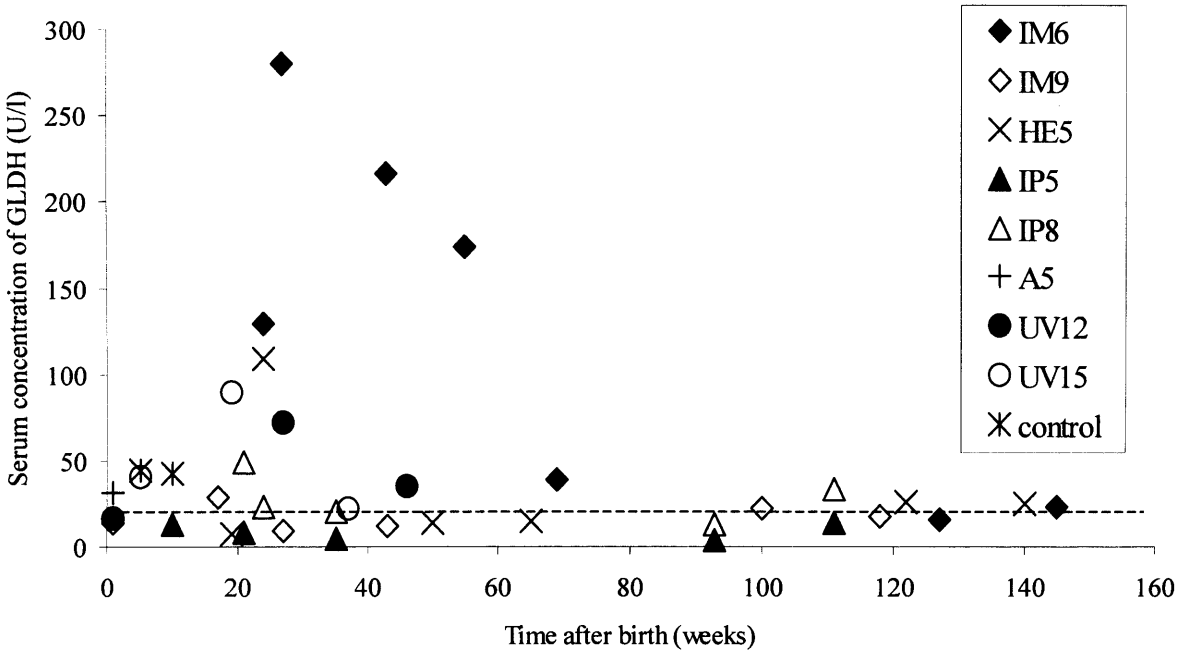


Figure D 32: GLDH concentration in the serum of sheep born after *in utero* early gestation injection of adhFIX vector.

The dotted line represents the upper limit of normal in adult sheep (20 U/l). IM: intramuscular, HE: intrahepatic, IP: intraperitoneal, A: intra-amniotic, UV: umbilical vein injection; control: 2 and 3 month old normal Romney lambs.

Gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP) are markers of cholestasis, GGT being associated with epithelial cells comprising the bile ductular

system and ALP associated with the canalicular membrane. Levels of GGT were high in the injected and non-experimental control lambs. GGT is present in colostrum and this may have resulted in the initial elevation up to 20 weeks of age when the lamb is suckling. The raised GGT level remained however beyond 1 year of age in the same animal that had a raised GLDH level (IP8), which suggested this animal may have a cholestatic problem (**Figure D 33**).

Serum levels of ALP fluctuate widely in normal ruminants and are less valuable than GGT in the evaluation of cholestatic disorders (Kaneko JJ et al., 1997). In addition, the bone isoenzyme would be elevated in neonates due to their rapid skeletal growth. ALP was not measured therefore in our sheep.

Bilirubin was not raised in the serum of any of the lambs. The levels of alanine aminotransferase (ALT) were normal which is not unexpected. There is only low level ALT activity in the hepatocytes of ruminants in contrast to rodent hepatocytes and therefore the level of this liver enzyme in the serum is not diagnostically useful.

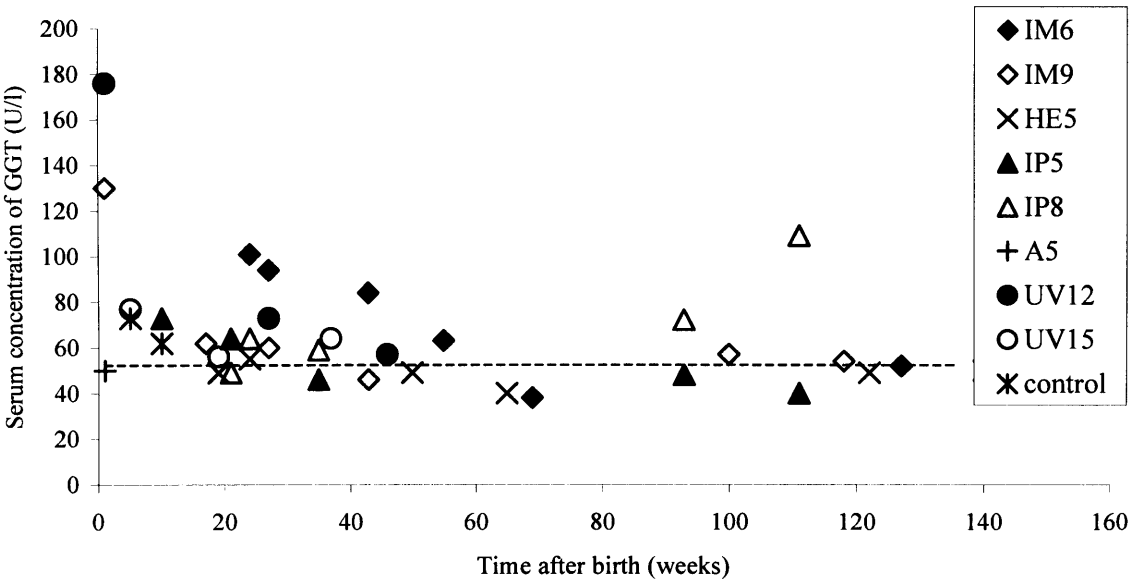


Figure D 33: GGT concentration in the serum of sheep born after *in utero* early gestation injection of adhFIX vector.

The dotted line represents the upper limit of normal in adult sheep (52 U/l). IM: intramuscular, HE: intrahepatic, IP: intraperitoneal, A: intra-amniotic, UV: umbilical vein injection; control: 2 and 3 month old normal Romney lambs.

D 5.3 Serum bile acids are raised

The level of liver enzyme activity observed does not always correlate with the degree of functional impairment and serum bile acid level is considered to be a better indicator.

The measurement of the serum total bile acid level in ruminants is difficult due to the wide range of reported normal values, hourly fluctuations up to 60μmol/L during feeding and occasional high levels in apparently healthy animals. Reference ranges for normal levels of serum bile acids in sheep have not yet been determined. The normal ranges quoted in the literature for studies investigating liver damage in sheep range from 20.2μmol/L (± SEM 1.78) (West HJ, 1987), 48 μmol/L (± SEM 3.3) (Anwer MS et al., 1976), to 74 μmol/L (± SEM 33μmol/L) (Sutherland RJ et al., 1992). In practice most veterinary biochemists use the normal range for adult cattle that has an upper limit of 60μmol/L (personal communication, Ms K Tennant, Department of Pathology & Infectious Diseases, Royal Veterinary College, UK).

To derive a normal range for our breed of sheep, we investigated the bile acid concentration in the blood of 10 normal adult non-pregnant Romney ewes (>1 year old) taken at the same time of the day as blood from our fetally injected sheep. The mean bile acid concentration was 39.8 μmo/l (± SEM 4.6, range 29.8 – 64.7 μmo/l).

From this data and published studies, nearly all the sheep injected *in utero* had abnormally high levels of serum total bile acids in the first year of life but these settled to within normal ranges subsequently **(Figure D 34)**

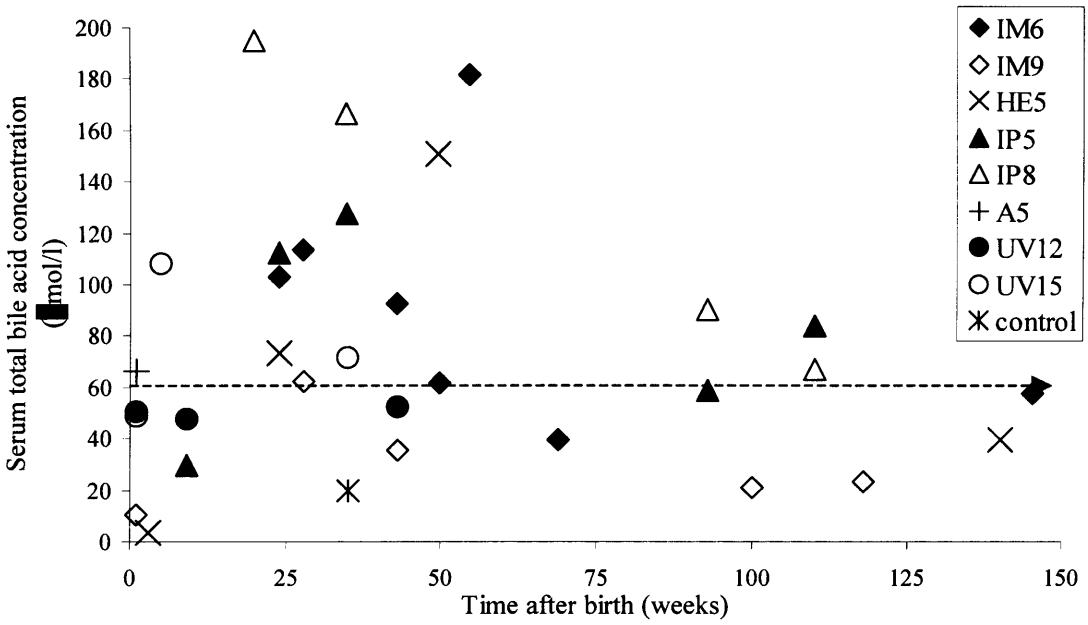


Figure D 34: Total bile acid concentration in the serum of sheep born after early gestation injection of adhFIX vector.

IM: intramuscular, HE: intrahepatic, IP: intraperitoneal, A: intra-amniotic, UV: umbilical vein injection; control: 3 month old normal Romney lamb. The dotted line represents the upper limit of normal bile acid concentration used in clinical practice (60 μmol/l).

The reason for this is unclear but generally there are low levels of bile acids in the fetus, and increased synthesis after delivery leads to a rise in the serum bile acids up to adult levels (Hardy KJ et al., 1980). In particular serum total bile acids reached their highest levels in the animal that had abnormal GLDH and GGT (IP8) and suggested chronic hepatic pathology with a reduced hepatic functional capacity.

D 5.4 Liver biopsy from one sheep injected as a fetus shows chronic liver pathology

We performed a liver biopsy on one of the sheep at 2 years of age that had the most abnormal liver function tests (IP8) to investigate the type of hepatocellular dysfunction observed. This showed chronic portal inflammation with cholestasis which was confirmed at post mortem examination a year later (**Section D 3**). These findings highlight the importance of long term follow up in any animal studies of fetal gene therapy in the future combining haematological and biochemical assessment of wellbeing with evidence from post mortem and histological examination.
In conclusion, the results suggest that all sheep born following early gestation injection initially had mildly deranged liver function, although by 1 year of age, this had resolved in all but one animal.

D 6. Adenovirus vector is not detectable in the sheep germline after early gestation administration

PCR analysis of semen samples from three male lambs born after early gestation intraperitoneal, intrahepatic or intramuscular injection of adhFIX and ovarian tissue from one female lamb born after intra-amniotic injection of adhFIX was carried out to investigate gene transfer to the sheep germline. No hFIX cDNA sequences were detectable on first round or nested PCR analysis of semen or ovarian tissue samples. It was not possible to test the other lamb born after intraperitoneal injection because it had bilateral undescended testes, a not uncommon finding in sheep flocks, and did not produce semen.

D 7 Discussion

D 7.1 Fetal size limits the gestational age at which ultrasound guided umbilical vein and intracardiac injection can be reliably and safely performed in the sheep fetus

Experiments prior to this work had shown that ultrasound-guided intravascular delivery of gene therapy could be easily achieved in the late gestation sheep fetus by injecting the UV as it passed through the fetal liver in the abdomen (Themis M et al., 1999). We studied UV and intracardiac injection from early gestation to achieve systemic delivery of gene therapy at an age when the sheep fetus is believed to be pre-immune, before 65 to 70 days of gestation.

D 7.1.1 Ultrasound guided injection of the umbilical vein

In clinical practice, injection of the UV at the placental cord insertion is the original site described (Daffos F et al., 1983) and the most widely used. Studies report fetal loss rates of up to 1.9% (Daffos F et al., 1985) although the risk of fetal loss may vary according to the indication for fetal blood sampling (Maxwell DJ et al., 1991).
Our attempts to inject the UV in the early gestation sheep fetus at a gestational age when it is believed to be pre-immune, were hindered by the small size of the fetus. A major obstacle to successful UV injection at 54 days of gestation was that blood flow in the umbilical cord vessels or intrahepatic UV was not detected by Doppler measurement with our machine. A more advanced machine with better Doppler capability might have allowed visualisation of blood flow. Even at the later gestational age of 60 days, it was not possible to visualise flow in the UV to confirm the correct injection site at the placental insertion.
We found that visualisation of the UV at the placental cord insertion in the fetal sheep was hampered by the short length of the umbilical cord, and division of the UV into numerous small branches to supply the cotyledons. Even in those cases where the placental cord insertion was adequately visualised, attempts to inject the UV were unsuccessful and we concluded that this route of intravascular injection should not be used in the fetal sheep. Newnham and colleagues had similar difficulties in the late gestation sheep and advocated intracardiac injection as an alternative route of fetal blood sampling (Newnham JP and Kelly RW, 1993). We believed that injection of the intrahepatic UV would be less traumatic than intracardiac injection at earlier gestations.

Injection of the intrahepatic UV in the fetal sheep also avoids the need to perform Doppler blood flow examination to confirm that an umbilical vein rather than an artery is being injected. This is important in the fetal sheep where there are normally two umbilical veins present.

Injection of the intrahepatic UV was attempted from 54 days of gestation but was abandoned due to severe narrowing of the UV, the difficulty with visualising the microbubble turbulence after vector injection and procedure related mortality. Injection at the slightly later gestation of 60 days allowed for fetal survival. The major problem at this gestational age was narrowing of the UV that occurred after the first injection attempt in almost every experiment. Even if the fetus was allowed to recover for a short time, the view of the UV was less good for subsequent injection attempts, reducing our success rate. More than one injection attempt was required in nearly half of the injected fetuses, and multiple injection attempts were associated with fetal death. A further problem was that intravascular delivery of the vector could not always be confirmed by microbubble visualisation. This was probably caused by dislodgement of the needle tip from the vessel when the syringe used to take fetal blood was switched to the syringe containing the viral vector. This could have been prevented by the use of a 3-way tap as applied in clinical practice during fetal blood transfusions. However because of the small circulatory volume of the fetus and small volumes of fluid injected in our experiments, the dead space of even a short piece of flexible tubing was believed to be too large. We considered instillation of PBS and observation of intravascular microbubbles as an alternative to withdrawal of fetal blood, but this would compromise visualisation of the UV should the needle tip not be correctly placed. While the ability to successfully inject the UV on the first attempt will improve with time as the operator becomes more experienced, the problems encountered if the first attempt fails and the difficulties with observing microbubbles, make this an unreliable route of vector delivery at this gestational age in fetal sheep.

Intrahepatic UV injection was more successful and reliable from 67 days of gestation. Although there were low numbers of fetuses injected between 67 and 70 days of gestation, subjectively the procedure was technically easier and therefore more repeatable. There was minimal morbidity and low mortality, which is similar to that observed in clinical practice (Nicolini U et al., 1990). The narrowing of the UV observed at 60 days was occasionally observed from 67 days of gestation but was less severe.

It is unclear whether it was the adenovirus vector or the procedure itself that caused the

hepatic necrosis observed nine days after intrahepatic UV injection at 60 days of gestation. Hepatic necrosis however, was not observed in one animal sampled 7 days after intrahepatic UV injection at 76 days of gestation. We concluded that the necrosis was probably as a consequence of UV narrowing seen at the earlier gestational age, that may have lead to hypoxia in the peripheral liver. A review of the literature identified only one case report of fetal hepatic necrosis following intrahepatic UV blood sampling (Sturgiss SN et al., 1996). The fetus in this case had severe growth restriction and the procedure was difficult to perform requiring repeated attempts to access the UV that probably contributed to the poor outcome. No long term alterations in the concentration of liver enzymes in the fetal blood have been shown following intrahepatic UV sampling (Nicolini U et al., 1990). In clinical practice, narrowing of the UV has not been documented and this may be because the placental cord insertion is preferentially sampled and intrahepatic UV injection is reserved for the 2nd trimester when the fetus is relatively larger.

Embryofetoscopy has been tested as an alternative technique for fetal blood sampling during the first trimester, in patients scheduled for termination of pregnancy. Umbilical cord puncture was successful in two out of three attempts at 14 weeks of gestation (Surbek DV et al., 2000) but was not achieved at 8 to 11 weeks of gestation (Surbek D et al., 1997). Early cordocentesis is not routinely used in clinical practice because it is associated with an increased risk of complications such as fetal bradycardia and haemorrhage. Fetal loss rates are related to gestational age and ranged from 5.2% at 12-18 weeks to 2.5% at 19-21 weeks of gestation in one series of cases of sampling at the placental cord insertion (Orlandi F et al., 1990).

The use of a needle guide has been advocated by some authors to reduce procedure-related losses during cordocentesis (Weiner and Okamura K, 1996) although most clinicians use the freehand technique which allows the needle to move with fetal or maternal movements and minimizes the risk of dislodgement. Intravascular pancuronium can be given to prevent fetal movements (Weiner CP et al., 1991). This was not required in our investigations because the ewe was anaesthetized.

An alternative site of UV injection in clinical practice is into a free loop of cord. This route is usually advocated only in cases of oligohydramnios when the placental cord insertion is less accessible because of the specific complication of cord haematoma and tamponade. One recent series of cases found sampling at the free loop of cord to be associated with a fetal loss rate of 3.2% at mid gestation (Tongsong T et al., 2000). In

our experience injection at the free loop of cord was successful in one fetus but we observed cord haematoma, and did not attempt this route of injection further.
In summary, delivery of gene therapy into the UV of the sheep fetus in mid-gestation is best performed using the intrahepatic UV route. In clinical practice, however, the placental cord insertion would be the route of choice because of the better visualisation of the placental cord insertion in the human as compared to the sheep fetus.

D 7.1.2 Ultrasound guided intracardiac injection is not compatible with fetal survival in the early gestation sheep fetus

Our failure to achieve successful intracardiac injection in the early gestation fetal sheep is not unexpected. Aspiration of blood from the left ventricle and vector injection was straightforward but the relatively large size of the needle compared to the heart resulted in immediate life-threatening trauma and haemorrhage. Ultrasound-guided cardiocentesis (Jauniaux E et al., 1999a) and intracardiac injection of radiolabelled fetal liver cells (Westgren M et al., 1997) has been performed on the human fetus at 12-17 weeks of gestation, corresponding to day 43 - 61 of gestation in sheep, prior to termination of pregnancy, but there is no data on the long-term complication rates. Intracardiac injection is rarely performed for clinical indications such as transfusion, and only later in pregnancy from 19 weeks of gestation onwards because of the high complication rate and associated mortality (Westgren M et al., 1988). Fetal intracardiac injection of viral vectors has been successfully performed by laparotomy and microsurgical techniques in mouse embryos at 10 days postcoitum (Christensen G et al., 2000). Ultrasound guided intracardiac injection using a 25 Gauge spinal needle in fetal rabbits in late gestation also had a high mortality rate of 25 – 40% 3 days after injection (Wang G et al., 1998).
In the fetal sheep, direct cardiac access by operative fetoscopy in mid to late gestation had an unacceptable 30% failure rate with 80% mortality (Kohl T et al., 2000b). The fetal loss rate from ultrasound guided intracardiac fetal blood sampling from 100 days of gestation can be reduced with operator experience to 4.5% (Newnham JP et al., 1989) (Newnham JP and Kelly RW, 1993). Pericardial haemorrhage was the cause of death in only one of seven fetuses that died, out of 76 that were sampled. The technique was refined to approach the heart from the inferior aspect that avoids the cardiac conduction system and reduced the mortality rate.
We concluded that in the early gestation fetal sheep (up to 14 weeks gestation in

humans), intracardiac injection was not suitable for clinical application of fetal gene therapy and that other routes into the fetal circulation would be safer alternatives.

D 7.2 UV delivery of viral vectors to the early and mid gestation fetal sheep achieves gene transfer and haematogenic spread

From previous work in our laboratory performed in late gestation fetuses, we knew that UV injection of adenovirus vector resulted in strong transgene expression in the fetal liver, spleen and adrenal gland. Therefore we anticipated that gene transfer to these tissues would be observed after early gestation UV injection. We did not examine fetal sheep liver tissues for the presence of Coxsackie Adenovirus Receptors but infection studies on the early gestation fetal liver *ex vivo* showed that adenovirus mediated gene transfer could be achieved. In the fetal mouse liver studies have shown homogeneous distribution of the Coxsackie Adenovirus Receptor and β5 integrin, which are necessary for adenovirus binding and internalization respectively (Bilbao R et al., 2003a). Because of the unreliability of the UV injection procedure at 60 days of gestation, we could only be sure that adenovirus vector had entered the fetal circulation in four fetuses. In two fetuses that were tested 2 and 9 days after injection, hFIX expression in the fetal plasma reached therapeutic levels and in the liver transgenic hFIX was detected by immunohistochemical analysis (**Figures D9** and **D10** respectively). The level of fetal liver tissue transduction suggests that the fetal liver was a major site of hFIX production. The remaining two fetuses were first tested for transgene expression at birth, 85 days after injection, and as expected from the short term nature of adenovirus mediated gene expression, no hFIX activity could be detected at these time points. PCR analysis of tissues from one fetus showed that the adhFIX vector had spread via the circulation to other fetal organs and expression in these ectopic locations may also have contributed to the hFIX levels observed. Human FIX sequences were detected on 1st round PCR analysis in the fetal adrenal and spleen, but in the liver only on nested analysis and this could represent variation in the sampling sites of tissue used for PCR analysis in this fetus. It is interesting to note however, that gene transfer to the adrenal gland was particularly evident in previous studies after UV injection in the fetal sheep at late gestation (Themis M et al., 1999) and at 60 days of gestation (Yang EY et al., 1999). We were unable to distinguish between the adrenal cortex and medulla because of the small fetal size, but in the late gestation study, β-galactosidase gene expression was mostly confined to the adrenal cortex (Themis M et al., 1999). Blood flow to the adrenal gland and particularly to the cortex increases at times of fetal stress such as

during haemorrhage (Toubas PL et al., 1981) or hypoxia (Richardson B et al., 1996), and needling of the fetal UV may provoke a stress response. Our data show that injection of the UV at 60 days of gestation narrowed the vessel probably resulting in transient hypoxia, and we believe that this caused the peripheral necrosis present in the fetal liver 9 days after injection.

Injection of the UV performed from 67 days of gestation onwards was more reliable and we confirmed that the vector had entered the fetal circulation by visualisation of microbubbles during injection in all but two cases. We also demonstrated colloidal carbon distributed in many of the fetal tissues after UV injection in one other case. Confident that we had achieved systemic delivery of the adenovirus vector, we expected to see gene expression particularly in the fetal liver, adrenal and spleen, but we were disappointed. In two experiments, we were concerned that the diluted vector used for the experiments may have degraded with time and a low dose was given inadvertently which may have lead to the negative results. It also became apparent that there was a problem with a fresh adlacZ vector preparation in use that failed to function *in vivo* despite good evidence of gene transfer to 293 human embryonic kidney cells *in vitro*. There did not appear to be any aggregation problem with viral particles in the preparation that can interfere with biological activity. Contamination of the vector could have prevented activity *in vivo* that did not occur *in vitro* because contaminants were diluted by the cell culture supernatant. This problem highlights the importance of testing vectors *in vivo* for biological activity as one of the stringent quality controls that will need to be in place for clinical application of gene therapy. Presently all vectors are pretested in adult mice by tail vein injection before use in sheep experiments.

Of seven fetuses that received biologically active adlacZ vector and survived to analysis, four showed no evidence of gene transfer. These four fetuses were aged between 68 and 87 days of gestation at injection and the dose of adenovirus received by these animals ranged from 2.5×10^{10} to 4.4×10^{11} p/kg. Positive β-galactosidase expression was only observed in one fetus aged 67 days of gestation that received sodium caprate pretreatment and in the two oldest fetuses injected at 95 days of gestation. It is unlikely that there is a gestational age specific response to adenovirus infection after umbilical vein injection, since we had already observed transgene expression after umbilical vein injection of adhFIX at 60 days of gestation. The sodium caprate pretreatment may have enhanced gene transfer. Vector complexation with DEAE dextran was used in some experiments and although this increased transduction

in general, it was not associated with positive transgene expression in these umbilical vein experiments.

It is more probable that there is a non-linear dose response to adenovirus infection. We observed strong transgene expression confirmed by positive immunohistochemical analysis for β-galactosidase in the fetal liver in one animal that received a high adenovirus dose of 2.7×10^{12} p/kg. The other animal received a lower dose of 8.5×10^{10} adenovirus p/kg and expression was low level and not confirmed by immunohistochemistry. There is evidence from studies in adult mice and primates of a non-linear dose response to intravenous adenovirus (Tao N et al., 2001). Temporary blockade of Kupffer cells by preadministration of a non-reporter adenovirus or by Kupffer cell depletion dramatically enhanced reporter gene expression over a very narrow dose range. The authors proposed that the reticuloendothelial system acted as a 'biological filter' to efficiently remove the majority of low doses of intravenously injected adenovirus prior to transduction of hepatocytes. This could be the explanation for the poor transgene expression we observed at the lower doses of adenovirus applied.

One other group from the Children's Hospital of Philadelphia, USA has studied injection of adenovirus vectors (AdCMVlacZ) into the UV of fetal sheep (Yang EY et al., 1999). They injected a similar adlacZ vector at laparotomy into the UV in a free loop of cord at 60 days of gestation. Strong expression of β-galactosidase was observed in fetal hepatocytes, the adrenal glands, kidneys and endocardium three days after injection and a dose response was observed. Significant fetal demise occurred at doses higher than 1×10^{11} pfu per fetus, equivalent to 1×10^{12} pfu/kg or 1×10^{13} p/kg, assuming each fetus weighs approximately 100g. This is a log10 dose higher than that used in our experiments, and although it is difficult to compare dosages from different studies because of biological variation in viral vector batches, it is likely that the higher dose applied resulted in better gene transfer.

Adenovirus is known to be a highly toxic vector. There is an initial response characterized by cytokine production (Zhang Y et al., 2001) followed by immune-mediated inflammation that targets vector-transduced cells (Yang Y et al., 1994). In our study a twin fetus that received a high dose of adenovirus (5×10^{12} p/kg) died shortly after injection, probably as a result of adenovirus toxicity. The previous study of UV injection in late gestation fetal sheep found that a dose of up to 4.8×10^{11} pfu per fetus, equivalent to 1-2 $\times 10^{11}$ pfu/kg was tolerated, but that 2.4×10^{12} pfu, equivalent to 1×10^{12} pfu/kg, led to intrauterine death. In that study, the adenovirus vector was titred by plaque assays on 293 cells that has now been superseded by the less variable optical

density method that was used in this study. The titres of adenovirus vector determined by optical density are assumed to be one order of magnitude lower than that from plaque assays (personal communication, Dr M Themis, Imperial College).

All these results indicate that there is a narrow therapeutic range in the sheep fetus over which umbilical vein injection of adenovirus results in gene transfer but without toxicity or fetal demise. This may be confined to the use of adenovirus vectors for gene therapy but should be studied for other more clinically appropriate vectors when these become available. Repeating our umbilical vein injection experiments at higher adenovirus vector doses might have produced better gene transfer. It is probable that the gestational age of a fetus affects the efficiency of vector infection and expression of transgenic proteins. A limitation to this work is that because of resource constraints associated with using a large animal model we were unable to demonstrate whether a gestation specific effect existed for umbilical vein injection. Early work with adenovirus vectors in mice showed that the gestational age of the fetal mouse at yolk sac vessel injection determined the pattern of organ expression (Schachtner SK et al., 1996). At 13 days post conception (dpc) the heart and liver were preferentially transduced and injection 2 days later resulted in widespread transduction of almost all organs, while at 18 dpc low levels of gene transfer were seen in the liver and lung. Particular susceptibility of cells to adenovirus infection appears to be an improbable explanation. In the fetal mouse, the pattern of adenovirus mediated transgene expression is unrelated to the expression of the CAR receptor or integrins that are necessary for adenovirus binding and internalization respectively (Schachtner SK et al., 1996). There may be other yet unidentified receptors or perhaps variability in the activity of promoters or transgene expression patterns through gestation that could be responsible for such development dependent variations in transgene expression. In the post-implantation mouse embryo, injection of adenovirus under the control of the CMV promoter resulted largely in endothelial gene transfer, while the RSV promoter lead to expression within the myocardium (Baldwin HS et al., 1997). It will be important for clinical application of prenatal gene therapy to explore experimentally the gestational age that best allows minimally invasive delivery and maximal gene transfer.

A critical factor that could affect vector dose after intravascular delivery is the fetal blood volume that as expected, increases with advancing gestational age. Data from red cell label experiments however, has shown that the blood volume normalized for fetal weight remains relatively constant through gestation (Brace RA, 1993). Dilution of the vector in the fetal circulation would thus be the same across gestational ages as long as

the same dose per kg fetus was applied. The fetal circulation is fully formed and functional by the end of the embryonic period and there are no significant changes in the blood flow over the gestational ages we investigated. In the fetal sheep, about 50% of the umbilical venous flow passes through the liver with approximately equal distribution between the left and right lobes (Edelstone DI et al., 1978). The other 50% flows through the ductus venosus directly into the inferior vena cava and then to the heart, bypassing the fetal liver. Delivery of gene therapy to the umbilical vein ensures its systemic distribution, as was borne out by the results of vector spread from tissue PCR analysis.

In small animals such as the fetal mouse, UV injection has a high mortality, and injection of the yolk sac vessel is used as an alternative route into the circulation. Indeed, yolk sac vessel injection of adenovirus vectors containing the hFIX gene into fetal mice resulted in therapeutic levels of hFIX expression (Waddington SN et al., 2003b). Long-term transgene expression was observed in the liver, heart, brain and muscle up to a year after delivery of lentivirus vectors containing the β-galactosidase gene into yolk sac vessels of fetal mice (Waddington SN et al., 2003c). This vector and route of injection was then used to achieve correction of the haemophilic phenotype in factor IX deficient mice, without the development of immune reaction to the transgenic hFIX protein and provided one of the first proofs of principle for therapeutic prenatal gene transfer (Waddington SN et al., 2004b).

The sheep fetus is believed to be capable of mounting an immune response between 65 and 70 days of gestation. This is also the time in gestation at which we were able to inject the UV reliably. Although we were unable to demonstrate good gene transfer using the adlacZ vector, it is likely that an alternative and preferably integrating vector would be able to transfer genes efficiently to the fetal liver and adrenal gland in particular.

D 7.3 Ultrasound guided intrahepatic delivery of gene therapy can be achieved in early gestation fetal sheep

Studies in the fetal mouse show that intrahepatic delivery of gene therapy vectors leads to transduction of hepatocytes and so we investigated this route of application in the fetal sheep for the first time using ultrasound guidance. As a technique, ultrasound-guided intrahepatic injection was easy to perform in the fetal sheep and had a low rate of early and late morbidity and mortality. There is likely to be a limit to the volume of

fluid that can be injected into the liver parenchyma before pathological damage is caused, although doubling the volume applied to 200μl did not have a significant effect. Ultrasound guided intrahepatic injection has not been used for therapeutic indications in the clinical setting. Fetal liver biopsy has been reported for prenatal diagnosis of inborn errors of metabolism such as ornithine transcarbamylase deficiency (Rodeck CH et al., 1982) and glucose 6 phosphatase deficiency (Golbus MS et al., 1988b) in which a liver sample was required to test localised enzyme expression. The availability of DNA analysis for some of these conditions has allowed prenatal diagnosis to be performed on chorion, fetal blood or amniocytes and has restricted the use of liver biopsy to uninformative families. The procedure is usually performed from the early second trimester onwards. An outer 17G cannula is used to access the uterus and to allow placement of an inner 19 Gauge needle into the fetal liver, where 10-25mg liver tissue is aspirated. The risk of complications such as haemorrhage is low and fetal loss is thought to be 1-2% (Vaughan JI and Rodeck CH, 2001).

Injection of fluid into the liver parenchyma is likely to be a less invasive procedure than liver biopsy. Two studies have investigated ultrasound guided intrahepatic injection of gene therapy vectors in the late gestation fetal rabbit and Rhesus monkey when no fetal losses were observed (Baumgartner TL et al., 1999,Lai L et al., 2002) and in another study, two fetal Rhesus monkeys that received late first trimester intrahepatic injection of retrovirus vectors survived to term (Tarantal AF et al., 2001c). We observed a fetal loss rate of 19%, which is likely to be due to the early gestational age at which procedures were performed. The necrosis observed within the liver parenchyma of one fetus may be related to the injection procedure itself but a local hepatotoxic effect of adenovirus cannot be ruled out.

D 7.4 Direct injection of adenovirus vector into the liver of early gestation fetal sheep does not result in significant gene transfer

Because the fetal intravascular injection route was unsuccessful in early gestation, we attempted to reach the fetal liver by injection of the liver parenchyma. This mode of injection, however, produced little or no hepatocyte gene expression as shown by immunohistochemistry and ELISA measurements for hFIX. Vector was present in the fetal and maternal liver and fetal heart only showing that very little haematogenic spread had occurred within the fetus. This was supported by the lack of systemic spread of colloidal carbon after intrahepatic injection.

First generation adenovirus vectors are highly immunogenic and are known to have a

tropism for hepatocytes. We observed haemorrhage and necrosis of the liver with surrounding inflammation around the site of vector injection in two fetuses. One possible reason for our findings may be that the strong tropism of adenovirus for hepatocytes caused significant infection surrounding the injection site and the resulting cellular 'over-load' of adenovirus infection and/or expression may have induced rapid cell death. Another explanation is that activation of the humoral or innate immune systems by adenovirus destroyed hepatocytes that had been successfully infected. In all remaining fetuses we observed only very little inflammation although it cannot be ruled out that the sites of vector injection might not have been analysed histologically in other fetuses due to sampling variations.

In the adult liver adenovirus gene therapy is limited by death of hepatocytes expressing the gene therapy product (Yang Y and Wilson JM, 1995). This elimination is caused by both necrosis and apoptosis mediated via the innate and cell-mediated immune response to the adenovirus such as tumor necrosis factor alpha and death domain receptors (Zhang H-G et al., 2002). Early induction of chemokines in the liver within 24 hours of vector delivery has been observed and is thought to be directly responsible for hepatic injury (Muruve DA et al., 1999). Self-limited vector-induced hepatitis was found in a clinical trial of adenovirus gene therapy treatment of partial ornithine transcarbamylase deficiency where the vector was infused into the right hepatic artery of subjects (Raper SE et al., 2002). To achieve efficient hepatocyte transduction by systemic intravenous delivery, high doses are required but the dose of adenovirus is of critical importance for its ability to cause injury. In baboons, increasing the dose of adenovirus vector delivered by intravascular infusion by a factor of 10 significantly increased transgene expression but resulted in severely abnormal blood, liver and biochemistry profiles and death within 48 hours (Morral N et al., 2002). A threshold effect was observed in mice for both transgene expression and hepatotoxicity but at a higher dose of adenovirus.

An immune response to the adenovirus vector might explain the poor gene transfer we observed. There is evidence of a pre-thymic population of immature lymphoid cells in the very early gestation fetal liver but these are no longer recognizable by 40 days of gestation, well before the age that fetuses in our study received intrahepatic injection. Lymphopoiesis occurs in the fetal thymus from 42 days of gestation and lymphocytes first appear in the peripheral blood from 55 days of gestation. Subsequently lymphopoiesis occurs in the spleen, lymph nodes, bone marrow and Peyer's patches as gestation advances (Al Salami M et al., 1985). Antibodies to the adenovirus vector were observed even 2 days after injection (see **Section G**), but they were at low level.

Kupffer cells, the resident macrophages in the hepatic sinusoid, appear to play a major role in the adult immune response to adenovirus infection because their depletion before vector administration results in higher hepatocyte transduction and a nearly linear dose response (Tao N et al., 2001). Macrophages in fetal tissues including the fetal liver develop before the initiation of bone marrow hematopoiesis. In the fetal mouse, macrophages capable of phagocytosis appear in the liver from 12 dpc (Naito M et al., 1997) and from 13 weeks of gestation human fetal Kupffer cells have the potential to respond to cytokines and lipopolysaccharide by increasing production of tumor necrosis factor-alpha and interleukin-1 beta (Kutteh WH et al., 1991). Thus the response of fetal Kupffer cells to the adenovirus vector may have contributed to the lack of gene expression we observed.

In contrast to our findings, studies in the late gestation fetal mouse (15 dpc) show that intrahepatic injection of adenovirus vectors resulted in high levels of transgene expression in the liver and distant organs (Lipshutz GS et al., 1999b,Lipshutz GS et al., 1999a) which declined over time (Lipshutz GS et al., 2000) in the absence of an immune response. The fetal liver in the mouse may also be particularly sensitive to adenovirus. One study found the maximally tolerised dose of adenovirus vector of 1 x 10^9 particles per fetus for intrahepatic injection to be at least one order of magnitude lower than that for other routes of delivery such as intramuscular or intra-amniotic injection (Mitchell M et al., 2000).

This is the only study of intrahepatic injection of viral vectors to the liver of fetal sheep. Ultrasound guided intrahepatic injection has been performed in two other large animal models. In the late gestation fetal rabbit, X-gal staining of the fetal hepatocytes was seen 2 days after ultrasound guided intrahepatic injection of adenovirus vectors containing the β-galactosidase gene (Baumgartner TL et al., 1999). The dose of vector applied, 2 x 10^8 pfu per animal, equivalent to 5 x 10^9 pfu per kg fetus assuming a fetal weight of 40g, was similar to the low dose vector used in our experiments. On closer scrutiny the results are not so encouraging. Of 12 fetuses injected, only 6 had evidence of β-galactosidase activity in the fetal liver, and in 2 of these, it was confined to the liver serosa which the authors believed was probably due to erroneous intraperitoneal rather than intrahepatic delivery.

In the early gestation fetal primate, intrahepatic delivery of HIV lentivirus vector resulted in lower levels of transduced hematopoietic progenitors in the liver, bone marrow and blood when compared with intraperitoneal delivery (Tarantal AF et al., 2001c). This is surprising since the fetal liver at this stage of pregnancy is the main site

of hematopoiesis. In addition, tissue distribution of the vector as assessed by PCR analysis, was more restricted than following intraperitoneal administration. Together the data suggest that the poor gene transfer observed in our study was not simply due to the use of adenovirus vector but the intrahepatic route itself may be an inhibitory factor. It is likely that the gestational age at injection and the type of vector applied are also factors influencing the gene transfer efficiency. In the late gestation rhesus monkey strong expression of transgenic enhanced green fluorescent protein was observed in hepatocytes one month after ultrasound-guided intrahepatic delivery of adeno-associated virus vectors (Lai L et al., 2002). Importantly vector genomes were detected throughout the fetal organs by PCR analysis supporting the concept that intrahepatic injection can achieve systemic spread. It is conceivable that delivery of a relatively large volume of fluid into the fetal liver in early gestation causes significantly more damage to the liver parenchyma than in late gestation. Delivery of adenovirus that is known to be hepatotoxic, may compound the physical damage. We concluded that direct hepatic injection with either low or high dose adenovirus vector was unable to achieve significant tissue expression of reporter genes in the early gestation sheep fetus.

D 7.5 Ultrasound guided intraperitoneal injection is a low risk procedure to the early gestation sheep fetus

We applied ultrasound guided intraperitoneal injection in the fetal sheep as an alternative to intravascular injection at early gestations for systemic delivery of gene therapy. The injection procedure was simple to perform, and an advantage of the technique over intrahepatic injection was that it was easy to observe microbubbles in the peritoneal cavity to confirm correct needle placement. There were 3 fetal deaths out of 15 fetal injections and the post mortem findings suggested these were probably related to the injection procedure or bacterial contamination of the vector rather than a toxic effect of the vector itself. The short term morbidity of the procedure was moderately low, and the technique was even successfully applied at 40 days of gestation in a few cases. In the medium to long-term there was significant intraperitoneal inflammation that could have been due to introduction of adenovirus or the procedure itself. Certainly in one animal that came to birth, there appeared to be plant material in the fibrous peritoneal adhesions that was probably introduced during the procedure. Although the fleece of the ewe is closely clipped over the abdomen before injection, it is extremely difficult to remove all the wool and the straw that is closely adherent to the fleece. Adenovirus is known to be highly immunogenic and its presence in the peritoneal cavity

may have caused the chronic inflammatory process detected in the other fetuses. In the other animal that came to birth there was evidence of hepatocellular dysfunction and cholestasis on serum biochemistry. Histological examination of the liver showed mild chronic portal tract inflammation that was also present in many of the ewes. There are a number of causes of hepatitis in ruminants including a wide variety of plant toxins such as alkaloids and mycotoxins, viral and fluke infections (Kaneko JJ et al., 1997).

In clinical practice, intrauterine blood transfusion was first performed in the 1960s by intraperitoneal injection for fetuses affected by haemolytic disease (Liley AW, 1963). In the 1980s the mortality rate from intraperitoneal transfusion was found to depend on the presence of fetal hydrops, with the survival rate in hydropic versus non-hydropic fetuses of 86% and almost 100% respectively (Harman CR et al., 1990,Watts et al., 1988). This is because ascites reduces the absorption of blood cells into the fetal circulation via the lymphatic drainage. Since then major advances in ultrasound technology have improved visualisation of the fetus such that direct intravascular transfusion is the route of choice in most cases after 18 weeks of gestation (Rodeck CH and Deans A, 2001,Harman CR et al., 1990). Intraperitoneal injection however, is still preferred for procedures before this gestational age when access to the fetal vasculature is hazardous or at any gestation if fetal positioning does not allow easy access to the fetal circulation. When intraperitoneal is combined with intravascular transfusion, an increased blood volume can be administered without overloading the fetal circulation, which allows a longer interval between transfusions (Nicolini U et al., 1989).

In the first trimester of pregnancy, ultrasound guided intraperitoneal injection has been used to apply *in utero* haematopoietic stem cell (HSC) transplantation for treatment of congenital disease in a number of cases (Westgren M et al., 2002,Flake AW et al., 1996,Muench MO et al., 2001,Touraine, 1999). Early application was thought necessary to take advantage of the immunological immaturity of the fetus and the space available in the developing bone marrow for engraftment of haematopoietic stem cells. One fetal death, due to graft versus host disease, has been reported 7 weeks after injection at 13 weeks of gestation (Bambach BJ et al., 1997), but no other fetal or neonatal complications have been observed.

In the fetal sheep, most studies of HSC transplantation or fetal gene therapy have been performed by intraperitoneal injection in the first trimester at laparotomy (Liechty KW et al., 2000,Zanjani ED et al., 1992,Westlake VJ et al., 1995,Porada CD et al., 1998). One study of HSC transplantation using ultrasound guided intraperitoneal injection between 45 and 60 days of gestation, found a fetal loss rate of 25% at term (6 out of 25

fetuses), the majority occurring up to 14 days after the procedure (Young AJ et al., 2003). The authors used a larger 20 Gauge needle which would be more traumatic and as anticipated, they observed a higher loss rate in twin pregnancies where both twins were injected. Survival in fetal primates was 100% following first or second trimester ultrasound guided intraperitoneal injection of HSCs or retrovirus vectors (Asano T et al., 2003,Shields LE et al., 1995,Tarantal AF et al., 2001c).

D 7.6 Intraperitoneal delivery of adenovirus vectors using ultrasound guided injection results in widespread gene transfer to the sheep fetus

Our poor results following intrahepatic delivery contrast those from intraperitoneal injection. We achieved therapeutic levels of hFIX in the fetal plasma 2 days after injection and low level hFIX expression was evident at birth and 5 months of age, over 8 months after the fetal injection. This was probably because several tissues were successfully transduced as shown by the wide haematogenic spread observed on first round and nested PCR analysis for the hFIX transgene and adlacZ vector when compared with other routes of adminstration at this early gestation. We localised β-galactosidase expression using immunohistochemical analysis to tissues that lined the peritoneal and pleural cavity and hepatocytes in the liver periphery. Many of these tissues are epithelial cell surfaces that the vector would have made contact with after injection and which provide a large surface area for viral transduction.

As expected, the level of hFIX gene expression fell significantly before birth. This was possibly due to the transient nature of adenovirus expression since the transgene is not inserted into the genome but remains episomal. Transcriptional inactivation of the CMV promoter driving hFIX expression may also be an important consideration for loss of gene expression (Löser P et al., 1998). Another likely contributor to the observed fall in hFIX concentration is the increase in the fetal blood volume over time as the fetus grows rapidly. Development of an immune response to the adenovirus or transgene is also a possibility and this is discussed further later.

We detected the adlacZ vector and the hFIX transgene in the placenta and the maternal liver, ovary and lung on PCR analysis, although no transgene expression was detectable in these tissues. Gene transfer to the placenta is not an issue because the placenta is disconnected from the body following delivery. Transduction of maternal tissues is a potential problem for integrating vectors when a route of vector application results in haematogenic spread. In the mother the main issue is with inadvertent transduction of

the ovary that conceivably could lead to germline alteration and this is discussed further in **Section D 7.9**.

In small animals, intraperitoneal injection of adenovirus vectors has been used for successful gene transfer to multiple tissues including the heart and liver in fetal mice (Bouchard S et al., 2003,Lipshutz GS et al., 1999c) As we observed, transgene expression after adlacZ administration is primarily confined to the liver capsule, the intestine and peritoneal cavity (Lipshutz GS et al., 1999b). Therapeutic levels of FVIII have been achieved after intraperitoneal injection of hemophiliac mice with an adenovirus vector containing the FVIII gene (Lipshutz GS et al., 1999c) but only transient phenotypic correction was observed.

In rats, ecotropic retroviruses were able to infect the fetal liver after intraperitoneal administration but only temporary transgene expression occurred. Partial hepatectomy reactivated expression for over a year and this is believed to be mediated via DNA replication activating expression of the viral promoter (Hatzoglou M et al., 1995).

When compared with adenovirus, intraperitoneal administration of AAV produces lower levels of transgene expression but over a longer time period (Schneider H et al., 2002,Bouchard S et al., 2003). Persistent peritoneal expression was observed 18 months after intraperitoneal injection of adeno-associated virus serotype 2 (AAV2) vectors containing the luciferase gene in fetal mice (Lipshutz GS et al., 2001b) and altering the AAV serotype and the promoter can further increase transgene expression (Lipshutz GS et al., 2003). Intraperitoneal injection has also been shown to be an effective route for administration of non-viral vectors such as cationic liposome-DNA complexes (Gaensler KML, 1999) and plasmid DNA (Gallot D et al., 2002), resulting in widespread transgene expression in multiple fetal tissues and types.

In large animal models intraperitoneal injection is an effective route of administration for integrating vectors although long-term transgene expression is still a concern Retrovirus vectors containing the α-L-iduronidase gene were delivered by ultrasound guided injection after exteriorisation of the uterus, into the peritoneal cavity or yolk sac of mid-gestation fetal dogs with canine α-L-iduronidase deficiency (muco-polysaccharidosis type 1). Widespread tissue transduction was seen and the fetal liver and kidney were targeted in particular (Meertens L et al., 2002). Surprisingly transgene expression did not persist beyond the neonatal period and this was thought to be due to the low dose of virus applied.

In early gestation fetal primates, Tarantal et al delivered retrovirus vectors by ultrasound guided intraperitoneal injection (Tarantal AF et al., 2001c) with the aim of gene transfer

to hematopoietic progenitors. Haematogenic spread of the vector was detected by PCR analysis of fetal tissues but transgene expression fell significantly at birth and was not detectable by 3 months of age. The loss of gene expression was probably due in part to methylation of the CMV-IE promoter element in the recombinant vectors. The finding that transgene expression in peripheral blood leukocytes *in vitro* could be reactivated by exposure to 5-azacytadine, a potent inhibitor of DNA methylase, confirmed this. In contrast, Porada et al demonstrated persistent gene marking of hematopoietic stem cells in the bone marrow and blood 5 years following delivery of Moloney murine leukemia viral vectors ($2 \times 10^5 - 1 \times 10^6$ particles) into the peritoneal cavity of early gestation fetal sheep at laparotomy (Porada CD et al., 1998). This was nevertheless low level with only 1% of peripheral blood leukocytes transduced and in 50% of animals, no transgene expression was found. When the titre of retrovirus was increased by 100 fold, the level of transduced peripheral blood leukocytes reached 6% (Tran ND et al., 2000) suggesting that viral titre is an important factor influencing gene transfer.

The hFIX level and the widespread distibution of the vector that we observed after intraperitoneal injection indicate that this is a universal and effective route for gene delivery in early gestation. The procedure is relatively straightforward, safe and reliable as compared with intravascular delivery and represents a useful therapeutic approach for *in utero* gene therapy in early gestation.

D 7.7 Ultrasound guided intramuscular injection is a low risk procedure in the early gestation sheep fetus

This is the first study to explore ultrasound guided intramuscular delivery of gene therapy in a large animal model at any gestational age. In small animals such as fetal mice, striated muscle can be used as an alternative site for transgene expression. We investigated this route of administration in early gestation when the fetal sheep is believed to be preimmune. At this age the volume of muscle present on the fetal thigh and buttocks was small and this initially presented difficulties at injection. We refined the procedure to place the needle in the muscle parallel to the fetal femur or pelvic bone to reduce backflow of the vector during injection. Despite this precaution, it is likely that some of the vector tracked from the injection site into the amniotic fluid. In addition, to increase the dose of vector that each fetus received, we performed a number of injections on one fetus, injecting only small volumes of vector at each injection site. Multiple injections will cause more trauma to the fetus but they are unavoidable at this early gestational age when the fetal muscle bulk is so small. Nevertheless, the morbidity

and mortality of the procedure was low and it carried the lowest mortality rate of all early gestation routes of administration, making it a potentially clinically useful application method.

The muscle haemorrhage and inflammation observed histologically in many of the fetuses is likely to have resulted from the injection procedure itself, although the adenovirus vector may have contributed. Intramuscular injection in late gestation fetal sheep was very straightforward with no complications observed. In early gestation the only significant pathology we observed were the dense perihepatic adhesions in one of the lambs that delivered. It seems unlikely that injection of the muscle could have lead to an inflammatory reaction in the peritoneal cavity and the adhesions might have been secondary to hepatitis that is common in ruminants (Kaneko JJ et al., 1997).

Ultrasound guided intramuscular injection has been used to deliver glucocorticoids to the mid - late gestation fetal sheep (from 104 days of gestation) by single or repeated injection and has a low complication rate (Jobe AH et al., 1996,Willet KE et al., 2001). In clinical practice intramuscular injection is not used therapeutically, although two pilot studies of intramuscular vitamin K (Larsen JF et al., 1978) or glucocorticoid injection (Ljubic A et al., 1999) in late gestation found no complications. Fetal muscle biopsy is performed for prenatal diagnosis of muscular dystrophies in families in which DNA studies are uninformative (Vaughan JI and Rodeck CH, 2001). Biopsies are usually performed from 18 weeks of gestation because of the risk of biopsy failure, and the complication rate is low (Evans MI et al., 1994). The optimum gestational age for intramuscular delivery of gene therapy in the human fetus would need to be a compromise between the safety and reproducibility of the procedure and the efficacy of gene transfer.

D 7.8 Therapeutic levels of hFIX transgene are detected in the fetal plasma after ultrasound-guided intramuscular injection of adenovirus vectors

Ultrasound guided intramuscular delivery of gene therapy in the early gestation fetal sheep achieved low but nevertheless therapeutic levels of hFIX transgenic protein in the plasma up to nine days after injection. Despite the large doses of adenovirus used, injection in the fetal muscle did not lead to major destructive or inflammatory reactions although a cellular infiltrate was observed in the muscle of three animals up to 9 days after injection. Positive β-galactosidase staining restricted to a small region at the site of injection was detectable. This limited area of expression may explain the rather low

level of hFIX expression in the blood at the two day time point. Haematogenic spread was also less than that observed after intraperitoneal delivery as evidenced by the small amount of β-galactosidase expression in fetal hepatocytes and lower level spread detected by PCR analysis.

In adult mice, binding of hFIX in the muscle may reduce its levels in the plasma. Experiments on intramuscular injection of adhFIX suggested transgenic hFIX was bound not only in the muscle fibres but also in the interstitial spaces and colocalized with collagen IV protein using immunofluorescent staining (Herzog RW et al., 1997). Human factor IX binds specifically to collagen IV (Wolberg A et al., 1997) in the extracellular matrix of endothelial cells *in vitro* (Cheung WF et al., 1996). In our study however, positive hFIX expression on immunohistochemistry was limited to the fetal myocytes only and not observed in the interstitial spaces. This could be due to the species difference or more likely the maturity of fetal striated muscle at 50 – 60 days of gestation.

Developmental changes in the fetal muscle also may have reduced hFIX expression. In the human fetal quadriceps muscle, myoblasts fuse into primary myotubes from the 6^{th} week of gestation and by 11 – 16 weeks of gestation, newly generated myoblasts surround and fuse with them to rapidly increase the number of cells in the muscle and to form secondary myotubes (Barbet JP et al., 1987). At the same time, equivalent to 36 to 58 days of gestation in the sheep fetus, numerous primary myotubes undergo degeneration and by 18 weeks of gestation, equivalent to 65 days in the sheep fetus, the first immature muscle fibers are observed (Fidzianska A and Goebel HH, 1991). Thus many transduced cells may undergo necrosis and death as a result of the normal development of striated muscle.

Muscle tissue is a promising target for gene therapy. Because mature muscle is composed of multi-nucleate post-mitotic myofibers, it might allow high-efficiency long-term expression of introduced genes. Muscle tissue is also highly vascularized and as such, may facilitate the systemic delivery of potentially therapeutic non-muscle products such as growth factors, clotting factors or erythropoietin for example. Transduction of mature as compared with immature muscle fibers is poor (Ascadi G et al., 1994,Acsadi G et al., 1994) and there appear to be a number of factors involved. The extracellular matrix acts as a physical barrier blocking the entrance of viral particles such as Herpes Simplex Virus (HSV) into mature myofibers (Huard J et al., 1996). In contrast, HSV particles can penetrate myofibers isolated from neonatal mice that are immature, and from mature homozygous dystrophic mice that have an abnormal

extracellular matrix. CAR expression is lower in adult compared with neonatal skeletal muscle. Increasing expression of the CAR receptor in adult muscle however, enhances susceptibility to adenovirus transduction (Nalbantoglu J et al., 2001). Fetal muscle may have advantages over adult muscle as a target for gene therapy. The basal lamina is immature (Borck C, 1977) facilitating access of viral vectors and there is a high level of CAR expression in fetal striated muscle (Bilbao R et al., 2003b). There are large numbers of myoblasts that fuse with developing myofibers and these may serve as intermediate carriers of genes, should integrating vectors be applied.

Studies of fetal gene transfer by intramuscular injection are confined to the mouse model. Injection of adenovirus vectors containing the β-galactosidase gene into the shoulder or hindlimb musculature resulted in persistent muscle transgene expression for up to 4 months and haematogenic spread to the liver was observed (Yang et al., 1999a,Mitchell M et al., 2000). Previous work in our group achieved *in vivo* hFIX expression at therapeutic levels up to 6 months after injection of adenovirus and AAV hFIX vectors in fetal mice (Schneider H et al., 2002). There were no antibodies to the hFIX transgene detectable in the serum at any time point after birth, which is similar to our findings in the fetal sheep (see **Section G**). In contrast to our results however, antibodies to the adenovirus vector were not detectable and there was no inflammatory reaction in the fetal muscle. This could be due to species differences or the relatively low dose of adenovirus vector applied. The absence of antibodies in the mouse study may also explain the long-term transgene expression observed.

Intramuscular injection of lentivirus vectors in fetal mice leads to transduction of the injected muscle groups with greatest efficiency from the Ebola and Mokola pseudotypes and expression persisted up to 10 weeks and 6 months respectively (MacKenzie TC et al., 2002). There was evidence of haematogenic or lymphatic spread from the injection site since cardiomyocytes and myocytes in the diaphragm were transduced after injection of the left hindlimb. This is encouraging when considering that these are some of the muscle groups that would need to be targeted for successful gene therapy of the life-threatening muscle wasting disorders such as Duchenne muscular dystrophy (DMD). These congenital diseases are caused by mutations in the dystrophin gene, the protein product of which aids membrane stabilization and force transduction from muscle fibres. A major problem with a gene therapy strategy for treatment of DMD is the very large size of the dystrophin gene (2.4Mb) although progress has been made in constructing a micro- and mini-dystrophin (3.6 - 14 kb) cassette. When this was applied in an adeno-associated virus vector to the leg muscle of neonatal and adult *mdx* mice it

led to normal myofiber histology and protected membrane integrity (Wang B et al., 2000). There is also a need to target this correct gene or sequence to most striated muscle groups in the body including the diaphragm, and cardiac muscle. A fetal approach offers a possible solution to this problem since correcting muscle precursor cells by gene transfer with a long term expressing vector would lead to continuous production of dystrophin in the adult. A recent study using VSV-G pseudotyped EIAV lentivirus containing the lacZ gene, combined intrathoracic, supracostal, intraperitoneal and intramuscular injection of three limbs and a single flank in the fetal mouse. This resulted in widespread gene expression in all injected muscles and also the diaphragm and heart (Gregory LG et al., 2004). Transgene expression was maintained for at least 5 months and no cellular or humoral immune response was detected in this time. Our demonstration that ultrasound-guided intramuscular injection is feasible in a large animal model with a low morbidity and mortality rate is a step towards possible clinical application of fetal gene therapy for treatment of muscular dystrophies.

D 7.9 Gene transfer to the germline

Vector spread is an important consideration when attempting *in utero* gene delivery. Ideally gene transfer should be restricted to the tissue of interest for production of the therapeutic protein. Although this is less significant for systemic production of hFIX, it is more important for diseases such as cystic fibrosis, Duchenne muscular dystrophy and glycogen storage disorders. It is also central to avoiding inadvertent gene transfer to the germ line that currently is ethically unacceptable.

There is concern that prenatal vector administration may carry a higher risk of inadvertent gene delivery to germ cells (Billings PR, 1999). The adenovirus vectors we used reached the fetal gonads after intraperitoneal, intramuscular, intragastric and intratracheal injection, albeit detected only by sensitive PCR. However analysis of the semen of rams born after *in utero* early gestation intraperitoneal, intramuscular or intrahepatic treatment showed no detectable vector. It is likely therefore, that despite the virus reaching the gonad by these routes of administration, it is either unable to reach or to infect the primordial germ cells. It is surprising that we did not detect transgenic DNA in the gonad following umbilical vein delivery at 60 days of gestation, because this route gives systemic vector spread. In the previous study on late gestation fetuses there was evidence of adenovirus vector in the fetal gonads after intravascular gene delivery, but no gene expression was detected by RT-PCR analysis. In keeping with another study of intra-amniotic delivery in fetal primates (Larson JE et al., 2000b), we

did not detect transgenic DNA in the fetal sheep gonad following intra-amniotic delivery, even at the very early gestation we studied.

Germline transmission has also been studied after early gestation intraperitoneal delivery of retrovirus vectors to fetal sheep (Porada CD et al., 1998,Tran ND et al., 2000). Proviral DNA was detectable in the testes and whole ejaculate of four fetally injected sheep. No sequences were present however in a pure population of sperm cells isolated from the ejaculate on five separate occasions, and more importantly, there was no transmission of the proviral DNA to the offspring of these animals. A similar risk of germline transduction occurs with adeno-associated virus (AAV) that can integrate at low level into the genome. No AAV sequences were detectable in the germline tissues of fetal mice receiving injection of AAV vectors via the intraperitoneal route nor the tissues of their progeny (Lipshutz GS et al., 2001b,Lipshutz GS et al., 2003). A clinical trial of gene therapy for hemophilia B using AAV vector was briefly suspended when vector genomes were detected in the semen of participants, although this was shown to be only a temporary finding (Manno CS et al., 2003).

The chances of germline transduction occurring in the mother are low because there is a blood-follicle barrier present in the ovary and the eggs are held in meiotic metaphase arrest until fertilization. Results from evaluation of maternal tissues in other studies of large animal fetal gene therapy are reassuring. No vector sequences were detectable in maternal tissues following intraperitoneal delivery of retro- and lentivirus vectors to early gestation Rhesus monkeys (Tarantal AF et al., 2001c). In the sheep, proviral DNA was present in the maternal ovary after early gestational intraperitoneal delivery of retrovirus vectors, but no vector sequences were detected in the tissues of her later offspring (Tran ND et al., 2000).

Compartmentalisation of the primordial germ cells in the gonads is complete by 7 weeks of gestation in humans and it is unlikely therefore that any therapy applied after this time would result in fetal germ-line transduction. Many of these issues are not confined to *in utero* or even adult gene therapy and concerns regarding germ-line transmission can be raised in particular for chemotherapy treatment (Schneider H and Coutelle C, 1999). Further studies using appropriate vectors for long-term gene expression which are more relevant to the aims of fetal gene therapy, such as integrative viruses, should be made on larger numbers of animals to evaluate this risk. An important and more recent strategy for tissue restricted and therefore safer gene transfer may be the use of tissue specific vector targeting (Engelstädter M et al., 2001).

E Prenatal gene therapy for cystic fibrosis

Cystic fibrosis is a systemic disease of most secretory epithelia and so the major affected organs are the lungs, pancreas, gut and liver. In these experiments we investigated minimally invasive methods of targeting gene therapy to the airways and small bowel of sheep fetuses since disease in the lungs and gut are leading causes of death from CF.

E 1. Ultrasound guided intra-amniotic delivery as a route to the fetal airways

In these experiments we were aiming to develop a minimally invasive ultrasound guided technique to deliver adenovirus vectors to the airways of sheep fetuses as early as possible in gestation and we began by investigating intra-amniotic delivery. Delivery of adenovirus vectors to the amniotic cavity results in transduction of the fetal skin (Schneider H et al., 1999). A number of authors have also tried to target viral vectors to the fetal airways via intra-amniotic injection. In small animals such as the fetal mouse, transduction of the airways can be achieved (Douar A-M et al., 1997), but the route is less successful in larger animals such as the fetal rabbit (Boyle MP et al., 2001) or fetal primate (Larson JE et al., 2000b) probably due to the dilution of the vector by the large volume of the amniotic fluid. We considered intra-amniotic injection to be the safest delivery technique and therefore we investigated its application earlier in gestation at 33-39 days (0.23-0.26 term), equivalent to 8 - 10 weeks of gestation in humans. Amniotic fluid from the early gestation fetal sheep had already been shown to have no effect on the ability of adenovirus vector to infect cells *in vitro* (Mike Themis, personal communication).

E 1.1 Ultrasound guided intra-amniotic injection is possible in early gestation fetal sheep

We applied adlacZ and adhFIX vectors to the amniotic cavity of fetal sheep at the earlier gestation of 33-39 days (n = 14). Our first attempt at intra-amniotic injection was complicated by the presence of the larger allantoic cavity and the relative flaccidity of the amniotic membrane at this gestational age. We overcame this initial difficulty entering the amniotic cavity in the fetal sheep by directing the needle close to the fetus and flicking it through the amniotic membrane (**Figure E 1**). In the fetal sheep the allantois expands rapidly from 28 days of gestation via the urachus to occupy both

horns of the uterus and contains fluid until term (Latshaw WK, 1987). The amnion begins to enlarge from 25 days but only stands away clearly from the fetus by 30 days of gestation (Cloete JHL, 1939). In addition the allantoic fluid volume (45.7 ml) is approximately ten times that of the amniotic fluid (2.3 ml) at 31 days of gestation but there is a rapid increase in the amniotic fluid volume, so that by 44 days of gestation, there is only a two fold volume difference (95.3 ml versus 38.8 ml respectively) (Wales RG and Murdoch RN, 1973). Correct needle placement was confirmed by withdrawal of amniotic fluid and by observing microbubbles around the fetus in the amniotic cavity on injection of 0.9% sodium chloride solution and vector. Intra-amniotic injection was achieved at the first attempt in all fetuses and excluding the time taken to complete first procedure (26 minutes), the mean time to successful injection in all other cases was 3 min 39 sec (± 3 min 11 sec, range 1- 10 min).

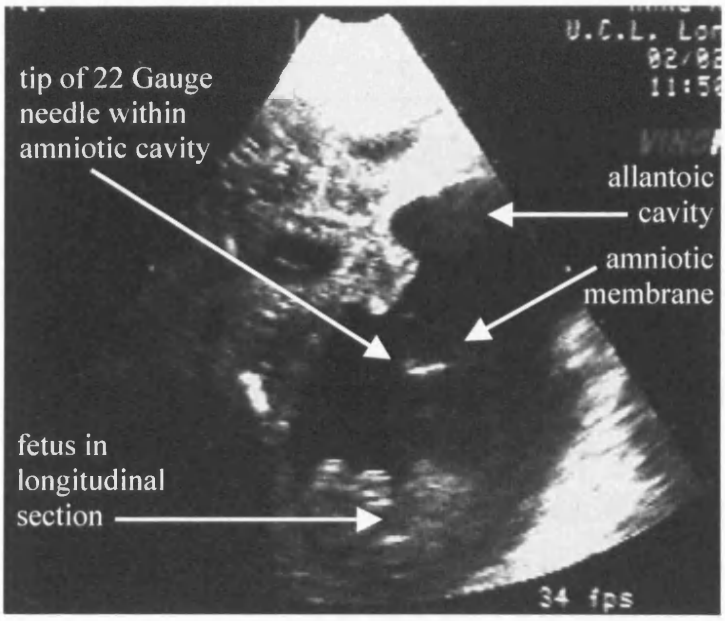


Figure E 1: Ultrasound-guided intra-amniotic injection in early gestation.

The ultrasonogram shows intra-amniotic delivery of adhFIX vector to a fetal sheep aged 33 days of gestation (A14).

A small volume of amniotic fluid was withdrawn before injection of the vector, and as can be seen in **Table E 1**, analysis of the osmolality and electrolytes confirmed this was amniotic rather than allantoic fluid. At this stage of gestation the composition of amniotic fluid is close to a dialysate of fetal plasma, while allantoic fluid is hypotonic since it has been filtered by the mesonephros, the primitive kidney that is active in fetal sheep from 17 days until full regression by 57 days of gestation.

Table E 1: Amniotic and allantoic fluid osmolality and biochemistry in early gestation sheep fetuses.

Fluid was obtained from fetuses aged 33 – 39 days of gestation. Measurement of K^+ concentration in amniotic fluid was inaccurate because at this gestation it is very high (normal range 17 ± 2 mmol/l) and the electrolyte analyser was unable to process the sample. For comparison, the osmolality and electrolytes in the allantoic fluid of one fetus removed at post mortem analysis is shown. *: denotes normal ranges from fetal sheep at 31 days of gestation (Wales RG and Murdoch RN, 1973).

Sheep	Na^+ (mmol/l)	Cl^-(mmol/l)	Osmolality (mOsm/kg water)
A2	140	142	292
A4	154	140	260
A5	147	135	292
A6	149	135	274
A7	159	145	201
A8	150	137	183
A9	155	142	288
A10	160	143	261
A11	131	114	283
A12	121	111	293
A13	127	111	262
A14	159	141	290
A15	132	115	306
A15 (allantoic fluid post op)	66	64	229
Amniotic fluid*	136 ± 14	124 ± 10	293 ± 3
Allantoic fluid*	89 ± 2	61 ± 5	214 ± 8

E 1.2 Ultrasound guided intra-amniotic injection has low morbidity and mortality

Fetal survival after intra-amniotic injection was 100% at 2 days following injection and 86% at term (**Table E 1**). One fetus of a twin pair that was still alive on day 5 after operation (ultrasound visible heart beat), was resorbed on day 9 at the time of post mortem sampling. This is a relatively common finding in normal early twin gestations in fetal sheep and may be unrelated to the procedure itself. Histological analysis of all fetal and maternal tissues was normal apart from that of the first fetus to be injected (A1), which showed evidence of acute bacterial chorioamnionitis. This was probably procedure related and the difficulties encountered entering the amniotic cavity are likely to have been a contributing factor.

Two lambs were left to delivery. Because of the foot and mouth epidemic it was not possible to access the ewes to assess fetal wellbeing by ultrasonography from mid-gestation onwards. One lamb aborted at 136 days of gestation as a fetus papyraceous

although fetal measurements at post mortem investigation suggested fetal demise at 80 days of gestation. Although this is commonly associated with chlamydial infection in sheep, there was no evidence of past or current infection in the maternal serum, or other causes of miscarriage such as toxoplasma infection. The other lamb delivered at term with multiple congenital skeletal abnormalities including torticollis and the animal was euthanased because of feeding difficulties and failure to thrive.

Table E 2: Ultrasound-guided intra-amniotic delivery of adenovirus vectors in the early gestation sheep fetus.

*: twin fetus pairs; d: days; Vol: volume of virus injected; p/kg: particles per kg; PM: post mortem.

Sheep	GA (d)	sampling	Vector	Vol (µl)	Dose (p/kg)
A1	33	2d	adlacZ	1000	4.4×10^{12}
A2	39	2d	adlacZ	300	1.8×10^{12}
A9*	35	2d	adhFIX	250	1.0×10^{13}
A10*	35	2d	adhFIX	250	1.2×10^{13}
A13	33	2d	adhFIX	100	4.0×10^{13}
A14	39	2d	adhFIX	100	4.0×10^{13}
A15	39	2d	adhFIX	100	4.5×10^{13}
A7*	36	9d	adhFIX	250	1.0×10^{13}
A8*	36	resorbed at 9d	adhFIX	250	1.0×10^{13}
A12	36	11d	adhFIX	250	1.0×10^{13}
A4	36	28d	adhFIX	250	1.0×10^{13}
A11	36	28d	adhFIX	250	1.0×10^{13}
A5	36	aborted at 100d	adhFIX	250	1.0×10^{13}
A6	36	birth	adhFIX	250	1.0×10^{13}

E 1.3 Therapeutic levels of hFIX are detected after early gestation intra-amniotic delivery of adhFIX

Because of their small size it was very difficult to take blood from fetuses 2 days after intra-amniotic injection, and plasma was only available from two of the five fetuses sampled; no hFIX expression was detected in the plasma of these fetuses. In those fetuses for which plasma was not available, hFIX was expressed in the amniotic fluid at levels of 610, 66.5 and 39ng/ml on day 2 after injection and may have been produced by the fetal keratinocytes and/or amniotic membranes. Therapeutic levels of hFIX were detected in the plasma of fetuses sampled on day 9 (103.5ng/ml), 11 (53.5ng/ml) and day 28 (49 ng/ml) after injection. Plasma from another fetus sampled 28 days after injection and a lamb sampled at birth had levels of hFIX below the reliable detection level of the assay (**Figure D 20, Table E 3**).

Table E 3: Transgene expression after ultrasound-guided intra-amniotic injection of adenovirus vectors in early gestation fetal sheep.

* denotes twin fetus; GA: gestational age; d: days; +++ indicates degree of transduction observed after X gal staining or β-galactosidase immunohistochemistry; nt: not tested; † indicates testing of amniotic fluid.

sheep	PM	GA (d)	Vector	Dose (p/kg)	X gal staining or hFIX level in plasma and/or †amniotic fluid ng/ml (%)	β-galactosidase immuno-histochemistry
A1	2d	33	adlacZ	4.4×10^{12}	+++	+++
A2	2d	39	adlacZ	1.8×10^{12}	+++	+++
A9*	2d	35	adhFIX	1.0×10^{13}	†66.5 (1.33%)	nt
A10*	2d	35	adhFIX	1.2×10^{13}	†39 (0.78%)	nt
A13	2d	33	adhFIX	4.0×10^{13}	†610 (12.2%)	nt
A14	2d	39	adhFIX	4.0×10^{13}	0, †0	nt
A15	2d	39	adhFIX	4.5×10^{13}	0, †0	nt
A7*	9d	36	adhFIX	1.0×10^{13}	103.5 (2.07%)	nt
A12	11d	36	adhFIX	1.0×10^{13}	53.5 (1.07%)	nt
A4	28d	36	adhFIX	1.0×10^{13}	49 (0.98%)	nt
A11	28d	36	adhFIX	1.0×10^{13}	0	nt
A6	birth	36	adhFIX	1.0×10^{13}	0	nt

E 1.4 β-galactosidase expression is observed in those fetal tissues in contact with the amniotic fluid after intra-amniotic delivery

X gal staining of fetal tissues 2 days after intra-amniotic adlacZ vector injection showed extensive expression in the fetal skin and amniotic membranes (**Figure E 2 A-C**). Immunohistochemistry showed positive β-galactosidase expression in fetal keratinocytes in the fetal skin, on the surface of the cord and in the epithelial lining of the nasal cavities (**Figure E 2 D-E**).

In summary we observed significant gene transfer to those fetal tissues that are in contact with the amniotic fluid together with therapeutic levels of the hFIX transgene in the circulation. This suggests that the amniotic route of application may be useful for targeting genes to the very early gestation fetus.

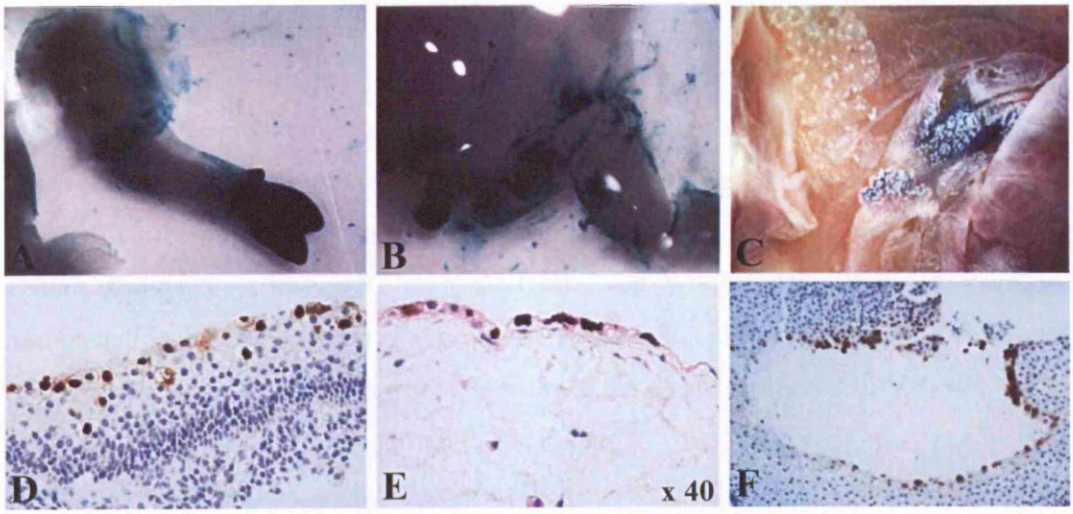


Figure E 2: β-galactosidase transgene expression after intra-amniotic delivery of adlacZ vector.

X gal staining (A-C) and immunohistochemical analysis (D-F, haematoxylin counterstain) 2 days after intra-amniotic delivery of adlacZ vector in early gestation. Positive transgene expression is seen in the fetal skin over the lower leg (A), thigh (B) and amniotic membranes (C) on X gal staining of one fetus (A1, 4.4 x 10^{12} p/kg), and in the keratinocytes of the fetal skin (D), surface of the umbilical cord (E) and epithelial lining of the nasal cavity (F) of another fetus (A2, 1.8 x 10^{12} p/kg). Original magnification of immunohistochemistry sections x 20 except where indicated.

E 1.5 Attempts to stimulate fetal breathing movements in mid-gestation fetal sheep

Transduction of fetal airways using intra-amniotic delivery may be improved by stimulating fetal breathing movements (FBM). Spontaneous FBM are known to start in the fetal sheep from about 50 days (Cooke IRC and Berger PJ, 1990) and in humans at about 10 weeks of gestation (de Vries JIP et al., 1986). In general animal studies have used fetal intravenous infusions of theophylline to study their effect on FBM. Intravenous theophylline administered to the ewe has been shown to increase FBM in the late gestation fetal sheep during normoxia or hypoxia (Moss IR and Scarpelli EM, 1981). In humans, intravenous infusion of theophylline in the mother has been shown to stimulate FBM thereby increasing inhalation of intra-amniotically injected surfactant (Cosmi EV et al., 1996).

Dr Suzy Buckley's work in our laboratory investigated adlacZ transduction and/or colloidal carbon intake into the fetal mouse lungs. Subcutaneous theophylline injection to the pregnant mother (2mg) did not result in any significant increase in adlacZ transduction of the fetal airways. However, when intra-amniotic adlacZ and theophylline (dose 40-160μg per fetus) was co-administered with maternal hypercapnia

(10% CO_2), fetal airways transduction appeared to increase, and quantitative levels of β-galactosidase expression analysed by ELISA reached statistical significance ($p=0.043$). Intake of intra-amniotic injected colloidal carbon was significantly increased ($p<0.01$) when co-administered with theophylline and maternal hypercapnia.

It was not known whether intra-amniotic administration of theophylline might have a similar stimulatory effect on FBMs in the fetal sheep. We tested this therefore, on twin mid-gestation sheep fetuses in which we had failed to inject the trachea in the fetal neck (CC4, see **Section E 2.6**) during development of the ultrasound-guided tracheal injection technique.

Theophylline (40mg) and colloidal carbon (500μl) were injected into the amniotic cavity of twin fetuses at 81 days of gestation. The dose of theophylline applied was determined by extrapolation from the fetal mouse data. A day 16 post conception fetal mouse weighs 1g and a sheep fetus aged 80 days of gestation would weigh approximately 500g. Since the optimum theophylline dose in the fetal mouse amniotic cavity was 80μg this gave a dose of 40mg theophylline into the fetal sheep amniotic cavity.

Both twins received an intra-amniotic injection since it was considered likely that theophylline would diffuse across the amniotic membranes. No FBM were seen in either twin, before or after theophylline injection. One twin that was alive 24 hours after operation was found dead at post-mortem 48 hours later. Histology of this twin showed meconium and small amounts of colloidal carbon in the airways, but none in the fetal gut; histology of the other fetus that was alive at postmortem analysis was normal. Although acute hypoxia is known to depress FBMs in sheep (Boddy K et al., 1974), gasping has been observed before death in the sheep fetus (Patrick JE et al., 1976) and this is likely to have caused the histological findings. It is also possible that theophylline which is known to stimulate FBMs during hypoxia (Bissonnette JM et al., 1990) may have had an effect. However no colloidal carbon was found in the lungs of the surviving twin fetus, suggesting that intra-amniotic theophylline at the dose applied in a normoxic fetus had no effect on FBMs. We had planned to perform further experiments in fetal sheep should we fail to achieve ultrasound-guided tracheal injection, but this was superseded by the successful development of this route of injection.

E 1.6 Haematogenic vector spread is observed after intra-amniotic injection in early gestation

We investigated the spread of vector in the fetal and maternal tissues after intra-amniotic delivery by PCR analysis (**Table E 4**). Because of the small size of fetuses sampled 2 days after injection and the difficulty identifying organs, we also analysed vector spread in a wide range of tissues from one animal sampled 28 days after vector delivery (A4). DNA from the vector or the transgene was detected in the fetal liver, skin, lung, heart, umbilical cord and membranes analysed by 1st round PCR analysis. Low levels of vector were also found in the maternal liver and gonads.

Table E 4: Vector spread after intra-amniotic delivery of adenovirus vectors to fetal sheep in early gestation.

Transgenic hFIX (hFIX) or adenovirus lacZ vector (adlacZ,) was detected in fetal and maternal tissues (n = 3) by PCR analysis after intra-amniotic injection of 3 fetal sheep sacrificed 2 days (A2, 1.8 x 10^{12} and A9, 1 x 10^{13} p/kg) or 28 days (A4, 1 x 10^{13} p/kg) after injection. Tissues that tested positive on 1st round PCR analysis (1st) were not generally subjected to nested PCR analysis (2nd). n: not available; m denotes maternal tissues; * indicates tissues tested from A4 fetus.

	hFIX (A9 or A4*)		AdlacZ (A2)	
Tissue	1st	2nd	1st	2nd
Liver	+	+	+	+
	$+^m$	$+^m$	$-^m$	$+^m$
Gonad	–	–	n	
	$+^m$	$+^m$	$-^m$	$+^m$
Lung	+	+	n	
	$+^m$		$-^m$	$+^m$
Placenta	+	+	+	+
Umbilical cord	+		+	+
Thymus	–*	–*	n	
Heart	+	+	n	
Spleen	–*	–*	n	
Small bowel	–*	–*	n	
Adrenal	–*	–*	n	
Skin	+	+	+	+

Fetal breathing and swallowing movements do not occur at this early stage in fetal sheep development and so it is not likely that the adenovirus vector accessed the lung or small bowel via this mechanism. In the fetal sheep the physiological hernia returns to the peritoneal cavity and the anterior abdominal wall closes by the 38th day of gestation (Cloete JHL, 1939). It is possible therefore that the peritoneal contents were exposed to the adenovirus vector although a physiological hernia was only apparent in one fetus aged 33 days of gestation at injection. The vector may also have crossed the fetal skin

which is not keratinized at this stage of development, and entered the lymphatic circulation and reached the heart and lung by this route.

E 1.7 The effect of adenovirus vectors on amniotic fluid biochemistry

We investigated whether intra-amniotic injection of adenovirus vector in early gestation fetal sheep affected the composition of the amniotic fluid. At 30 - 41 days of gestation the mesonephros is the main functioning kidney and the fetal urine drains into the allantoic cavity. The amniotic fluid on the other hand, is close to a dialysate of fetal plasma and therefore its biochemistry and osmolality would provide us with some information about the effect of the adenovirus vector on fetal well-being.
The concentration of sodium and chloride ions and the osmolality of amniotic fluid were compared before and after intra-amniotic adenovirus vector delivery in 4 fetuses. There was no significant difference detected between the two samples (paired Students *t*test) indicating that the adenovirus vector had no significant effect on amniotic fluid biochemical composition.

E 2 Ultrasound guided tracheal injection to deliver gene therapy to the fetal airways

Having failed to achieve significant airways transduction by intra-amniotic delivery, we proceeded to develop a method of injecting the fetal trachea directly. We also wanted to investigate how transduction of the fetal airways might be improved since previous studies have shown poor epithelial airway cell gene transfer.

E 2.1 Tracheal fluid from the fetal sheep does not impair adenovirus transfection in vitro

Prior to *in vivo* experiments, we tested the adlacZ vector *in vitro* and *ex vivo*. The aim of these experiments was to determine whether fetal tracheal fluid affected adenovirus transfection of an epithelial cell line. An initial experiment was done to determine the optimum dose range of the adenovirus vector. AdlacZ vector was incubated with tracheal fluid from a non-injected fetus aged 84, 97 or 116 days of gestation, or with PBS, for 30 minutes at room temperature. Serial dilutions of the vector in tracheal fluid were then made with PBS. These were applied to four 24-well plates containing human bronchial epithelial cells (HBE, 7 x 10^4 cells per well) cultured to 30% confluence to achieve 10^8, 10^7, 10^6, 10^5, 10^4 or 10^3 adenovirus particles per well. The plates were

exposed to X-gal stain 48 hours after transfection and the number of blue cells per well counted. At the two highest adenovirus concentrations it was not possible to accurately count the number of blue cells per well. Examples of positively stained HBE cells at different virus concentrations are shown in **Figure E 3.**

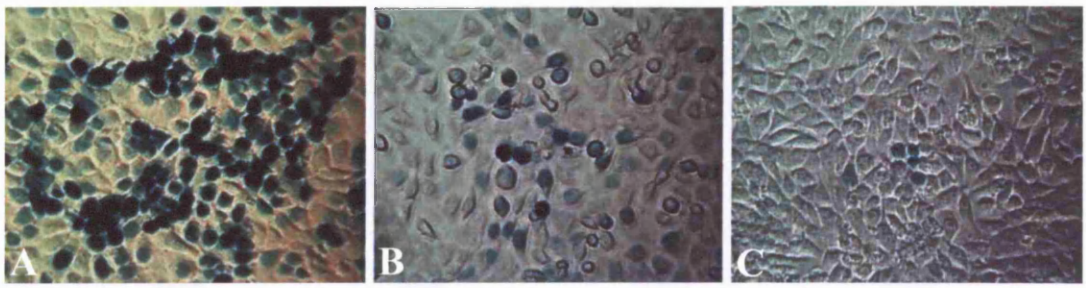


Figure E 3: HBE cells expressing β-galactosidase after infection with adlacZ vector.

AdlacZ vector was pre-incubated with tracheal fluid from a fetal sheep aged 84 days of gestation for 30 minutes and was then serially diluted with PBS and applied in the following concentrations (particles per well) (A) 10^7, (B) 10^6 and (C) 10^5. X-gal solution was applied 48 hours after infection. Original magnification is x 4.

Having determined the optimum dose range of the adenovirus vector, the experiment was repeated in triplicate using the following concentrations of adenovirus particles per well: 10^7, 10^6, 5 x 10^5, 10^5, 10^4 or 10^3. At the two highest adenovirus concentrations it was not possible to accurately count the number of blue cells per well. The results are displayed in **Figure E 4** as mean ± SD. Preincubation of the adlacZ vector with fetal tracheal fluid for 30 minutes significantly enhanced infection of HBE cells when compared with preincubation with PBS (two-way analysis of variance, $p = 0.004$). As can be seen, tracheal fluid taken from a sheep fetus aged 84 days of gestation had the most enhancing effect.

This enhancement effect is possibly due to factors in the fetal tracheal fluid that improve adenovirus infection of HBE cells in culture. The fetal tracheal fluid might contain growth factors that could enhance division of the HBE cells in culture. The small volume of fetal tracheal fluid (1 μl at the highest concentration of adenovirus vector) applied to each well however, makes this unlikely to be the cause of the enhancement effect. We concluded that incubation of adlacZ vector with fetal tracheal fluid for 30 minutes had no inhibitory effect on its ability to infect epithelial cells in culture.

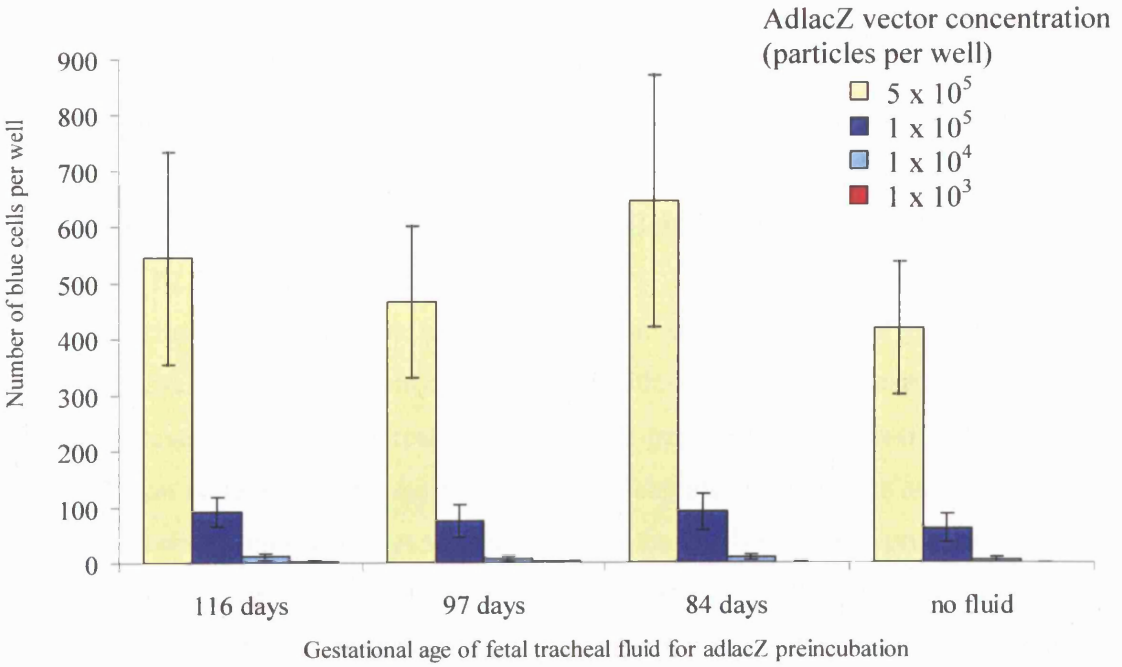


Figure E 4: The effect of fetal tracheal fluid on adenovirus infection of epithelial cells *in vitro*.

AdlacZ vector was pre-incubated for 30 minutes with tracheal fluid from fetal sheep aged 116, 97 or 84 days of gestation, or with PBS. The adlacZ vector was serially diluted with PBS and applied to HBE cells *in vitro*. Cells were stained with X-gal solution 48 hours later and the number of blue cells per well counted, presented as mean ± SD.

E 2.2 Adenovirus vector efficiently transfects the fetal sheep airways *ex vivo* and gene transfer can be improved by sodium caprate and DEAE dextran

We then performed experiments *ex vivo* to investigate adenovirus mediated gene transfer to the fetal sheep trachea through gestation. Samples were taken from fetal sheep at 78-113 days of gestation that were either uninjected (n = 2) or had received intramuscular (n = 1), umbilical vein (n = 1) or intracranial (n = 1) injection with adenovirus vector. We determined the effect of sodium caprate and DEAE dextran, either singly or in combination, on adenovirus transfection. In the adult mouse, pretreatment of the trachea with sodium caprate and complexation of the adenovirus vector with DEAE dextran had been shown to significantly enhance gene transfer to the airway epithelium (Gregory LG et al., 2002). Sodium caprate acts by reversibly opening tight junctions allowing the adenovirus vector access to the coxsackie adenovirus receptor (CAR) that is situated on the basolateral surface of the airway epithelial cells. The polycation DEAE dextran, complexes with the adenovirus vector, reducing its

negative charge and improving CAR receptor binding. Samples of the fetal trachea taken at post mortem examination were opened and cut into four sections that were randomly assigned to four different treatment groups. After 48 hours of incubation with vector the tracheal samples were stained with X gal solution overnight and the results of transgene expression analysed quantitatively by β-galactosidase ELISA (**Figure E 5, Table E 5**).

Complexing the adlacZ vector with DEAE dextran enhanced levels of gene transfer by 41% on average. Pre-treatment of the airways with sodium caprate before application of uncomplexed adenovirus increased the level of β-galactosidase expression significantly by 87% on average. The combination of sodium caprate pre-treatment and DEAE dextran complexed adenovirus further increased the levels of gene expression observed by 90% on average when compared with adenovirus alone. All treatments with enhancers resulted in significantly higher transduction levels than that obtained with adenovirus alone.

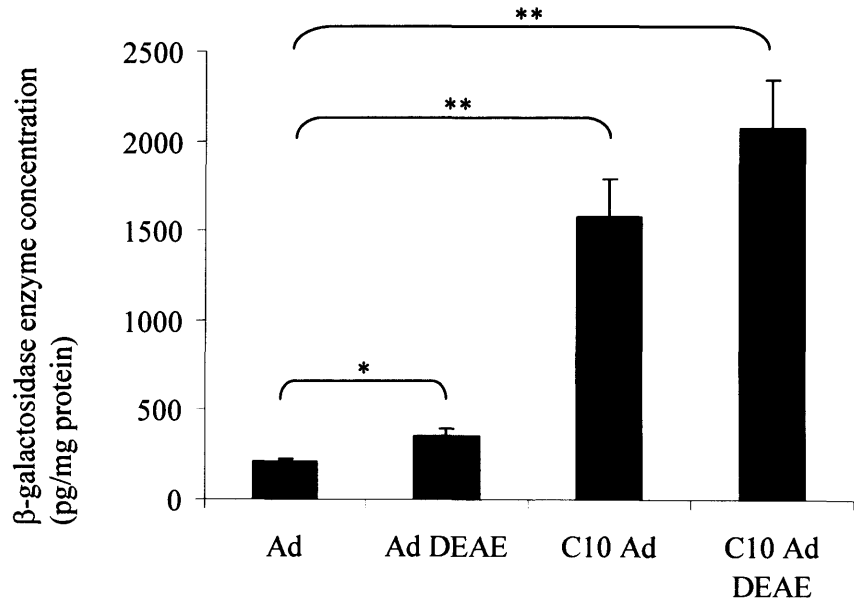


Figure E 5: Enhancement of adenovirus-mediated gene transfer to the fetal tracheal epithelium *ex vivo*.

Sections of the trachea from 5 sheep fetuses were pre-treated with 50mM Na-caprate (C10) for 10min prior to addition of either adenovirus alone (Ad) or complexed with DEAE dextran (DEAE). β-galactosidase transgene expression was analysed quantitatively using ELISA analysis. Results are expressed as averages ± SEM (n = 5). * $p < 0.05$ and ** $p < 0.01$ comparing each of the enhancers and combination of Na-caprate pre-treatment and DEAE dextran complexed virus with virus alone.

Table E 5: Enhancement of adenovirus-mediated gene transfer to the fetal tracheal epithelium *ex vivo*.

Age (days)	β-galactosidase enzyme concentration (pg/mg protein)			
	adlacZ alone	adlacZ-DEAE	C10 adlacZ	C10 adlacZ-DEAE
78	268	298	1349	1873
78	196	374	983	2250
83	203	461	1532	2963
89	234	212	1864	1941
113	196	374	983	2250

E 2.3 Experiments investigating injection of the fetal sheep trachea at post mortem

The fetal airway epithelium can be more directly targeted for gene transfer by injection of the lung parenchyma or the trachea. Ultrasound-guided injection of adlacZ vector through the uterine wall into the lung parenchyma was performed in a pilot experiment in a sheep fetus aged 98 days of gestation (personal communication Torvid Kiserud, Charles Coutelle). This resulted in low level β-galactosidase expression confined to the site of injection assessed by X-gal staining of the lungs. There was also significant haemorrhage present in the alveoli and small airways. Experiments delivering viral vectors to the fetal sheep trachea via catheters placed at laparotomy or fetoscopically result in gene transfer to the airway epithelium (Yang EY et al., 1999). We investigated the feasibility of percutaneous ultrasound-guided injection of the trachea in fetal sheep. Before attempting to inject the fetal sheep trachea *in vivo* we studied tracheal injection at post mortem in some fetal sheep available from other investigators. This allowed us to obtain measurements of the fetal trachea and to determine the direction of fluid flow in the trachea after injection. We believed that injecting the trachea in the fetal neck would be the best approach *in vivo* since a transthoracic approach might lead to damage to the large vessels in the fetal chest. As we were aiming to target the medium airways with a gene therapy vector for treatment of cystic fibrosis, we decided to pass a catheter down a needle inserted into the trachea in the neck. We also wanted to determine whether a catheter passed down beyond the needle tip, would direct injected fluid distally down the fetal airways, rather than proximally towards the fetal larynx. Different sized catheters were tested by passage down a 16 or 17 Gauge needle. A

catheter measuring 1.02mm outer diameter and 0.58 inner diameter (Sims Portex, UK) fitted down a 17 Gauge needle into the trachea. A 23 Gauge needle could then be fitted into the non-injected end for instillation of the vector. Narrower catheters were available to fit finer needles, however their small diameter meant fluid instillation took a significant length of time; more than 10 minutes for 5ml fluid.

E 2.3.1 A catheter placed via the needle in the fetal trachea at post mortem passes down the airways and directs fluid distally to the peripheral airways

Four fetal sheep were examined (**Table E 6**). The lungs and trachea were exposed by removal of the anterior chest wall and neck and the trachea measured from the top of the larynx to the carina. With the fetus lying flat on its back, a 17 Gauge needle was inserted into the trachea 2cm distal to the larynx and a measured length of catheter (ID 0.58mm, OD 1.02mm, Sims Portex, UK) was passed down. Methylene blue dye solution was injected down the catheter and the volume injected was noted when it could be seen coming out of the fetal mouth. This volume was a crude estimate of the volume of the airways. The catheter was clamped in the trachea, the needle removed and the lungs and trachea dissected *en bloc*. The trachea and main bronchi were opened and the position of the catheter noted. Tissue staining with methylene blue was observed at dissection.

Table E 6: Post mortem insertion of a catheter and needle into the trachea of late gestation fetal sheep.

Sheep fetus	Age (days)	Length of trachea (mm)	Volume of methylene blue injected (ml)	Catheter position at dissection	Tissue staining (methylene blue)
blue PM1	135	120	20	L main bronchus	pericardial and L pleural cavity
blue PM2	135	130	20	R main bronchus	all airways
blue PM3	140	130	20	20mm above carina	all airways
blue PM4	120	125	5	20mm above carina	large and medium airways

In the first fetus to be examined (blue PM1) we wanted to evaluate how easy it was to pass the catheter beyond the carina. An 18cm length of catheter was passed with some difficulty down the needle and methylene blue dye was instilled. At dissection the

catheter had passed down the L main bronchus, through the L upper lobe and perforated the visceral pleura. Methylene blue dye stained the pericardial and L pleural cavities but was not present in the fetal airways. This highlighted the potential danger of passing a catheter too far into the fetal airways. Repeating this experiment (blue PM2), the catheter passed with ease beyond the carina into the R main bronchus and methylene blue dye was spread throughout the fetal airways. In two further experiments, the catheter was passed until the tip was estimated to be just above the carina. There was widespread staining of the airways in one animal (blue PM3) into which 20ml could be injected. In the other animal (blue PM 4) however, only 5ml volume of methylene blue dye fluid could be instillled before it came out of the fetal mouth and staining was limited to the large and medium airways (**Figure E 6**). This was probably an underestimate of the volume of the airways. It emphasized however, the importance of volume in delivery to the fetal airways.

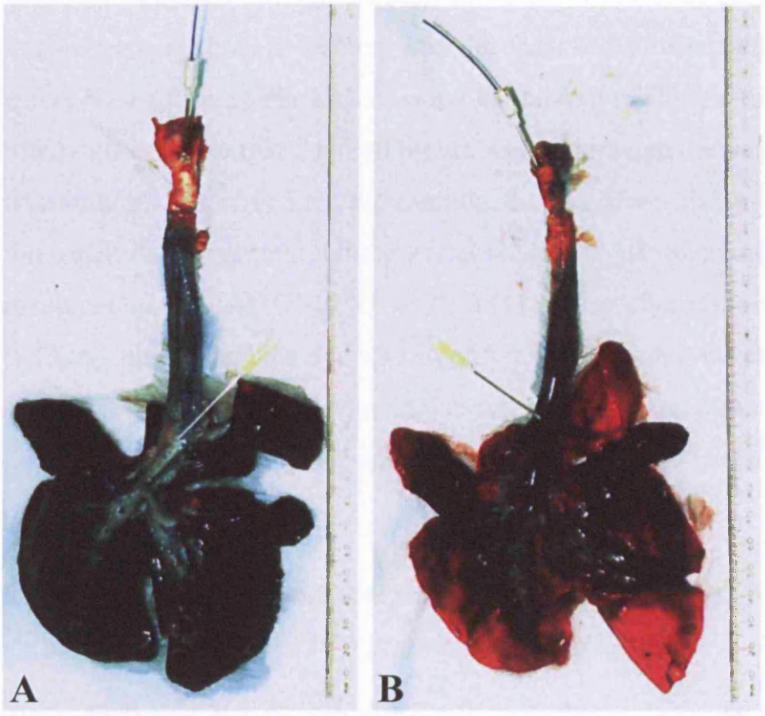


Figure E 6: Spread of methylene blue dye after tracheal instillation *ex vivo*.

The lungs and trachea of fetal sheep after *ex vivo* instillation of methylene blue down a catheter placed in the fetal trachea. The position of the tip of the catheter above the carina is indicated by the yellow needle in each case. (A) Widespread staining of the airways after instillation of 20ml dye in a sheep fetus aged 140 days of gestation (blue PM3). (B) Staining is limited to the large and some medium airways after instillation of 5ml dye in a sheep fetus aged 120 days of gestation (blue PM4).

E 2.3.2 Removal of tracheal fluid at post mortem does not result in net movement of fluid into the fetal trachea

We considered that dilution of the vector in the tracheal fluid was a potential problem for gene transfer. To avoid this, a large volume of tracheal fluid could be removed prior to vector injection but we were concerned that this might lead to influx of amniotic fluid through the fetal mouth. On the other hand it was possible that the epiglottis could close off the trachea by the creation of a vacuum within the trachea.

We explored this at post-mortem on a fetal sheep (100 days of gestation) from another investigator. Normally the late gestation fetus has breathing movements which allows tracheal fluid to efflux from the trachea. Findings at post mortem cannot be directly extrapolated to the *in vivo* situation because of the lack of muscular activity. Fetal breathing movements however, would be unlikely to happen while the fetus is anaesthetized. A 16 Gauge intravenous cannula (Becton Dickinson, Franklin Lakes, NJ, USA) was inserted through the fetal skin under ultrasound guidance into the fetal trachea just proximal to its entry into the chest and sutured in place. The fetus was submerged flat in a large sink of water containing methylene blue dye and the fetal head tilted to the side so that the fetal mouth was underneath the water surface. Tracheal fluid was withdrawn slowly from the cannula, the end of which was held above the surface of the water. At this gestation the tracheal volume is 3.85ml and the lung liquid volume measures up to 40ml (Olver RE et al., 1981). Even after removal of 30ml of tracheal fluid, no methylene blue dye was seen in it, showing that the fluid bathing the fetus was not entering the trachea via the fetal mouth. We concluded that amniotic fluid was not likely to enter the fetal sheep trachea at times when fetal breathing movements were not occurring.

E 2.4 The fetal sheep trachea can be visualised from mid-gestation using ultrasound

To develop a method of injecting the fetal trachea directly we first investigated the dimensions of the trachea in mid to late gestation fetal sheep, the gestational age at which we believed injection was most achievable and clinically relevant. Ultrasound visualisation and measurement of the fetal trachea has been investigated in the sheep at 70 days of gestation (Kalache KD et al., 2001). In this study with the animal awake, clear images of the trachea were possible in 14 out of 16 ewes examined and results were correlated with measurements obtained stereoscopically at post mortem. The

inner-to-inner diameter of the trachea in the distal third of the fetal neck measured 1.3 ± SD 0.28 mm (range 0.95 - 1.7 mm).

Measurements of the trachea (inner-to-inner diameter) in the fetal chest and in the distal third of the neck were taken in fetuses aged from 67 days of gestation from animals used in umbilical vein gene therapy injection experiments and from animals used by colleagues (**Figure E 7**). Ewes were also scanned prior to surgery to detect fetal number, to confirm viability and gestational age and tracheal measurements were obtained where possible. From 80 days of gestation the tracheal diameter in the fetal chest or neck measured at least 3 mm, and by 100 days of gestation it had increased to 5mm which we thought would be sufficiently large to permit ultrasound guided percutaneous injection.

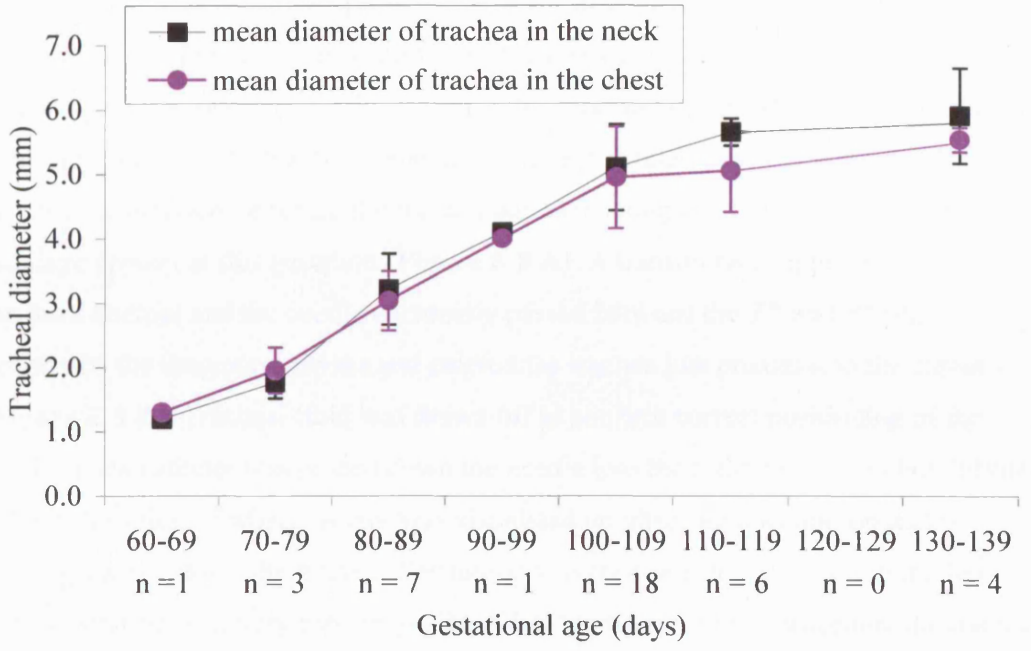


Figure E 7: Ultrasound measurement of the fetal trachea in the mid to late gestation sheep.

The inner-to-inner diameter of the trachea was measured at the fetal chest and at the distal third of the fetal neck.

E 2.5 Ultrasound guided injection of the fetal sheep trachea is achieved in late gestation

The first ultrasound-guided experiments used 3 late gestation sheep fetuses to determine whether injection of the trachea, and insertion of a catheter into the fetal airways was possible. The trachea could be easily visualized in the fetal neck and thorax in

longitudinal section and Doppler flow was used to image the blood vessels in the neck and chest. Experimental details are given in **Table E 7.**

Table E 7: Ultrasound guided injection of the fetal trachea in late gestation sheep.

Sheep	Age (days)	Injection site	Total no. attempts		Time to successful injection (minutes)
			thorax	neck	
IT1	137	thorax	1	2	35:00
IT2	138	thorax	1	2	106:00
IT3	138	thorax	1	2	46:00

E 2.5.1 A transthoracic route of injection is successful in late gestation

In the first fetus (IT1), two attempts were made initially to inject the trachea within the fetal neck using a 17 Gauge needle (Cook, UK), both unsuccessful. Visualisation of the fetal trachea during injection was difficult because of shadowing from the fetal wool that was dragged with the needle into the subcutaneous tissues. The needle also tended to either glance off the trachea in the neck where it is relatively less supported by subcutaneous tissue, or tented the trachea due to the tough fully formed tracheal cartilage present at this gestation (**Figure E 8 A**). A transthoracic approach was used on the third attempt and the needle eventually passed between the 2nd and 3rd rib, penetrated the lung parenchyma and entered the trachea just proximal to the carina (**Figure E 8 B**). Tracheal fluid was drawn off to confirm correct positioning of the needle and a catheter was passed down the needle into the right main bronchus (**Figure E 8 C**). Injection of adlacZ vector was visualized on ultrasound as microbubbles passing distally down the trachea. Perflubron was then injected after which the lung parenchyma became very echogenic. On videotape review of the procedure the trachea appeared to dilate slightly although no measurements were taken at the time of injection.

In the second fetus (IT2), injection of the trachea in the neck met with similar problems. Attempts to penetrate the tracheal cartilage with an 18 and 20 Gauge needle resulted in displacement of the trachea to the side. Following the attempt with an 18 Gauge needle, an intercostal vein was accidentally perforated and an echogenic area within the tracheal lumen thought to be a blood clot, was seen extending down from the site of needle entry to the carina. The trachea was finally accessed through the fetal thorax using a 17 Gauge needle between the 2nd and 3rd rib but the catheter would not pass beyond the end of the needle. The adlacZ vector complexed with DEAE dextran was instilled directly down the needle.

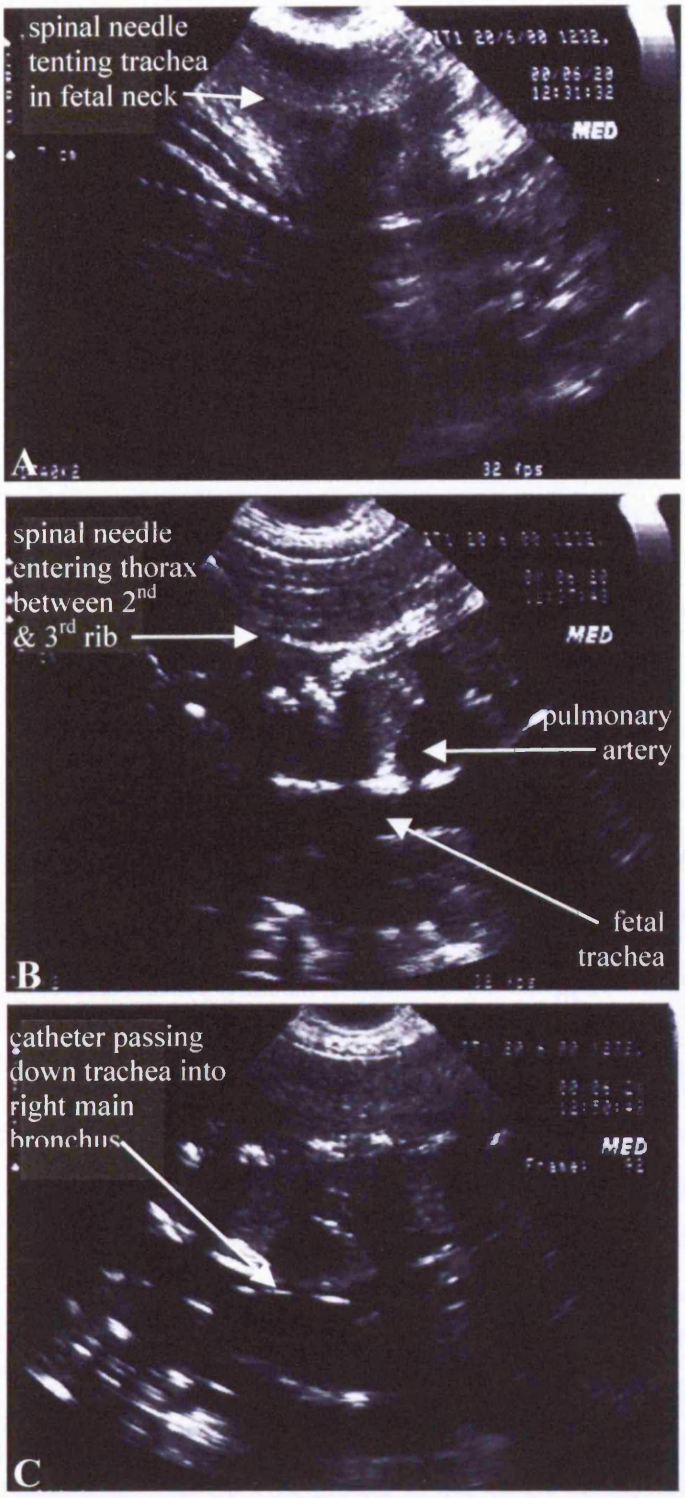


Figure E 8: Ultrasound-guided injection of the trachea in late gestation fetal sheep.

Ultrasonongrams of a sheep fetus at 137 days of gestation (IT1) during injection the fetal trachea. (A) shows tenting of the fetal trachea during an attempt to insert a 17 Gauge spinal needle into the trachea in the fetal neck. The successful transthoracic approach (B) passed the needle through the 2nd and 3rd ribs into fetal thorax and (C) a catheter was inserted through the needle down the trachea into the right main bronchus prior to delivery of adlacZ vector.

In the third fetus (IT3) a 17 Gauge needle was injected into the trachea through the fetal upper thorax after two attempts to place it in the neck were unsuccessful. The catheter was successfully passed into the left main bronchus but became bent back on itself preventing fluid instillation. The catheter was removed but the sharp needle tip sliced off a section of catheter as it was pulled up. A second attempt to site the catheter was similarly unsuccessful and resulted in a second section of catheter remaining *in situ.* The adlacZ vector complexed with DEAE dextran was instilled down the needle itself followed by perflubron. Mild tracheal dilation was again noted on video review following the procedure.

The main difficulties encountered during this set of experiments were probably related to the fetal gestation. The fetal skin, which at this gestation is covered with wool, was difficult to penetrate, obscured the view and distorted the fetal anatomy as it was dragged deep into the subcutaneous tissues by the needle tip. The tracheal cartilage was similarly difficult to penetrate. The trachea in these fetuses was accessed in the upper thorax via the transthoracic route between the 2nd and 3rd rib using a 17 Gauge needle on the first attempt.

E 2.5.2 Ultrasound guided transthoracic injection of the fetal trachea in the chest of late gestation sheep results in minimal trauma

Post mortem examination of the second fetus (IT2) 48 hours after surgery showed a blood clot on the outer surface of the upper $^2/_3$ of the oesophagus and within the tracheal lumen measuring 50mm long extending from the injection site in the fetal neck to the carina. There was no evidence of the injection site in the chest on post-mortem analysis of all three fetuses. At dissection of the third fetus (IT3), a cut section of catheter was found bent back on itself in the left main bronchus and a short piece was also found at the site of tracheal perforation (**Figure E 9 A and B**). The two fetuses injected with perflubron (IT1 and IT3) had a hyperaemic trachea and a fine fibrinous exudate on the posterior surface of both lungs (**Figure E 9 A**). Histology showed focal squamous metaplasia together with a small focus of ulceration at the site of injection, and both fetuses had fibrinous thickening of the pleura. Histological analysis of the fetal lung, lower airways and other fetal and maternal organs of all these fetuses was normal.

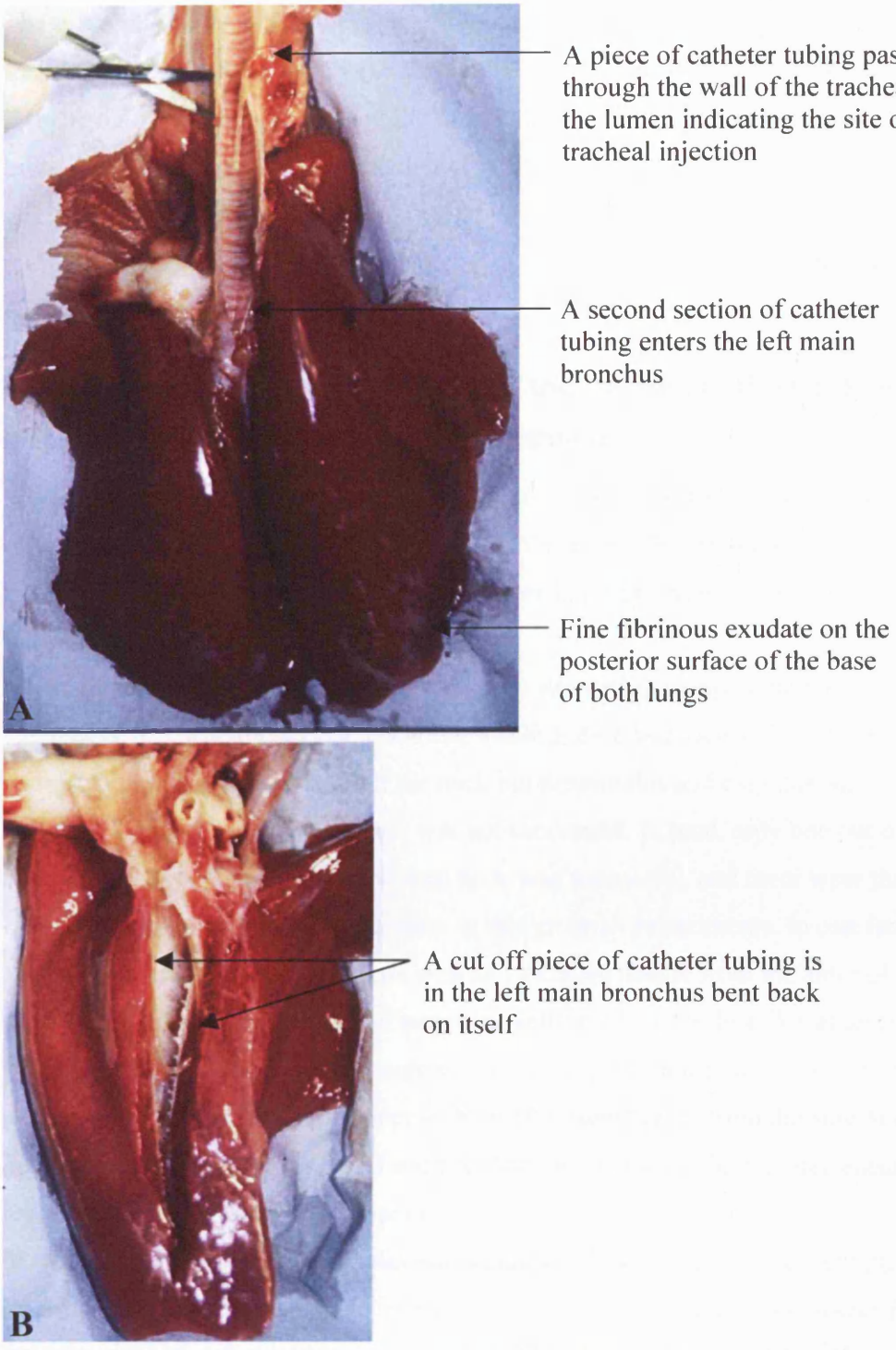


Figure E 9: Post mortem findings after ultrasound-guided tracheal injection of adlacZ vector via a catheter.

At post mortem analysis two days after placement of a fine catheter tube into the trachea and instillation of adlacZ vector and perflubron into the fetal trachea (138 days of gestation), (A) the tracheal epithelial lining was hyperaemic, and there was a fine fibrinous exudate on the lower posterior surface of both lungs. Two sections of catheter were cut off on removal of the needle and can be seen within the tracheal lumen in (A) and (B).

The use of the catheter did not appear to improve vector distribution since virus was seen instilling the distal airways even in the fetus injected without the catheter. There were also difficulties with passing the catheter down the trachea, and catheter parts being cut off, which was a common observation in fetal balloon valvuloplasty (Kohl et al., 2000). We therefore abandoned the use of a catheter while we determined the best route for tracheal injection at earlier gestations and explored the use of enhancing agents further.

E 2.6 Ultrasound guided injection of the trachea in the neck is unsuccessful in mid-gestation fetal sheep

We believed that injection of the trachea in mid-gestation would be easier at this stage of fetal development because there was no fetal wool to obscure the view and the tracheal cartilage was less well developed. More importantly, this is a clinically more relevant gestation for therapeutic intervention.

We began our experiments in mid-gestation by attempting to inject the fetal trachea in the neck (n = 3, 82-103 days of gestation, **Table E 8**). Clear views of the fetal trachea were obtained as it passed through the neck but despite this and easy passage of the needle to the tracheal wall this route was not successful. In total, only one out of 12 attempts to inject the trachea in the fetal neck was successful, and there were three failed attempts at intrathoracic injection in this group of experiments. In one fetus (102 days of gestation, IT6) two attempts with an 18 Gauge needle from the anterior of the neck were unsuccessful because of lateral movement of the trachea. A further two attempts were made using a 22 Gauge needle but this bent under the pressure applied to penetrate the trachea. A fifth attempt with an 18 Gauge needle from the side of the fetal neck was successful. Post mortem and histological analysis 48 hours after injection showed no signs of trauma or inflammation.

In two other fetuses (103 and 82 days of gestation, IT5 and CC4) there were multiple attempts via the neck (3 attempts in one and 4 in the other) and thoracic routes (2 and 1 attempt respectively) that were unsuccessful. After failing to inject the trachea of CC4, this fetus was used with its cotwin to study stimulation of fetal breathing movements (see earlier). The fetuses that received tracheal injection however, were dead at 24 (IT5) and 48 hours (CC4) after the procedure. Death in one fetus (IT5) was probably related to haemorrhage following accidental needle insertion into the vena cava which occured with the fifth injection attempt. At post mortem analysis there was a large blood clot measuring 50 x 15 x 15mm in the right pleural cavity. In the second fetus (CC4), a

small blood clot was observed in the fetal neck at the site of an injection attempt but no cause of death was found. Bacterial culture from both was negative.

Table E 8: Ultrasound guided injection of the trachea in the fetal neck of mid-gestation sheep fetuses.

Sheep	Age (days)	Injection site	Total no. attempts		Time to successful injection (minutes)
			thorax	neck	
CC4	82	failed	1	4	failed
IT6	102	neck	0	5	68:20
IT5	103	failed	2	3	failed

The main reason for failing to access the fetal trachea using this route was the relative mobility of the trachea within the neck that meant that it could slip sideways as soon as the needle tip was pressed against it. We decided therefore, to attempt a transthoracic approach in mid-gestation.

E 2.7 Ultrasound guided injection of the trachea in the chest is successful in mid-gestation fetal sheep

Having established that ultrasound-guided transthoracic injection of the trachea was possible in late gestation fetal sheep, we performed experiments to develop this transthoracic injection technique at mid-gestation (n = 34, 81-116 days of gestation). We began by injecting adlacZ vector (n = 21) together with different combinations of agents that we had shown *in vitro* and *ex vivo* to enhance adenovirus mediated gene transfer. In addition we applied the fluorocarbon perflubron that has been shown to enhance transduction in the lungs of adult rodents and monkeys (Weiss DJ et al., 1999,Weiss DJ et al., 2002a) in the hope that this might flush the vector distally towards the peripheral airways.

Having determined the optimum combination on adenovirus transfection *in vivo* (see later) we performed experiments injecting adenovirus containing the human CFTR transgene (adhCFTR, n = 11, 1 x 10^{11} – 2 x 10^{12} p/kg). All fetuses injected with adhCFTR received pretreatment with sodium caprate and DEAE dextran complexation with the adenovirus. Finally we used a novel vector, Sendai virus containing the lacZ transgene (Sendai lacZ, n = 2). For the lacZ and hCFTR adenovirus vectors, post mortem analysis was performed at a few different time points after injection (**Table E 9**). Data on the injection technique from all transthoracic injections are presented together.

The results of adenovirus vector mediated lacZ transgene expression and its enhancement with DEAE dextran and sodium caprate and the effect of perflubron are described in **Section E 2.8**. Results of Sendai vector mediated lacZ transgene expression are described in **Section H 4.2**. Antibodies to detect human CFTR against a sheep background for hCFTR immunohistochemistry were not available at the time of this thesis. Testing of a panel of 9 antibodies to hCFTR on mixtures of human and sheep nasal epithelial cell lines has revealed two that did not react to endogenous sheep CFTR (personal communication, Dr H Davidson, Medical Genetics, University of Edinburgh) and this is the focus of ongoing studies.

Table E 9: An overview of transthoracic injection experiments in mid-gestation fetal sheep.

Vector	Gestational age at injection (days)	Sampling (post mortem)	Fetal number
AdlacZ	81	2 days	4
AdlacZ	82	16 days	1
AdlacZ	100 – 115	2 days	14
AdlacZ	100 – 115	Birth	1
AdhCFTR	81 – 82	Birth	2
AdhCFTR	100 – 115	2 days	2
AdhCFTR	100 – 115	17-21 days	3
AdhCFTR	100 – 115	Birth	2
Sendai lacZ	100 – 104	2 days	2

E 2.7.1 Transthoracic injection of the fetal trachea is reproducible and can be achieved in a short time

Injection of the trachea using the transthoracic route was achieved in 31 out of 34 fetuses **(Table E 10)**. A 22 Gauge needle was used to inject one fetus aged 81 days of gestation (IT4) and an 18 Gauge needle was tested on two fetuses at 108 and 115 days of gestation (IT9 and IT10). A 20 Gauge spinal needle however, provided the optimum combination of flexibility to manoeuvre between the ribs in the thorax, stiffness to enable the needle to be 'flicked' through the tracheal wall and size to allow easy tissue penetration. The first mid-gestation fetus to be injected via the transthoracic route was aged 81 days of gestation. Because of the difficulties encountered we developed the technique further in fetuses aged at least 3 weeks older (102 days of gestation) and returned to the earlier gestation once the technique had been established.

Table E 10: Ultrasound-guided injection of the fetal trachea in the chest in mid gestation fetal sheep.

Results are displayed in chronological order.*indicates twin fetuses; Time: time to successful injection; Poor view: poor ultrasound view of fetal trachea due to fetal lie.

Sheep	Age (days)	No. attempts		Time (min)	Poor view	Complications during procedure
		thorax	neck			
IT4 co twin	81*	0	0	failed	Y	
IT4	81*	1	0	7:00		
IT7	102	1	0	10:00		
IT8	109	2	1	57:51		
IT9	108	2	0	52:00	Y	
IT10	115	1	0	3:42		
IT11	116	1	0	37:00		needle dislodged
IT12	109	1	0	7:19		
IT13	116	1	0	9:03		
IT14	109	1	0	10:05		
IT15	109	1	0	05:27		
IT16	102	1	0	02:59		
IT17 cotwin	102*	1	1	failed	Y	
IT17	102*	1	1	12:30		
IT18	102	1	0	12:00		
IT19	102	1	0	03:59		
IT20	102	1	0	04:13		
IT21	81	5	0	68.19		tracheal collapse
IT22	81*	1	0	5:32		
IT23	81*	1	0	4:17		
IT24	82	1	0	9:30		
IT25	102*	1	0	3:39		
IT26	102*	1	0	3:36		blood vessel accident (chest)
IT27	102	1	0	19:55		blood vessel accident (chest)
IT28	102*	1	0	19:41	Y	
IT29	102*	1	0	13:32		
IT30	81	1	0	2:23		
IT31	113	1	0	23:03		blood vessel accident (chest)
IT32	113	3	0	failed	Y	blood vessel accident (chest)
IT33	110	1	0	2:44		
IT34	82	1	0	4:00		
IT35	100	2	2	51:35	Y	blood vessel accident (neck)
IT36	100	1	0	8:24		
IT37	104	1	0	8:55		

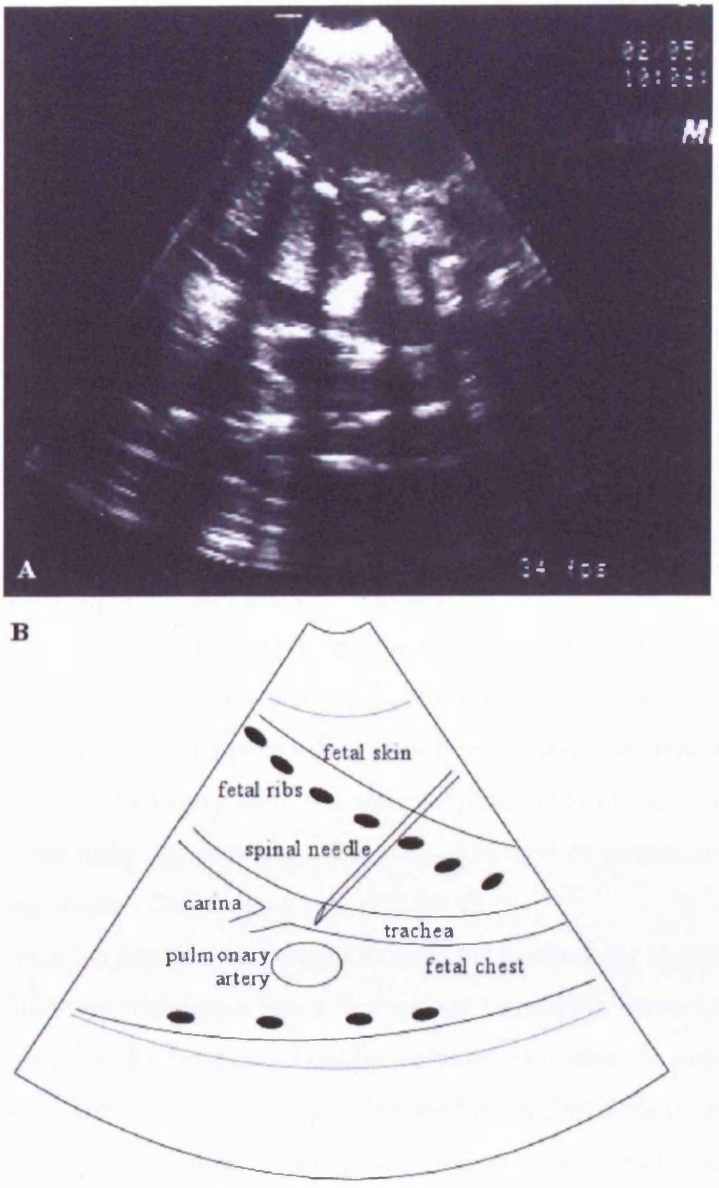


Figure E 10: Ultrasound-guided injection of the trachea using the transthoracic route.

An ultrasonogram (A) and diagram (B) showing injection of the trachea using the transthoracic route in a sheep fetus (114 days of gestation). A 20 Gauge spinal needle is inserted into the fetal thorax between the 3rd and 4th rib, penetrates the lung parenchyma and enters the fetal trachea just proximal to the carina.

The trachea in the fetal neck and chest was scanned in the coronal plane. **Figure E 10** shows an ultrasonogram of a typical injection using the transthoracic route. The fetus was lying right side up and the needle passed anterior to the fetal shoulder and arm, through the chest wall between the 3rd and 4th ribs, into the fetal trachea just superior to the carina avoiding the great vessels. In this fetus the pulmonary trunk was posterior to the trachea which made tracheal access more straightforward. In 6 fetuses (18%) there

was a poor view of the trachea, defined as inability to visualize part or all of the trachea from the distal third of the neck to the carina. This fetal position was observed in nearly two-thirds of cases (22 out of 34 fetuses) and in only 2 fetuses was there a poor view of the trachea, which was caused by the fetal humerus obscuring it. Tilting the ewe on the theatre table and moving the humerus with the needle improved the view.

For the remaining experiments (12 out of 34 fetuses), the fetus was lying so that the pulmonary trunk (n = 10) or aorta (n = 2) was anterior to the trachea and in these cases the needle was passed only between the 2nd and 3rd rib so as to penetrate the trachea superior to the arch of the aorta. In this position there was a poor view of the trachea in 4 fetuses caused by the fetal scapula obscuring the trachea. Positioning of the great vessels anterior to the trachea was significantly associated with a poor view of the fetal trachea (Chi-squared test, $p<0.0025$).

The gestational age at injection was also associated with the quality of the ultrasound view of the trachea. In the younger age group (81 – 83 days of gestation), there was only one case of a poor view out of 8 injected fetuses that was in a twin pregnancy (IT4) and occurred early on in our series. All the other instances of poor view (n = 5) occurred in the older age group (n = 26, 100 – 116 days of gestation) and this was statistically significant (Chi-squared test, $p<0.0005$).

Once the needle was thought to be in the trachea, the trocar was removed and tracheal fluid was withdrawn into a 2ml syringe to confirm correct needle placement. Occasionally fluid could not be withdrawn because the needle tip was in the tracheal wall. While a vacuum was maintained in the 2ml syringe the needle tip was moved slightly, positioning the tip in the tracheal lumen which was confirmed by withdrawal of tracheal fluid. The gene therapy vector and transduction enhancing agents were instilled into the fetal trachea and could be seen flowing down the bronchii as microbubbles (**Figure E 11**). Following injection of perflubron, the small airways and lung parenchyma became very echogenic and this obscured the ultrasound view. However there was no change in the fetal heart rate or in the breathing movements, which were absent while the ewe was under anaesthesia.

It was not possible to scan the fetal mouth during vector instillation to detect movement of fluid out of the trachea. However only a small volume of fluid was occasionally seen flowing proximally in the trachea towards the larynx, suggesting that very little, if any injected viral vector escaped from the trachea into the amniotic fluid.

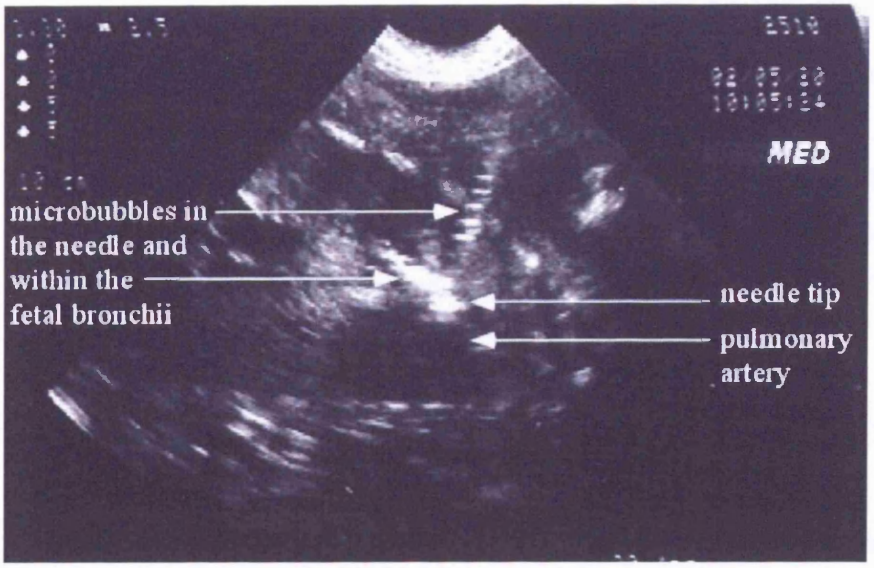


Figure E 11: Ultrasound-guided delivery of adlacZ vector to the fetal trachea.

Microbubbles are visualised passing down a 20 Gauge needle, into the trachea and down the bronchii of a fetal sheep (109 days of gestation) on instillation of adlacZ vector.

There were three procedure failures (8.8%). Two occurred in fetuses from twin gestations in which fetal positioning prevented a good view of the trachea. In one fetus (IT4 cotwin) lying deeply with the back anterior, the trachea could not be adequately visualized and in the other fetus also positioned with its back anterior (IT17 cotwin) the procedure was abandoned after attempts to access the fetal trachea in the thorax and neck failed. The other procedure failure occurred in a singleton fetus (IT32) lying back up in which the right fetal scapula obscured the view of the trachea despite maximum tilting of the ewe on the table and efforts to move the scapula with the needle. The pulmonary artery positioned anterior to the trachea was accidentally punctured and a large blood clot filled the trachea masking it from the surrounding soft tissues. The procedure was abandoned. Post mortem examination showed a large blood clot extending from the upper trachea to the medium airways, and 15ml blood in the right pleural cavity.

In 28 out of 31 successful procedures (90%), transthoracic injection was achieved at the first attempt. The mean time taken to complete the injection procedure after a successful first attempt was 11 minutes 31 sec (SD 13 minutes 31 sec, range 2 minutes 40 sec – 68 minutes 19 sec). In two fetuses complications following successful first injection prolonged the injection procedure to 37 minutes and 68 minutes 19 sec after needle dislodgement (IT11) and tracheal collapse (IT21) respectively. Excluding these cases,

successful first attempts were straightforward in 26 out of 28 fetuses and completed in a mean time of 8 minutes 22 sec (SD 5 minutes 39 sec, range 2 minutes 40 sec – 23 minutes 3 sec). Of three fetuses that failed transthoracic injection at the first attempt, a second attempt was successful in all three. There were attempts in two fetuses (IT8 and IT35) to inject the trachea in the fetal neck that failed before a second attempt to inject the trachea in the fetal chest was successful.

E 2.7.2 The transthoracic route to inject the fetal trachea has a low complication rate

Complications during the transthoracic tracheal injection procedure are listed in **Table E 10** and can be classified into three main types: tracheal collapse during injection, needle dislodgement during vector instillation and blood vessel accident.
Tracheal collapse occurred in one fetus aged 81 days of gestation. A 20 Gauge needle was passed with ease into the fetal chest and was thought to be in the tracheal lumen. Tracheal fluid was not aspirated however, and therefore the needle tip was repositioned while a vacuum was maintained using a 2ml syringe. Tracheal fluid was suddenly withdrawn (1.5ml) when the needle was correctly positioned in the trachea and the trachea collapsed, falling off the needle tip. Over the following hour three unsuccessful attempts to inject the trachea in the chest were made. As fluid reaccumulated, the trachea was more easily seen and the fifth injection attempt was successful. This was the first 81 day old fetus to be injected after the technique had been established in older fetuses. As can be seen from **Table E 11**, the volume of the tracheal and lung fluid increases by almost five and ten fold respectively between 85 and 105 days of gestation.

Table E 11: The volume of the lung and tracheal liquid in fetal sheep through gestation.

Values are given as the mean ± standard error of the mean (Olver RE et al., 1981).

Gestational age (days)	Lung liquid volume (ml)	Tracheal volume (ml)
74.1 ± 0.68	0.955 ± 0.220	0.295 ± 0.027
84.8 ± 0.55	2.993 ± 0.368	0.831 ± 0.079
96.1 ± 0.93	9.298 ± 1.937	1.603 ± 0.170
105.2 ± 1.6	34.57 ± 4.92	3.85
113.4 ± 0.7	49.17 ± 3.42	3.69
125.31 ± 0.72	69.52 ± 7.09	5.07
134.9 ± 1.03	85.41 ± 16.47	5.37 ± 0.64
126.4 ± 0.42	76.23 ± 4.99	not available
140.0 ± 0.19	122.6 ± 3.75	not available

The vacuum created by the syringe during the first injection attempt resulted in withdrawal of approximately half the fluid volume of the lungs and trachea, causing tracheal collapse. Following this complication, the plunger on the syringe was withdrawn enough so as to remove only 500μl fluid and this complication has not recurred.

There was one incidence of needle dislodgement from the trachea in the middle of viral vector instillation. This occurred in a fetus aged 116 days of gestation (IT11) during a straightforward transthoracic injection procedure. After instillation of sodium caprate and viral vector the needle tip was observed in the subcutaneous tissues proximal to the trachea. The needle was reinserted into the trachea to allow delivery of perflubron. Histological analysis of tissues showed no evidence of trauma in the subcutaneous tissue surrounding the fetal trachea and there was no positive X-gal staining or positive β-galactosidase immunohistochemical localization in the tissues around the trachea. Following this complication we reviewed our delivery procedure and considered attaching an extension set and three-way stopcock (Connecta™ Plus 3, Becton Dickinson, Helsingborg, Sweden) to the end of the needle for instillation of gene therapy vectors as used in fetal blood transfusion procedures clinically. This would minimize movement of the needle after correct placement. The dead space of the extension set and three-way stopcock was large (500μl) in comparison to the total volume of fluid injected into the trachea, especially in the earlier gestation fetal sheep. We were concerned that use of an extension set could reduce the amount of viral vector delivered and since we were already using large doses requiring a considerable preparation time, we did not believe their use in this context was justifiable. No further needle dislodgments occurred despite continuing to use individual syringes to deliver the viral vector and enhancing agents. For clinical application however, use of an extension set and three-way stopcock would be a safer procedure.

Accidental damage to a blood vessel occurred in 5 fetuses (15%) but this lead to significant morbidity in only two cases (6%). Major vessel damage occured in one fetus (IT32) in which the pulmonary artery was punctured resulting in a large haematoma within the trachea and the procedure was abandoned. In one twin fetus (IT26, chest vessel puncture) a left sided pleural effusion measuring 27 x 36mm was noted on ultrasound scan the day after surgery; on the second day, large bilateral pleural effusions and ascites were present **(Figure E 12 A and B)**.

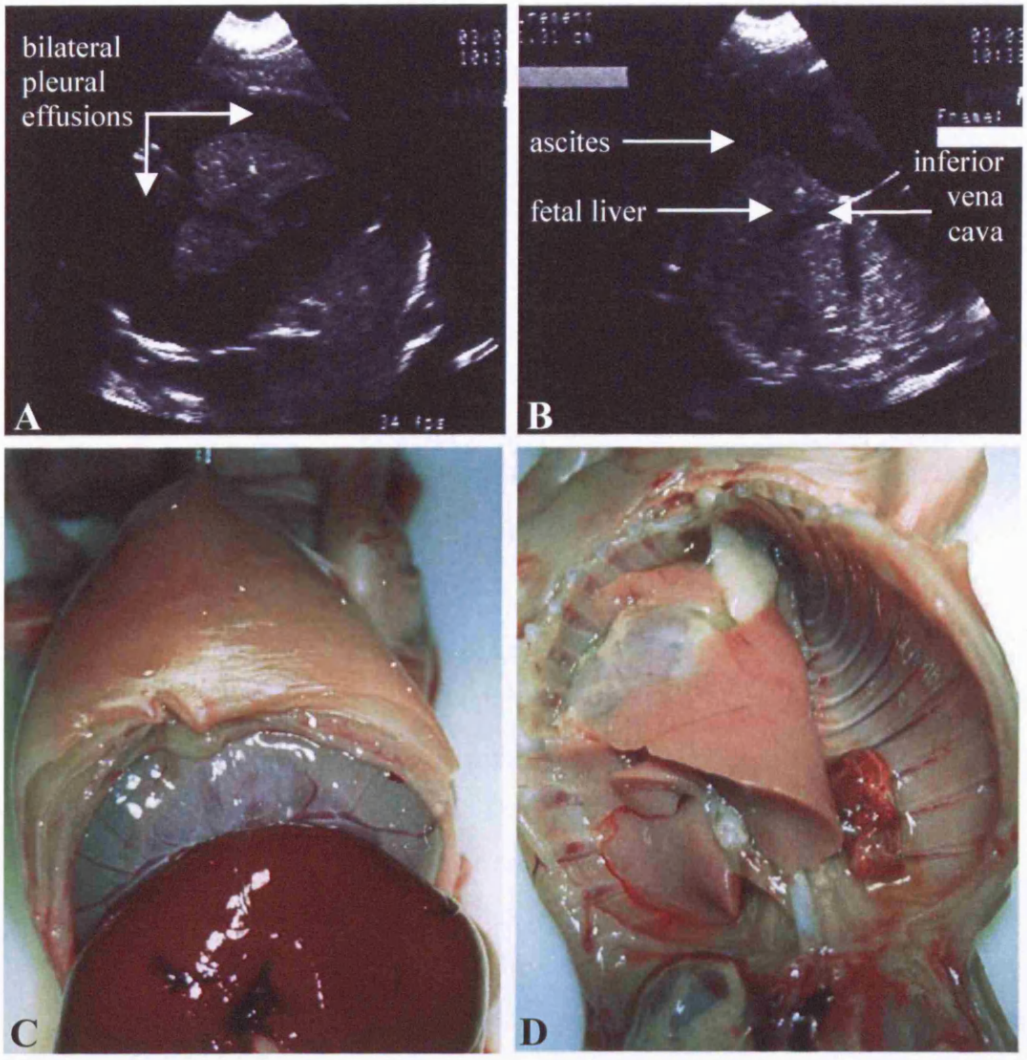


Figure E 12: Complications after ultrasound-guided transthoracic tracheal injection in mid-gestation.

Two days after ultrasound-guided transthoracic tracheal injection (102 days of gestation, IT26) during which a vessel was punctured in the fetal chest, ultrasonograms show (A) bilateral pleural effusions in the thoracic cavity in transverse section and (B) ascites and a pleural effusion either side of the fetal diaphragm in a transverse section of the upper peritoneal cavity. At post mortem examination there is (C) subcutaneous oedema in the skin over the abdomen and thorax and a large bilateral pleural effusion distending the diaphragm, and (D) an organised blood clot in the left pleural cavity with clear pleural fluid.

Post mortem analysis that day showed a severely hydropic fetus with right (50ml) and left pleural effusions (60ml) **(Figure E 12 C)**. A small blood clot measuring 30 x 20 x 5mm was present in the left pleural cavity **(Figure E 12 D)** although no vessel damage was visible. The pleural and ascitic fluid was of low protein content, very low cellularity with rare erythrocytes and occasional mononuclear cells which is consistent with it being a transudate. A full blood count taken at post mortem examination showed that the haemoglobin concentration was lower in this fetus (12 g/dl) than in of its co-

twin (14.7 g/dl) but still within the normal range. Overall these results suggest that the haemorrhage caused by the procedure was small, but was sufficient to compromise cardiac function causing heart failure.

In three cases there was damage to a minor vessel in the chest (2 fetuses, IT27, IT31) or neck (1 fetus, IT35). Post mortem examination of these fetuses 2 days after surgery (neck vessel puncture) or 17 days after surgery and 1 day after birth (chest vessel puncture) showed no haematoma, inflammation or scar tissue. It is interesting to note that no blood vessel accidents occurred in fetuses aged 81 – 82 days of gestation.

E 2.7.3 Ultrasound guided transthoracic injection of adenovirus vectors and transduction enhancing agents into the fetal trachea results in some morbidity

Post mortem examination and histological analysis of tissues was performed on all fetuses 2, 16 or 17 days after injection or 1 day to 1 week after birth (**Table E 12**). Post mortem and histological examination was completely normal in 15 (48%) and 8 (28%) out of 29 injected fetuses respectively. The maternal and fetal organs other than the lungs and trachea were normal in all cases.

The most common abnormal findings at post mortem examination were pleural and/or pericardial adhesions (5 fetuses, 17%). These were generally small and all were situated at the upper lobes of the lung probably at the site of injection through the pleura (**Figure E 13 A**). A visible injection site was seen in the fetal skin, chest wall or lung in 4 fetuses (14%) and occurred only in fetuses aged 81 – 82 days of gestation (**Figure E 13 B**). This probably reflects the relative larger size of the needle compared to the fetus at this earlier gestation. The mean fetal weight was 428 ± 57 g and 2100 ± 849 g at 81 – 82 and 100 – 116 days of gestation respectively.

Clear pericardial fluid (1.5ml) and a thickened yellow pericardium was observed in one fetus (IT31) at post mortem analysis. Histological analysis of the pericardium was normal, and Giemsa stain of the pericardial fluid showed no evidence of inflammatory cells, suggesting this was a transudate rather than an inflammatory exudate.

Haemorrhagic lungs were observed at post mortem or histological analysis in 3 fetuses (10%) aged 81 – 82 days of gestation (**Figure E 13 C**). These fetuses received large volumes of vector and enhancing agents: 10.5 ml (IT4), 7.5 ml (IT21) and 6 ml (IT22). Following this observation, the total volume delivered to the trachea of younger fetuses was reduced to 1.2 ml and lung haemorrhage was not seen subsequently.

Table E 12: Post mortem and histological analysis of mid-gestation fetal sheep after transthoracic injection of adenovirus vectors.

†: adhCFTR vector; h: hrs; d:days; wk: week; PN: postnatal, PM: time of post mortem after injection; C: sodium caprate; D: DEAE dextran; P: perflubron; GA: gestational age at injection.

Sheep	PM	C	D	P	GA (d)	Post mortem	Histology
IT4	2 d	-	+	-	81	visible injection site haemorrhagic lung	ulcerated focus in trachea, haemorrhagic lung focus
IT21	2 d	+	+	-	81	visible injection sites pleural fluid + clot	haemorrhagic injection site, necrosis + lung haemorrhage
IT22	2 d	+	+	-	81	visible injection site haemorrhagic lung	haemorrhagic injection site in lung
IT23	2 d	+	+	+	81	visible injection site	normal
IT24	16 d	+	+	-	82	pleural adhesions, thick pericardium	thick pleura + pericardial lymphocyte infiltration
IT30†	12 h PN	+	+	-	81	pleural -pericardial adhesion	normal
IT34†	1 wk PN	+	+	-	82	consolidation + mucus pasteurella pneumonia	confluent bronchopneumonia
IT35†	2 d	+	+	-	100	normal	normal
IT7	2 d	+	+	-	102	normal	normal
IT16	2 d	+	-	-	102	normal	foamy material
IT17	2 d	+	+	-	102	normal	granular material
IT19	2 d	+	+	-	102	normal	normal
IT20	2 d	+	+	-	102	normal	granular material
IT25†	2 d	+	+	-	102	normal	normal
IT26†	2 d	+	+	-	102	bilateral pleural effusions pleural clot	pigmented material
IT9	2 d	+	+	+	108	white grainy patches	focal acute inflammation small airways + mucosa
IT8	2 d	+	+	-	109	normal	mild focal neutrophilia
IT12	2 d	+	-	+	109	white grainy patches	pigmented material
IT14	2 d	-	+	-	109	normal	pigmented material
IT15	2 d	-	-	-	109	normal	pigmented material
IT10	2 d	-	+	-	115	normal	normal
IT11	2 d	-	+	+	116	white grainy patches	normal
IT13	2 d	-	-	+	116	normal	pigmented material
IT28†	17 d	+	+	-	102	normal	meconium in alveoli
IT29†	17 d	+	+	-	102	pleural-pericardial adhesion	meconium in alveoli
IT31†	17 d	+	+	-	113	pericardial fluid, thick pericardium	mild alveolar + bronchiolar neutrophilia
IT18	6 h PN	+	+	-	102	small pleural adhesion	pleural thickening
IT27†	1 d PN	+	+	-	102	normal	few alveolar neutrophils
IT33†	1 d PN	+	+	-	110	normal	few alveolar + bronchiolar neutrophils

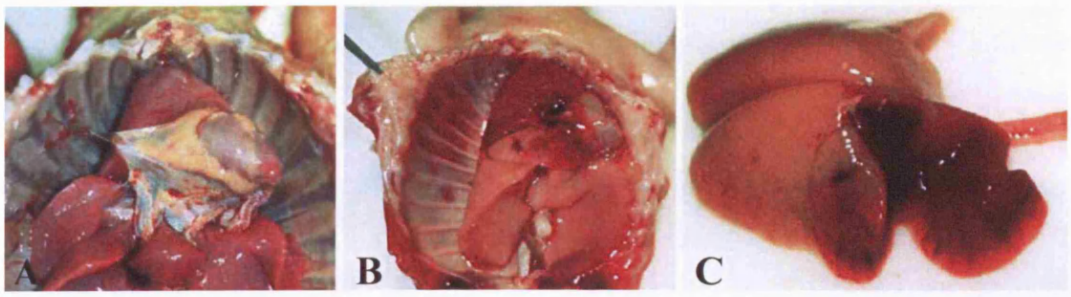


Figure E 13: Post mortem findings after ultrasound-guided transthoracic tracheal injection of mid-gestation fetal sheep.

(A) A pleural-pericardial adhesion 17 days after injection at 102 days of gestation (IT29). (B) A visible injection site on the inner chest wall and (C) haemorrhage in the upper right lobes of the lung at the site of injection 2 days after injection at 81 days of gestation (IT21).

Other findings were white grainy patches on the lung bases (3 fetuses, 10%) that were only seen in fetuses injected with perflubron as in the late gestation fetuses. A pleural clot was seen in two fetuses (7%), one complicated by tracheal collapse during injection and the other with an accidental blood vessel puncture in the chest. One of the five lambs born following tracheal injection of adhCFTR vector developed pasteurella pneumonia 6 days after birth which was seen on post mortem analysis as lung consolidation (see below).

In two fetuses (7%, IT10 and IT22) there was X gal staining observed on the outer surface of the visceral pleura (**Figure E 18**). This suggested that the visceral pleura was perforated either during the tracheal injection procedure itself or during delivery of the fluid. The injection procedure was straightforward in both cases and fluid was observed passing down the tracheal lumen. A large volume of aqueous fluid however, was delivered to both fetuses, 15 ml to IT10 (115 days of gestation) and 6ml to IT22 (81 days of gestation) and we hypothesized that this was responsible for the pleural perforation. To prevent this occurring again, the total aqueous volume applied to the fetuses was reduced.

On histological examination, pigmented or foamy granular material was commonly seen within the lumen of the small and medium airways (8 fetuses, 28%, **Figure E 14 A, B & C**) but there was no associated inflammatory infiltrate. Four of these fetuses had received a combination of sodium caprate and DEAE dextran complexed adenovirus. The other four fetuses however, had received various combinations of therapy (virus, perflubron or DEAE dextran alone or sodium caprate and perflubron combined). In two cases (IT15 and IT17) there was an uninjected cotwin available for comparison and

small amounts of granular material were observed in the airways. Small amounts of meconium were occasionally observed in the alveoli without an inflammatory infiltrate (2 fetuses, 7%).

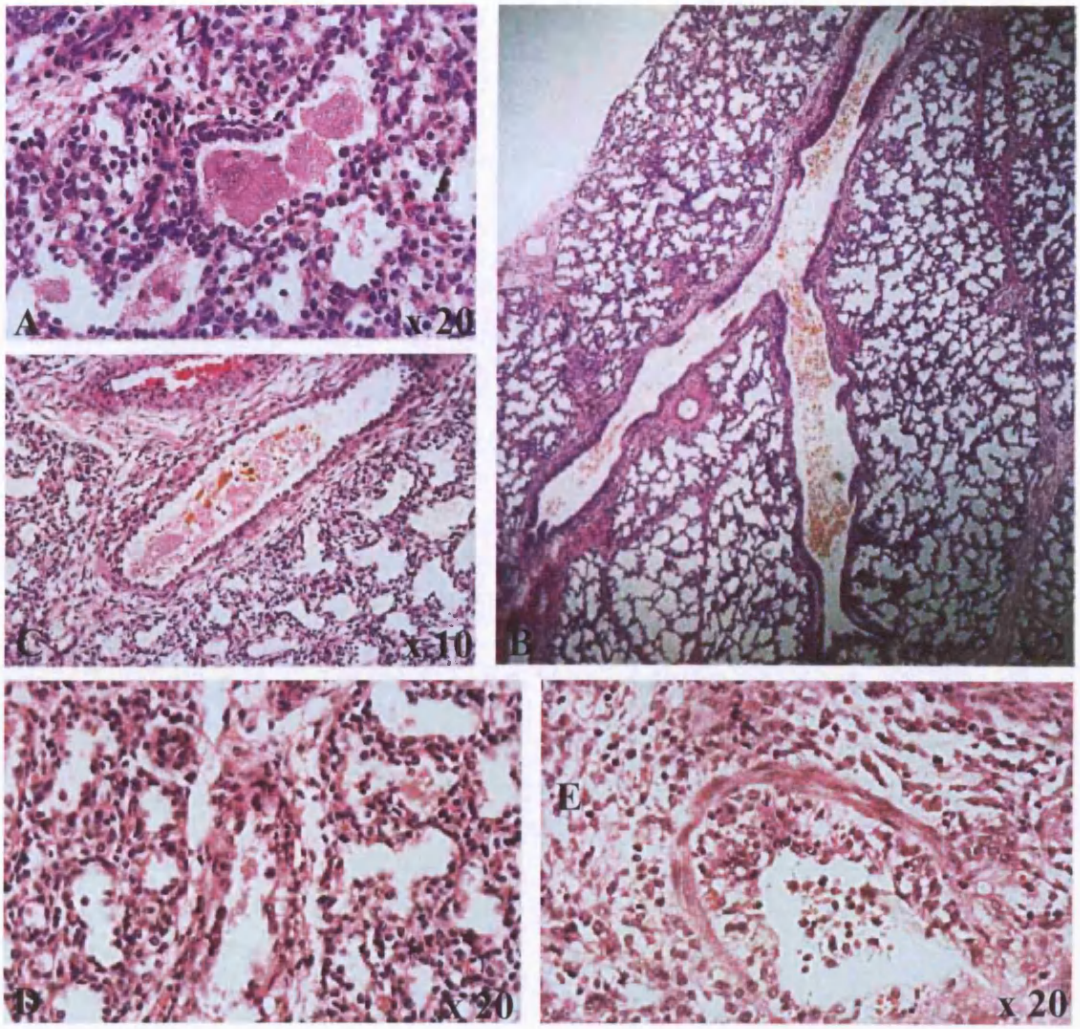


Figure E 14: Histological findings in the fetal airways after tracheal injection of adlacZ vector.

Light microscopy (H & E stain) of the trachea and airways shows in the lumen (A) non-pigmented foamy material after injection of adlacZ and sodium caprate (IT16), (B) pigmented material after injection of adlacZ and perflubron (IT13), (C) pigmented and non-pigmented foamy material after injection of adlacZ, sodium caprate and perflubron (IT12). Pigmented material was also present in the lumen of airways after injection of adlacZ vector alone.
(D) and (E) There is focal fluid in the alveoli and bronchioles with widespread focal acute inflammation in the mucosa of small airways following injection of sodium caprate, adlacZ DEAE dextran complex and perflubron (IT9). Original magnifications are as indicated. Fetal sheep were aged 102-116 days of gestation at injection.

A neutrophilia was seen in the alveoli and/or bronchioles of 3 fetuses and 2 lambs (17%). This was mild in four animals all of which had received sodium caprate and DEAE dextran complexed adenovirus. In the fifth animal that had received perflubron in addition to sodium caprate and DEAE dextran (IT9), histological analysis showed widespread focal acute inflammation in the mucosa of the small airways (**Figure E 14 D & E**).

E 2.7.4 Health of lambs following transthoracic injection in mid-gestation

Lambs born following tracheal injection were examined after birth (**Table E 13**).
No signs of respiratory distress were noted in any lamb immediately following birth. Blood was analysed for haematology, biochemistry, liver function and bile acids from all lambs 6 hours after delivery and results were normal.

Table E 13: Vital signs in lambs born after transthoracic injection of the trachea.

†: use of adhCFTR, PN: postnatal.

Sheep	Time of examination	Resp rate (/min)	Pulse rate (/min)	Oxygen saturation (%)
IT18	6 hrs PN	76	170	90
IT30†	12 hrs PN	78	190	92
IT27†	1 day PN	70	190	88
IT33†	1 day PN	64	160	92
IT34†	6 hrs PN	80	186	90
	6 days PN	100	158	87
	7 days PN	120	160	92

A non-injected twin (IT33 cotwin) was found dead in the morning 24 hours after a straightforward delivery. The lamb had been well and feeding normally in the 12 hours after birth and post mortem examination revealed a large retroperitoneal haematoma extending from the upper pole of the kidney to the pelvis. There were no other abnormalities and microbiological culture of tissues showed mixed bacterial growth including Pasteurella. The most likely cause of death was thought to be crushing by the ewe, especially since she had retained the placenta that can lead to infection and unsteadiness (M Sheldon, personal communication).
One lamb (IT34) developed a tachypnoea 6 days after delivery but was otherwise well with no temperature and was feeding normally. Post mortem examination had been

planned to take place 1 week after birth to allow for long term investigation of antibody responses to the gene therapy. This showed dense consolidation of the right lungs and the peripheral left lungs, with profuse thick green mucus in the large, medium and small airways (**Figure E 15 A**). There was confluent pneumonia on histological examination (**Figure E 15 B**) and microbiological analysis showed Pasteurella and Escherichia coli in 2 lungs lobes and a pure growth of Pasteurella from the mucus.

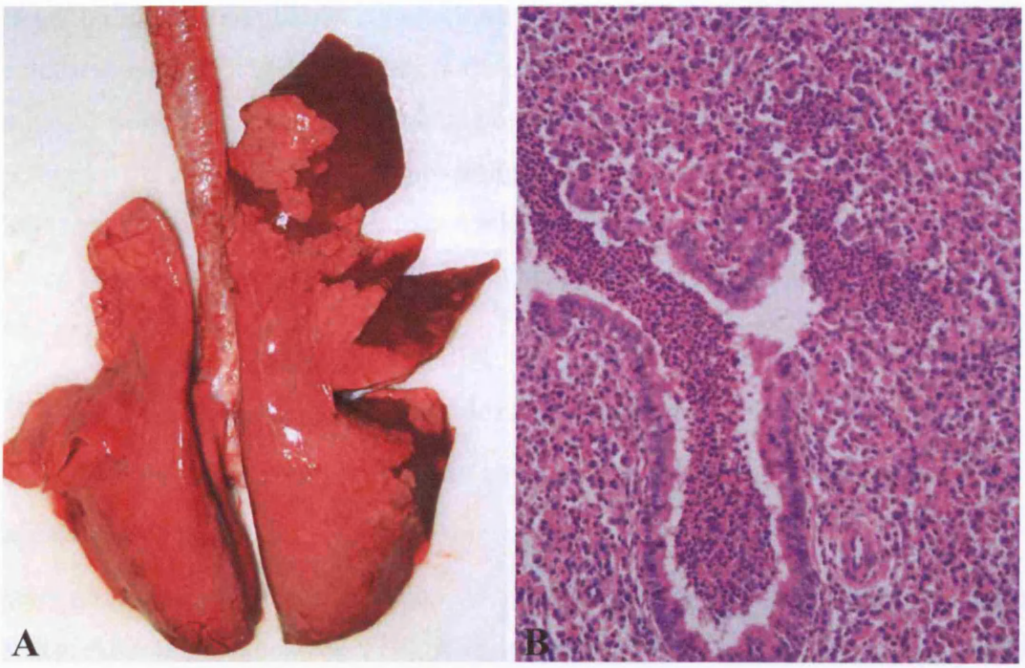


Figure E 15: Evidence of pneumonia in a lamb that received fetal tracheal injection.

Post mortem and histological findings (H & E stain) in the lungs of a 1 week old lamb that had received tracheal injection of adhCFTR (2 x 10^{12} p/kg) and transduction enhancing agents at 82 days of gestation. (A) There is dense consolidation of the right lungs and (B) confluent bronchopneumonia with neutrophilia in the upper right lobe of the lung (x 40).

Pasteurella haemolytica is a common cause of pneumonia and death in lambs and is a commensal in domestic sheep. Ewes were routinely vaccinated a few weeks before they were due to deliver so that their colostrum can passively immunize the lamb when it starts to suckle. Factors such as stress and handling increase their susceptibility but more importantly, respiratory viruses such as adenovirus 6 and bovine adenovirus type 2 predispose the lamb to secondary pasteurella infection (Brogden KA et al., 1998). It is possible that low level inflammation caused by the adenovirus vector predisposed the lamb to pneumonia despite maternal vaccination. In neonatal rats, tracheal instillation of adenovirus induced only mild non-significant inflammation, but airways morphometric

analysis showed dose-dependent lung growth impairment (Waszak P et al., 2002). This highlights the importance of long-term postnatal evaluation of the effect of prenatal gene therapy.

E 2.8 Adenovirus mediated transgene expression in late and mid-gestation fetal airways can be enhanced

In adult rodents a significant stimulation of adenovirus mediated gene transfer has been achieved in the airways, as already outlined in **Section A 5.5**, by complexing the virus with the polycation DEAE dextran, by pretreating the airways with sodium caprate (Gregory LG et al., 2002) or by application of the inert fluorocarbon perflubron (Weiss DJ et al., 1999). In order to to improve adenovirus reporter gene transfer to the fetal sheep airway epithelium we therefore explored enhancement of gene transfer in the late and mid-gestation fetal sheep.

E 2.8.1 Combinaton of DEAE dextran and perflubron enhances adenovirus mediated transgene expression in late gestation fetal airways

We first investigated the effect of DEAE dextran and perflubron in late gestation fetal sheep. After application of 2 x 10^{10} p/kg adlacZ vector to the trachea of a late gestation fetal sheep (IT1, 137 days) and instillation of 10ml perflubron to flush the vector distally, we did not observe any positive transgene expression with X-gal staining or β-galactosidase immunohistochemical analysis. Similarly complexing the adlacZ vector (IT2, 3.1 x 10^{10} p/kg, 137 days) with DEAE dextran (5μg/ml) did not result in any detectable transgene expression. Combination of vector complexation with DEAE dextran (5μg/ml) followed by perflubron instillation (10ml) resulted in hazy light blue X-gal staining along the upper and medium airways and some stripey dark blue staining in the trachea (IT3, 138 days, 2.2 x 10^{10} p/kg, **Figure E 16**). Immunohistochemical analysis for β-galactosidase expression demonstrated one positively stained nucleus in the trachea (data not shown).

Although the combination of DEAE dextran complexation and perflubron instillation resulted in detectable transgene expression, we believed that the large volume of lung and tracheal fluid, approximately 100ml at this stage of gestation, would have a large dilutional effect on the vector. We therefore performed further experiments in smaller fetuses in mid-gestation

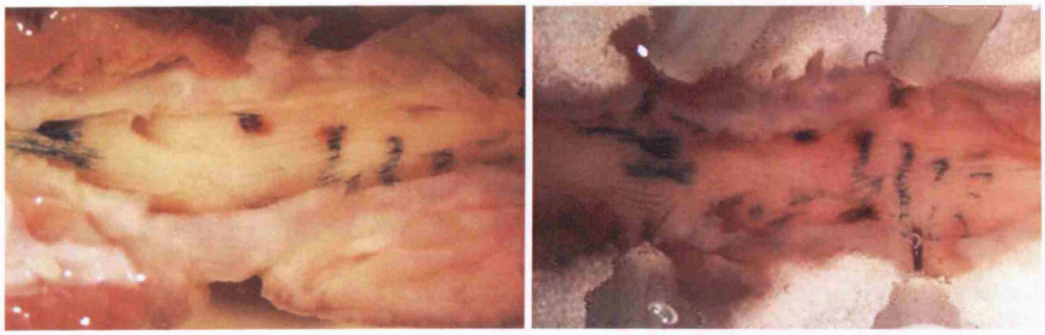


Figure E 16: β-galactosidase transgene expression after ultrasound-guided tracheal injection.

Two days after tracheal delivery of adlacZ vector complexed with DEAE dextran and perflubron (IT3, 138 days of gestation), application of X-gal solution gives low level staining seen as stripey dark blue staining. Original magnification x 4.

E 2.8.2 Low level transgene expression in the mid-gestation fetal airway epithelium is observed following tracheal instillation of adenovirus vector alone

To determine the effect of adenovirus tranduction alone we applied a dose of 1×10^{12} adlacZ vector particles to the trachea of one fetus (IT15) from a twin pregnancy in mid-gestation. Low level and dispersed β-galactosidase expression that appeared to be limited to the peripheral airways or parenchyma was detected 48 hours after injection (**Figure E 17 A**).

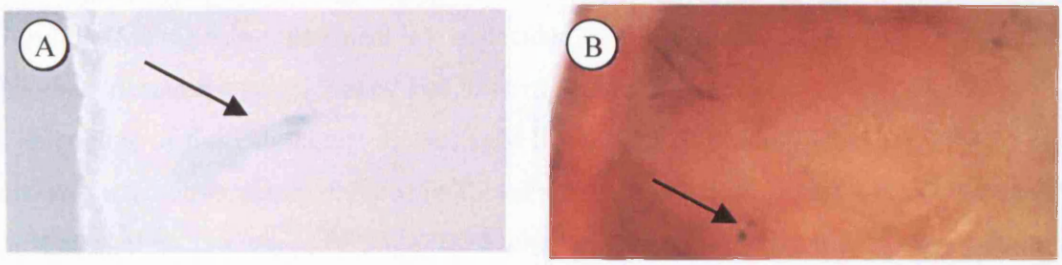


Figure E 17: The effect of DEAE dextran on β-galactosidase expression in the fetal airways.

(A) A pale blue area of X-gal staining of the fetal trachea 2 days after instillation of adlacZ virus alone (IT15, 4.9×10^{11} p/kg) shows only low level β-galactosidase expression. (B) Complexing the adlacZ vector with DEAE dextran (IT14, 5.3×10^{11} p/kg) had very little effect on the visible transgene expression in the fetal trachea as seen by the patchy blue X-gal staining (arrowed). Original magnifications x 4.

Tissue samples taken from the right side of the lung gave β-galactosidase enzyme concentrations of 55pg/mg protein, as determined by ELISA (**Table E 14**), but there

was no detectable β-galactosidase protein in the left side of the lung. There was no positive staining on immunohistochemistry for β-galactosidase.

E 2.8.3 The polycation DEAE dextran enhances adenovirus mediated transgene expression in the mid-gestation fetal sheep airways

Having shown that gene transfer to the airways could be improved in the late gestation fetal sheep, we devised a series of experiments to evaluate the effects of transduction enhancing agents on gene transfer in mid-gestation and to determine the optimum combination (**Table E 14**). We used the same dose of adenovirus vector (1×10^{12} particles) for each injection and the number of particles per kg was calculated for each fetus from the weight at post mortem 2 days after injection. The β-galactosidase enzyme content of the lung parenchyma was measured quantitatively using an ELISA assay.
One of the fetuses (IT10) received an injection of 5ml PBS, 5ml adlacZ complexed with DEAE dextran (5μg/ml) followed by 5ml PBS, a total aqueous fluid volume of 15ml. X-gal histochemistry showed faint staining of the large airways with stronger staining of the left upper lobe small airways (**Figure E 18 A and B**). There was also strong staining of the visceral pleura of the right upper lobe that suggested the injected fluid ruptured the lung and pleura leading to staining of the visceral pleura (**Figure E 18C and D**). Immunohistochemistry for expression of β-galactosidase was negative. The injection procedure itself was straightforward and the only feature of this experiment different to others was the higher total volume of aqueous fluid injected, 15ml versus 10ml. Following this experiment it was decided to inject a maximal aqueous volume of 10ml in subsequent animals aged 100 days of gestation and above.
The volume of fluid applied to fetuses aged 81-82 days of gestation was initially 7.5 ml and this was subsequently reduced to 1.2 ml after haemorrhagic lungs were detected at post mortem examination. PBS was used where necessary to make up the total volume of fluid applied in place of sodium caprate or DEAE dextran.
Four fetuses received application of adlacZ vector complexed with DEAE dextran (5μg/ml, IT4, IT6, IT10 and IT14). Some weak staining was observed in the fetal lungs (IT14, **Figure E 17 B**) similar to that observed with adenovirus on its own and there was no macroscopically visible staining in the trachea or main bronchii. However, quantitative ELISA determination of β-galactosidase was positive in each of the samples taken from both the left and right sides of the lungs showing an enzyme

concentration ten fold increased above that achieved with the non-complexed virus (285 pg/mg versus 28 pg/mg, **Table E 14**).

Table E 14: The effect of different enhancers on adenovirus infection of mid-gestation fetal airways.

GA = gestational age of fetus (days). For transgene expression,– indicates negative result. + to +++ indicates degree of transduction observed after β-gal staining or immunohistochemistry. (+) indicates single positive cells or sparse staining. d: days; h: hours; PN: postnatal; C: sodium caprate; D: DEAE dextran (5μg/ml); P: perflubron, T: trachea; PA: peripheral airways; L: left hand side; R: right hand side; mean: average; nt: not tested.

Sheep	PM (d)	GA (d)	C	D	P	p/kg	β-gal staining		β-gal ELISA (pg/mg protein)			β-gal immuno	
							T	PA	L	R	mean	T	PA
IT15	2	109	–	–	–	4.9×10^{11}	–	(+)	0	55	28	–	–
IT13	2	116	–	–	+	3.8×10^{11}	–	(+)	0	114	57	–	–
IT4	2	81	–	+	–	2×10^{13}	–	(+)	nt	nt	nt	–	–
IT6	2	102	–	+	–	3.8×10^{12}	–	–	nt	nt	nt	–	–
IT10	2	115	–	+	–	8.3×10^{11}	(+)	+	nt	nt	nt	–	–
IT14	2	109	–	+	–	5.3×10^{11}	–	(+)	273	297	285	–	–
IT11	2	116	–	+	+	3.7×10^{11}	–	(+)	108	184	196	–	–
IT16	2	102	+	–	–	8×10^{11}	++	(+)	2576	3212	2894	–	–
IT12	2	109	+	–	+	5.4×10^{11}	+	(+)	3766	4038	3902	(+)	(+)
IT7	2	102	+	+	–	7.5×10^{11}	+	+	nt	nt	nt	–	–
IT8	2	109	+	+	–	4.8×10^{11}	++	++	nt	nt	nt	++	++
IT17	2	102	+	+	–	7.4×10^{11}	(+)	(+)	372	526	449	–	(+)
IT19	2	102	+	+	–	8.3×10^{11}	–	–	0	0	0	–	–
IT20	2	102	+	+	–	6.9×10^{12}	–	–	0	0	0	–	–
IT21	2	81	+	+	–	2×10^{12}	++	+++	17715	15750	16733	++	++
IT22	2	81	+	+	–	2.2×10^{12}	+	+	212	340	276	++	+
IT9	2	108	+	+	+	4.2×10^{11}	+	+++	nt	nt	nt	++	+++
IT23	2	81	+	+	+	2.2×10^{12}	+	++	7235	4915	6075	++	+++
IT24	16	82	+	+	–	2.2×10^{12}	–	–	584	688	636	nt	nt
IT18	6h PN	102	+	+	–	8×10^{11}	–	–	0	0	0	–	–

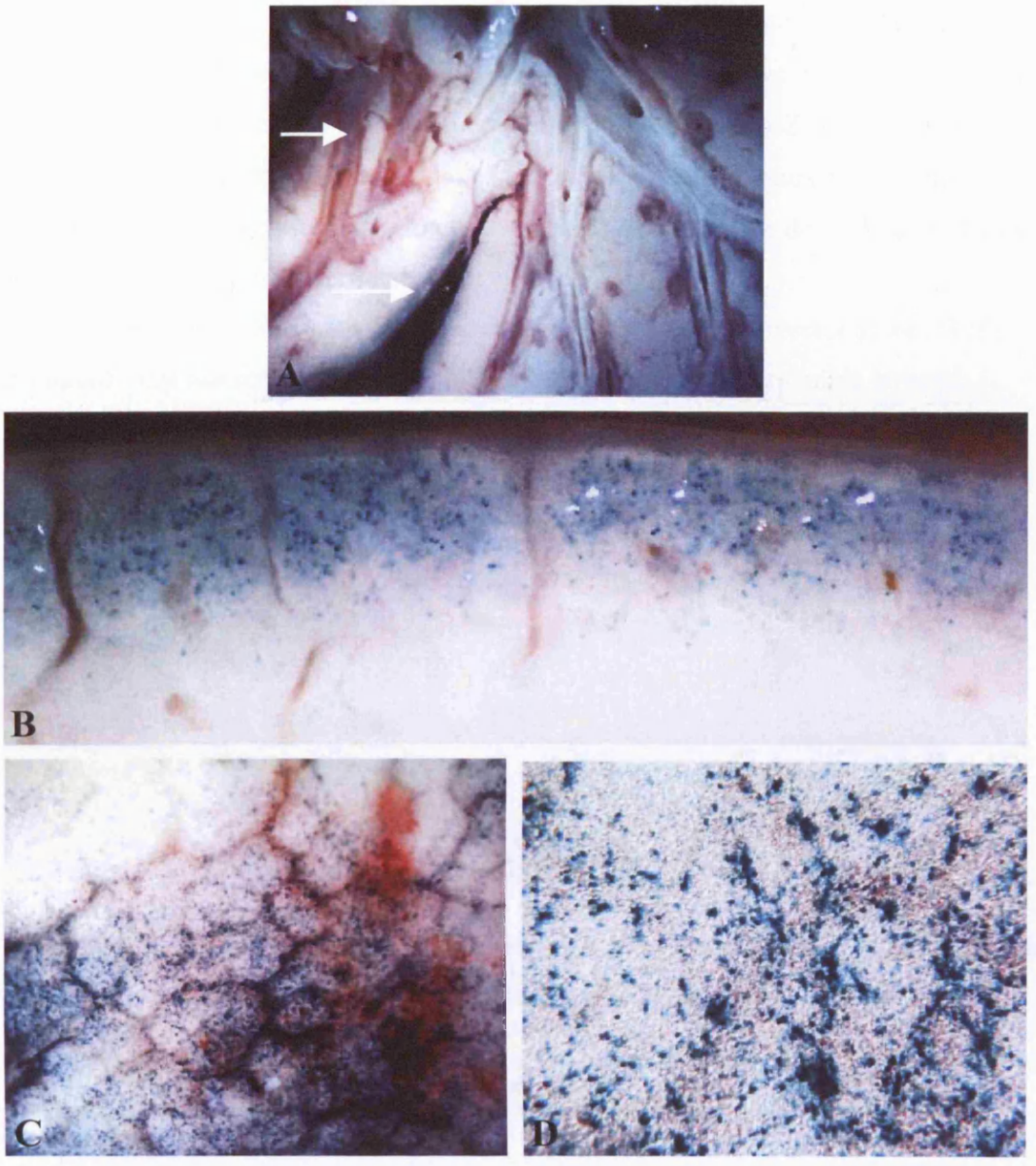


Figure E 18: β-galactosidase transgene expression in the airways after delivery of adlacZ vector complexed with DEAE dextran.

X-gal staining of the trachea and lungs after intratracheal injecton of adlacZ (9.3 x 10^{11} p/kg) and DEAE dextran complex in a fetal sheep (IT10, 115 days of gestation). There is (A) faint staining of the large airways and (B) stronger staining of the left upper lobe small airways seen here in cut section. (C) Staining of the right upper lobe was confined to the visceral pleura when this was peeled off the lung (D) which suggested that during tracheal injection the right upper lobe and visceral pleura had ruptured. Original magnifications x 4.

E 2.8.4 Sodium caprate enhances adenovirus mediated transgene expression in the mid-gestation fetal sheep airways

The optimal dose of sodium caprate (Na-caprate) for pre-treatment of the airways was unknown to us. A dose of 30mM has been shown to enhance adlacZ gene transfer in

adult human airway epithelial cells *in vitro* (Coyne CB et al., 2000) and adult mouse airways *in vivo* (Gregory LG et al., 2002). We therefore compared the effect of 30mM (IT7) or 100mM Na-caprate (IT8) used in combination with adlacZ complexed with DEAE dextran (5μg/ml) and showed the higher dose to be associated with enhanced levels of airway transduction (**Figure E 19 A and B**). The higher dose of 100mM was used for all subsequent experiments.

Pre-treatment with Na-caprate followed by instillation of adlacZ vector alone (IT16) produced clear macroscopic staining of the tracheal epithelium and main bronchi. Levels of transduction as shown by the β-galactosidase ELISA assay were 90 fold higher as compared with virus treatment alone and 10 fold higher than the combination of adlacZ complexed with DEAE dextran (**Table E 14**).

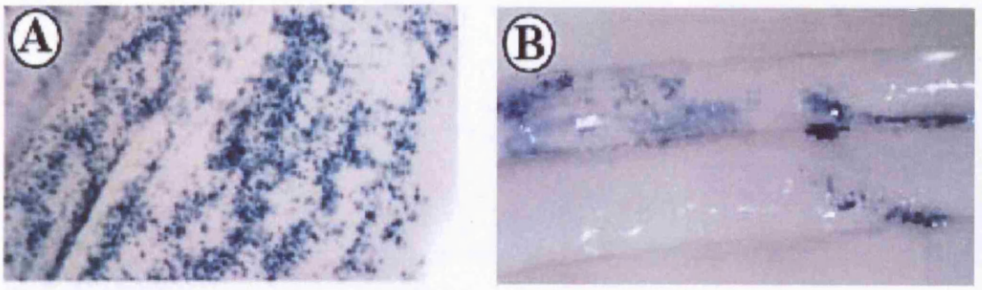


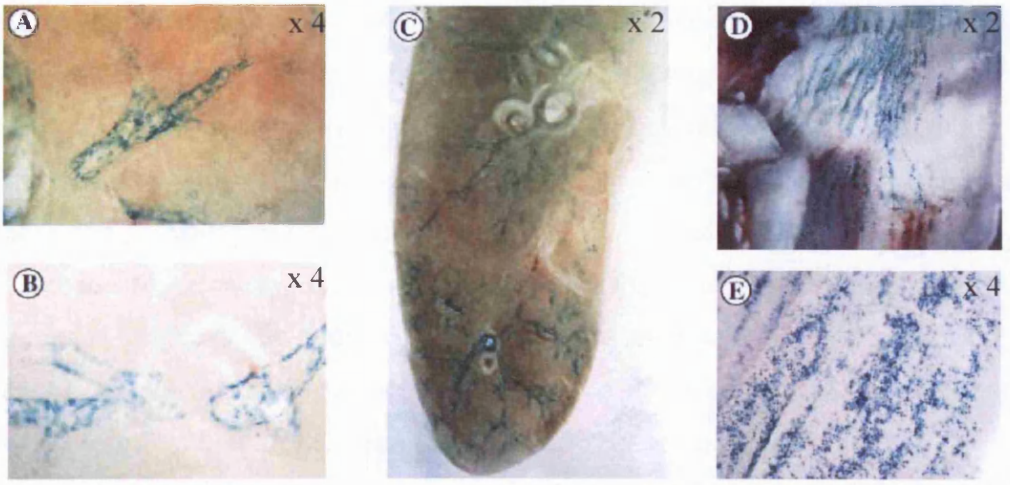
Figure E 19: The effect of different concentrations of Na-caprate pre-treatment on adlacZ mediated tranduction of the tracheal epithelium.

Two fetuses received sodium caprate pre-treatment at (A) 100mM sodium caprate (IT8) or (B) 30mM concentration (IT7) followed by adlacZ vector (4.8 and 7.5 x 10^{11} p/kg respectively) complexed with DEAE dextran. The higher dose of Na-caprate results in more widespread X-gal staining. Original magnifications x 4.

E 2.8.5 Sodium caprate pre-treatment and DEAE dextran complexation significantly improve adenovirus mediated transgene expression in the mid-gestation fetal airways

The combination of Na-caprate pre-treatment and virus complexed with DEAE dextran was then investigated. Collectively, these transduction enhancing agents resulted in significant β-galactosidase staining of the trachea and also marked transgene expression in the epithelial cells lining the large, medium and small airways observed both macroscopically and histologically in fetuses treated at both 109 (IT8, **Figure E 20**) and 81 (IT21) days of gestation (data not shown).

Panel 1:



Panel 2:

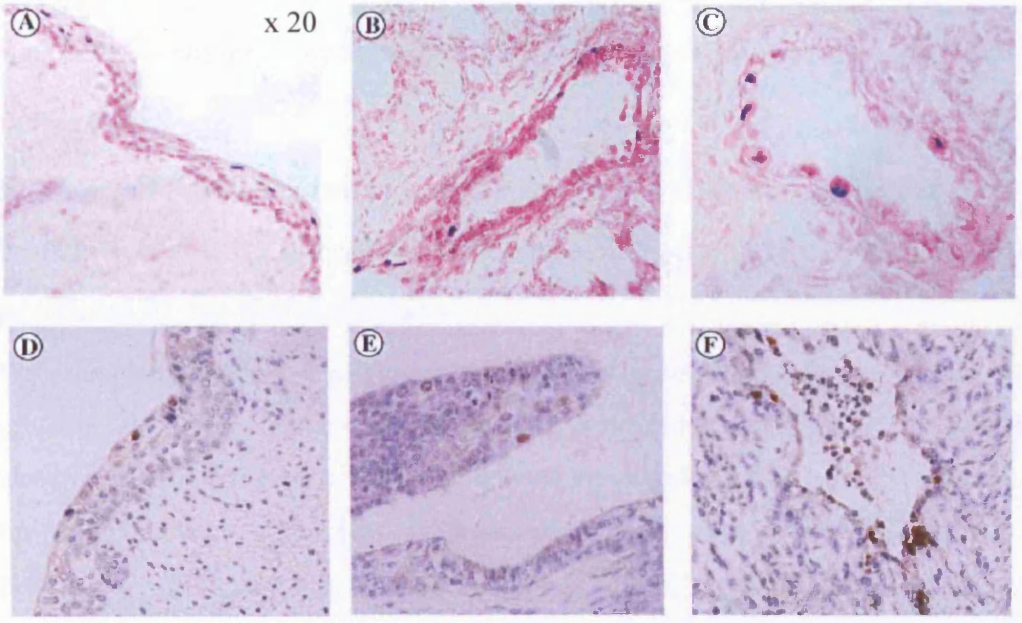


Figure E 20: The effect of pre-treatment with sodium caprate on DEAE dextran complexed adenovirus mediated β-galactosidase expression in the fetal sheep airways.

Panel 1 shows widespread gene expression on X-gal staining in the small (A), medium (B) and large (C) airways and also the main bronchi (D) and trachea (E).
Panel 2 shows widespread X-gal staining (A-C) and immunohistochemical localisation (D-F, haematoxylin counterstain) of β-galactosidase expression throughout the fetal airways. (A & D) show sections through the fetal trachea, (E) the bronchiolar epithelium and (B, C & F) the distal airway epithelium. Original magnifications x 40 except where indicated. The fetal sheep was 109 days of gestation (IT8, 4.8 x 10^{11} adlacZ p/kg).

The observable increase in marker gene expression using this combination of enhancers was reflected as a 600-fold increase in expression of β-galactosidase protein, when

determined by ELISA, compared with virus alone in the lungs. The combination of Na-caprate pre-treatment with DEAE dextran complexed adenovirus was repeated in five further fetuses. In one of these cases (IT22) X gal staining of the visceral pleura suggested pleural perforation and quantitative β-galactosidase analysis was of low level. In three other cases (IT17, IT19 and IT20) there was a low level of airways transduction observed probably due to use of a frozen preparation of adlacZ vector that was subsequently shown to have a low titre. A further fetus came to birth for investigation of perinatal morbidity and immune response and was analysed 6 hours after delivery (IT18). As anticipated, β-galactosidase expression was not detectable probably because of the length of time from injection procedure to tissue sampling.
In conclusion, we have shown that pre-treatment of the airways with 100mM sodium caprate combined with application of adlacZ vector complexed with DEAE dextran, significantly enhances gene transfer to the mid-gestation fetal sheep airway epithelium.

E 2.8.6 Perflubron administration following adenovirus vector instillation results in redistribution of transgene expression from the proximal to the distal airways

We examined the effect of perflubron on adlacZ mediated gene expression in the mid-gestaton fetal airways since we had shown this enhanced gene transfer in late gestation airway epithelia. For each of the combinations investigated, addition of perflubron had little effect on the overall levels of β-galactosidase expression in the lung tissue. As can be seen from **Table E 14**, in the presence of perflubron, β-galactosidase expression was low (IT13, 57 pg/mg) and levels were similar to adlacZ vector alone as measured by ELISA (IT15, 55 pg/mg). Instillation of adlacZ/DEAE dextran complexes followed by perflubron (IT11, 196 pg/mg) was not observably different to that of adlacZ/DEAE dextran alone (IT14, 285 pg/mg). Equally perflubron did not appear to enhance gene expression further when used in combination with Na-caprate pre-treatment and uncomplexed adlacZ vector (IT12, 3902 pg/mg with perflubron and IT16, 2894 pg/mg without perflubron).
However, the addition of perflubron after Na-caprate pre-treatment and administration of DEAE dextran complexed virus did alter the overall pattern of gene expression. There was reduced transduction in the trachea and larger bronchi while expression in the very peripheral airway epithelia was substantially enhanced (**Figure E 21**).

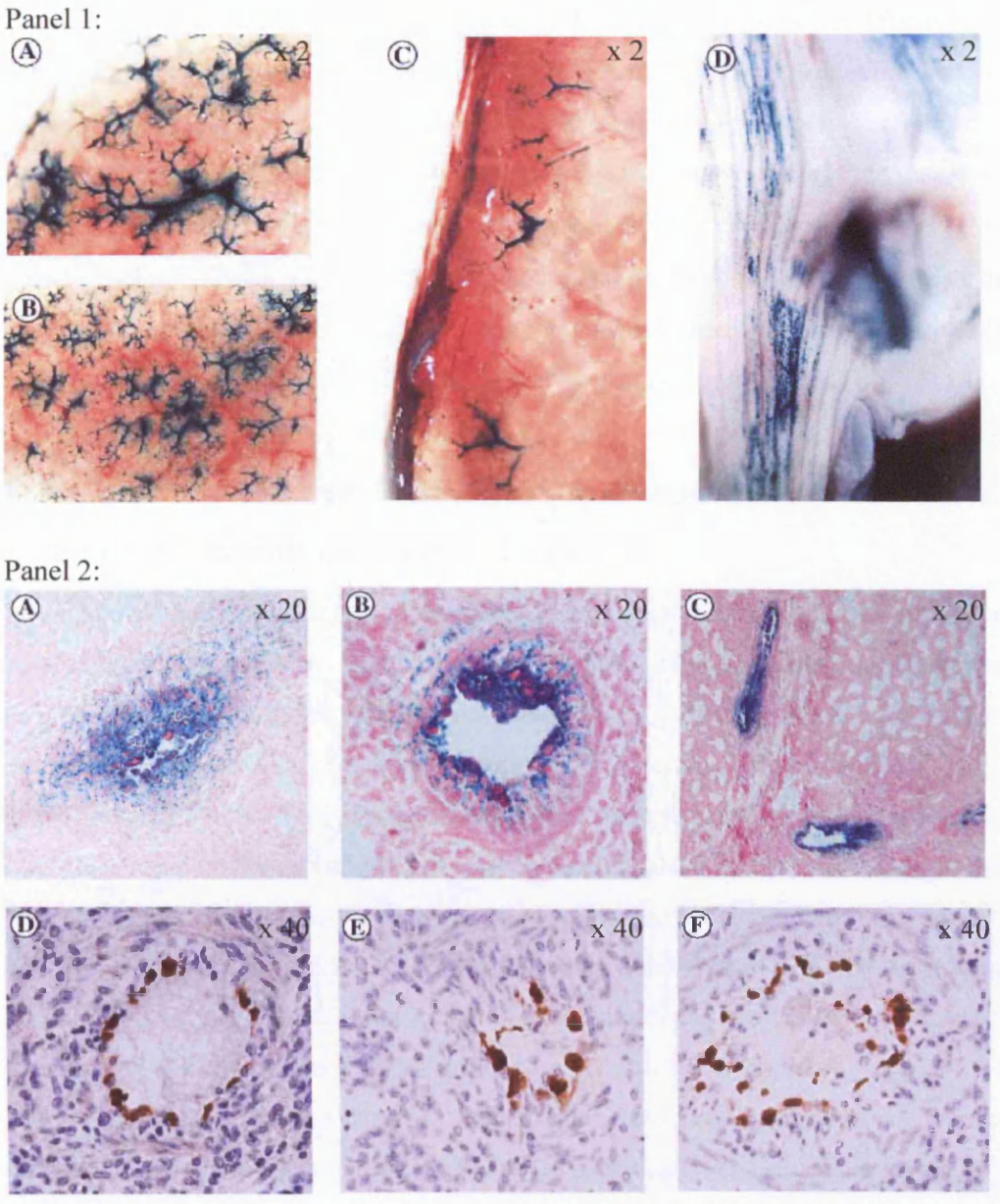


Figure E 21: The effect of perflubron instillation on adenovirus-mediated β-galactosidase expression in the fetal airways.

The airways of a fetal sheep (108 days of gestation) were treated with 100mM sodium caprate and DEAE dextran complexed adlacZ (IT9, 4.2 x 10^{11} p/kg).
Panel 1: X-gal staining of the peripheral airways in transverse sections (A & B) and longitudinal section (C) showing gene expression is limited to the terminal branches of the bronchial tree. Some staining of the bronchioles was also observed (D).
Panel 2 shows X-gal staining (A-C) and immunohistochemical localisation (D-F, haematoxylin counterstain) of β-galactosidase expression in the peripheral airways. Original magnifications as indicated.

Macroscopically and histologically, intense blue staining could be seen in the small airways that exceeded the epithelial cell layer and spread into deeper tissues layers. We were concerned this might be an artefact of excessive chromogen production due to the

high local enzyme activity. β-galactosidase immunohistochemistry was therefore used to subsequently determine the exact histological location of transgene expression and showed a high density of stained cells restricted to the epithelial layer. Perflubron effectively altered the distribution of transgene expression, restricting it to the very peripheral airways resulting in intense transduction of epithelial cells in this region. Addition of perflubron did not further enhance total lung β-galactosidase levels using the combination of Na-caprate pre-treatment and DEAE dextran complexed virus (IT23, 6075 pg/mg with perflubron; IT21, 16733 pg/mg without perflubron, **Table E 14**).

E 2.8.7 Instillation of perflubron results in significant short-term dilatation of the mid-gestation fetal trachea

In the late gestation fetal sheep experiments, it was noted that perflubron instillation resulted in slight dilatation of the trachea. In the mid-gestation sheep fetus dilatation throughout the length of the fetal trachea was seen immediately following injection of perflubron and persisted for at least 5 minutes after injection (**Figure E 22**).
The effect of perflubron on the tracheal diameter was measured and compared with mid-gestation fetal sheep that did not receive perflubron injection. The maximal internal diameter (inner edge to inner edge) was measured using ultrasound with the fetal trachea viewed in longitudinal section (**Table E 15**). In the fetal neck the trachea was measured in the distal third, just proximal to the fetal thorax and in the chest it was measured at the site of injection, just above the level of the pulmonary artery.
In fetal sheep aged 108-116 days the tracheal diameter in the chest increased from a mean of 6.2mm (SD 1.7) to 13.1mm (SD 1.4) significantly, (paired Students t test, $p = 0.016$). In the fetal neck the diameter of the trachea increased from a mean of 6.8mm (SD 1.4) to 10.7mm (SD 1.0) but this change was not statistically significant. Perflubron had a similar dilatation effect on the trachea of one fetal sheep aged 81 days of gestation (IT23) that received perflubron injection.

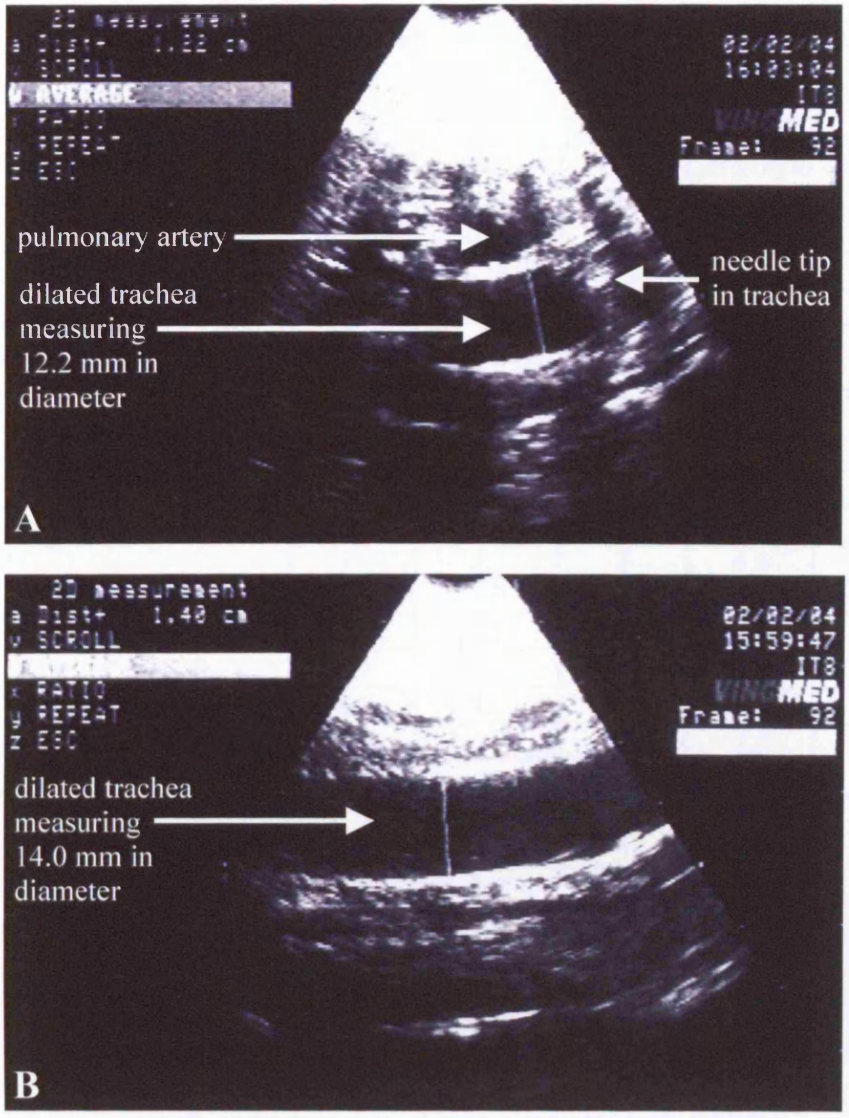


Figure E 22: The effect of perflubron instillation on the diameter of the fetal trachea.

Injection of 5ml perflubron (109 days of gestation, IT9) resulted in dilatation of the trachea in the fetal chest (A) from 6.0mm before to 12.2mm after injection and in the fetal neck (B) from 6.0mm before to 14.0mm after injection

Table E 15: The effect of perflubron on the diameter of the fetal trachea.

na = not available

Sheep	Perflubron volume (ml)	tracheal diameter in neck (cm)		% change	tracheal diameter in chest (cm)		% change
		before	after		before	after	
100-116 days of gestation							
IT9	5	6.0	14.0	133	6.0	12.2	105
IT11	5	na	na	–	4.5	13.4	198
IT12	5	5.8	9.0	55	5.8	14.8	155
IT13	5	8.6	9.0	5	8.6	11.7	36
IT10	0	na	na	–	4.6	4.5	-2
IT14	0	5.1	5.3	4	4.2	4.0	-5
IT15	0	5.2	6.0	15	5.0	4.9	-2
IT16	0	5.0	5.1	2	4.6	5.6	22
IT17	0	3.5	3.9	11	3.9	4.0	3
IT18	0	na	na	–	na	na	–
IT19	0	5.4	5.5	2	4.1	4.6	12
IT20	0	4.1	4.3	2	4.6	5.1	11
IT25	0	4.5	4.5	0	4.5	4.7	4
IT26	0	4.6	4.4	-4	4.2	4.7	12
IT27	0	3.9	3.4	-13	3.4	4.3	26
IT28	0	3.8	4.0	5	3.8	3.4	-11
IT29	0	3.9	4.0	3	3.6	4.0	11
IT31	0	5.8	5.8	0	5.8	5.8	0
IT33	0	5.5	5.5	0	5.2	5.5	6
IT35	0	4.1	4.8	22	4.0	4.4	10
IT36	0	4.7	4.7	0	4.4	4.5	3
IT37		5.3	5.0	-6	5.4	5.1	-6
81-82 days of gestation							
IT23	1.5	2.5	3.2	28	2.5	3.3	32
IT21	0	2.9	2.8	-3	2.8	2.8	0
IT22	0	2.6	2.7	4	2.6	2.4	-8
IT24	0	3.2	3.0	7	3.3	3.1	-6
IT30	0	3.0	3.1	3	2.1	2.3	10
IT34	0	3.1	3.4	10	3.3	3.4	3

E 2.8.8 Dilatation of the fetal trachea at post mortem following instillation of liquids is related to the viscosity of the liquid

We explored the hypothesis that perflubron acted as a piston to push fluid distally down the airways because of its high density. We compared tracheal instillation of three fluids with different densities: PBS (1 g/ml), 5% glycerol (1.26 g/ml) and perflubron (1.92 g/ml) in a non-injected sheep fetus (102 days of gestation) at post mortem. The fetal trachea was visualized in the chest using ultrasound. A 19 Gauge Venflon was inserted

into the fetal trachea in the neck and the fluids were injected. The volume at which fluid was seen coming out of the fetal mouth was noted and the diameter of the trachea was measured before and after injection of each solution.

After injection of 15ml PBS fluid was seen coming out of the mouth and there was no change in the diameter of the trachea in the chest. Injection of 5ml 5% glycerol increased the tracheal diameter by a third (3.6mm to 5.0mm) and the trachea remained dilated for 30 seconds. Finally, administration of 5ml perflubron resulted immediately in a tripling of the tracheal diameter (3.6mm to 9.1mm) that remained dilated for 90 seconds. A second injection of perflubron confirmed these findings. Although there was a small leakage of perflubron (2ml) from the fetal mouth during the second injection, the majority of fluid moved distally in a column down the airways. It appears therefore, that it is the density of perflubron that causes it to act as a piston and push fluid down the trachea to the distal airways. Although a balloon tipped catheter placed in the trachea would be able to prevent all injected fluid passing proximally, we saw very little movement of injected fluid proximally. Variations in the volume of perflubron used may thus allow targeting of injected fluid to different areas of the airways.

E 2.9 The effect of adenovirus and Sendai vectors and transduction enhancing agents on fetal tracheal fluid

E 2.9.1 Cytological analysis of fetal tracheal fluid

Fetal tracheal fluid was analysed for evidence of an inflammatory reaction at post mortem examination and compared with samples obtained prior to injection of vector. Broncho-alveolar lavage fluid (BAL) was also obtained at post mortem examination (**Table E 16**). The most florid inflammation was seen in two cases that were examined 17 days after surgery (IT31) and one day after birth (IT27). In the remaining fetuses very little inflammatory reaction was observed, even when post mortem examination was delayed longer than two days after injection. Occasional epithelial cells were observed commonly in both the pre and post treatment samples and many of these were pyknotic. Red blood cells (RBCs) were seen in one sample pre-op (IT33). In nine fetuses there were RBCs observed in post-op samples, in half of which they were numerous (**Figure E 23 A**). Neutrophils were observed in post-op tracheal fluid or BALF of four fetuses (**Figure E 23 A and B**) and this infiltrate was dense in the two animals that came to birth (IT31 and IT34), which correlated with histological findings of an alveolar neutrophilia.

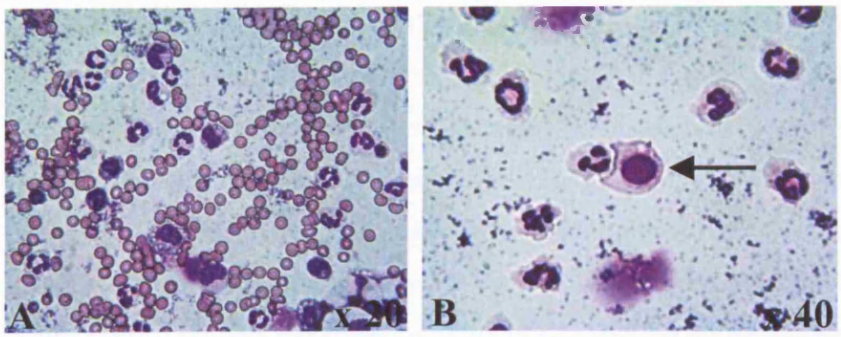


Figure E 23: Cytological analysis of bronchoalveolar lavage fluid after fetal tracheal injection.

Samples of BALF taken at post mortem examination were stained with Wright-Giemsa. (A) Red blood cells and neutrophils were seen 17 days after tracheal injection in one fetus (IT31). (B) A monocyte (arrowed) and many neutrophils are seen 1 day after delivery of a lamb that was injected in mid-gestation (IT27).

Table E 16: Cytological analysis of tracheal and broncho-alveolar lavage fluid by Wright-Giemsa stain.

GA: gestational age at injection; d: days; PN: postnatal; h: hours; C: sodium caprate; D: DEAE dextran; P: perflubron; E: epithelial cell; RBC: red blood cell; M: monocyte; N: neutrophil; (+) indicates few cells per high power field; + to +++ indicates occasional to many cells per high power field.

Sheep	PM (d)	GA (d)	C	D	P	Vector	Tracheal fluid		BAL fluid
							Pre-op	Post-op	
IT21	2	81	+	+	–	adlacZ	E+	E+	E+; RBC(+)
IT22	2	81	+	+	–	adlacZ	E+	M+; RBC(+)	RBC(+)
IT23	2	81	+	+	+	adlacZ	nt	RBC+++; M(+)	M(+); RBC(+)
IT24	16	82	+	+	–	adlacZ	nt	E+	E+
IT35	2	100	+	+	–	adhCFTR	E+	E+	E+
IT25	2	102	+	+	–	adhCFTR	E+	E+; RBC+	E+; RBC+
IT26	2	102	+	+	–	adhCFTR	E+	M+; RBC+++; E++	N+; RBC+++; E++
IT28	17	102	+	+	–	adhCFTR	E(+)	E+	E(+)
IT29	17	102	+	+	–	adhCFTR	E+	E+	E+
IT31	17	113	+	+	–	adhCFTR	nt	E+; RBC +++; N+	RBC+++; N+++
IT27	1d PN	102	+	+	–	adhCFTR	nt	E+; RBC++; N+++	E+; RBC++; N+++; M+
IT33	1d PN	110	+	+	–	adhCFTR	E+; RBC++	E++; RBC ++	E++; RBC ++
IT30	12h PN	81	+	+	–	adhCFTR	E+	E++	E++
IT34	7d PN	82	+	+	–	adhCFTR	E+	E+; M++; N+++	E+; M++; N+++
IT36	2	100	–	–	–	Sendai lacZ	E(+)	E(+)	E(+); RBC (+)
IT37	2	104	–	–	–	Sendai lacZ	M+	E++; M(+)	E++; M(+)

Monocytes were observed in the fluid from five fetuses (**Figure E 23 B**), in low numbers in four animals (2-3 per high power field), but there was a dense infiltrate in the remaining animal, in which post mortem and histological analysis demonstrated confluent bronchopneumonia. Tracheal and BALF fluid taken from a non-injected sheep fetus at 100 days of gestation demonstrated numerous epithelial cells but no RBCs, neutrophils or monocytes were visible. Thus there appears to be a low grade inflammatory process associated with tracheal injection that could be due to the viral vectors or the procedure itself. We did not have access to tracheal or BALF fluid from a normal newborn lamb for comparison. Fluid from adult airways does not normally contain inflammatory cells but it is possible that neonatal airways contain low levels of inflammatory cells in response to antigenic stimuli. In two of the animals that came to birth however, there were significant numbers of inflammatory cells in the airways fluid.

E 2.9.2 Biochemical analysis of fetal tracheal fluid

The solute composition and osmolality of fetal tracheal fluid before injecton and at post mortem examination was measured to determine whether adenovirus vectors had any effect. The concentration of Na^+, Cl^- ions and the osmolality were not significantly different and were within the normal ranges for mid to late gestation ovine fetal tracheal fluids (Adamson TM et al., 1969). The K^+ concentration was significantly higher after exposure to adenovirus vector than before (8.12 ± 1.5 versus 4.48 ± SD 0.26 mM/kg water respectively, paired Students *t*test $p<0.001$, **Table E 17**).

Table E 17: The concentration of K+ ions in fetal tracheal fluid.

Sheep	[K^+] mM/kg water	
	before adenovirus	at post mortem examination
IT24	4.29	6.06
IT25	4.52	8.62
IT26	4.17	10.22
IT28	4.7	8.89
IT29	4.5	6.27
IT31	4.92	8.09
IT35	4.27	8.74

This could reflect an increase in the secretion of K^+ ions into the tracheal fluid but was more likely due to the release of K^+ ions from dying cells. It is interesting to note

however, that the normal range for K^+ ions in mid to late gestation ovine fetal tracheal fluid is 6.3 ± SE 0.7 which is in between the measurements obtained before and after adenovirus injection (Adamson TM et al., 1969).

E 2.10 Spread of adenovirus vector in fetal and maternal tissues following intratracheal administration

We explored the spread of transgene to the fetal and maternal organs by PCR analysis of tissues in one late gestation fetus (**Table E 18**). The first fetus analysed (IT3) was one of the first tracheal injection experiments in which there was very low-level transgene expression and we hoped that PCR analysis would provide a highly sensitive analysis of the spread of the vector. AdlacZ was detected in all tissues investigated but only after nested PCR analysis, indicating low levels of vector spread.

Table E 18: Vector spread following adenovirus vector delivery to the airways of late gestation fetal sheep.

Transgenic adenovirus β-galactosidase cDNA was detected by PCR analysis in fetal and maternal tissues 2 days after ultrasound guided tracheal injection of adlacZ vector with or without transduction enhancing agents. IT3 (138 days of gestation) received 2.2 x 10^{10} pfu/kg adlacZ complexed with DEAE dextran (5μg/ml) followed by 10ml perflubron. ^{m} denotes maternal tissues.

Tissue	1st round PCR	Nested PCR
Gonad	–	+
	$-^m$	$+^m$
Placenta	–	+
Upper trachea	–	+
Lower trachea	–	+
Lower bronchial tree	–	+
Soft palate	–	+
Upper oesophagus	–	+

The presence of vector, albeit at low level, in the placenta, fetal and maternal gonad suggests that haematogenic spread may have occured. It is possible that direct exposure of the placenta to the adenovirus vector occurred during withdrawal of the needle from the uterus. This is unlikely however, since the vector was flushed through with perflubron or PBS prior to removal from the fetus, and it is doubtful that the placentome through which the needle passed was sampled at post mortem. The vector could have reached the fetal circulation during transthoracic injection in which trauma to minor blood vessels is inevitable. An alternative route into the fetal circulation is via the lymphatic drainage of the fetal airways.

E 3 Placement of balloon-catheter systems and occlusive balloons in the trachea using ultrasound-guided injection

This series of experiments was designed to explore placement of balloon-catheter systems or occlusive balloons into the fetal trachea using the ultrasound-guided transthoracic injection technique. Using fetoscopy, tracheal obstruction with a detachable balloon has been shown to improve gene transfer to the fetal airway epithelium after tracheal instillation of adenovirus vectors (Sylvester KG et al., 1997). Tracheal obstruction with fetoscopically placed detachable balloons is being used for the treatment of congential diaphragmatic hernia (CDH) in the fetal sheep animal model (Deprest JA et al., 1997a) and clinically (Harrison MR et al., 1998). The morbidity from fetoscopy is significant however, because of the relatively large diameter of the puncture site in the fetal membranes that may lead to premature rupture of the membranes and preterm labour and its associated problems. We wanted to investigate whether balloon-catheter systems or detachable balloons could be placed in the trachea using ultrasound-guidance alone. This might improve vector mediated gene transfer to the fetal airway epithelium and the technique might also be adapted for clinical use in the treatment of CDH.

E 3.1 Current balloon-tipped catheter systems are too wide for placement via a needle into the late gestation fetal sheep trachea at post mortem

Using a modified Seldinger technique we considered that it might be possible to pass a balloon-tipped catheter over a wire into the trachea. A 16 Gauge needle was thought to be the largest size suitable for ultrasound-guided injections. Larger needles could result in more trauma to the fetus and they would approach the diameter of fetoscopes that are capable of placing a balloon with minimal fetal trauma down the trachea through the fetal mouth. Two balloon-tipped catheter systems were tested at post mortem in late gestation fetal sheep but were too large to fit a 16 Gauge needle (1.29mm). In addition, both systems had a maximal balloon diameter less than the tracheal diameter in mid-gestation. For example, both a ureteropelvic junction occlusion balloon catheter and an overwire ureteroscopy balloon catheter (Cook Urological, IN, USA) measured 3.0 French in diameter (1mm) and had balloons that could only inflate to 4mm, less than the diameter of the fetal trachea at 100 days of gestation. Coronary artery balloon catheters have been successfully used in ultrasound-guided balloon valvuloplasty of human

fetuses (Kohl T et al., 2000a). However the catheter diameter of one commonly used system (CrossSail™, Guidant, CA, USA) was 3.0 mm and had a maximal balloon diameter of 4.0 mm.

E 3.2 A detachable occlusive balloon can be passed through a 15 or 16 Gauge needle

We investigated whether a detachable balloon as used in the treatment of congenital diaphragmatic hernia could be passed down a needle. The balloons tested were goldvalve embolisation balloons (GVB 16, CathNet-Science SA, Paris, France) that are used by Professor Jan Deprest's group in Belgium for treatment of CDH in the fetal sheep animal model and clinically (personal communication). The GVB16 balloon inflates to a maximal length of 21mm and a maximal diameter of 8mm. This is sufficient to occlude the trachea of a late gestation fetal sheep (Evrard VA et al., 1997). The deflated balloon is mounted on a 3 French catheter tapered to a 2.5 French tip containing a mandrel guidewire (Minitorquer detachable balloon catheter with teflon tip, CIFN35, CathNet-Science SA, Paris France). For fetoscopic application this is then passed down an 8 French (2.66mm) guiding catheter through the fetoscope. We tested whether the GVB16 balloon attached to its mounting catheter alone would fit down a variety of needles used in obstetrics and gynaecology (**Table E 19**).

As can be seen, the balloon could be backloaded up the bevel of a 17 Gauge Chiba needle but would not pass down through it (**Figure E 24 A and B**). The balloon passed with some difficulty down the 16 Gauge needles, but easily passed down the 15 and 14 Gauge needles. The mounting catheter fitted down all needles that were sized 17 Gauge or larger. We were concerned that the larger needles that had not been designed with a cutting bevel might have difficulty penetrating the fetus and we therefore tested out all the needles (17, 16 and 15 Gauge) that had fitted the balloon and catheter.

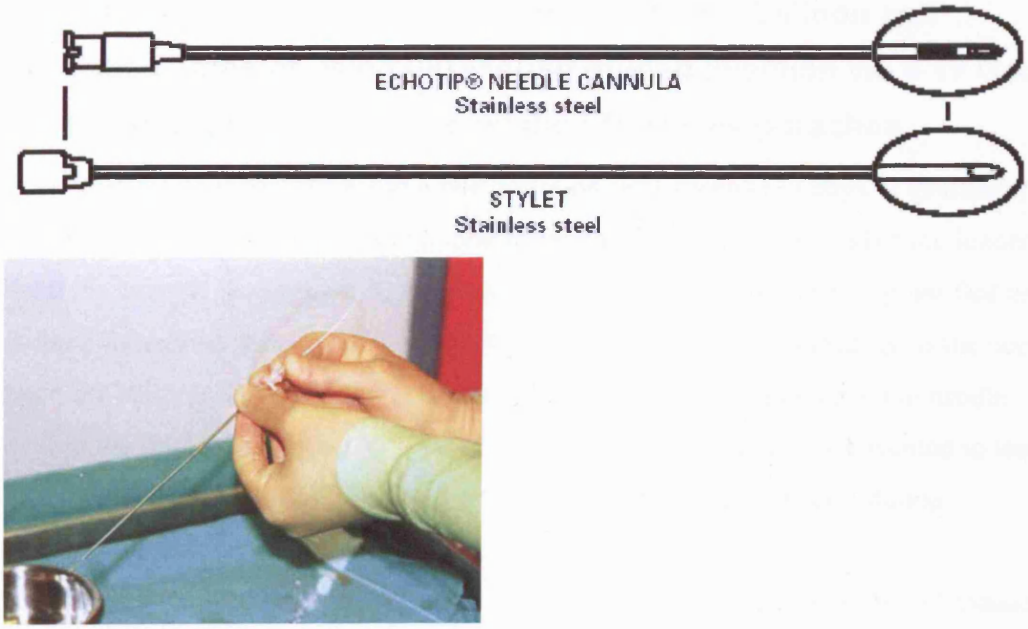


Figure E 24: Testing a 17 Gauge Chiba needle (Cook USA Ltd).

(A) Diagram of a Chiba needle which has a bevelled stylet and needle cannula allowing good soft tissue penetration. (B) Backloading of a 17 Gauge Chiba needle. The mounting catheter is passed through the needle and a deflated GVB16 balloon is placed on its tip, before being withdrawn into the bevel end of the needle for ultrasound guided transthoracic injection.

Table E 19: Needles tested for placement of occlusive goldvalve embolisation balloons in the fetal trachea.

Needle type	Needle size (Gauge)	Manufacturer	Passage down needle	
			GVB16 balloon	Mounting catheter
Spinal	18	Cook Ob/Gyn, Spencer, Indiana, USA	No passage	No passage
Chiba	17	Cook Ob/Gyn, Spencer, Indiana, USA	No passage down needle; balloon can be back loaded up the bevel	Passes easily
Epidural	16	Braun Melsunger AG, Germany	Balloon passes with difficulty; curved tip is a problem	Passes easily
IV cannula	16	Becton Dickinson, Helsingborg, Sweden	Balloon passes with difficulty	Passes easily
Pencil tip	16	Somatex, Teltow, Germany	Balloon passes with difficulty	Passes easily
Pencil tip	15	Somatex, Teltow, Germany	Balloon passes easily	Passes easily
IV cannula	14	Becton Dickinson, Helsingborg, Sweden	Balloon passes easily	Passes easily

E 3.3 Attempts to place a detachable occlusive balloon and mounting catheter using ultrasound guided injection via a 17 Gauge Chiba needle fail in the late gestation fetal sheep trachea

We began *in vivo* experiments in a late gestation fetal sheep (137 days of gestation) using the 17 Gauge Chiba needle (Cook Ireland Ltd, Limerick, Ireland) back loaded with the balloon mounted on its catheter. This needle has a sharp bevel point that makes it ideal for cutting through soft tissues. Unfortunately due to backloading of the needle with the balloon, we would be unable to confirm correct positioning of the needle within the fetal trachea by aspiration of tracheal fluid. In addition, we wanted to test whether the bevel point might damage the balloon and/or the catheter during positioning.

Firstly we tested the mounting catheter and mandrel guidewire alone. The 17 Gauge Chiba needle containing its trocar was passed into the fetal trachea via the chest wall between the 2nd and 3rd ribs. The trocar was removed and tracheal fluid was aspirated to confirm correct positioning in the trachea. The mounting catheter and its mandrel guidewire were passed with ease into the fetal trachea as far as the carina. The catheter was flushed with PBS (5ml) and microbubbles were seen within the trachea. The catheter was then removed without damage, followed by the needle.

The 17 Gauge Chiba needle was backloaded with a deflated GVB16 balloon on its mounting catheter and mandrel guidewire. The needle was reinserted through the fetal chest and appeared to be in the trachea, although it was not possible to confirm this by aspiration of tracheal fluid. The balloon was advanced with some difficulty beyond the needle tip and inflated with 8ml fluid to its maximal dimensions measured on ultrasound (22.3mm long x 8.9mm diameter). The needle was removed from the trachea and the inflated balloon was detached by a sharp pull on the mounting catheter.

It was difficult to determine whether the balloon was within the fetal trachea or just alongside it on ultrasound examination of the fetal chest (**Figure E 25 A**). The inner-to-inner diameter of the trachea distal to the balloon measured 6.4 mm before surgery, 6.3 mm immediately after surgery and 5.5 mm 24 hours after the procedure. We concluded that the balloon was positioned just outside the trachea and was probably compressing it. This was confirmed at post mortem examination 24 hours after surgery (**Figure E 25 B**).

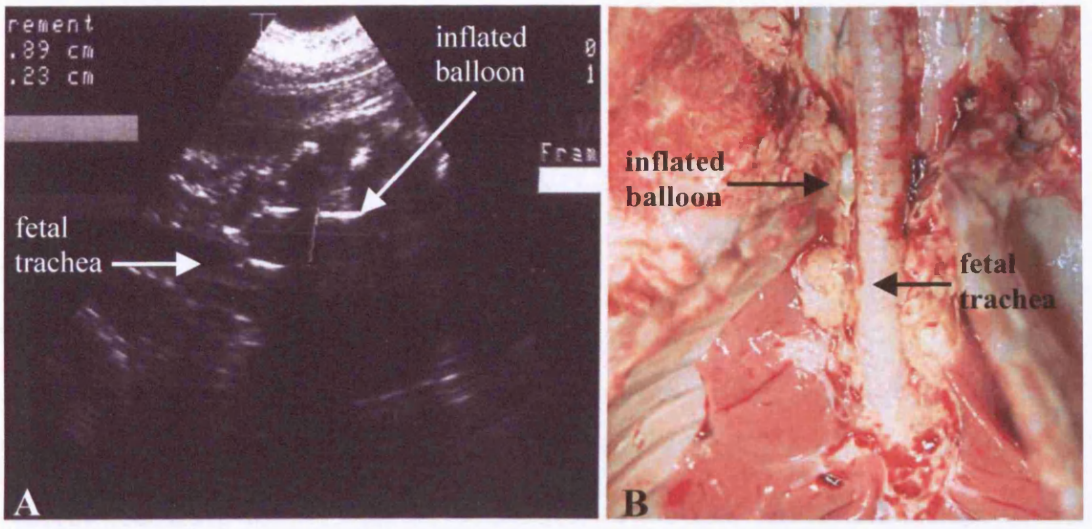


Figure E 25: Failed attempt to occlude the trachea with a detachable balloon.

After ultrasound-guided transthoracic injection, (A) an ultrasonogram shows the fetal trachea in longitudinal section with the inflated balloon that appears to be alongside. (B) At post mortem examination 24 hours after injection the inflated balloon was actually positioned on the right side of the fetal trachea

Dissection of the fetal neck showed the balloon was slightly posterior and to the right of the trachea with the oesophagus to its left. The upper part of the balloon was at the level of the 1st rib. There was a small blood clot on the left side of the trachea at the injection site but no other abnormal findings.

Although we failed to place the balloon in the fetal trachea, this experiment demonstrated that a detachable balloon could be passed down a needle without a guiding catheter, and inflated under ultrasound guidance.

E 3.4 Attempts to place a detachable occlusive balloon and mounting catheter using ultrasound guided injection via a 15 Gauge pencil tip needle fail in the mid gestation fetal sheep trachea

A second experiment was performed in a mid-gestation fetal sheep (102 days of gestation). Using a 15 Gauge pencil tip needle (Somatex, Teltow, Germany) we aimed to aspirate tracheal fluid to confirm correct needle placement before positioning the balloon in the trachea. The balloon passed with difficulty down a 16 Gauge pencil tip needle and we were concerned that it would be damaged, and therefore we chose to use the larger gauge instead.

There were three attempts to pass the 15 Gauge pencil tip needle into the fetal chest and all were complicated by excessive tenting of the skin. On the second attempt, the needle was positioned superior to the 1st rib. A large haematoma developed posterior to the

trachea which distorted the trachea anterior and obscured the view. A third attempt to place the needle between the 1st and 2nd rib failed.

We believed the pencil tip point of the needle was not able to cut through the soft tissues. We decided therefore to place a 30cm long 18 Gauge spinal needle (Cook Ob/Gyn, Spencer, Indiana, USA) into the trachea and use it as a guide down which to pass the outside cannula of the 15 Gauge pencil tip needle. The 18 Gauge spinal needle was positioned in the trachea and tracheal fluid withdrawn. It was not possible however, to pass the outside cannula of the 15 Gauge pencil tip needle beyond the subcutaneous tissues of the neck and the fetal anatomy was distorted significantly to obscure the view. This was because the outside cannula was not sharp enough to cut through the tissues.

E 3.4.1 A detachable occlusive balloon is successfully placed in the fetal trachea under ultrasound guidance

Having failed to access the fetal trachea using a 15 Gauge pencil tip needle in the mid-gestation sheep fetus, we decided to have a further attempt in the same fetus using a 17 Gauge Chiba needle, backloaded with the inflatable balloon and mounting catheter. The needle passed through the 1st and 2nd rib into the fetal chest and once the tip was thought to be in the fetal trachea, the catheter and balloon were advanced into the trachea up to the carina. A good view of the balloon within the trachea was obtained. The balloon was inflated with 8ml PBS and the needle was removed from the fetal chest but remained in the fetal subcutaneous tissues. A sharp pull was made to the catheter to detach it from the balloon but as this was done, the balloon deflated. The mounting catheter was removed with the deflated balloon still attached and on examination there was a small hole 3 cm from the distal end of the mounting catheter. We believed it likely that during the sharp pull on the catheter to detach the balloon, the needle point had perforated the catheter causing the fluid to leak out of the balloon.

Using a new mounting catheter and after checking the integrity of the balloon, the procedure was repeated with the 17 Gauge Chiba needle. The balloon was placed and inflated within the fetal trachea and the needle was removed completely from the ewe's abdomen before the inflated balloon was detached from the mounting catheter, to avoid perforation. The inflated balloon measuring 18mm in length and 5.5mm in diameter completely occluded the fetal trachea (**Figure E 26 A**), which measured 5.1mm in the chest and neck prior to surgery. The time taken from needle insertion to successful insertion of the inflated balloon, removal of the needle and mounting catheter was 10

minutes 45 seconds. The previous attempt that failed due to catheter perforation took 21 minutes 48 seconds from needle insertion to removal of the deflated balloon.

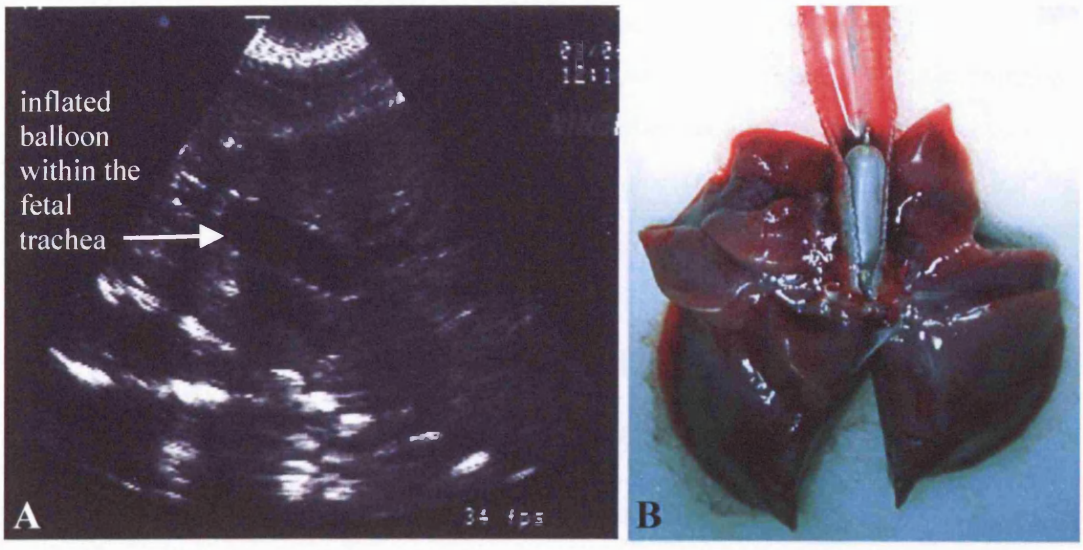


Figure E 26: Successul occlusion of the fetal trachea by ultrasound-guided injection.

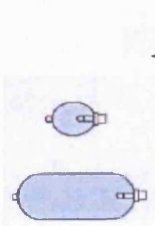
A detachable inflated balloon was placed in the trachea of a mid-gestation fetal sheep using ultrasound-guided injection. (A) Ultrasonogram showing the inflated balloon within the fetal trachea superior to the carina. (B) At post mortem examination the inflated balloon is positioned 5mm from the carina.

Because of the trauma to the sheep fetus from multiple injection attempts, post mortem examination was performed immediately after successful balloon placement. There were multiple puncture sites visible on the right side of the neck and posterior shoulder associated with small haematomas. In the fetal neck there was a large blood clot posterior and to the right of the trachea. On opening the chest there was a large amount of blood in the thoracic cavity and the root of the neck. The inflated balloon measuring 20mm long and 5.5 mm wide was positioned in the fetal trachea 5mm from the carina **(Figure E 26 B)**.

This result confirmed that it was possible to place and inflate a detachable balloon within the trachea of mid-gestation fetal sheep by ultrasound-guided injection. For future clinical application, it was important to develop a needle and balloon catheter system that would allow confirmation that the needle tip had been correctly positioned in the trachea, before deploying the balloon. This could be achieved either by using a smaller balloon or a larger needle.

A balloon of smaller volume was tested in the needles (GVB15, **Figure E 27**). Although the manufacturer stated the deflated diameter was the same as the GVB16

balloon, it could be passed down a 17 Gauge Chiba needle after prelubrication with paraffin. Unfortunately the inflated length of the GVB15 balloon is shorter than that of the GVB16 (8mm compared with 21mm) and this results in more pressure damage on the tracheal epithelium limiting its use in the treatment of CDH (personal communication, Professor Jan Deprest, Leuven University, Belgium). We concentrated our investigations therefore, on finding a more suitable needle.



Code	Ref.	Deflated balloon Diam.	Deflated balloon length	Inflated balloon Diam.	Inflated balloon length	Max. volume	Recommended guiding catheter size
1032828	GVB 15	1.5 mm	3.0 mm	6.0 mm	8.0 mm	0.1 ml	GC 7F/95 cm
1032853	GVB 16	1.5 mm	6.5 mm	8.0 mm	21.0 mm	0.8 ml	GC 8F/95 cm

Figure E 27: Gold valve balloons used for fetal tracheal occlusion.

The GVB16 balloon (CathNet-Science SA, Paris, France) is used clinically for fetal tracheal occlusion and has an inflated length nearly 3 times that of the GVB15 balloon.

E 3.4.2 Occlusion of the trachea is achieved by ultrasound guided placement of a detachable balloon in the fetal sheep trachea

We investigated alternative needle systems used in other clinical specialities and tested a 16 Gauge Kellett needle (Rocket Medical plc, Watford, UK) that is used for liver biopsy in gastroenterology. This 3-way needle system included an outer flexible plastic catheter, an inner metal sleeve with a sharp tapered end and a diamond faceted stylet (**Figure E 28**). A locking mechanism allowed the needle parts to be secured as one unit during initial injection. The stylet could be retracted by twisting it back into the metal sleeve and then on removal of the stylet, the outer catheter and inner metal sleeve remained secured together. Prelubrication of the outer plastic catheter with perflubron allowed the balloon and catheter to pass with ease.

We tested this needle system in a fetal sheep aged 102 days of gestation using its cotwin as a control animal. The 16 Gauge Kellett needle was introduced into the fetal trachea under ultrasound guidance at the first attempt. The stylet was retracted 2mm and the needle then advanced a little further to ensure the tip of the outer plastic catheter was in the trachea before the stylet was removed. To confirm correct needle placement, tracheal fluid was withdrawn through the inner metal sleeve that was then removed

leaving the flexible outer plastic catheter in place. The deflated balloon on its mounting catheter was passed down this into the trachea and was directed towards the carina.

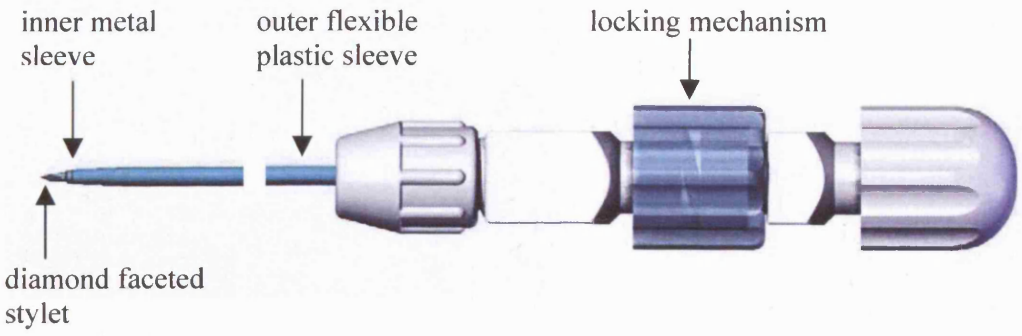


Figure E 28: Diagram of a 16 Gauge Kellett needle.

Picture courtesy of Rocket Medical plc, Watford, UK.

Once in place, the balloon was inflated and the mounting catheter removed. The balloon measured 26.6mm long and 7.4mm in diameter (**Figure E 29**). The time taken from needle insertion, successful inflation of the balloon to removal of the needle and mounting catheter was 17 minutes. The ultrasound dimensions of the trachea and thorax at balloon insertion and at post mortem analysis are shown in **Table E 20**. The balloon dimensions did not change significantly during the post operative period but the trachea above the balloon narrowed and the trachea below the balloon widened.

Table E 20: Changes in the lungs and airways after tracheal occlusion.

(A) Ultrasound and (B) post mortem measurements of the fetal chest and trachea following ultrasound guided placement of an occlusive balloon in the fetal trachea.

A: Ultrasound measurements	Injected fetus		Control fetus	
	at surgery	at PM	at surgery	at PM
Chest height (mm)	48.5	135	50.3	103
Chest circumference (mm)	165	246	164	218
Trachea below balloon (mm)	4.2	6.8	4.3	6.0
Trachea above balloon (mm)	4.1	4.0	4.7	6.5

B: Post mortem measurements	Injected fetus	Control fetus
Lung weight (g)	510	84
Lung-to-body-weight ratio	0.145	0.027

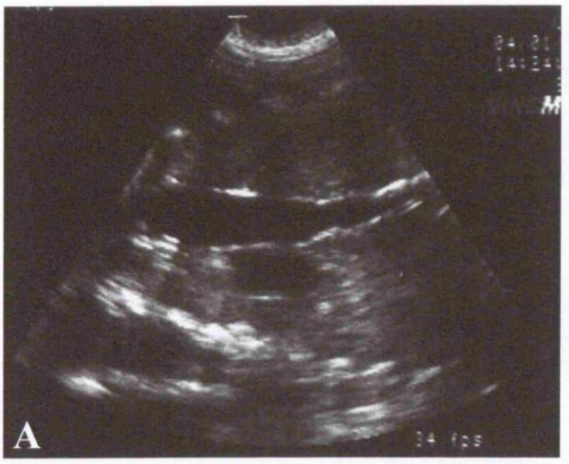


Figure E 29: Occluison of the fetal trachea by ultrasound-guided injection.

The ultrasonogram of the fetal chest shows the inflated detached balloon within the fetal trachea immediately after transthoracic tracheal injection.

Ultrasound scanning immediately prior to post mortem examination 21 days after surgery, showed ascites with a deepest pool of 23mm and bilateral pleural effusion. The injected fetus had lightly meconium stained liquor that was reduced in volume compared to the control animal. On opening the peritoneal cavity there was 100ml of clear ascites and the diaphragm was distended into the abdominal cavity (**Figure E 30 A and B**). There were pronounced rib markings on all surfaces of the lungs (**Figure E 30 C**). The lung weight and lung-to-body-weight ratios were over six and five times more in the injected fetus than the control fetus respectively, confirming that lung growth had occurred (**Table E 20 B**). Histological analysis of the lung parenchyma showed dilated small airways and alveoli when compared with control (**Figure E 30D and E**). There was no evidence of trauma, adhesions or inflammation.
These results confirmed that the balloon had been inflated correctly within the fetal trachea. We concluded that ultrasound guided balloon placement was technically feasible.

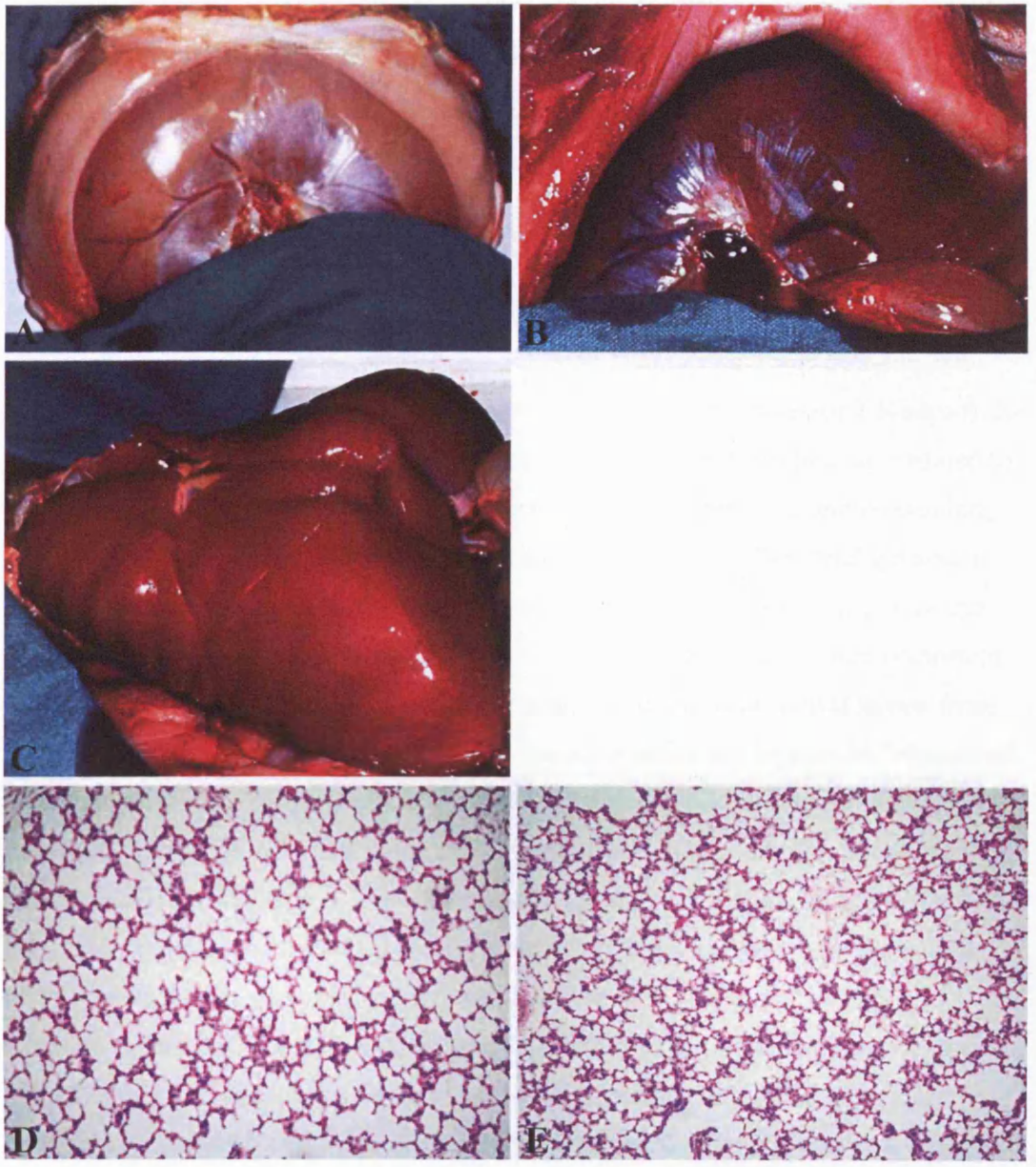


Figure E 30: Post mortem and histological findings after ultrasound guided fetal tracheal occlusion with a balloon.

At post mortem examination (A) the diaphragm was distended into the abdominal cavity in the injected fetus but not in the control fetus (B). (C) The lung of the injected fetus showing rib markings on all surfaces. Histological sections (H & E) of the lung showed dilated small airways and alveoli of the injected lung (D) when compared with the control lung (E); original magnification x 10.

E 4 Ultrasound guided delivery to the fetal gut

As well as airways disease, cystic fibrosis leads to pathology in the gut that manifests as meconium ileus, a life-threatening condition that occurs in 10-15% of neonates with cystic fibrosis. Gene transfer to the fetal gut may be able to prevent this occurring. In the late gestation fetal sheep (112, 117, 120 and 125 days of gestation), previous experiments by our group had shown that ultrasound guided injection of adenovirus vectors containing the β-galactosidase gene into the fetal stomach was possible, with minimal morbidity and no mortality (personal communication, Professor T Kiserud). X-gal staining of the fetal gut demonstrated very low level tissue transduction confined to the villi of the small bowel, particularly the terminal ileum (personal communication, Professor T Cook). We considered that the relatively large size of the fetal gut and its maturity may have contributed to these poor results. We also wanted to target the gut prior to the gestational age at which the fetus is thought to become immune competent. In the human fetus, tiny areas of fluid can be seen within the small bowel lumen from 13 weeks of gestation but ultrasound guided injection would not be possible because of their small size. By 25 - 30 weeks of gestation the average diameter of the small and large bowel lumen is 1.8 and 8.0 mm respectively which would permit ultrasound guided injection (Parulekar S, 1991), although widespread visualisation and peristalsis is typically only associated with bowel obstruction. The human fetal stomach may be visible on ultrasound examination of the abdomen from 9 weeks, and usually by 14 weeks of gestation. Thus we designed a series of experiments to investigate ultrasound guided delivery of adenovirus vectors to the fetal sheep stomach in early gestation.
We hypothesized that instillation of the fluorocarbon perflubron into the fetal stomach after vector injection might flush the vector distally into the small and large bowel, in a similar way that perflubron acted in the fetal airways after tracheal injection. We aimed to transduce the cells in the intestinal crypts that are the site of CFTR expression (Kälin N et al., 1999). Experiments on adult rat intestine have shown that lumenal distension of the small bowel with PBS results in shortening of the villi and widening of the intervillus spaces (Sandberg J et al., 1994). Thus perflubron instillation might also aid vector delivery to the crypt spaces.

E 4.1 Gastric fluid from the fetal sheep does not impair adenovirus transfection *in vitro*

The aim of these experiments was to determine whether fetal gastric fluid affected adenovirus transfection of an epithelial cell line *in vitro*. We used human bronchial epithelial (HBE) cells because there are no gastric epithelial cell lines available and our intention was to test the stability of the adenovirus vector in fetal sheep gastric fluid, rather than its ability to infect gastrointestinal epithelium *per se*.

An initial experiment was performed to determine the optimal dose range of the adenovirus vector. AdlacZ vector was incubated with gastric fluid from a non-injected fetus aged 60, 83 or 113 days of gestation or PBS for 30 minutes at room temperature. Serial dilutions of the vector in gastric fluid were then made with PBS. These were applied to four 24-well plates containing HBE cells (7 x 10^4 cells per well) cultured to 30% confluence to achieve 10^8, 10^7, 10^6, 10^5, 10^4 or 10^3 adenovirus particles per well. The plates were exposed to X-gal stain 48 hours after transfection and the number of blue cells per well counted.

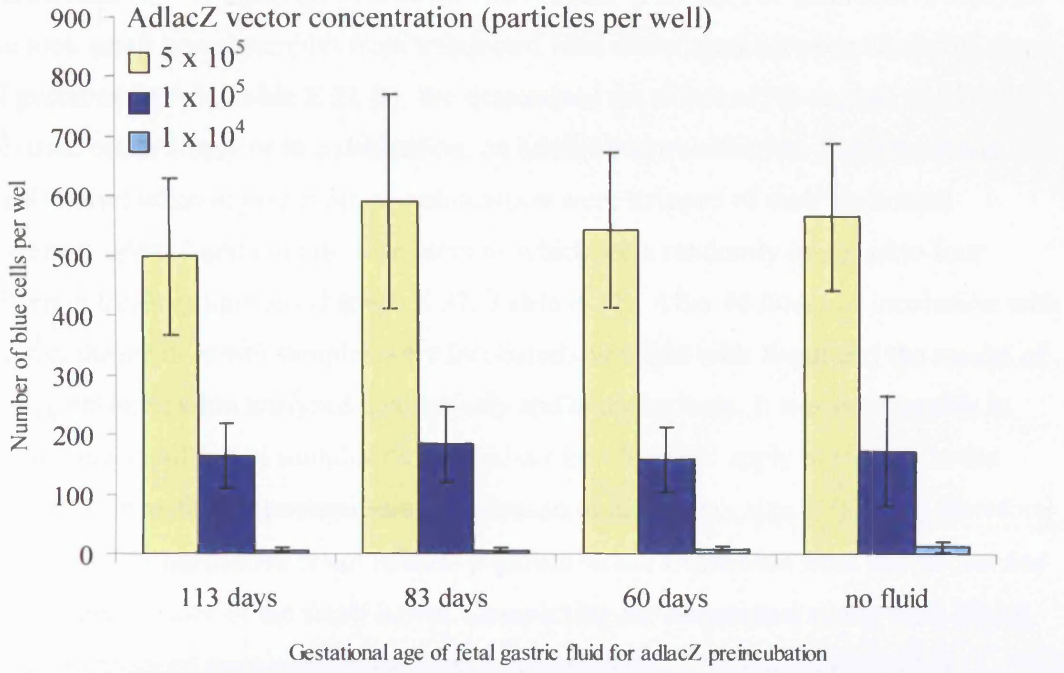


Figure E 31: The effect of fetal gastric fluid on adenovirus infection of epithelial cells *in vitro*.

AdlacZ vector was pre-incubated for 30 minutes with gastric fluid from fetal sheep aged 113, 83 or 60 days of gestation, or with PBS. The adlacZ vector was serially diluted with PBS and applied to HBE cells *in vitro*. Cells were stained with X-gal solution 48 hours later and the number of blue cells per well counted, presented as mean ± SD.

At the two highest adenovirus concentrations it was not possible to count the number of blue cells per well. There were no blue cells in the wells with the lowest concentration of adenovirus vector (10^3 particles per well). The results are displayed in **Figure E 31** as mean ± SD. Having determined the optimum dose range of the adenovirus vector, the experiment was repeated in triplicate using the following concentrations of adenovirus particles per well: 10^7, 10^6, 5 x 10^5, 10^5, 10^4 or 10^3. Preincubation of the adlacZ vector with fetal gastric fluid for 30 minutes had no significant effect on adenovirus infection of HBE cells when compared with preincubation with PBS (two-way analysis of variance).

E 4.2 Adenovirus vectors efficiently transfect the fetal sheep gut *ex vivo*

We performed experiments *ex vivo* to investigate adenovirus mediated gene transfer to the fetal sheep gut through gestation. For qualitiative analysis, small bowel samples were taken from fetal sheep at 59, 78, 83, 89 and 113 days of gestation that were either uninjected (n = 2) or had received intramuscular (n = 1), umbilical vein (n = 1) or intracranial (n = 1) injection with adenovirus (**Table E 21 A**). For quantitative analysis we took small bowel samples from uninjected fetal sheep aged between 58 and 65 days of gestation (n = 5, **Table E 21 B**). We determined the effect of Na-caprate and DEAE dextran, either singly or in combination, on adenovirus transfection. Samples of the small bowel taken at post mortem examination were stripped of their peritoneal covering, opened and cut into four sections which were randomly assigned to four different treatment groups (**Figure E 32, Table E 21**). After 48 hours of incubation with vector, the small bowel samples were incubated overnight with X-gal and the results of transgene expression analysed qualitatively and quantitatively. It was not possible to culture the small bowel samples on a liquid-air interface and apply the vector to the luminal side as for the tracheal samples because of their small size. It is likely therefore, that the final quantitative result reflects β-galactosidase expression from the serosal and the luminal surface of the small bowel. Complexing the adenovirus vector with DEAE dextran enhanced gene transfer the most, whether alone (71% mean increase) or following Na-caprate pretreatment (78% mean increase). Na-caprate pretreatment only enhanced gene transfer by 28% on average, less than was seen in the fetal trachea.

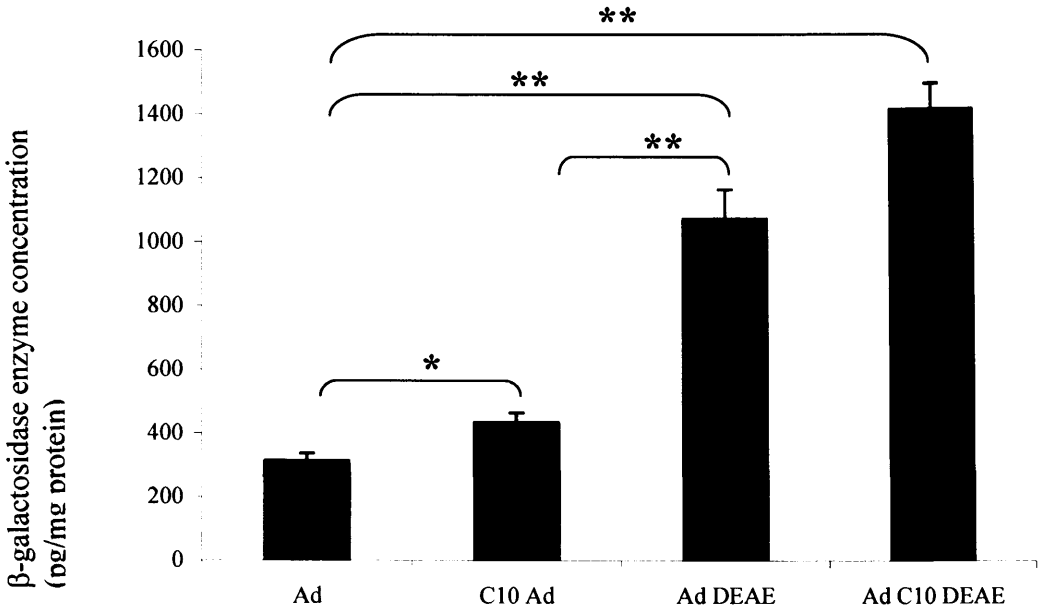


Figure E 32: Enhancement of adenovirus-mediated gene transfer to the fetal gastrointestinal epithelium *ex vivo*.

Sections of the small bowel from 5 sheep fetuses were pre-treated with 50mM Na-caprate (C10) for 10min prior to addition of either adenovirus alone (Ad) or complexed with DEAE dextran (DEAE). β-galactosidase transgene expression was analysed quantitatively using ELISA analysis. Results are expressed as averages ± SEM (n = 5). * $p < 0.02$ and ** $p < 0.0003$ comparing each of the enhancers and combination of Na-caprate pre-treatment and DEAE dextran complexed virus with virus alone, or virus and Na-caprate.

Table E 21: Enhancement of adenovirus-mediated gene transfer to fetal small bowel epithelium *ex vivo*.

(A) Qualitative analysis

Age (days)	X-gal staining of small bowel			
	adlacZ alone	C10 adlacZ	adlacZ-DEAE	C10 adlacZ-DEAE
59	(+)	+	++	+++
79	(+)	+	++	+++
83	(+)	+	++	+++
89	(+)	+	++	+++
113	(+)	+	++	+++

(B) Quantitative analysis

Age (days)	β-galactosidase enzyme concentration (pg/mg protein)			
	adlacZ alone	C10 adlacZ	adlacZ-DEAE	C10 adlacZ-DEAE
58	242.22	355.7	813.62	1254.64
61	364.44	496.5	1158.94	1583.59
62	315.87	512.04	1045.76	1362.83
62	287.68	389.56	980.55	1245.93
65	359.42	420.04	1365.9	1630.73

A limitation to the quantitative analysis of transgene expression after *ex vivo* and *in vivo* application was that some of the β-galactosidase expressing cells were on the serosal surface of the stomach, small or large bowel. We can therefore not exclude that the different effect of Na-caprate and DEAE dextran on transgene expression in the fetal gut *ex vivo* may be due to transduction of the serosa. We tried to reduce this effect after *in vivo* application by removing the peritoneal covering on tissue samples prior to analysis. Nevertheless it is likely that the true level of β-galactosidase protein expressed by the gut lumen is lower than we measured.

E 4.3 Measurement of the fetal sheep stomach through gestation using ultrasound

A literature search revealed there was no information about ultrasound visualisation of the stomach or its dimensions in the fetal sheep through gestation. We therefore used ultrasound to visualise and measure the fetal sheep stomach in early gestation.
Firstly video recordings from all fetal sheep experiments in early gestation were analysed. They recorded a general fetal anatomical survey, the fetal measurements including the abdominal circumference taken at the level of the fetal stomach, and the injection procedure. Ewes were also scanned prior to surgery to detect fetal number, to confirm viability and gestational age. From these records we evaluated whether the fetal stomach was visualised by ultrasound (**Figure E 33**).
The fetal stomach was not visualized in any fetus aged 40 days of gestation or less. Only three fetuses aged between 40 and 50 days of gestation were available for evaluation and so no conclusions can be drawn at this age range. From 50 days of gestation more data was available. Between 51 and 54 days of gestation the fetal stomach was visualised in 35 out of 43 fetuses (81%) but from 55 days of gestation, the fetal stomach was always visible.
Secondly, the dimensions of the fetal stomachs were measured prospectively in all early gestation fetal sheep experiments from November 2002 – June 2003. The widest anteroposterior (AP) and transverse (Trans) diameters (inner-to-inner) were measured in the coronal plane and the widest longitudinal (Long) diameter was measured (inner-to-inner) in the sagittal plane. The rumen was seen in all fetuses as a prolate ellipsoid. The reticulum was also occasionally seen, situated caudal to the rumen in the longitudinal plane and medial to it in the coronal plane (**Figure E 34**). The omasum and abomasum were not visible. The measurements were grouped according to gestational age (**Figure**

E 35). A description and diagram of the development and anatomy of the fetal sheep stomach is given in **Section A 7.3**.

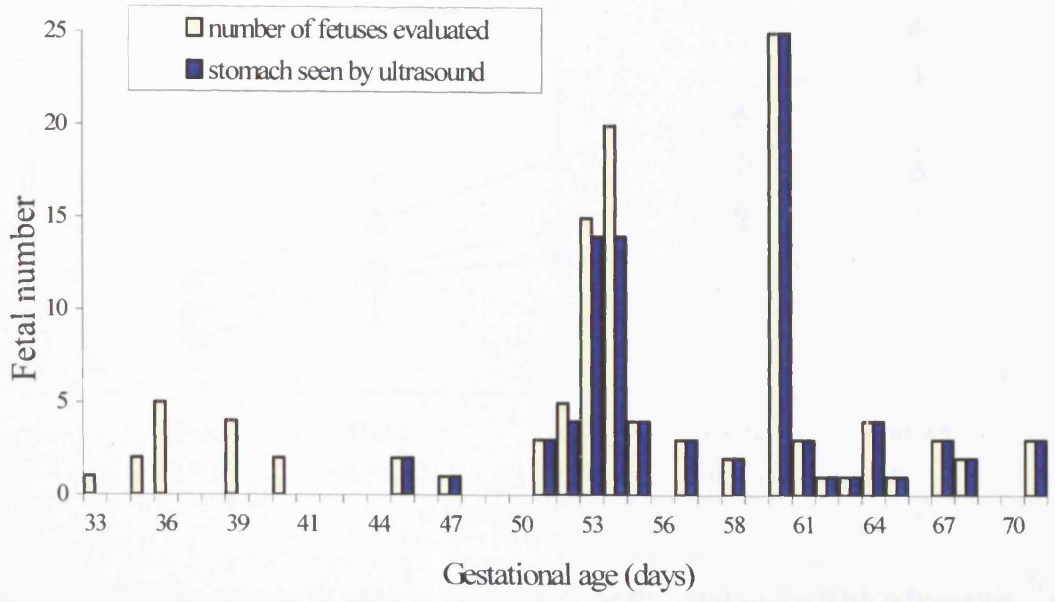


Figure E 33: Visualisation of the stomach in the early gestation sheep fetus by ultrasound.

Video recordings were reviewed from early gestation sheep experiments in which an anatomical survey of the fetus was conducted. Ewes were also scanned prior to fetal surgery to detect fetal number, confirm viability and the gestational age. The fetal stomach was identified as a sonolucent area in the upper left quadrant of the fetal abdomen (see Figure E 34).

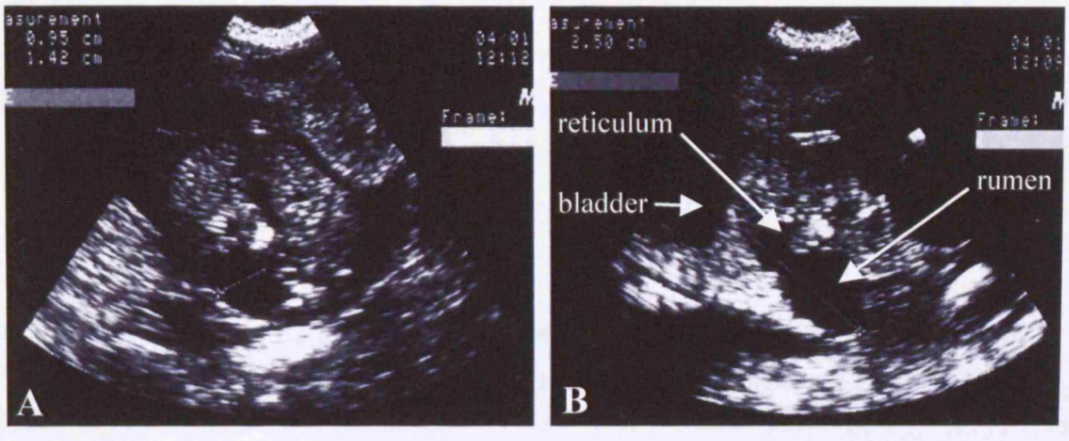


Figure E 34: Measurement of the early gestation fetal sheep stomach by ultrasound.

(A) With the fetal abdomen in coronal section the AP and Trans diameter are measured and (B) in the sagittal plane the Long diameter is measured.

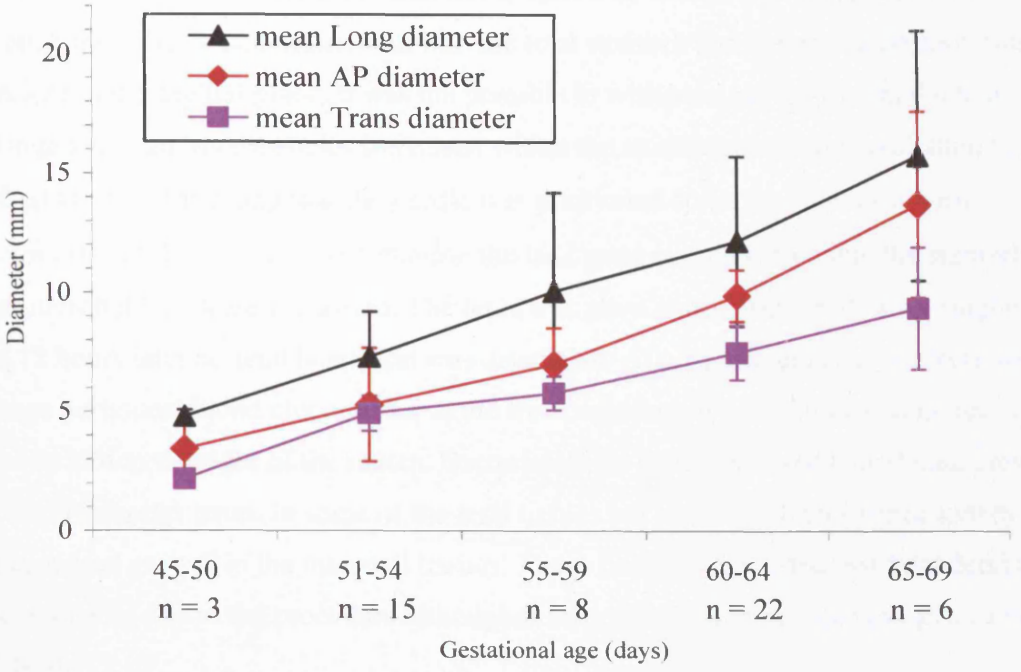


Figure E 35: The change in gastric dimensions in the fetal sheep with advancing gestational age.

The mean value ± 1.0 SD of the AP, Trans and Long diameter of the fetal stomach are illustrated. The number of fetuses measured at each time point is shown.

The fetal stomach dimensions increased linearly from 45 days to 69 days of gestation and the largest diameter at any time point was the longitudinal diameter. From 55 – 60 days of gestation all measurements were on average above 5 mm and from 60 days of gestation the mean AP diameter was approaching 10 mm.

We believed these findings would influence our ability to inject the fetal stomach by ultrasound guidance. The fetal stomach was reliably seen in fetuses aged from 55 days of gestation and the fetal stomach would need to be large enough to allow removal of a small amount of gastric fluid to confirm correct needle placement. Thus we decided the first attempt to inject the fetal stomach should be from 55 days of gestation.

E 4.4 Ultrasound guided intragastric injection is achievable in the early gestation fetal sheep

E 4.4.1 Gastric injection before 60 days of gestation is unsuccessful

The first attempt to inject the fetal sheep stomach was in a fetus aged 55 days of gestation. Only the rumen was visible and it measured 2.7 x 3 x 5 mm (AP x Trans x

Long). With the fetal abdomen in transverse view a 22 Gauge needle was advanced through the anterior abdominal wall into the fetal stomach and the needle position was checked in the sagittal plane. It was not possible to withdraw any gastric fluid into a syringe although microbubbles were seen within the stomach lumen on instillation of 100 µl saline, confirming that the needle was positioned correctly. The adenovirus vector (100 µl, 2 x 10^{11} p/kg) containing the lacZ gene was delivered into the stomach and microbubbles were visualised. The fetus was alive immediately following surgery but 12 hours later no fetal heart beat was detectable. At post mortem analysis there was a large peritoneal blood clot anterior to the liver, adjacent to the umbilical vein, and a smaller clot to the right of the rumen. Bacteriological culture showed a moderate growth of campylobacter jejuni in some of the fetal tissues but not in the fetal liver or kidney and a mixed growth in the maternal tissues. These findings suggested that fetal demise was probably due to the procedure although infection could not be ruled out as a cause of death.

E 4.4.2 Gastric injection at or after 60 days of gestation is successful

Because of the difficulty injecting the fetal stomach at 55 days gestation and our observation that the AP diameter was approaching 10 mm at 60 days we decided further stomach injections would be performed from this gestational age onwards. Injection was attempted in 11 fetuses from 7 ewes with success in 10 (91%, **Figure E 36**). In these fetuses the needle was confirmed to be correctly positioned within the fetal stomach by withdrawal of 100µl of gastric fluid. The one injection failure was in a triplet pregnancy in which the other two fetuses were successfully injected. In this case visualisation of the fetal stomach was inadequate due to fetal positioning. The needle was thought to be within the stomach lumen but attempts to withdraw gastric fluid failed. This suggested that the needle was not correctly positioned and we did not attempt to inject the vector. Occasionally it was difficult to see microbubbles on ultrasound following injection of 100µl fluid into the fetal stomach during early experiments, although review of the video recordings confirmed their presence in all cases. In later experiments (G6 onwards) the volume of Na-caprate and adenovirus vector administered was increased from 100µl to 200µl which improved microbubble visualisation. In most cases (8 out of 11 injections), the fetus was positioned so that the needle transgressed the abdominal cavity only a small distance before entering the stomach. In three cases, the fetus was lying on its left side and to reach the stomach, the

needle had to pass through the fetal liver. These cases were not associated with failed injection, haemorrhage or infection.

Overall the mean time to successful gastric injection was 7 minutes 21 seconds (SD 6 minutes 44 seconds, range 29 seconds to 13 minutes 34 seconds). In 2 twin fetuses the first attempt at gastric injection failed due to a combination of fetal positioning and operator inexperience but a second attempt was successful in each case. In 8 fetuses the injection procedure was achieved at the first attempt in a mean time of four minutes 56 seconds (SD 2 minutes 41 seconds, range 29 seconds to 8 minutes 18 seconds).

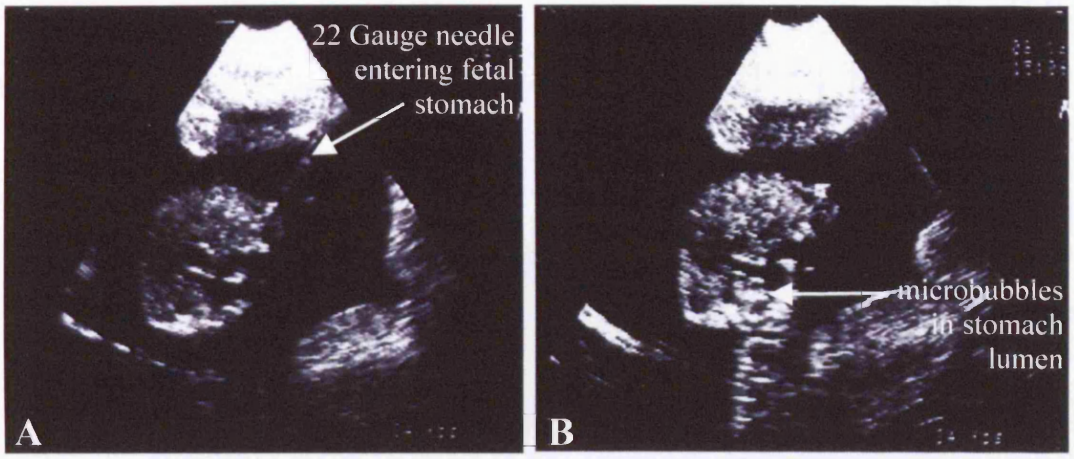


Figure E 36: Ultrasonograms showing injection of the stomach in a fetal sheep aged 61 days of gestation.

With the fetal abdomen in coronal section, (A) the needle was inserted into the fetal stomach through the anterior abdominal wall and 100µl gastric fluid was removed to confirm correct needle placement. (B) The vector was injected and microbubbles were observed in the stomach lumen (G3).

E 4.5 Effect of gastric injection on ultrasound visualisation of the fetal stomach

E 4.5.1 Fetal stomach volume

The fetal stomach was measured in three planes immediately before and after gastric injection. The stomach volume was calculated as that of a prolate ellipsoid: $4/3\pi(AP/2)*(Trans/2)*(Long/2)$, a figure generated by the revolution of an ellipse around its major axis (Zimmer EZ et al., 1992). This equation is used to calculate the volume of the human fetal stomach on ultrasound examination. We decided that it provided a good approximation to the fetal sheep stomach because at this gestational age only 2 stomach cavities were visible. The mean stomach volume before injection

was 0.8ml (±SD 0.47). As expected, injection of the largest volume of perflubron (1500µl) resulted in the largest increase in stomach volume (**Figure E 37**). On average the stomach volume increased by 116%, 68%, 289% and 347% after injection of vector and Na-caprate alone, 300µl, 1000µl and 1500µl perflubron respectively.

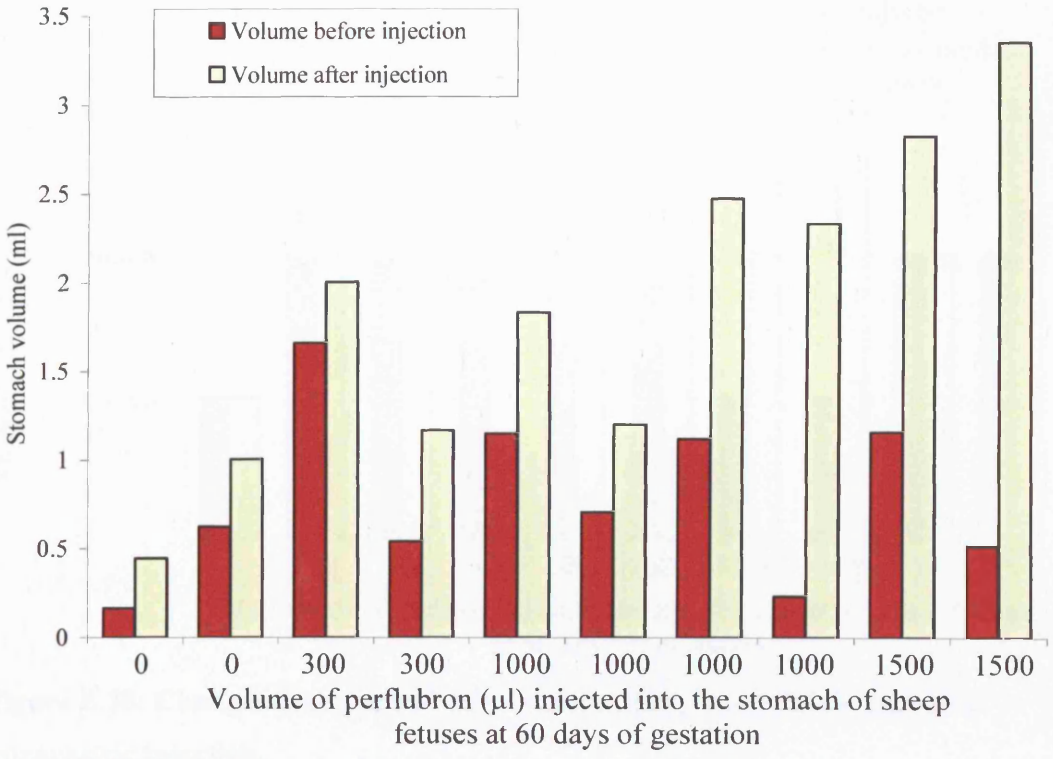


Figure E 37: The change in stomach volume with gastric injection.

Fetuses (60 days of gestation) received intragastric adenovirus vector with or without DEAE dextran complexing and Na-caprate pretreatment, followed by injection of different volumes of perflubron.

E 4.5.2 Number of fetal stomach chambers visualised by ultrasound

The number of stomach chambers visualized by ultrasound was determined before and after gastric injection. The sagittal view of the fetus was found to be the best for determining which stomach chambers could be seen. Studies of the development of fetal sheep (Latshaw WK, 1987) and examination of our early gestation sheep fetuses at post mortem confirmed our ultrasound finding that at 60 days gestation, the rumen was the largest stomach chamber and the reticulum was commonly visible.

Following injection of the rumen, in 6 out of the 10 animals injected, there was an increase in the number of stomach chambers seen by ultrasound (**Figure E 38**). This was particularly observed in those fetuses that received perflubron after injection of the adenovirus vector and Na-caprate. In two fetuses that received 300µl and 1000µl

perflubron all four stomach chambers became visible (**Figure E 39**) and the dense perflubron could be seen lining the omasum and abomasum after injection. We concluded that injection of perflubron into the rumen distended the fetal stomachs distal to the injection site.

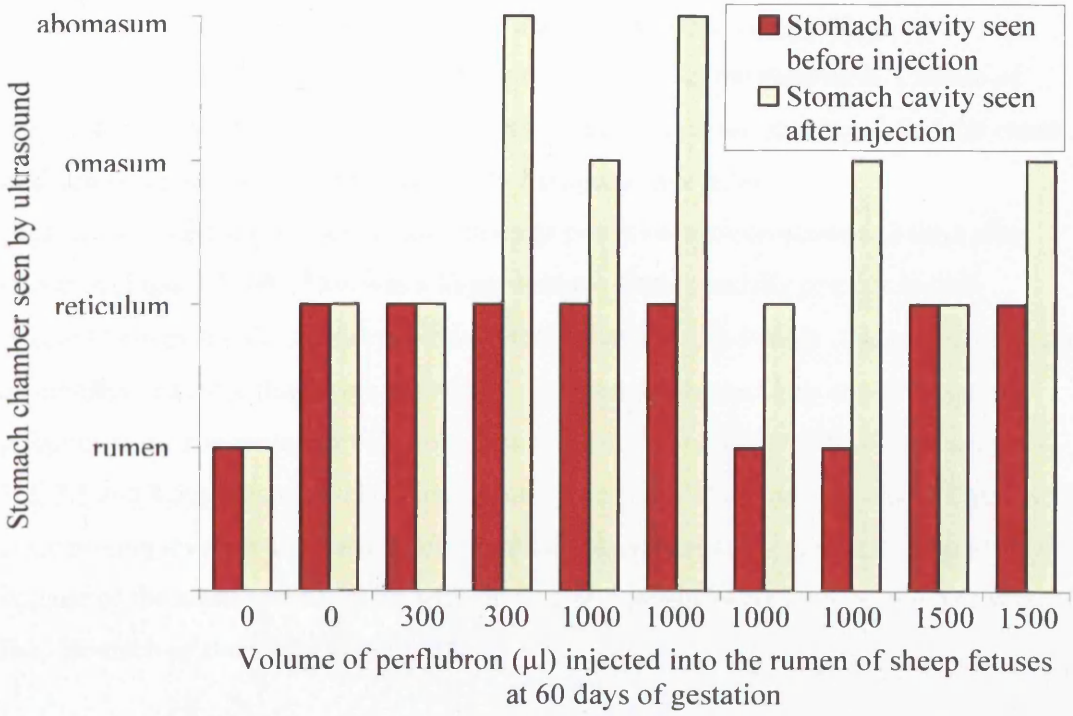


Figure E 38: Changes in the visualisation of fetal sheep stomach cavities after intragastric injection.

Fetuses received intragastric adenovirus vector with or without DEAE dextran complexing and Na-caprate pretreatment, followed by injection of different volumes of perflubron.

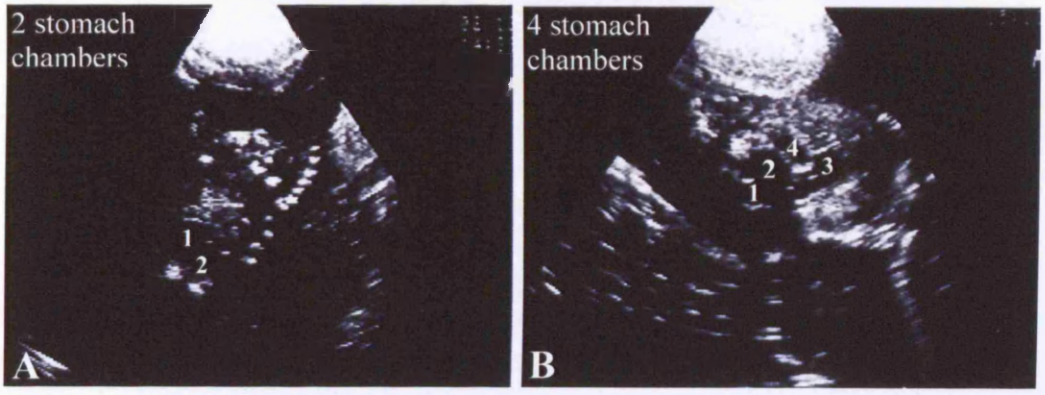


Figure E 39: The increase in the number of stomach chambers visualised after gastric injection.

Ultrasonograms showing a sheep fetus (61 days of gestation) in coronal section. (A) Two stomach chambers can be visualised before surgery, and (B) four stomach chambers after surgery.

E 4.6 Gastric injection has a low morbidity and mortality

There was one fetal death of a twin fetus (G12) 24 hours after injection in which both twins received gastric injection (90% fetal survival). Post mortem analysis was performed 2 days after injection to allow sufficient time for maximal transgene expression in the surviving twin. This showed a large blood clot posterior to the right lobe of the liver and extending to the left of the midline in the dead twin. Culture of fetal tissues showed no evidence of bacterial infection and we concluded that the cause of death was procedural rather than due to iatrogenic infection.

Clear ascitic fluid was present in all fetuses at post mortem examination 2 days after injection **(Figure E 40)**. This was a larger volume than is usually present in non-injected fetuses and those injected via alternative routes (50-100µl). The protein content of samples of ascitic fluid were analysed to determine whether they showed signs of inflammation. The protein levels from four fetuses, G5, G10, G9 and G11 measured 3, 3.2, 7.5 and 8.5 g/l respectively. These results imply that the fluid was only a transudate, since protein levels must reach 25g/l to indicate an inflammatory process. In general the volume of the ascites was highest with the largest volume of perflubron injected into the fetal stomach as shown in **Table E 22**.

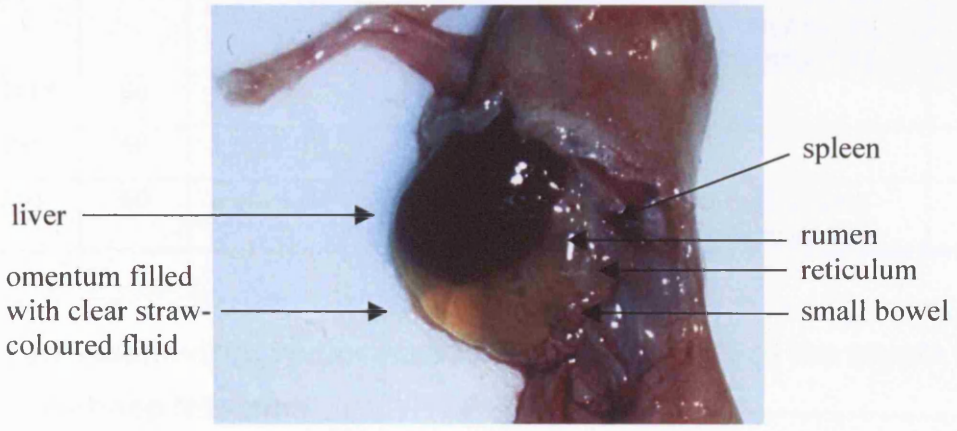


Figure E 40: Peritoneal fluid after intragastric injection of adlacZ vectors.

The peritoneal cavity of a sheep fetus aged 62 days of gestation at post mortem examination 2 days after ultrasound guided gastric injection of gene therapy vectors. The fetus received Na-caprate (100 µl, 100 mM), adlacZ (100 µl, 9.5 x 10^{12} p/kg) complexed with DEAE dextran (5µg/ml), and perflubron (300 µl). Clear straw-coloured fluid can be seen distending the omentum and also within the rumen and reticulum.

It is likely that the perflubron itself contributed to the ascitic volume by leaking out of the stomach after gastric injection. In two fetuses there were other significant findings at post mortem examination. In the fetus with the largest volume of ascites (8ml) a small

pleural effusion was also observed. This may have occurred secondary to the compression effect of the ascites on the venous return to the heart, leading to cardiac failure. In another fetus, fine peritoneal adhesions were seen. Culture of the fetal liver and ascites showed a pure growth of coagulase negative staphylococcus that was most likely introduced during the injection procedure.

Table E 22: Post mortem findings after gastric injection of adenovirus vector and transduction enhancing agents.

Fetuses were examined 2 days after injection; dex (5μg/ml): DEAE dextran, cap (100mM): Na-caprate pretreatment, perf: perflubron, *denotes addition of colloidal carbon (1:2 dilution) with adenovirus vector.

Sheep	Age (days)	Vector and enhancing agents				Post mortem findings in addition to clear ascitic fluid	Ascites (ml)
		adlacZ (p/kg)	dex	cap (μl)	perf (μl)		
G2	62	1×10^{10}	-	100	-	-	1
G5	60	1.6×10^{13}	-	-	-	-	2
G3	61	1×10^{13}	-	100	300	-	2
G4	60	9.5×10^{12}	+	100	300	-	2
G6	60	1.4×10^{13}	+	200	1000	-	0.5
G8*	60	1.0×10^{13}	+	200	1000	Fine adhesions from liver to anterior abdominal wall	1
G11*	60	1.1×10^{13}	+	200	1000	-	6.5
G9*	60	1.0×10^{13}	+	200	1500	-	3
G10	60	8.8×10^{12}	+	200	1500	Small pleural effusion (0.2ml)	8

E 4.7 Adenovirus vector transfects the villi but not the crypts in the fetal sheep intestine

Results of *ex vivo* transfection experiments had shown us that adenovirus mediated gene transfer to the fetal sheep gut was possible at 60 days gestation. A series of experiments were conducted to evaluate the effect of the fatty acid Na-caprate and the polycation DEAE dextran on adenovirus transduction of the fetal gut. X-gal staining and β-galactosidase immunohistochemical analysis of tissues from the fetal gastrointestinal system was performed on fetuses 2 days after gastric injection (**Table E 23 A**). The amount of β-galactosidase expression was also determined using ELISA analysis (**Table E 23 B**).

Table E 23: β-galactosidase transgene expression after intragastric injection of adlacZ vectors.

(A) X-gal staining and β-galactosidase immunohistochemical analysis and (B) ELISA analysis of β-galactosidase expression (pg/mg protein) in the fetal gut.

Tissues were analysed 2 days after injection of Na-caprate (cap, 100mM), adenovirus vectors (adlacZ) with or without DEAE dextran (dex, 5μg/ml) and different volumes of perflubron (perf) into the stomach of fetal sheep at 60 days of gestation. * denotes addition of colloidal carbon (100 μl, 1:2 dilution) with adenovirus vector. For transgene expression, + to +++ indicates degree of transduction observed after β-gal staining or immunohistochemistry; (+) indicates single positive cell or sparse staining.

Table E23 A	Age (days)	Vector and enhancing agents				X-gal staining: small bowel	β-gal immuno: small bowel
Sheep		adlacZ (p/kg)	dex	cap (μl)	perf (μl)		
G5	60	1.6×10^{13}	-	-	-	+	++
G2	62	1×10^{10}	-	100	-	(+)	+
G3	61	1×10^{13}	-	100	300	(+)	(+)
G4	60	9.5×10^{12}	+	100	300	+	(+)
G6	60	1.4×10^{13}	+	200	1000	+++	+++
G8*	60	1.0×10^{13}	+	200	1000	+++	+++
G11*	60	1.1×10^{13}	+	200	1000	+++	+++
G9*	60	1.0×10^{13}	+	200	1500	+++	+++
G10	60	8.8×10^{12}	+	200	1500	+++	+++

Table E23 B		G5	G3	G4	G6	G8*	G11*	G9*	G10
dex (5μg/ml)		-	-	+	+	+	+	+	+
cap (μl)		-	100	100	200	200	200	200	200
perf (μl)		0	300	300	1000	1000	1000	1500	1500
Oesophagus	Upper				224	1129	11	41497	1124
	Lower				722	629		20375	601
Stomach	Rumen	3496		373	714	1528	923	11318	8751
	Reticulum	77			1584	3448		8864	2204
	Omasum				1568	2138		10684	568
	Abomasum				538	10322		13188	5984
Duodenum					518	1411		8394	174
Small bowel	Upper $^1/_3$				551	671		3091	174
	Middle $^1/_3$				318	341		2601	104
	Lower $^1/_3$	775			1378	578		4108	3818
Caecum					315	3654		811	2257
Transverse colon					498	3002		20375	2264

In the first experiment (G2) only Na-caprate and the adenovirus vector were injected. X-gal staining showed weakly positive transgene expression in the villi of the small bowel that was not quantified by ELISA (data not shown). Immunohistochemical analysis of β-galactosidase expression (**Figure E 41 A - E**) showed scattered positive cells in the villi of the distal small bowel, the spleen and the serosa surrounding the reticulum, small bowel and pancreas. There were many positive cells in the myocardium, and strong staining of subcapsular hepatocytes. There was no transgene expression observed in the crypts of the small bowel.

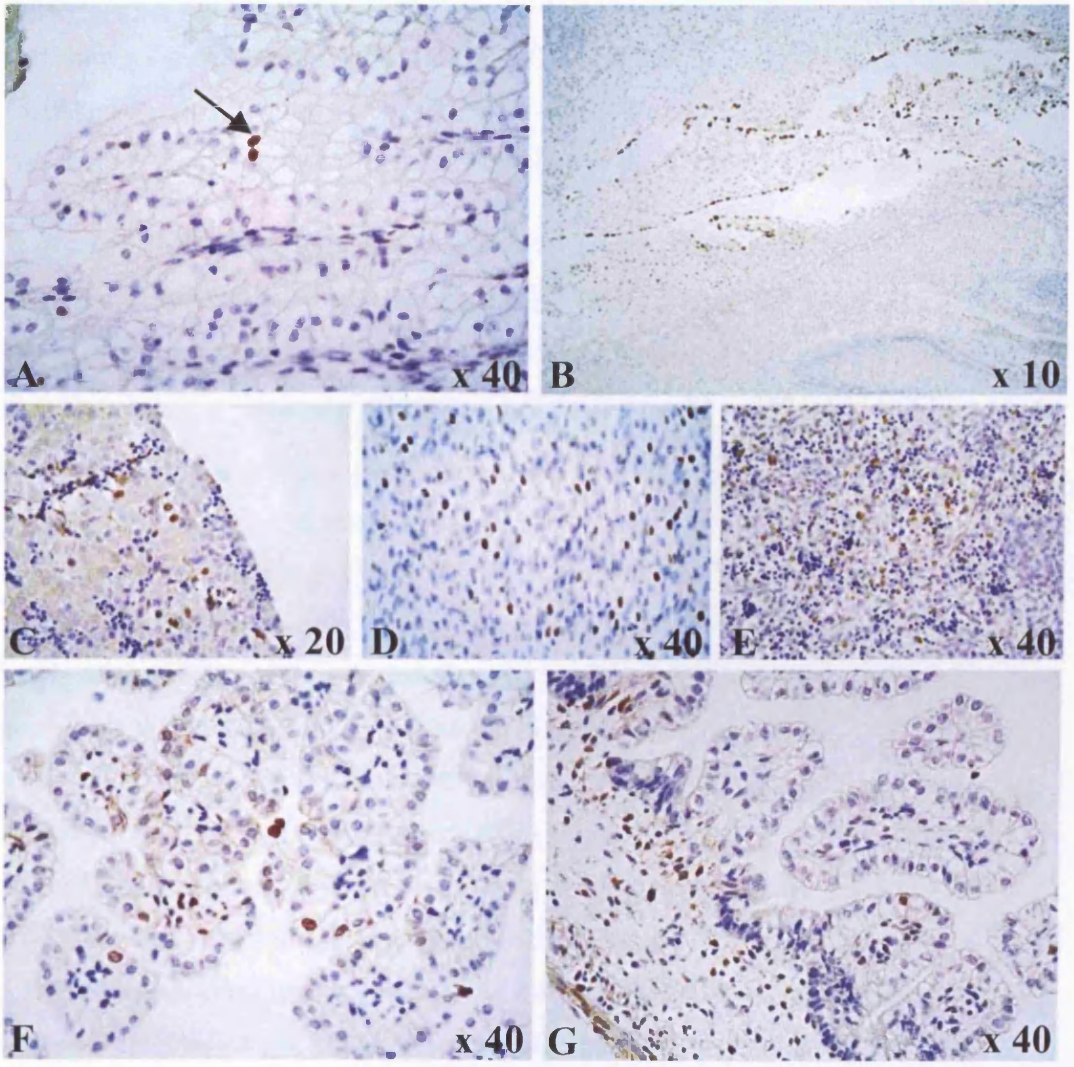


Figure E 41: β-galactosidase transgene expression after intragastric injection.

Two days after injection of Na-caprate and adlacZ vector (G2, 1 x 10^{10} p/kg), immunohistochemical analysis (haematoxylin counterstain) shows scattered positively stained (A) villi of the distal small bowel (arrowed) and (B) serosa of the reticulum. Strong positive staining of (C) subcapsular hepatocytes, (D) the myocardium and (E) the spleen. Increasing the dose of adlacZ vector without Na-caprate pretreatment (G5, 1.6 x 10^{13} p/kg) resulted in a small improvement in β-galactosidase expression in the proximal small bowel villi (F) but no transduction of the epithelial cells in the crypts was observed (G). Original magnifications are as indicated.

To improve adenovirus mediated transgene expression we decided to increase the dose of vector applied. From our ultrasound measurement study, the fetal stomach volume measured between 0.5 – 1.5ml at 60 days of gestation and the gastric fluid would dilute the vector considerably. Therefore we increased the adenovirus vector dose from 1 x 10^{10} p/kg to approximately 1 x 10^{13} p/kg, a similar dose to that applied by intramuscular injection.

Increasing the adenovirus vector dose alone (G5) without Na-caprate pretreatment resulted in a small improvement in the level of transgene expression in the small bowel but positive staining was confined to the villi and was not observed in the epithelial cells lining the crypts (**Figure E 41 F, G**). Quantifying the level of β-galactosidase by ELISA showed maximum transgene expression at the site of injection (rumen) and there was low level expression in the small bowel up to the distal third (**Table E 22 B**).

E 4.8 Instillation of the fluorocarbon perflubron improves distribution of adenovirus mediated transgene expression to the distal intestine but does not affect transduction of the intestinal crypts

In this series of experiments we wanted to evaluate the effect of the fluorocarbon perflubron on spread of the adenovirus vector distally beyond the gastric injection site through the small and large bowel. We considered that the lack of crypt transduction might result from an inability of the vector to reach the crypts, and that perflubron might distend the small bowel and improve crypt access. We therefore decided to inject increasing volumes of perflubron into the fetal stomach after delivery of the adenovirus vector. Because we did not know the maximum volume of perflubron that the fetus would tolerate, we began by injecting 300μl which would increase the stomach volume by approximately a third (mean stomach volume before injection = 0.8ml). We then injected 1000μl and 1500μl which would approximately double and triple the stomach volume respectively. To test whether this would improve crypt access we administered colloidal carbon (100 μl, 1:2 dilution) with the adenovirus vector in some fetuses. In all these experiments the volume of Na-caprate and adenovirus vector administered was increased from 100μl to 200μl to improve visualisation of microbubbles during stomach injection. We considered it unlikely that this small increase in volume relative to the gastric volume would affect gene transfer results.

Injection of 300µl perflubron after pretreatment with Na-caprate and delivery of adenovirus vector alone (G3) resulted in only low level transgene expression (**Figure E 42**) which had extended down to the level of the transverse colon but was below the level of detection by ELISA. Complexing the adenovirus vector with DEAE dextran (5µg/ml, G4) increased the amount of gene transfer to a level of 373 pg/mg protein by ELISA analysis but maximal expression was still local to the injection site at the fetal rumen. Immunohistochemical analysis for β-galactosidase expression was negative in both fetuses. These results were in agreement with the findings from *ex vivo* transfection experiments in which complexing the adenovirus vector with DEAE dextran improved gene transfer to the fetal gut. To achieve maximal gene transfer therefore, further experiments used DEAE dextran complexed adenovirus vector.

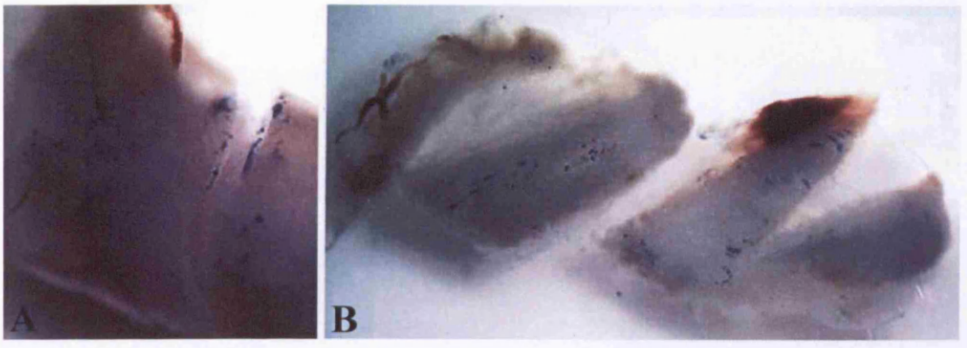
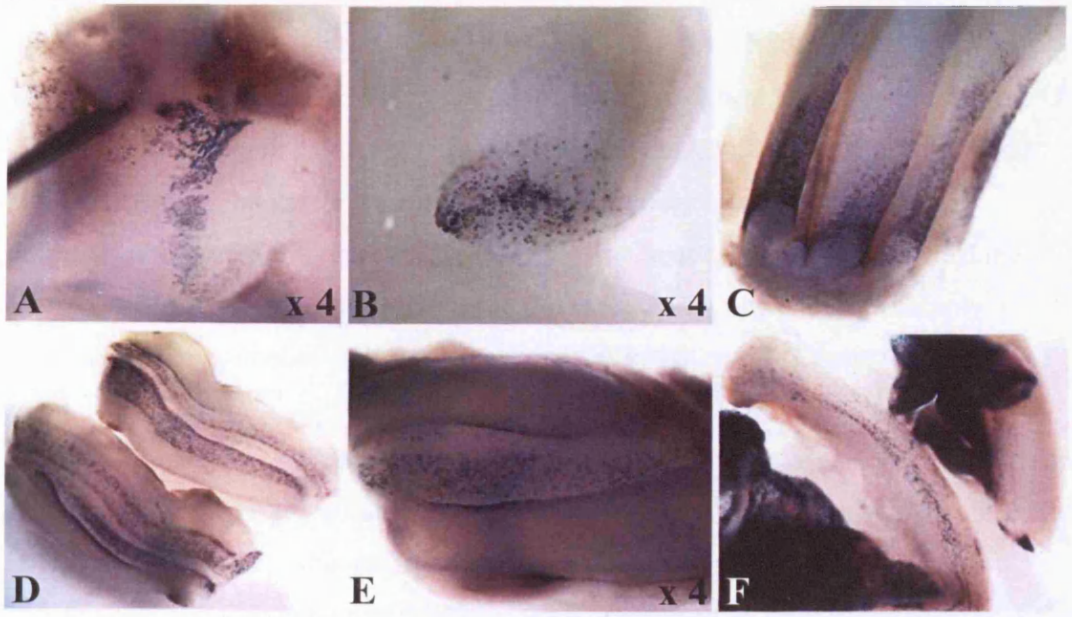


Figure E 42: β-galactosidase transgene expression after intragastric injection.

X-gal staining of the fetal sheep gastrointestinal tract 2 days after Na-caprate pretreatment and injection of adlacZ vector with perflubron (300µl) into the stomach at 61 days of gestation (G3, 1 x 10^{13} p/kg). There is low level staining in the (A) oesophagus and (B) duodenum. Original magnification x 4.

Increasing the volume of perflubron substantially increased the spread and amount of adenovirus mediated gene transfer to the fetal gut. Administration of 1000µl perflubron to the stomach of two triplets (G6 and G8, **Figure E 43, Panel 1 A and B**) resulted in high levels of X-gal staining. Immunohistochemical analysis for β-galactosidase expression confirmed these results (**Figure E 43, Panel 2 A – C**) and quantification by ELISA analysis showed positive expression throughout the length of the fetal gut that was maximal in the fetal reticulum of G6 (1584 pg/mg protein) and abomasum of G8 (10322 pg/mg protein). Examination of the non-injected co-triplet, G7 showed no positive X-gal staining and no β-galactosidase expression on ELISA analysis.

Panel 1:



Panel 2:

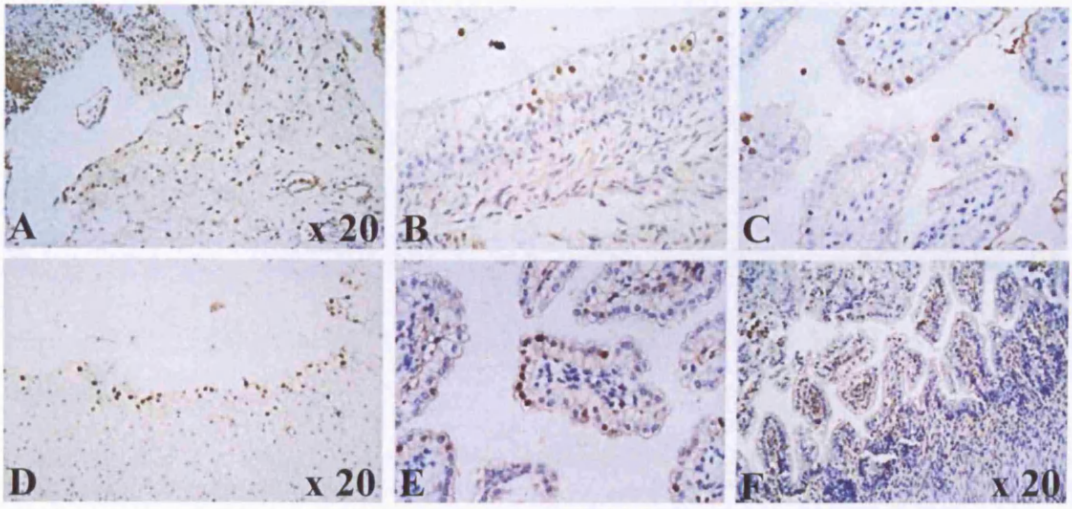


Figure E 43: The effect of perflubron instillation on adlacZ mediated gene transfer to the early gestation sheep gastrointestinal epithelium.

All fetuses received pretreatment with Na-caprate and adlacZ ($8.8 \times 10^{12} - 1.4 \times 10^{13}$ p/kg) complexed with DEAE dextran.
Panel 1: X-gal staining of the (A) rumen (G6) and (B) abomasum (G8) after instillation of 1000μl perflubron and of the (C) lower oesophagus, (D) rumen, (E) distal small bowel and (F) rectum after instillation of 1500μl perflubron (G9). Original magnifications x 2 except where indicated.
Panel 2: β-galactosidase expression in the (A) serosa of the rumen (G6), (B) omasum and (C) proximal small bowel (G8) after instillation of 1000μl perflubron, and in the (D) rumen (G9) and (E) and (F) in the proximal small bowel (G10) after instillation of 1500μl perflubron. No staining was observed in the epithelial cells lining the crypts (F). Original magnification x 40 except where indicated. Haematoxylin counterstain was used in all immunohistochemistry analysis.

Increasing the perflubron volume instilled to 1500μl in a set of twins increased the level of expression in one twin (G9) but had no effect on the other twin (G10). LacZ

expression was seen throughout the gastrointestinal system, as for G6 and G8 in which 1000μl perflubron was given (**Figure E 43, Panel 1 C - F**). Maximal expression in G10 was observed in the rumen (8751 pg/mg protein). The highest level of expression in G9 was in the upper oesophagus (41497 pg/mg protein) suggesting that as well as being directed distally, the vector was also flushed proximally up the oesophagus. β-galactosidase expression detected by immunohistochemical analysis confirmed the findings from X-gal staining (**Figure E 43, Panel 2 D-F**), but staining was only observed in the epithelial cells of the villi and not in the crypts (**Figure E 43, Panel 2 F**).

In addition there was positive staining in the nucleus and cytoplasm of the endothelium and subendothelium lining the portal vein branches in the left lobe of the liver of both fetuses (**Figure E 44**). This probably represents gene transfer to the portal vein endothelium and also uptake of β-galactosidase expressed by transduced gastrointestinal epithelium.

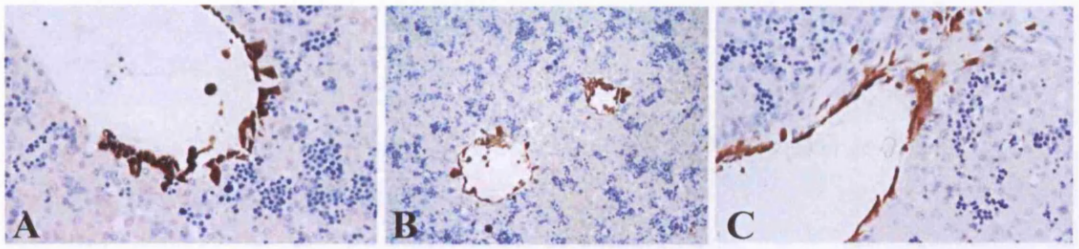


Figure E 44: β-galactosidase transgene expression in the liver after intragastric injection.

Immunohistochemical analysis of β-galactosidase expression (haematoxylin counterstain) in the left lobe of the liver 2 days after ultrasound-guided injection of Na-caprate, DEAE dextran complexed adlacZ ($8.8 \times 10^{12} - 1 \times 10^{13}$ p/kg) and perflubron into the stomach of two early gestation fetal sheep. Strong transgene expression is seen in the nucleus and cytoplasm of the endothelium and subendothelium lining the portal vein branches in G9 (A and B) and G10 (C). Original magnification x 40.

Colloidal carbon was injected with the adenovirus vector (100 μl, 1:2 dilution) in one of the triplets (G8) and one of the twins (G9). Low powered microscopic examination of the opened gut showed colloidal carbon in the lumen of the small and large bowel down to the rectum. On histological examination there were a few dots of carbon in the crypt of the small bowel in one fetus (G8) but none in the other. This demonstrated that vector was able to reach the intestinal crypts but only in very small quantities. Colloidal carbon was seen within the lumen over the length of the gastrointestinal system confirming that the vector spreads distally when large volumes of perflubron are given.

A small amount of X-gal staining was seen on the serosal surface of the stomachs and the small and large bowel of the fetuses that received 1000μl and 1500μl perflubron (G6-G11) suggesting that some of the injected fluid escaped from the fetal rumen into the peritoneal cavity after injection. Although we did not observe microbubbles passing from the fetal rumen into the peritoneal cavity immediately after stomach injection, it was not unexpected because of the amount of stomach distension we achieved that would increase intragastric pressure. The majority of X-gal staining was seen within the lumen. Prior to X-gal staining and β-galactosidase ELISA the peritoneal covering was stripped from the small and large bowel. It cannot be ruled out however, that serosal transgene expression contributed to the total β-galactosidase expression observed on ELISA analysis of the fetal stomach cavities.

To conclude, sodium caprate pretreatment and DEAE dextran complexation improved adenovirus mediated gene transfer to the gastrointestinal epithelium *in vivo* but lacZ expression was limited to the intestinal villi. Flushing with perflubron gave transgene expression throughout the length of the gut but did not result in gene transfer to the intestinal crypts.

E 4.9 Atropine, a suppressor of fetal gastrointestinal motility, is instilled with the adenovirus vector

We speculated that relaxation of the fetal small bowel smooth muscle might improve vector access to the intestinal crypts. Fetal hypoxia and intragastric atropine, an anticholinergic agent, have been shown to suppress upper gastrointestinal motility in late gestation fetal rabbits (Sase M et al., 2002,Acosta R et al., 2002). In adult humans atropine relaxes the gastrointestinal smooth muscle from stomach to colon and it is believed to act via muscarinic receptors. No data exists for the fetal sheep, but fetal gastric peristalsis appears as early as 14 weeks of gestation in humans (Sase M et al., 1999). This is approximately equivalent to 52 days gestation in the fetal sheep and we believed therefore, that atropine would have some effect on the fetal gut even at 60 days of gestation.

We investigated the effect of atropine on adenovirus mediated gene transfer to the gut of twin fetal sheep (G11 and G12). Both fetuses received an intragastric injection of Na-caprate (100μl, 100mM), colloidal carbon (1:2 dilution) and adenovirus vector complexed with DEAE dextran (5μg/ml), and 1000μl perflubron. In addition, one fetus (G11) received intragastric atropine (0.04μg/g estimated fetal body weight) with the Na-

caprate pretreatment. The dose was the same as that used in the late gestation fetal rabbits (Acosta R et al., 2002).

Unfortunately the fetus that did not receive atropine died 24 hours after injection (G12) and it was not possible therefore, to compare the results of the two fetuses. The fetus that received intragastric atropine (G11) was analysed 48 hours after injection. On examination of the opened bowel with low power microscopy, colloidal carbon could be seen within the lumen of the distal third of the small bowel but not in the caecum or colon. These results can be compared with those of the fetus that received the same volume of Na-caprate, vector, perflubron and colloidal carbon (G8) in which colloidal carbon could be seen within the lumen of the entire length of the gastrointestinal system. X-gal staining and β-galactosidase immunohistochemical analysis was negative although β-galactosidase expression was detected in the rumen on ELISA analysis. We concluded that atropine probably had a small relaxant effect on gastrointestinal motility even at this early gestation but did not affect transgene expression in the intestinal crypts. Indeed, addition of atropine may have reduced the efficiency of adenovirus infection and gene transfer.

E 4.10 The effect of adenovirus vectors and transduction enhancing agents on fetal gastric fluid

E 4.10.1 Biochemical analysis of fetal gastric fluid

The concentration of solutes and the osmolality of fetal gastric fluid before adenovirus delivery and at post mortem analysis were compared to determine whether the injection of the viral vector had any effect. There were no significant differences in values from samples before injection or those taken at post mortem. No normal ranges for ovine fetal gastric fluid were available at this early gestation (60 days) but all samples fell within the normal ranges for late gestation fetal ovine gastric fluid (Sherman DJ et al., 1996).

E 4.10.2 Cytological analysis of fetal gastric fluid

Fetal gastric fluid was analysed for evidence of an inflammatory reaction at postmortem analysis and compared with samples obtained prior to injection of vector. There were no epithelial or inflammatory cells observed in pre or post-injection gastric samples.

E 4.11 Spread of adenovirus vector in fetal and maternal tissues following intragastric administration

We explored the spread of transgene to the fetal and maternal organs by PCR analysis of tissues from one fetus (G6). AdlacZ was detected throughout the gastrointestinal system and all fetal organs tested suggesting that systemic spread of the vector had occured (**Table E 24**). This could have been via haematogenic or lymphatic spread.

Table E 24: Vector spread after intragastric injection of adlacZ vector to fetal sheep in early gestation.

AdlacZ vector was detected by PCR analysis of fetal and maternal tissues taken from a fetus aged 60 days of gestation sacrificed 48 hours after intragastric injection (G6, 1.4 x 10^{13} p/kg). Tissues that tested positive on 1st round PCR analysis (1st) were not generally subjected to nested PCR analysis (2nd).

Tissue		1st	2nd
Stomach chamber	Rumen	+	
	Reticulum	+	
	Omasum	+	
	Abomasum	+	
Duodenum		–	+
Small bowel	Proximal ⅓	+	
	Middle ⅓	+	
	Distal ⅓	+	
Caecum		+	
Transverse colon		+	
Rectum		+	
Liver		+	+
Gonad		+	+
Lung		+	+
Heart		–	+
Spleen		+	+
Kidney		+	
Placenta		–	–

E 5 Discussion

E 5.1 Ultrasound guided intra-amniotic injection is safe at early gestation in the fetal sheep

In this study, we performed intra-amniotic injection of the fetal sheep at 33 – 39 days of gestation equivalent to a human gestational age of 8 – 10 weeks. This is the earliest gestational age that ultrasound-guided intra-amniotic injection has been conducted in the fetal sheep. Short-term survival after injection was excellent, suggesting that the procedure carries a low complication rate, in keeping with evidence from clinical

studies performed at this gestation. From these experiments it was not possible to differentiate the effect of the injection procedure from the effect of early gestation intra-amniotic adenovirus vector on fetal morbidity and mortality. Our results however, are comparable to the only other study on ultrasound-guided intraamniotic injection in early gestation fetal sheep (aged 38 – 98 days of gestation), in which retrovirus vectors or saline were delivered, resulting in the loss of 5 out of 18 fetuses (Galan HL et al., 2002). The long-term morbidity and mortality was more likely to be related to application of the adenovirus vector. This is of concern since both fetuses that were left to deliver developed complications. No infective cause was identified for the fetus that aborted in late pregnancy and the other lamb had multiple congenital skeletal abnormalities. Congenital abnormalities are not uncommon in sheep and may be due to teratogenic agents in food or be genetic in origin. Akabane virus, an arthropod borne Bunyavirus is a major cause of ruminant congenital malformations in Australia, the Middle and Far East. The congenital effects that include arthrogryposis, hydrocephalus, microencephaly or encephalomyelitis were not present in our lamb, and the infection is not found in the United Kingdom (Charles JA, 1994). Pestiviruses are the only infectious agents occurring in the United Kingdom that cause such congenital abnormalities (Nettleton PF and Entrican G, 1995) and they are not present in our flock. The possible role of the adenovirus vector cannot be excluded, particularly since it was given before the completion of organogenesis. In the fetal primate, ultrasound guided intraamniotic injection of adenovirus vectors in mid-gestation resulted in one fetal death from candida infection 10 days after injection. The remaining 5 animals analysed from 10 to 30 days after injection showed no developmental abnormalities (Larson JE et al., 2000b) but there is no data on long term follow-up in the fetal primate.

Adenovirus infection is associated with pathology *in utero*. Adenovirus vectors cause developmental delay and disorganised cleavage of mouse embryos after the 2-cell stage (Gordon JW, 2002) and bovine adenovirus infection has been associated with unexplained fetal abortion in cows (Bartha A and Mate S, 1983). In the human, adenovirus is the most common viral pathogen identified in amniotic fluid and other fetal tissues from abnormal pregnancies (Wenstrom KD et al., 1998,Van den Veyver IB et al., 1998). Echogenic liver lesions with or without fetal hydrops have also been observed in patients with positive PCR results for adenovirus in the amniotic fluid (Baschat AA et al., 2003). Although it is unlikely that adenovirus would be the vector of choice for prenatal gene therapy, our results and the evidence from the literature

highlight the important concept that any vector used in clinical application must have an excellent safety profile in the fetus and mother.

In clinical practice, intra-amniotic injection has been used primarily to sample amniotic fluid to aspirate fetal cells for prenatal diagnosis. This procedure is performed between 15 - 17 weeks of gestation and most fetal medicine units quote a procedure-related additional risk of spontaneous miscarriage of 0.5 - 1%. Early amniocentesis at 11 – 13 weeks of gestation is technically straightforward but has a higher rate of complications including miscarriage, amniotic fluid leakage, and orthopaedic deformity (CEMAT group, 1998). Intra-amniotic injection is also used to drain excess amniotic fluid that can occur for example, in twin to twin transfusion syndrome (TTTS). Amnioreduction is usually performed from 16 weeks of gestation when severe TTTS may first present and even aggressive amniodrainage is associated with a low 1.5% complication rate (Wee LY and Fisk NM, 2002). Delivery of therapeutic compounds into the amniotic cavity has been performed for a few fetal conditions in mid or late gestation, including thyroxine for the treatment of fetal hypothyroidism (Collins FS and Watson JD, 2003) and surfactant for the prevention of neonatal respiratory distress syndrome (Cosmi EV et al., 1996). Intra-amniotic injection at gestations earlier than 11 weeks has usually been performed to sample amniotic fluid for research purposes only, on women who subsequently underwent surgical termination of pregnancy (Jauniaux E et al., 1999b) and the associated complication rate is therefore unknown.

The optimum gestational age for clinical application of intra-amniotic fetal gene therapy must be a balance therefore between the risks of an earlier procedure and targeting the fetus for the congenital disease to be treated. For gene therapy of some genetic conditions for example skin diseases such as epidermolysis bullosa, earlier exposure of the fetus may result in better gene transfer because of immaturity of the developing skin. For cystic fibrosis gene therapy however, transport of the vector into the airways relies on fetal breathing movements that only appear from the end of the first trimester.

E 5.1.1 Alternative fluid compartments in the fetus: the extraembryonic coelom

The exocoelomic cavity or extraembryonic coelom surrounds the embryo and fetus during most of the first trimester and could be used for delivery of gene therapy. Intra-allantoic injection is straightforward in the fetal sheep. Indeed in our study, during intra-amniotic injection, the needle first entered the allantoic cavity before passing through the amniotic membrane. Ultrasound-guided transabdominal coelomic injection has been

studied in the sheep fetus at 40 – 45 days of gestation for stem cell transplantation. Low level stem cell engraftment was achieved after delivery of human haematopoietic stem cells but the procedure had a 33% miscarriage rate by mid-gestation (Noia G et al., 2004). The relatively large fluid volume would reduce the efficacy of coelomic delivery for gene therapy. There would also be preferential transduction of the placenta since there is no anatomical barrier between the mesenchyme of the placental fetal plate and the extraembryonic coelom (Jones CPJ and Jauniaux E, 1995). This was observed after injection of adenovirus vectors into the extraembryonic coelom of the fetal rat (Laurema A et al., 2004). Giant cells of Reichert's membrane, the parietal lining of the fetal rat yolk sac exhibited a high level of transduction. No transgene expression however was detectable in the fetal or maternal liver, and PCR analysis confirmed that vector spread was restricted to Reichert's membrane.

In the human embryo, the extraembryonic coelom can be visualised from 5 to 12 weeks of gestation and sampling of coelomic fluid or coelocentesis can be performed by ultrasound guided transvaginal puncture with a 96% success rate between 6 and 10 weeks of gestation (Jurkovic D et al., 1993). The high failure rate of cell growth from coelomic fluid has limited its use in prenatal diagnosis to DNA analysis (Jauniaux E et al., 2003). In addition, a study of short-term safety of coelocentesis in women undergoing elective termination of pregnancy for psychosocial reasons found a miscarriage rate of 25%, 2 to 13 days after the procedure (Ross J et al., 1997). Long-term safety has not been assessed in the human fetus, but a study in the baboon showed a loss rate of one out of 9 fetuses up to the third trimester after ultrasound-guided transvaginal coelocentesis at 40 days of gestation (equivalent to 8 weeks of gestation in the human) (Santolaya-Forgas J et al., 1998). For these reasons, coelocentesis is not used currently for routine prenatal diagnosis. Coelomic delivery may be suitable for targeting gene transfer to the placenta in early gestation, but it is unlikely to be a suitable route for fetal gene therapy.

E 5.2 Ultrasound guided intra-amniotic injection in early gestation fetal sheep does not transfer genes to the airways

We investigated intra-amniotic application of adenovirus vectors because this route has been shown to allow gene transfer to the fetal airways (Douar A-M et al., 1997,Larson JE et al., 2000b,Boyle MP et al., 2001). In addition we wanted to test whether tissues transduced via this route of injection could act as an ectopic source of hFIX production. Therapeutic plasma concentrations of hFIX were detectable up to 11 days after injection

and immunohistochemical analysis showed positive expression of β-galactosidase in the fetal skin and nasal cavities. This suggests that transduction of keratinocytes *in utero* may be able to deliver proteins to the circulation as well as to treat hereditary skin disease such as epidermolysis bullosa.

At the gestational age that we chose for injection, the abdominal wall is still open prior to the return of the herniated intestinal loops to the peritoneal cavity which occurs in fetal sheep between 32 and 40 days of gestation (Cloete JHL, 1939). We hypothesised this would allow vector entry into the peritoneal cavity and although not observed by immunohistochemistry, PCR analysis showed vector presence in the fetal liver. We did not however detect vector in any of the other intraperitoneal organs even by sensitive nested PCR analysis.

We did not see any significant airway or gastrointestinal tissue transduction after ultrasound-guided intra-amniotic delivery of adenovirus vectors to early gestation fetal sheep. Studies of intra-amniotic delivery in other large animal models have had more promising results. Ultrasound-guided injection of adenovirus vectors in mid-trimester rhesus macaque fetuses resulted in significant transgene spread to tissues coming into contact with amniotic fluid but low level transgene expression in the fetal airways and intestine (Larson JE et al., 2000b). Application of a retrovirus producer cell line had similar results (Bennett M et al., 2001). Short-term gene transfer to the fetal trachea and pulmonary epithelium was found after intra-amniotic injection of AAV in late gestation fetal rabbits (Boyle MP et al., 2001). At its peak however, transgene expression was only detected in 1 in 500 cells and most of these were in the alveoli that are not the site of CFTR expression. Long-term low level gene expression in the lung, intestines and kidney was seen 16 months after delivery of AAV containing the GFP reporter gene to a mid-gestation rhesus macaque (Garrett DJ et al., 2003).

The different result we observed is most likely due to the early stage of gestation in our study. At this early gestation we were unable to take advantage of fetal breathing movements or swallowing to distribute the vector to the airways or gut since these first occur at around 50 days of gestation (Cooke IRC and Berger PJ, 1990) and are only common in the last third of gestation (Ross MG and Nijland MJ, 1998). The dilution of the vector in the relatively larger volume of amniotic fluid is probably responsible in general for the low transfection levels that are achieved. In fetal sheep the amniotic fluid volume increases ten-fold from 2mls at 31 days to 20mls at 38 days of gestation (Cloete JHL, 1939), thus the gestational age of vector delivery is of critical importance.

We did nevertheless observe β-galactosidase expression in the nasal cavity, and transduction of the fetal skin was most likely responsible for blood levels of hFIX, which reached about 2% of normal human adults. At this stage of development in the human fetus, the immature epidermis undergoes remodelling by programmed cell death to be replaced by mature keratinocytes to form the definitive four-layer arrangement by mid-gestation (Polakowska RR et al., 1994). This remodelling could explain some of the lower values of hFIX at later investigation points in spite of the lack of anti-hFIX antibodies. It may also be due to the dilution of the protein in the blood volume of the growing fetus that increases rapidly by 10 fold between day 31-38 of gestation (Cloete JHL, 1939). In therapeutic terms 1% of the normal hFIX level would be sufficient to convert a severe form of Haemophilia B into a mild condition. However the transient nature of expression from the adenovirus would require re-application in late gestation, where delivery to the liver by intra-umbilical injection would be more effective in achieving high enough hFIX levels to avoid perinatal haemorrhagic complications. Intra-amniotic application of adenovirus vectors has been investigated extensively in small animal models such as the fetal rat (Sekhon HS and Larson JE, 1995,Garrett DJ et al., 2003), mouse (Douar A-M et al., 1997,Holzinger A et al., 1995,Larson JE et al., 1997,Larson JE et al., 2000a,Mitchell M et al., 2000,Sekhon HS and Larson JE, 1995,Mitchell M et al., 2000,Larson JE et al., 1997,Larson JE et al., 2000a,Sekhon HS and Larson JE, 1995,Holzinger A et al., 1995) and guinea pig (Senoo M et al., 2000). Therapeutic plasma concentrations of hFIX have also been achieved in fetal mice after intra-amniotic injection of adenovirus vectors carrying the hFIX gene (Schneider H et al., 1999). In general, transgene expression is maximal in those tissues that are in contact with the amniotic fluid, namely the amniotic membranes and the fetal skin with less transduction of the gut and the mucosae. In larger animals, intra-amniotic application of adenovirus vectors has generally been performed in mid to late gestation, using highly invasive laparotomy techniques (Holzinger A et al., 1995,Iwamoto HS et al., 1999) but has only achieved limited gene transfer. The only other study of intra-amniotic prenatal gene therapy to be performed at a comparable gestational age applied an amphotropic retrovirus producer cell line to fetal sheep (Galan HL et al., 2002) but no gene transfer could be demonstrated. The authors concluded this was specific to fetal sheep since the same vector produced limited gene transfer to mid-gestation fetal macaques (Bennett M et al., 2001). However the gestational age differences in these two studies is an important factor to consider.

The low level transgene expression that we and others observe in the lungs shows that intra-amniotic injection is not a suitable route to target gene transfer to the airways for prenatal gene therapy of cystic fibrosis, even in early gestation. In 1997 Larson et al claimed that the CF-phenotype in CFTR-knockout mice could be cured by short-term prenatal expression of CFTR from an adenovirus vector (Larson JE et al., 1997). Recent work in our laboratory aimed to reproduce this report but could not substantiate this claim (Buckley SMK et al., 2003). Intra-amniotic delivery however, may be suitable for prenatal gene therapy of skin disorders such as dystrophic epidermolysis bullosa (Horn HM and Tidman MJ, 2002). We have also shown that this route of injection results in transduction of tissues that can be used as an ectopic source of proteins such as hFIX for treatment of congenital diseases requiring systemic correction.

E 5.3 The trachea can be reached by a minimally invasive ultrasound guided injection technique

Results of gene transfer to the fetal airways are poor following gene delivery to the amniotic cavity (Boyle MP et al., 2001) or injection of the lung parenchyma (Tarantal AF et al., 2001a). Direct instillation of viral vectors into the trachea has been more successful. This has been attempted in fetal sheep using highly invasive surgery such as hysterotomy and placement of catheters down the fetal airway or fetal tracheotomy (Vincent MC et al., 1995,McCray PB et al., 1995). Another group used fetoscopic bronchoscopy in fetal sheep at laparotomy (Sylvester KG et al., 1997,Yang EY et al., 1999) and although this technique could be developed for percutaneous use, endoscopic procedures are known to have a significant complication rate (Deprest JA et al., 1997b). To target the fetal trachea using an ultrasound guided percutaneous injection method we firstly studied its sonographic appearance from mid to late gestation. At 100 days of gestation the tracheal diameter was wide enough to permit injection and the fetus was not too large that fetal parts obscured the view. Injection before 70 days of gestation is most likely not achievable because of the small size of the trachea and therefore targeting of gene therapy to the preimmune fetal sheep trachea is probably not possible via this route.

We first believed that injection of the trachea would be more easily achieved in the fetal neck than in the thorax, and might be associated with fewer complications. In late gestation sheep, attempts to inject the trachea in the fetal neck were hampered by the fleece and the tracheal cartilage and we eventually successfully injected the trachea via a transthoracic route. To our surprise however, injection of the trachea in the fetal neck

was also unsuccessful in the mid-gestation sheep. Despite being clearly visualized, the trachea was extremely mobile and slipped sideways from the needle as it approached. Altering the needle dimensions did not improve our success rate. The trachea within the fetal neck is superficial and relatively unsupported with the oesophagus placed posterior and fatty subcutaneous tissue placed laterally. The sheep neck is longer than that in the human and so the sheep trachea may be relatively more mobile. This approach would probably also be more difficult in the human fetus because of the more flexed attitude adopted by the human fetus.

The best route to the trachea in the mid-gestation sheep fetus was found to be via the thoracic cavity. The procedure was reproducible and of short enough duration for application in a clinical setting. As anticipated, fetal position was important for access to the trachea and was made easier when the fetus was lying with its right side anterior so that the great vessels were positioned posterior to the trachea. The gestational age was also a determining factor and there appeared to be a window of access in gestation, from approximately 80 days when the trachea was wide enough for injection, up to 120 days of gestation when it became increasingly obscured by the fetal humerus and scapula as the fetal head entered the pelvis.

The most common complication during the procedure was accidental vessel perforation leading to haemorrhage, although this caused significant morbidity in only 6% of cases. Poor visualisation of the trachea was a major factor and could be improved by careful selection of the gestational age for injection, and during the procedure, by tilting the mother on the table. Perforation of a major vessel within the chest can lead to catastrophic haemorrhage as we observed in one fetus, and may be severe enough to cause death. Even damage to a minor vessel can have serious consequences. In our series, one fetus developed bilateral pleural effusions that in the long term might have required drainage with all its attendant complications. Although uncommon, vessel perforation is associated with serious morbidity and therefore transthoracic injection of the trachea should only be performed on fetuses that are suffering from a life-threatening condition.

The other complication was needle dislodgement during fluid injection that could be prevented in clinical practice by an extension set and three-way stopcock. Passing a catheter down the needle into the trachea would also prevent needle dislodgement but was associated with other complications such as tracheal perforation and damage to the catheter on the needle tip. The importance of tracheal volume was highlighted in one case when too much fluid was removed from the trachea leading to its collapse. This

becomes most important in earlier gestations when the volume of the trachea is small. We considered that removal of tracheal fluid might improve airways transduction by reducing dilution of the vector in tracheal fluid. The problems caused by the tracheal collapse however, lead us to abandon this technique.

It is noteworthy that the injection site was not identifable forty-eight hours after the procedure either macroscopically or microscopically in fetuses aged 100 days of gestation or older. This illustrates that the fetus is able to heal rapidly without scarring following minor trauma. At the younger gestation of 80 days the injection site was always visualised but use of a smaller gauge needle may prevent this.

E 5.3.1 An occlusive balloon can be placed in the fetal sheep trachea by percutaneous ultrasound guided injection

With the aim of improving gene transfer to the fetal airway epithelium, we adapted our transthoracic injection technique to place and inflate an occlusive balloon within the fetal trachea. None of the balloon tipped catheter systems available would fit down a needle suitable for intratracheal injection. We considered whether a modified Seldinger technique used in interventional radiology, could be used to place a guide wire along a needle that had been injected into the fetal trachea under ultrasound guidance. After removal of the needle, a balloon-tipped catheter would then pass over the guide wire into the fetal trachea. However it was thought unlikely that this system would succeed because of the distance to the trachea and the tough tissues involved, and we could not locate a suitable catheter. We therefore investigated a detachable balloon system that has been used in the fetus for the treatment of CDH.

Studies in animal models of CDH have used a variety of occlusive devices in the fetal trachea including a polymeric foam insert (Skarsgard ED et al., 1996), a foam filled endotracheal tube (Bealer JF et al., 1995), and detachable silicone (Harrison MR et al., 2001) or latex balloons (Flageole H et al., 1997). The detachable balloon systems that were developed for interventional neurovascular treatments (Higashida RT et al., 1991) proved the best for maintaining tracheal occlusion with minimal damage to the fetal trachea.

We showed that a detachable inflated balloon could be placed within the fetal trachea by ultrasound-guided injection alone. Whether the percutaneous technique is adopted for use in treatment of CDH depends on a risk benefit analysis comparing it with the currently used fetoscopic technique. With improvements in balloon technology it may be possible to use a smaller gauge needle that would be less traumatic to the fetus.

A randomized trial concluded that fetoscopic tracheal occlusion did not improve survival or morbidity rates in fetuses with CDH when compared with standard care (Harrison MR et al., 2003). Premature rupture of the membranes and preterm delivery were more common in the group receiving the fetoscopic intervention, and the benefits of tracheal occlusion on pulmonary function were probably outweighed by the adverse effects of earlier delivery. Recent data from the Eurofoetus Consortium suggest that the use of a narrower fetoscope may reduce the complication rate (Jan Deprest, personal communication). A wholly percutaneous approach might further reduce the risk of preterm delivery such that intervention in CDH may prove beneficial overall.
A feasibility study published in 2002 has shown that occlusion of the fetal trachea might be achieved through the mouth using an ultrasound guided guidewire and detachable balloon system in the fetal sheep (Fauza DO et al., 2002). After exteriorisation of the uterus at laparotomy, a catheter was introduced into the amniotic cavity and under ultrasound guidance, a remotely steerable guide wire was fed past the vocal cords into the trachea. Using the Seldinger technique, a catheter and an occlusive detachable balloon were placed in the trachea. Further developments in catheter steering technology may allow this to be achieved without the need for laparotomy.
The transthoracic tracheal injection technique may be of further use in the treatment of CDH. Reversible tracheal occlusion has been found to improve type II pneumocyte density in fetuses with CDH while maintaining the beneficial effect on lung growth. Unplugging of the balloon by puncture has been performed in fetal sheep under tracheoscopic vision (Flageole H et al., 1998). Recently it has been performed clinically in a few fetuses with CDH treated by balloon occlusion using the transthoracic ultrasound guided technique developed here (Deprest J et al., 2004).

E 5.4 Adenovirus mediated gene transfer to the fetal airway epithelium is enhanced by Na-caprate and the polycation DEAE dextran

In keeping with other studies, we found that the sheep airway epithelia is very resistant to adenovirus transduction *in utero* (Vincent MC et al., 1995,Yang EY et al., 1999,Iwamoto HS et al., 1999). We believed that this was due to the lack of adenovirus receptors on the apical side of the airway epithelia and we used two strategies to enhance virus transduction. Initially we applied the virus with the polycation DEAE dextran that forms complexes with the negatively charged adenovirus. This reduces the repulsive charge interaction between the virus and the anionic sialylated glycoproteins

and glycolipids on the epithelial cell surface, which present a barrier to luminal vector delivery (Arcasoy SM et al., 1997a). We did not observe any macroscopically visible transduction in the trachea or main bronchi, although the quantitative measurement of total lung β-galactosidase expression did show a ten-fold increase over that of adenovirus-only administration. Our second approach was to expose the airways to Na-caprate prior to virus application to allow virus penetration to the basolateral surface of the epithelial cells, where the CAR and adenovirus binding integrins are present in higher abundance (Walters RW et al., 1999). This pharmacological pre-treatment, that temporarily and reversibly opens the epithelial tight-junctions, resulted in widespread staining of the trachea and main bronchi and increased virus mediated transgene expression in the lungs by more than 90-fold. The combination of virus and DEAE dextran complexing and Na-caprate pretreatment resulted in significantly enhanced gene transfer to the trachea and main bronchi as well as the large, small and medium airways. The synergy we observed between DEAE dextran and Na-caprate is presumably due to facilitated attachment and cellular uptake of the complexed adenovirus at the basolateral versus the apical cell surface.

Our findings were validated quantitatively using an *ex vivo* tracheal culture system. The enhancement we observed was independent of gestational age since the results were similar from each of the five experiments performed on tracheal tissues from fetuses aged between 72 and 106 days of gestation. Indeed, tight junctions are present and functional in the lung of the fetal sheep aged 69 days of gestation (Olver RE et al., 1981). We did not examine fetal sheep airways for the presence of adenovirus receptors because it was beyond the scope of this thesis. Studies in the fetal mouse lung however, have shown expression of the Coxsackie Adenovirus Receptor and β5 integrin particularly by the airway epithelial cells (Bilbao R et al., 2003a).

E 5.5 Perflubron administration following adenovirus vector instillation alters the pattern of transgene expression in the fetal airways

In an attempt to increase gene delivery further we also applied the fluorocarbon perflubron, Overall we saw redistribution from the upper to the peripheral airways, where expression was enhanced at the expense of tracheal and the large and medium airway epithelia. This redistribution of expression is most likely due to flushing of the vector solution further down the airways by the water immiscible perflubron that increases the virus concentration locally in the peripheral airways. It has also been

proposed that perflubron transiently increases the permeability of epithelial tight junctions which could explain the high intensity of local transgene expression in the extreme periphery (Weiss DJ et al., 2003).

The finding that perflubron did not affect levels of β-galactosidase expression in each combination was not that surprising when considering the difference between fetal and adult airways. In one study on gene transfer to adult rodent airways where there is an air-tissue interface, instillation of perflubron following adlacZ vector delivery increased levels of β-galactosidase activity by 5 to 6 fold and more uniform distribution of β-galactosidase expression was observed (Weiss DJ et al., 1999). The low surface tension of the fluorocarbon allows it to spread through communicating small airways and over alveolar walls (Shaffer TH et al., 1992) resulting in a less patchy distribution of tissue transduction when compared to virus alone. This is particularly effective where there is lung damage already present (Weiss DJ et al., 2001). In the fetus however, a liquid-tissue interface exists already and therefore this effect of perflubron is likely to play a smaller part.

Perflubron administration may not provide an advantage to the potential treatment of CF *in utero* that requires predominantly upper and medium airway gene transfer. The pathological process in cystic fibrosis starts in the distal airways where a combination of abnormally thick mucus and chronic bacterial infections lead to damage. The main site of CFTR expression in the adult lung however, is in the serous tubules of the submucosal glands (Engelhardt JF et al., 1992) with only low level expression in the surface epithelium. One of the major difficulties facing cystic fibrosis gene therapy in adults is targeting of these glands which are more proximal. In the human fetal lung submucosal glands develop from around 15 weeks of gestation and successful transduction of submucosal glands using AAV in a mid-trimester human fetal tracheal organ culture system have shown proof of principle (Lim F-Y et al., 2002). Variations in the volume of perflubron may allow targeting of injected fluid to different areas of the airways necessary for CF gene therapy.

The gestational age at vector delivery is likely to be critical to the efficiency of airways gene transfer and to targeting of cells in the epithelium. In this study we administered the adenovirus vector during the late pseudoglandular phase, up to 90 days of gestation and the early canalicular phase of lung development, from 90 to 125 days of gestation. From quantitative measurement of β-galactosidase expression, transgene expression was higher at the earlier than at later gestation. This may simply reflect the higher local

concentration of vector that can be achieved at the younger age but also suggests that gene transfer may be more efficient at the earlier pseudoglandular stage. Similarly lentivirus mediated gene transfer was found to be more efficient at the pseudoglandular stage when compared with the earlier embryonic stage after lung parenchyma injection of fetal macaques (Tarantal AF et al., 2001a). Application of adenovirus vectors to late gestation fetal sheep results in gene transfer to type II alveolar pneumocytes as would be expected (Sylvester KG et al., 1997).

The gestational age is probably also critical for targeting an integrating vector to the stem cells of the airway epithelium for permanent transgene expression. Although the common epithelial airway stem cell has not yet been identified there are several stem cell subsets which are capable of reconstituting a fully differentiated tracheobronchial epithelium (Zepeda ML et al., 1995,Engelhardt JF et al., 1995). In the fetal sheep submucosal glands begin as gland buds during the pseudoglandular stage of lung development (Smolich JJ et al., 1978) similar to humans (Thurlbeck WM et al., 1961). It is not until the saccular stage that tubules are found, after the stage of gestation of our experiments. Targeting of submucosal gland bud progenitors may be possible using the lymphoid enhancing factor 1 (LEF1) of which expression has been shown to be important for submucosal gland development (Duan et al., 1998).

Transgene expression may be limited by the fetal immune response to it. In the fetal sheep lung, adenovirus mediated gene transfer faded coincident with the development of cellular inflammation and serologic evidence of antiadenovirus antibody production over a few weeks (McCray PB et al., 1995,Vincent MC et al., 1995). In our experiments the sampling time point of two days after injection is insufficient time for them to mount an effective immune response and long term studies are required to evaluate this. An important area to investigate is the effect of this potential treatment on the fetal airways and other organs. The pigmented material seen in the airway lumen is thought to be epithelial slough since it was seen in both injected and uninjected fetuses. There was evidence of inflammation in the pleura and the pericardium on post mortem or histological examination in some fetuses. Injection of a needle is traumatic and will have caused much of the inflammation observed but the adenovirus vector used in these experiments is highly inflammatory and probably contributed largely to these findings. Significant pulmonary inflammation consisting of cellular infiltrates and fibrous tissue was observed after tracheal administration of adenovirus (McCray PB et al., 1995,Vincent MC et al., 1995) and retrovirus (Pitt BR et al., 1995) vectors to late gestation fetal sheep. In all these studies however, the vector was applied using highly

invasive laparotomy and catheter techniques that will be associated with more inflammation.

Whitened plaques identified microscopically as fibrous thickening of the pleura, were seen on the posterior surface of the lungs of fetuses that received perflubron. Focal inflammation in the lumen of the airways was also more evident in these fetuses. This may be due to impurites such as hydrogen fluoride that are present in non-clinical grade perflubron that we used (Weiss DJ et al., 2002b). Perflubron which is used in liquid ventilation of neonates, is unlikely to cause any long-term damage of the airways (Shaffer TH et al., 1992) and it has been shown to reduce inflammation in acutely damaged lungs (Younger JG et al., 1997). Transient inflammation however, has been observed after combination of perflubron with adenovirus vectors in normal and damaged adult lungs (Weiss DJ et al., 2001). In adults, perflubron is quickly cleared from the airways although it may persist for a few weeks following liquid ventilation of premature infants (Leach CL et al., 1996). There are no studies on its use in fetal airways although it is likely that very little will remain *in situ* because of the net efflux of fluid from the fetal airways. Na-caprate has been used to enhance delivery of antibiotics to the rectal mucosa in humans with no ill effects (Lindmark T et al., 1997) although there are as yet no long-term studies on its use in fetal or adult airways.

It has been suggested that gene transfer to between 5 and 10% of airway epithelia may be sufficient to achieve therapeutic correction of the defective chloride channel function in cystic fibrosis (Johnson LG et al., 1992). Given the widespread gene transfer throughout the airways under optimal conditions and the density of transduced cells in the individual airways the achieved level of gene expression may be sufficient to provide a therapeutic effect when applying a CFTR expressing vector. Ovine CFTR has a similar pattern of expression during development to the human gene and there is 90% sequence identity within the coding region, making detection of human CFTR on an ovine background potentially difficult. Using antibodies specific to hCFTR on a sheep background we are currently developing a protocol to analyse hCFTR expression in our fetal sheep samples.

E 5.6 Ultrasound guided intragastric injection is achievable in the early gestation fetal sheep with low short term morbidity and mortality

The aim of intragastric delivery of viral vectors was to target the gene therapy to the small and large bowel in early gestation, at an age when the fetus is considered to be

preimmune. Ultrasound examination of the fetal sheep abdomen from 31 days of gestation showed that the stomach could be visualized from 40 days but it was always observed from 55 days of gestation. In one study in the human using transabdominal scanning, the stomach could be seen in 98% of fetuses from 14 weeks of gestation; indeed, non visualisation of the stomach at this age was associated with an abnormal pregnancy outcome (Pretorius DH et al., 1988). Transvaginal sonography (TVS) in the human fetus permits earlier visualisation of the stomach from 9 weeks, and it was seen in almost all cases at 12 weeks of gestation, equivalent to 46 days of gestation in the fetal sheep (Bronshtein M et al., 1992). TVS has not been used in the fetal sheep but it is likely that fetal anatomy would be visible earlier in gestation by this method. Transrectal sonography has been used to detect pregnancy in sheep but has a lower accuracy than transabdominal scanning (Watt BR et al., 1984) and is associated with rectal perforation.

The development and anatomy of the sheep stomach is very different to that of the human because of its more specialized function. By ultrasonography the human stomach is elliptical in its longitudinal section and spherical on transverse section. Although there is a wide variation in stomach volume due to dynamic filling and emptying (Zimmer EZ et al., 1992), over long periods of evaluation the dimensions remain relatively constant (Goldstein I et al., 1987). Using three dimensional reconstruction from two dimensional ultrasound measurement, the stomach volume has been shown to increase linearly in the latter half of pregnancy (Nagata S et al., 1994). The four chambers of the sheep stomach develop at different rates through gestation. In early pregnancy at the stage of gestation we studied, the rumen is the most prominent chamber and it was observed in all fetuses where the stomach was visible. The reticulum was the next chamber most likely to be visualised, but the other two chambers were not seen routinely. Because of this, we approximated the shape of the stomach by ultrasonography to be a prolate ellipsoid, as in the human fetus. The largest diameter of the stomach was also found to be the longitudinal measurement, and the dimensions increased linearly over the gestational ages that were measured.

This is the first report of ultrasound guided intragastric injection in a large animal model in early gestation. From the stomach measurements obtained, we decided that intragastric injection should be possible from 55 days of gestation since the AP and transverse diameters measured on average 5mm. We considered however, that the small stomach size might not permit withdrawal of some gastric fluid to confirm correct needle placement and this was our experience in one fetus at this age. To improve our

chance of success, we moved forward in gestation to 60 days, when the AP diameter was approaching 10mm on average and reached our goal. Intragastric injection was achieved in all but one case and in a relatively short time. Despite differences in the stomach anatomy, the shape of the fetal sheep rumen is very similar to that of the whole stomach in the human fetus. We expect therefore, that injection of the stomach in the human fetus would be just as straightforward.

An advantage of the procedure over other routes such as intramuscular or intrahepatic injection, was that the needle position could be confirmed prior to injection by withdrawal of a small volume of gastric fluid. The stomach could be seen expanding during instillation of viral vectors and transduction enhancing agents. As would be anticipated, the volume and the number of stomach chambers visualized rose with increasing volumes of fluid instillation, although there was very little difference between instillation of 1000 and 1500 μl fluid.

The short term morbidity and mortality from the procedure was low. Measurement of the protein levels in the ascitic fluid demonstrated that it was most likely due simply to leakage of the fluid after gastric instillation into the peritoneal cavity rather than inflammation. As anticipated, we observed haemorrhagic and infective complications but further experiments are needed for a more accurate assessment. In a clinical setting the risk of trauma to intraperitoneal organs might be reduced by injecting the fetal stomach only when it was positioned uppermost, and closest to the uterine wall. It is likely that procedures would be done without maternal general anaesthetic or sedation, and therefore the injection could be performed when the fetus had moved into the optimum position. We performed post mortem and histological analysis 2 days after injection but longer term follow up, including allowing some lambs to deliver, is necessary to evaluate the effect of intragastric injection on the development of the fetal stomach and its function in postnatal life. Peritonitis and intraperitoneal adhesions are likely to be the most common complications.

There is one published study of ultrasound guided intragastric injection of late gestation fetal rabbits (Brandt et al., 1997b). The authors successfully injected the stomach in 70% of cases (18 out of 26 cases). This was lower than our success rate of 91% (10 out of 11 cases) and may be due to the double needle technique that the authors used in which they placed a 20 Gauge spinal needle into the fetal abdominal cavity and then advanced a 26 Gauge needle through this into the fetal stomach. Air (100μl) was injected to confirm correct needle placement before barium (500μl) was instilled. The

presence of barium in the peritoneal cavity of injection failures suggested that inadvertent intraperitoneal injection had occcurred, although it might also have leaked out of the stomach after intragastric delivery. Their survival rate four hours after injection was 100% and and there was no histological evidence of trauma or haemorrhage. Thus, in the short term, ultrasound guided intragastric injection appears to be of low risk to the fetus and mother, although further work is needed to determine longer term outcomes.

E 5.7 Gene transfer to the small and large bowel of fetal sheep can be achieved by ultrasound-guided intragastric injection

Genetic diseases affecting the gastrointestinal tract are rare but in the case of cystic fibrosis, can have severe consequences, and treatment before birth may eliminate intestinal obstruction and its deleterious effect on nutritional status. The intestine could also be used as an alternative site for expression of critical genes in somatic gene therapy treatment of liver disorders. As a first step towards gastrointestinal fetal gene therapy, we have shown that the gut epithelium can be targeted in early gestation for gene transfer by a minimally invasive injection procedure in the fetal sheep.
Prior to our research, very little work had been done on gene transfer to the fetal gut. Gene therapy studies targeting the adult gut epithelium use various methods such as intraluminal (Foreman PK et al., 1998) or transrectal injection (Arenas et al., 1996) to deliver vectors to the small or large bowel epithelium respectively. Such methods bypass the stomach and involve flushing of the bowel with saline to prevent exposure of the vector to gastric acid and proteases that affect vector stability (Shao G et al., 2003). Our preliminary *in vitro* studies showed that gastric fluid from early and mid gestation fetal sheep did not inhibit adenovirus mediated gene transfer to an epithelial cell line. This is in agreement with studies on the ontogeny of gastric acidity which show that acid secretion begins in the fetal sheep stomach from 120 days of gestation (Shulkes A et al., 1985) and in the human fetus, gastric acid, intrinsic factor and gastrin can be secreted only from the middle of the second trimester (Kelly EJ and Brownlee KG, 1993).
Before administering adenovirus *in vivo* we analysed the effect of enhancers using an *ex* vivo small bowel culture system, similar to that used in the fetal trachea. The best gene transfer was obtained when both Na-caprate pretreatment and complexation of the adenovirus with DEAE dextran were applied successively. DEAE dextran appeared to enhance gene transfer more to the fetal small bowel than to the fetal tracheal epithelium.

Conversely Na-caprate, either alone or in combination with DEAE dextran, had less of an enhancing effect on transduction of the fetal small bowel. This is an interesting finding, since much of the work on the mechanism of action of Na-caprate has been done on human intestinal epithelial (Caco-2) cells (Anderberg EK et al., 1993,Lindmark T et al., 1998) (Chao AC et al., 1999). In addition, Na-caprate can be used clinically to promote intestinal absorption of hydrophilic drugs such as ampicillin across the gastrointestinal mucosa (Lindmark T et al., 1997). The different effects of Na-caprate and DEAE dextran on fetal small bowel and airway epithelial cells that we observed may be due to the maturity of the epithelium at the time of application, since the agents were applied at 60 and 80-110 days of gestation respectively. In our *ex vivo* fetal small bowel experiments, we quantified the enhancement of adenovirus mediated gene transfer at around 60 days of gestation. Although we only subjectively assessed gene transfer in older samples, we did not observe any major differences. The gut epithelial surface has a similar negatively charged glycocalyx with sialic acid residues in the adult and during fetal development (Hill RR and de Bruyn G, 1991) and this may explain the enhancing effect we observed with DEAE dextran.

Our findings also suggest that even at 60 days of gestation, tight junctions are functional in the fetal sheep gut epithelium. This is supported by research in the human fetal colon showing that the structural components of tight junctions are already present by 10 weeks of gestation (Polak-Charcon S et al., 1980) and functional tight junctions can be observed in fetal rat small bowel epithelium at 16 days of gestation (term = 22 days) (Colony PC and Neutra MR, 1985). It is possible that tight junctions in the small bowel epithelium at 60 days of gestation may be more leaky than those in the airway epithelium of older sheep fetuses.

Delivery of adenovirus vector alone resulted in gene transfer to the fetal gut epithelium *in vivo* at the site of injection. We showed that flushing the vector and enhancing agents with the fluorocarbon perflubron improved gene transfer to the small and large bowel epithelium. Increasing the volume of perflubron applied led to transduction of the gut more distal to the site of injection, and application of 1ml was sufficient to reach the transverse colon. The small intestine is the area of the adult human gut where maximum CFTR expression occurs, with lower levels in the colon (Strong TV et al., 1994,Trezise AE and Buchwald M, 1991). A similar pattern of CFTR expression is seen in the gut of second trimester human fetuses (Trezise AE et al., 1993). Thus intragastric injection of viral vectors can target CFTR gene transfer to the relevant tissues with the aim to prevent meconium ileus in cystic fibrosis.

Throughout the fetal gut, adenovirus mediated transgene expression was seen only in the epithelial cells of the villi and not in the cells lining the crypts. This is important for transduction of stem cells and in cystic fibrosis gene therapy. In the adult mouse, multipotent stem cells residing in the base of the crypts of Lieberkühn give rise to the four cell lineages in the small intestine (Winton and Ponder, 1990). Stem cell transduction is the 'holy grail' in gene therapy, since this would ensure that all progeny were corrected for a congenital disease. There is a decreasing gradient of CFTR expression along the crypt to villous tip axis in the adult and fetal gut, with the highest levels of expression in the crypts and only weak or undetectable expression in the top one-third of the villi (Trezise AE et al., 1993,Strong TV et al., 1994). Gene expression in the villi but not in the crypts was observed after oropharyngeal application of adenovirus to late gestation fetal rabbits (Wu Y et al., 1999). Similar results were also found in the adult gut epithelium after application of adenovirus (Foreman PK et al., 1998). Intestinal mucus present in the adult bowel, may prevent vector access to the crypts and flushing the bowel with mucolytics improves crypt transduction (Cheng et al., 1997b). We did not consider mucus to be a problem in the fetal sheep gut at the gestational age used in our experiments. Mucous cells are only seen in the small intestine of small animals in late gestation (Smith T et al., 1985,Trier and Moxey PC, 1979) and in human fetuses from 17 weeks of gestation (Verma KB, 1979).

We hypothesized that the crypts might be inaccessible to the adenovirus vector because of their deep location. The presence of colloidal carbon in the small bowel crypts of one animal after co-injection with the vector suggested that the vector was able to reach the crypts but only in low amounts. Attempts to relax the smooth muscle in the bowel wall using intragastric atropine resulted in gene transfer closer to the site of injection suggesting that bowel peristalsis was slowed, but no expression was detected in the crypts. Subcutaneous injection of glucagon, which is known to reduce small intestine contractility, was not successful in targeting adenovirus mediated gene transfer to the crypt cells in the adult mouse (Cheng et al., 1997a).

Physical distension of the bowel lumen should improve access to the crypts, since it causes shortening of the villi and widening of the intervillus space (Sandberg J et al., 1994). When sufficient adenovirus vector was instilled into clamped off sections of adult rodent small bowel to distend the lumen, gene expression was observed in the crypts (Hamilton TE, 1997). Transgene expression was also seen in the crypts after rectal instillation of plasmids but the method involved extensive flushing of the colon before vector delivery which may have dilated the bowel sufficiently to allow the vector

to penetrate the crypts (Westbrook et al., 1994). We hoped that perflubron instillation would have the same distending effect but it was probably applied at too high a level to have any effect on the small bowel. The increase in the stomach volume and the number of cavities observed showed that we did distend the fetal stomach.

Adenoviruses are not able to infect immature cells such as stem cells efficiently and this may have resulted in the expression pattern we observed in the fetal gut. In the adult mouse, modifications to first generation adenovirus by covalently attaching activated monomethoxypoly (ethylene) glycols (MPEGs) that stabilize the vector during oral administration, led to improved levels of gene transfer throughout the gut when compared with the unmodified vector. Notably, gene expression was observed in the crypts of the small bowel (Cheng X et al., 2003). In another study, the same group showed that coadministration of adenovirus with beta cyclodextrins, that enhance the transport of macromolecules across the intestinal epithelium, lead to concentrated expression in the crypts (Croyle MA et al., 1998). Application of lentivirus vectors that are capable of transducing stem cells may reveal whether the lack of crypt expression we observed is due to a failure of viral infection or an inability of fetal stem cells to express the transgene.

In the adult mouse a single multipotent stem cell maintained in the crypts of Lieberkühn gives rise to absorptive enterocytes and to the three types of secretory cells: goblet, enteroendocrine and Paneth, that are resident in the gut (Winton and Ponder, 1990). The descendants of the multipotent stem cell undergo several rounds of cell division in the midportion of the crypt. Members of the Paneth cell lineage differentiate and remain in the base of each crypt for approximately 20 days (Cheng H, 1974). The remaining cell lineages differentiate as they translocate from a crypt to the apex of a surrounding villus where they exfoliate and this sequence takes between 2 to 5 days depending on the type of cell (Cheng H and Leblond CP, 1974). In fetal mice the crypt stem cells are initially polyclonal and become monoclonal by the third week of postnatal life (Ponder BA et al., 1985). This could be a problem for fetal gene therapy if the efficiency of crypt stem cell transduction is poor. There is no data on the kinetics of cell movement up the villus from the crypt in the fetal gut. Studies of the human fetus shows that cell proliferation in the crypt-villus axis peaks at 8 –10 weeks and falls to a plateau by 18 weeks of gestation (Arsenault P and Menard D, 1987,Arsenault P and Menard D, 1989). Although growth of the crypt-villus axis also occurs at this stage of development, the data suggest that the transit time of cells from crypt to villus is at least as fast as in the adult gut. Therefore it

is possible that the gene expression we observed in the villi of the fetal sheep intestine resulted from migration of epithelial cell daughters of a transduced crypt stem cell. Our finding of significant transgene expression in the endothelium and subendothelial lining of portal vein branches in the fetal liver probably represents transduction of the portal vein endothelium. PCR analysis following intragastric injection showed widespread distribution of the vector in the majority of fetal tissues that probably occurred via lymphatic drainage of the intestine. The intestine has been suggested as an alternative site for expression of critical genes. *In vitro* studies show that intestinal epithelial cells infected with retrovirus vectors can secrete significant levels of transgenic factor VIII and IX (Lozier JN et al., 1997) and transgenic secreted alkaline phosphatase was detected in the circulation of adult rats after luminal instillation of adenovirus (Foreman PK et al., 1998). Intragastric injection is therefore a potential route of administration of fetal gene therapy to achieve systemic correction of congenital disease.

F Prenatal gene therapy for mucopolysaccharidoses

In utero gene delivery to the fetal brain may be useful for the treatment of congenital diseases that affect the brain such as storage disorders eg mucopolysaccharidosis type VII and Tay-Sachs disease, or to prevent hypoxic-ischaemic brain damage in fetuses. Some congenital diseases may affect the brain from early in fetal development (Burlet P et al., 1998). Studies in the fetal sheep from 60 days of gestation show that the permeability of the blood brain barrier, which prevents larger molecules from entering the neurocortex, decreases through gestation (Stonestreet BS et al., 1996,Dziegielewska KM et al., 1979). We therefore used early gestation fetal sheep for these experiments to take advantage of the immaturity of the blood brain barrier. The fetal skull sutures would be wider and the fetal skull would be softer and more easily penetrated if it proved difficult to place the needle directly through a skull suture. Any damage due to passing the needle through the parenchyma might be more easily repaired at early gestation. We chose to inject the vector into the lateral ventricle rather than into the brain parenchyma, since this would reduce the effect of pressure damage on the developing neurons. Also we believed that it would be easier to see the needle tip in the sonolucent ventricle and could visualise microbubbles confirming it was correctly positioned. Therefore we investigated gene delivery to the fetal brain by injection of adenovirus vectors containing the lacZ gene into the lateral ventricles of the early gestation fetal sheep.

F 1 Adenovirus efficiently transfects the neurocortex of early gestation fetal sheep ex vivo

Before applying our vector to fetal sheep *in vivo*, we tested whether adenovirus was able to transfer genes to the fetal sheep neurocortex *ex vivo*. The fetal neurocortex of one non-injected animal (55 days of gestation) was taken at post mortem examination, sectioned and infected with 1 x 10^8 particles adlacZ vector. X-gal staining 48 hours after application showed scanty β-galactosidase expression (data not shown).

F 2 Visualisation of the fetal brain in early gestation fetal sheep

A search of the literature revealed two studies on ultrasound visualisation of the fetal brain in late gestation sheep. One group had developed an experimental model of hydrocephalus in fetal sheep by ultrasound-guided injection of kaolin into the cisterna magna (Cambria S et al., 1979). In another study, blood flow velocity in the cerebral

circulation was evaluated by Doppler ultrasound examination of fetal sheep during maternal hypoxia (Gunnarsson GO et al., 1998). However no studies had been done on ultrasound visualisation or injection of the early gestation sheep fetal brain. We began these experiments therefore, by evaluating the ultrasound view and measurements of the fetal brain in early gestation.

We retrospectively reviewed ultrasound examinations of the fetal brain including measurements of the biparietal diameter and occipito-snout length in a series of experiments recorded on videotape (n = 95) to decide whether the lateral ventricles could be clearly seen (**Figure F 1**). The axial view of the brain at the level where the biparietal diameter is measured gives a good view of the lateral ventricles in the human fetus and is also the view in which ventricular size is measured (Griffin D, 2002).

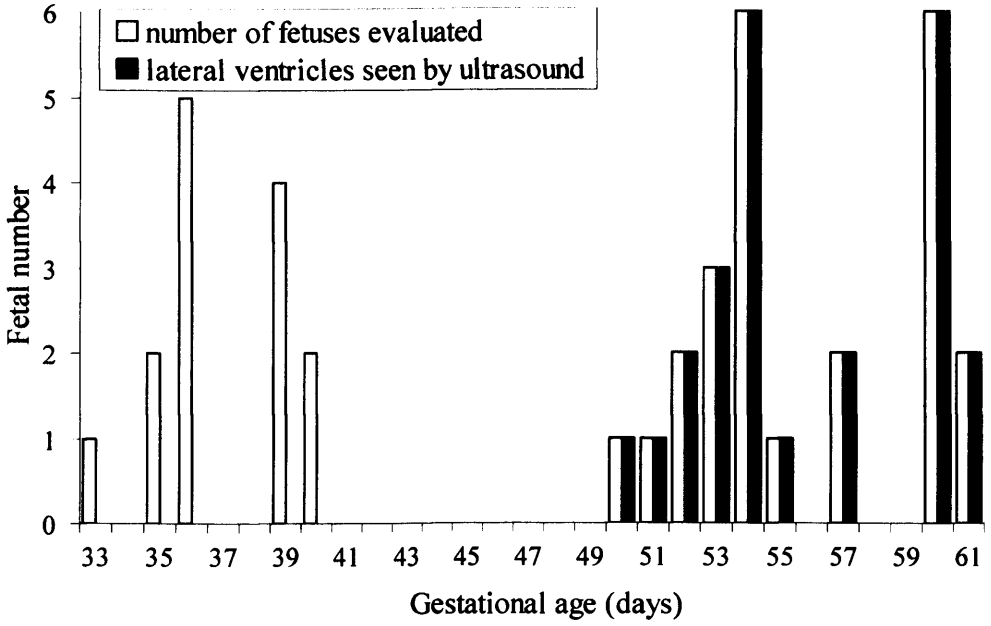


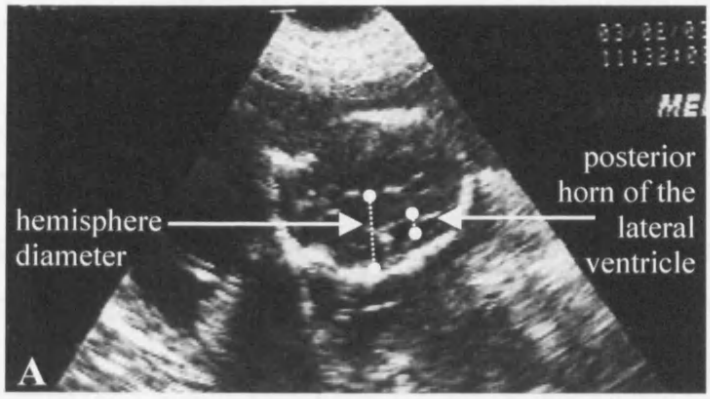
Figure F 1: Visualisation of the lateral ventricles in the early gestation sheep fetus by ultrasound.

Videotape recordings were reviewed from early gestation sheep experiments in which an anatomical survey of the fetus was conducted and fetal biometry was performed. The lateral ventricles were identified as two translucent areas in an axial scan of the fetal head.

The lateral ventricles could not be identified in fetuses aged 40 days of gestation or less. There was no data available for the age range 41 to 49 days of gestation because no fetal experiments had been performed at these gestational ages. We attempted to visualise the lateral ventricles in fetuses aged between 41 and 49 days of gestation by sonography of awake ewes a week prior to surgery, but because of difficulties restraining the ewes visualisation of the fetal head was not adequate and no conclusions could be drawn. The

lateral ventricle was seen in all fetuses aged 50 days of gestation and older, equivalent to 13 weeks gestation in humans. The coronal and lamdoidal skull sutures were visualised with the skull in coronal section.

Measurements of the cerebral hemisphere and the posterior horn of the lateral ventricle were performed in 10 fetuses between 52 and 60 days of gestation (**Figure F 2**).



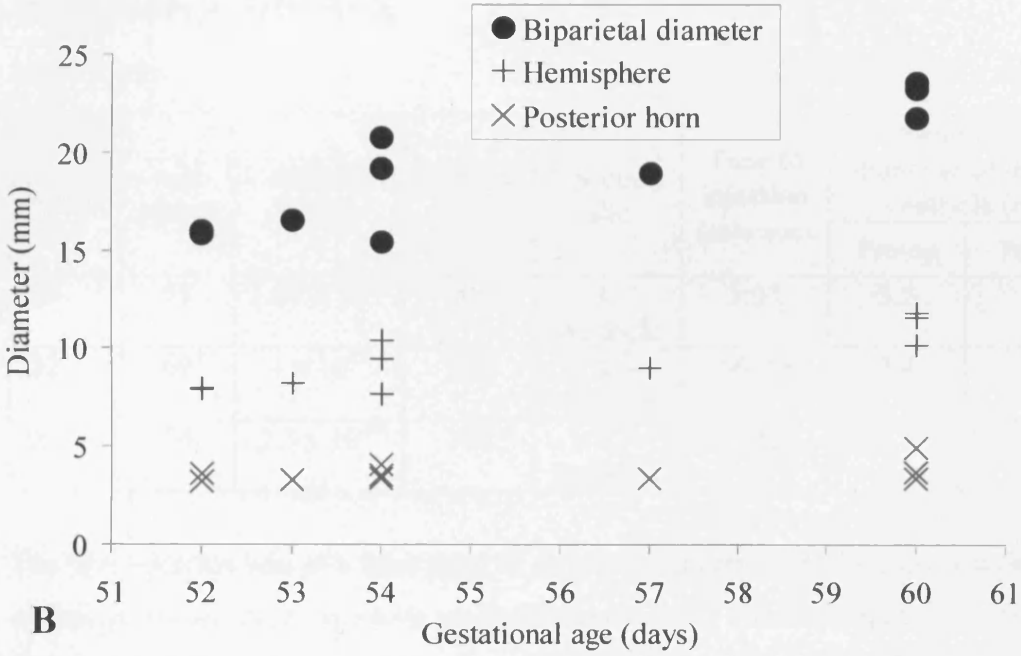


Figure F 2: Skull and brain measurements in the early gestation sheep fetus by ultrasound.

(A) The distal hemisphere diameter (inner to inner) and the posterior horn of the lateral ventricle were measured as indicated in a fetus aged 57 days of gestation (B1, Table F1) and (B) plotted with gestational age, together with the biparietal diameter.

As in the human fetus, the distal hemisphere was chosen for measurement because of disturbance of the ultrasound beam as it passes through the convex proximal skull bone.

The fetal skull and brain were assessed for the best possible route of injection. The fetal orbits and eyes in the sheep are more prominent and laterally placed than in the human. To avoid damaging the eyes it was thought best to direct the needle through the coronal suture in a posterior direction into the posterior horn of the lateral ventricle. In addition visualisation of the anterior horn of the lateral ventricle was difficult in early gestation due to the prominance of the fetal orbits. The posterior horn measured on average 3.7 mm that was considered to be adequate for ultrasound-guided injection.

F 3 Ultrasound guided injection of the fetal lateral ventricle is achievable in early gestation but technically difficult

In these experiments we aimed to pass the needle through a skull suture line so as to cause minimal trauma and to deliver adenovirus vectors into the lateral ventricle. Experimental details are shown in **Table F 1**

Table F 1: Ultrasound-guided injection of adlacZ vectors into the lateral ventricle of early gestation fetal sheep.

L: left; R: right.

Sheep	Age (days)	AdlacZ (p/kg)	Volume (μl)	Injection site	Time to injection (min:sec)	Posterior horn diameter of injected ventricle (mm)	
						Pre-op	Post-op
B1	57	1.67×10^{11}	100	L ventricle	5:08	3.5	3.6
B2	60	1×10^{12}	200	L & R ventricle	56:09	3.4	3.4
B3	54	2.3×10^{12}	100	R ventricle	5:02	3.5	3.5

The first injection was of a fetus aged 57 days of gestation (B1), since this was in the middle of the age range in which we had measurements. The fetal head was very superficially situated close to the maternal anterior abdominal wall, left side anterior and the lateral ventricles were clearly seen and measured. At this gestation the choroid plexus was prominently sited within the lateral ventricle. Under ultrasound guidance, a 22 Gauge spinal needle was advanced into the uterus, passed with ease through the left coronal suture posterior to the left eye, and entered the posterior horn of the left ventricle (**Figure F 3**). AdlacZ vector was injected through the needle which was then flushed with 40μl PBS and removed. Microbubbles were seen within the posterior horn on injection of the vector and PBS.

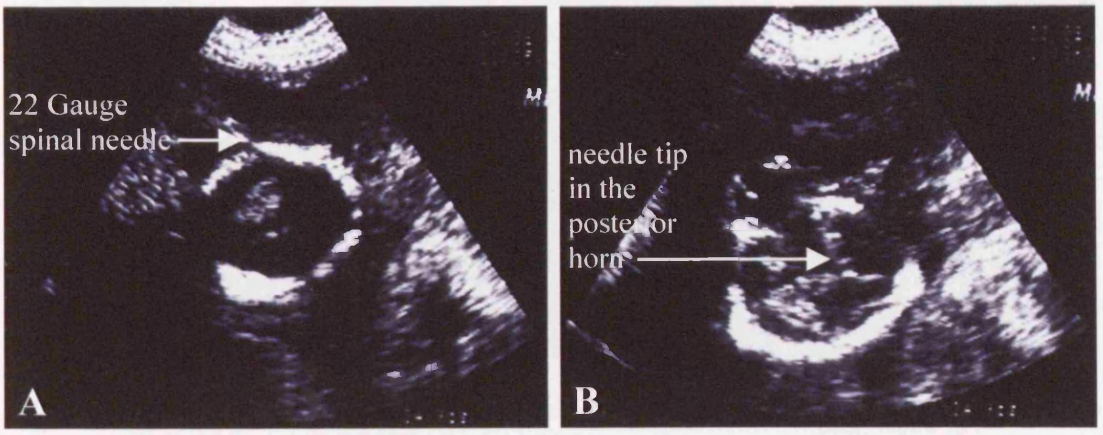


Figure F 3: Ultrasound-guided injection of the lateral ventricle in a sheep fetus aged 57 days of gestation (B1).

Ultrasonograms of a sheep fetus (57 days of gestation, B1) showing (A) with the fetal skull in transverse section, a 22 Gauge needle positioned in line with the left coronal suture and (B) inserted into the posterior horn of the left lateral ventricle.

We repeated the intraventricular injection successfully in two more fetuses, although in both cases this was technically more demanding. Although the first fetus (B2, 60 days of gestation) was well positioned with the head superficially placed and the left side anterior, the first two injection attempts via the left coronal suture flexed the head on the neck laterally and then forward (**Figure F 4 A**). It appeared that the skull was thicker and the coronal suture was more difficult to enter. There were three further unsuccessful attempts in which the needle entered the scalp but skimmed over the skull because of the shallow angle of inclination of the needle. Efforts to move the fetal head into the correct position with the needle tip resulted in it drifting around in the amniotic fluid. At the sixth attempt the needle passed through the left coronal suture and into the left lateral ventricle (**Figure F 4 B**).

AdlacZ vector (100µl) was injected but appeared to leak back out of the needle track. The needle was therefore advanced across the midline into the right lateral ventricle and a further dose of vector was given (100µl) that did not leak. Microbubbles were observed after each injection. During the procedure fetal blood was seen coming from the scalp at the site of failed injection attempts.

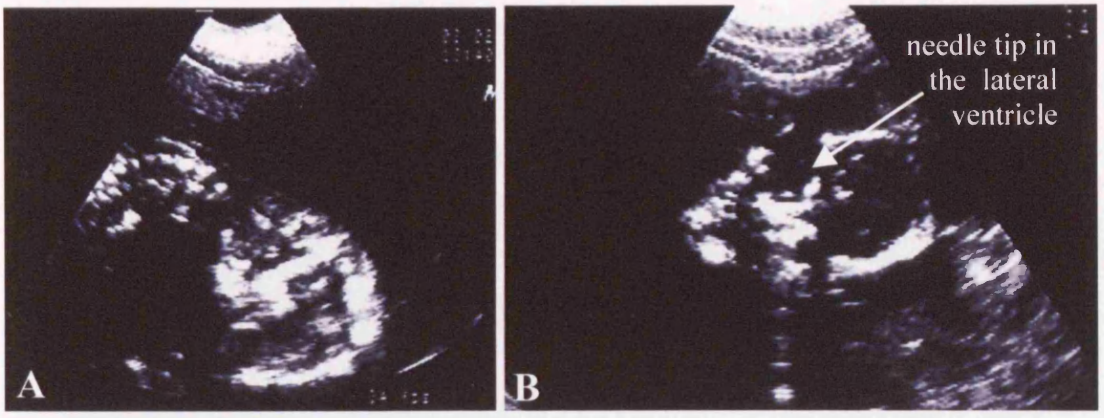


Figure F 4: Flexion of the fetal head during ultrasound-guided injection of the lateral ventricle.

Ultrasonograms of a sheep fetus aged 60 days of gestation (B2). (A) The first two attempts to inject the fetal skull failed but resulted in severe lateral flexion of the fetal head. (B) On the sixth attempt the needle successfully passed through the left coronal suture into the left lateral ventricle.

Because of the difficulty injecting the second fetus, our third injection attempt was in a younger animal (B3, 54 days of gestation) in which we expected the skull to be thinner. The fetal head was positioned right side up with the face slightly anterior which resulted in difficulty lining up the coronal suture with the needle. We were reluctant to reposition the head because of our previous experience and therefore the needle was injected into the skull, through which it passed with ease, into the posterior horn of the right ventricle where the vector was delivered. Bleeding from the scalp was seen at the site of injection.

In all fetuses the posterior horn was more translucent after injection probably as a result of displacement of the choroid plexus with fluid. The diameter of the posterior horn did not increase significantly (**Table F 1**) although this is probably a reflection of the small volume of fluid given.

F 4 Ultrasound guided injection of the fetal lateral ventricle results in minimal short-term morbidity

All three fetuses survived the injection procedure and post mortem analysis was performed 2 days after injection. In two fetuses (B2 and B3) the injection site(s) could be seen as small petechial haemorrhages in the scalp (**Figure F 5A**). A moderate amount of haemorrhage lined the skull of B2 and the meninges were adherent to the underlying neurocortex. This probably reflected the technical difficulties encountered during injection. Histological analysis of the skull of this fetus showed thickened

meninges with reactive fibroblastic tissue but few inflammatory cells (**Figure F 5B**). Histological examination of the brain in all fetuses showed no inflammatory infiltrate or other abnormalities (**Table F 2**).

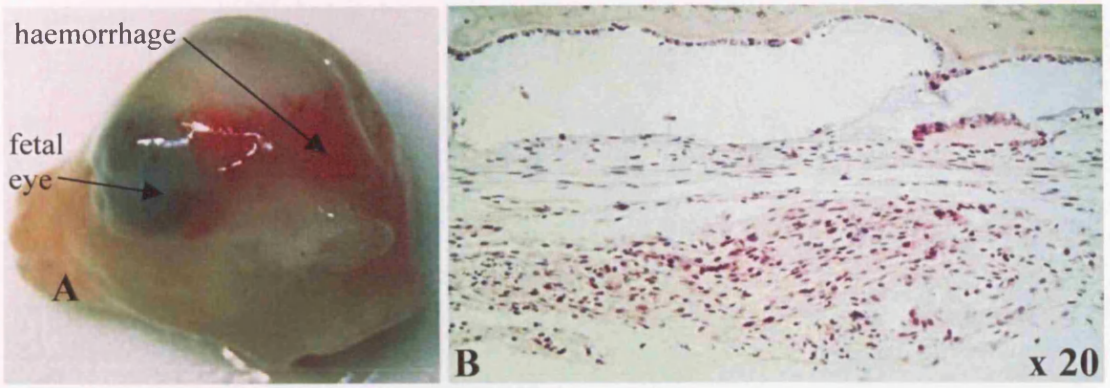


Figure F 5: Post mortem and histological findings after ultrasound-guided injection of the lateral ventricle.

Two days after injection of the lateral ventricle (60 days of gestation,B2), (A) small petechial haemorrhages and a skin laceration are seen in the left scalp at the site of injection. (B) On light microscopy (H & E staining) the meninges is thickened with reactive fibroblastic tissue but few inflammatory cells.

Table F 2: Ultrasound-guided injection of adlacZ vectors into the lateral ventricle of early gestation fetal sheep.

Post mortem and histological findings, and β-galactosidase transgene expression 2 days after injection.

Sheep	Post mortem findings	Histological analysis	β-galactosidase expression	
			X-gal staining	immuno
B1	normal	normal	choroid plexus, lateral ventricle ependyma & cortex	negative
B2	scalp & skull haemorrhage, adherent meninges	meningeal fibroblasts	choroid plexus	negative
B3	scalp petechial haemorrhages	thickened meninges	negative	negative

F 5 Successful transgene expression is observed in the fetal brain after ultrasound-guided injection of the fetal lateral ventricle in early gestation

X-gal staining performed on frozen sections of the fetal cortex, cerebellum and upper thorax was positive in two fetuses (B1 and B2, **Figure F 6**).

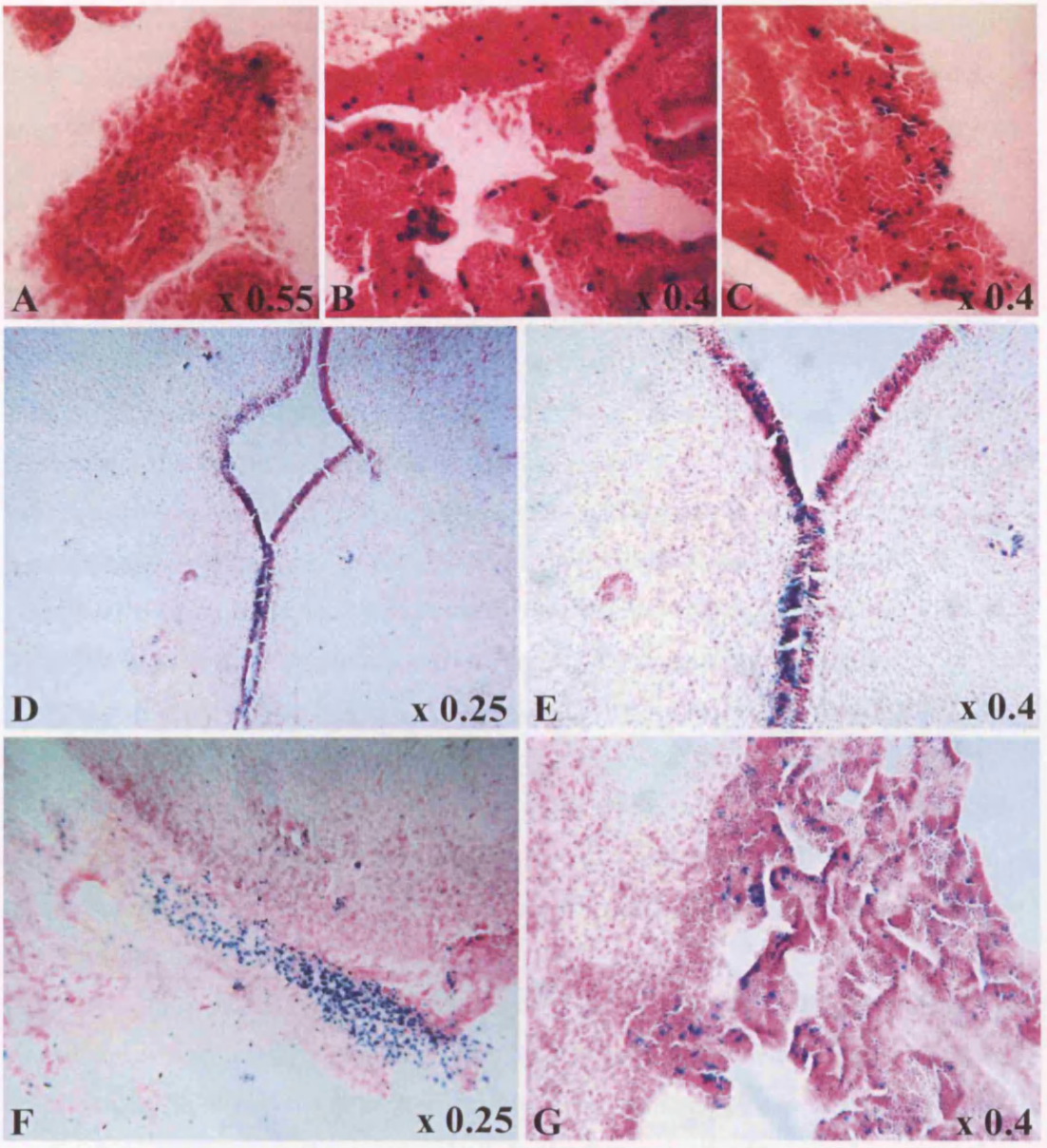


Figure F 6: β-galactosidase transgene expression after ultrasound-guided adlacZ injection into the lateral ventricle of early gestation fetal sheep.

X-gal staining of the fetal brain 2 days after injection of a fetus (57 days of gestation, B1), positive staining is seen in the choroid plexus of (A) the anterior, (B) the middle and (C) the posterior lateral ventricle. There is also staining of the ependymal lining of the lateral ventricle (D and E) and (F) of the cortex of the occipital region of the brain which may represent the needle track. (G) In a fetus aged 60 days of gestation (B2) there is staining of the choroid plexus in the posterior third of the lateral ventricle near the site of injection. Original magnifications as indicated.

In B1 there was extensive staining of the choroid plexus throughout the fetal cortex (**Figure F 6 A-C**), and positive transgene expression in the ependymal cells lining the lateral ventricles (**Figure F 6 D and E**). There was also staining of developing cortical neurones in the occipital cortex probably along the needle track (**Figure F 6 F**).

In B2 positive X-gal staining was confined to the choroid plexus in the posterior third of the left lateral ventricle (**Figure F 6 G**). X gal staining of the fetal spinal cord and β-galactosidase immunohistochemical analysis of the brain and spinal cord were negative.

F 6 Discussion

F 6.1 Ultrasound guided intraventricular injection is achievable in the early gestation sheep fetus

This is the first study of percutaneous intraventricular injection in the early gestation fetal sheep. Ultrasound examination of the fetal sheep head showed that the lateral ventricles were consistently visualised from 50 days of gestation, equivalent to 13 weeks gestation in humans. At this gestation, one study showed that complete visualisation of the head and brain structures was achieved in 80% and 82% of human fetuses scanned transabdominally and transvaginally respectively (Braithwaite JM et al., 1996). In clinical practice however, transvaginal sonography allows better visualisation of the fetal brain in the first trimester and is recommended for patients at risk of congenital brain abnormalities (van Zalen-Sprock RM et al., 1995). The coronal skull suture, through which we hoped to direct our needle, was visible in the sheep fetus at this gestation and the diameter of the posterior horn of the lateral ventricle was adequate to permit needle injection.

Our results showed that ultrasound guided placement of a needle within the lateral ventricle was achievable in early gestation but was technically demanding. The major problem was flexion of the fetal skull on the neck as an attempt was made to pass the needle through the coronal suture or skull. In the first case, the fetal head in coronal section was positioned perpendicular to the ewe's abdomen so that the needle passed directly through the coronal suture into the lateral ventricle with no flexion. In the subsequent two fetuses, the fetal head was not so well positioned and attempts to reposition one fetus using the needle was unsuccessful. Injection through the skull rather than the coronal suture however, was relatively straightforward. The morbidity observed at post mortem examination 2 days after injection probably reflected the difficulties encountered during injection and further experiments are needed to evaluate the mortality and long term morbidity of the procedure

In clinical practice intracranial or intraventricular injection is rarely performed. Therapeutic drainage of fetal hydrocephalus was first attempted in 1981 when repeated ultrasound guided cephalocentesis was performed in a fetus aged 25 weeks of gestation

(Birnholz JC and Frigoletto FD, 1981). Ventriculo-amniotic shunting was then developed, but the results of the "International Fetal Surgery Registry" were very poor and since then, most medical centres have discontinued the practice (Manning FA et al., 1986). In Brazil, where termination of pregnancy is not permitted, repeated cephalocentesis is still performed to reduce the intracranial pressure to within normal limits which some believe is of benefit to neurological development (Cavalheiro S et al., 2003). The only complication observed in this study was a small haemorrhage in the needle track in a few cases and there were no procedure related deaths.

There is one other study in the fetal sheep in which ultrasound guided brain injection has been performed (Cambria S et al., 1979). The authors injected kaolin into the cisterna magna under ultrasound guidance at laparotomy in late gestation to create an experimental hydrocephalus with low morbidity and mortality. In the mid-trimester fetal Rhesus monkey, ultrasound guidance has been used to place a thermocouple into the tentorium through the posterior fontanelle via a 20 Gauge needle for measurement of brain temperature (Tarantal AF et al., 1993). Ultrasound guided intraventricular injection of lentivirus vectors has also been performed in the first trimester but no information about the morbidity or mortality of the technique is available (Tarantal AF et al., 2001b). Ultrasound backscatter microscopy (UBM) provides excellent visualisation of the brain in the fetal mouse (Cheng H and Bjerknes M, 1984) and has been used to guide injection of the brain parenchyma at laparotomy with 34% survival (Olsson M et al., 1997).

In our hands, intraventricular injection was achievable but too technically demanding in early gestation to consider clinical application. Injection in mid-gestation may be easier to perform because there is less liquor and therefore less passive fetal movement on the needle tip. However, it would be more important to place the needle through a suture line because of the bone density and there might be difficulty gaining an adequate view of the fetal brain because the fetus is usually in cephalic presentation at this gestation.

F 6.2 Gene transfer to the early gestation fetal sheep brain can be performed using ultrasound guided intraventricular injecton

Many life-threatening congenital diseases considered as candidates for fetal gene therapy affect the fetal brain from early in development. Targeting genes to the brain however, is difficult because the blood brain barrier effectively prevents access to the brain after intravascular injection. Indeed, we were unable to show any gene transfer to the early gestation fetal sheep neurocortex after intraperitoneal or intramuscular

injection in which haematogenic spread of vector was observed. Injection of adenovirus vectors into the lateral ventricle resulted in significant transgene expression in the choroid plexus and low level expression in the ependymal cells lining the lateral ventricles. There was limited gene transfer to the neurocortex at the site of the needle track but in effect, only those tissues in direct contact with the vector were transduced. We had already shown that early gestation fetal neurocortex could be infected by the adenovirus vector and express transgenic protein *ex vivo*. In adult brain the capacity of adenovirus vectors to penetrate through cell layers is limited. After intraventricular injection in the adult rodent brain, only ependymal cells expressed transgenic protein and, in contrast to our findings, no transduction of the choroid plexus was observed (Akli S et al., 1993,Bajocchi G et al., 1993). It has been postulated that the limited spread of vector after parenchymal injection is affected by cellular organization and the degree of myelinization of the injection area (Hermens WTJMC et al., 1997).

In the fetal mouse, preliminary data on delivery of adenovirus and AAV vectors to the 4^{th} ventricle via intracranial injection of the fetal mouse gave good gene expression with no morbidity and good survival (American Society of Gene Therapy conference abstract 2001 (Lipshutz GS et al., 2001a)). In our case, the lack of adenovirus expression in the neurocortex is probably partly due to the dilution of the vector in the cerebrospinal fluid and the large surface area of cells lining the ventricles that will have 'mopped up' the vector. In addition, there appears to be a CSF-brain barrier present specifically during early fetal but not in adult life that may have prevented adenovirus penetration. This barrier of 'strap junctions' has a tight junction-like appearance of close and continuous contact between neighbouring cells that spirals from the ventricular pole of the cells towards the subependymal zone (Mollgard K and Saunders JM, 1986). In the fetal sheep it functions in early gestation at 60 days and has prevented the movement of protein and horseradish peroxidase across the neuroependyma after ventricular perfusion (Balslev Y et al., 1997,Fossan G et al., 1985). By 125 days of gestation however, ependymal permeability reaches adult levels (Fossan G et al., 1985) and the strap junctions are replaced by mature gap junctions that are present in adult brains (Mollgard K et al., 1987). Thus we might expect to see more extensive penetration of the neurocortex after ventricular injection of adenovirus vectors in late gestation.

Targeting genes to the brain via systemic administration is difficult because the blood brain barrier effectively prevents access to the brain. There was until recently a widely held belief that the fetal blood brain barrier is immature and therefore should be relatively permeable to proteins and other small molecules. It is however now accepted

that the barrier between the blood and the brain develops from very early stages of brain vascularisation and it is made up of tight junctions (Saunders NR et al., 2000). In the fetal sheep, even at 30 – 40 days of gestation, very little intravenous plasma protein penetrates the lateral ventricular CSF (Cavanagh ME et al., 1983). At 12 weeks of gestation there is evidence of tight junction formation in the endothelium of human brain microvessels (Virgintino D et al., 2004) and permeability studies also support the concept that the human fetal blood brain barrier matures early (Bell JE et al., 1991). A number of agents are able to increase the permeability of the blood brain barrier including sodium caprate (Awazu S et al., 1999), hyperosmotic mannitol (Muldoon LL et al., 1995) and VEGF (Young PP et al., 2004) but there are concerns about their toxicity.

We did not observe any toxicity or inflammation within the injected neurocortex or lateral ventricle although there was an inflammatory reaction localized to the skin and meninges at the site of the injection. This lack of response is probably because the two day time point to sample the brain was too soon for an inflammatory reaction to develop. Although it is generally believed that the brain is an immunoprivileged site, delivery of adenovirus vectors into the adult rat brain parenchyma lead to invasion by macrophages and cytotoxic T cells from four and eight days respectively after injection (Hermens WT and Verhaagen J, 1997). A humoral immune response to the transgenic protein and to adenovirus was also seen (Kajiwara K et al., 2000). In our study there was no evidence of cytotoxicity of the adenovirus on the fetal brain tissue even at doses of 2×10^{12} p/kg (1×10^{11} particles). Injection of 10^7 pfu adenovirus vector into the brain parenchyma of chicken embryos similarly had no toxic effect at 24 hours (Thakur A et al., 2001). By contrast, in the adult mouse brain cytotoxicity has been observed when doses of 10^7 pfu, equivalent to 10^8 particles or higher have been applied to the parenchyma (Akli S et al., 1993). The lack of cytotoxicity we observed probably reflects the dilutional effect of the cerebrospinal fluid on the vector although it is possible that the fetal neurons are less susceptible to the cytotoxic effect of adenovirus.

This is the only study to investigate delivery of adenovirus vectors into the brain of a large animal fetal model. Gene transfer assessed using PCR analysis of transgenic protein in the fetal brain tissue was low level after intraventricular delivery of lentivirus vectors into the early gestation fetal rhesus monkey lateral ventricle (American Society of Gene Therapy conference abstract (Tarantal AF et al., 2001b)). Most of the research on gene transfer to the fetal brain has used injection of retrovirus vectors to analyze cell lineage and migration of neural progenitors. Vectors have been delivered to the neural

tube of chicken embryos (Golden JA et al., 1997) or into the lateral ventricle of fetal rats (Walsh C and Cepko CL, 1988) and mice (Luskin MB et al., 1988). This results in gene transfer to dividing cells and allows them and their progeny to be followed along migration pathways. Thus application of integrating vectors, especially lentiviruses that are able to infect non-dividing cells, should result in more widespread gene transfer. Interestingly retrovirus mediated gene transfer to brain cells from fetal sheep aged 60 to 80 days of gestation *in vitro* was shown to be at least an order of magnitude more efficient than gene transfer to kidney, muscle, lung or skin cultures (John HA, 1994). The author suggested this may be due to areas of active genome transcription in brain cells into which retrovirus vectors might preferentially integrate, rather than an effect of cell proliferation.

Early treatment of lysosomal storage diseases with early and severe CNS involvement may be of critical importance, and this is one of the rationales behind fetal as opposed to neonatal gene therapy. Evidence from studies of gene therapy to neonatal mouse models of lysosomal storage diseases supports this concept. In neonatal twitcher mice, a model of Krabbe disease, intraventricular delivery of adenovirus vectors containing the therapeutic gene to newborn animals was more effective at correcting neurological symptoms and the enzyme defect at the cellular level than injection two weeks after birth (Eto Y et al., 2004). Adeno-associated vectors have also proved capable of long-term prevention of pathology in beta-glucuronidase-deficient mice (Passini MA et al., 2003).

It is unlikely that delivery of gene therapy to the fetal ventricle will become clinically acceptable in early or even late gestation because of technical difficulties with the procedure. Techniques to transfer genes to the fetal brain may prove useful however for investigating the mechanism and possible treatment of conditions such as perinatal brain injury (Peebles DM and Wyatt JS, 2002).

G The fetal humoral immune response to ultrasound-guided injection of adenovirus gene therapy

The rationale that fetal gene therapy may avoid the development of immune reactions to the vector or transgene product depends critically on the timing of gene delivery and the route of administration. Some authors propose that there is a 'window of opportunity' in the first third to half of pregnancy during which time introduction of foreign genetic material may not produce an immune response but may allow development of tolerance (Billingham RE et al., 1956,Binns R, 1967). This time is thought to be up to 60 – 75 days of gestation in the fetal sheep although the immune response depends on the type of antigen used and there is also wide variation between individual fetuses (Fahey KJ and Morris B, 1978,Fahey KJ and Morris B, 1974).

We investigated the humoral immune response of fetal sheep that had received ultrasound-guided adenovirus vector injection. We studied the levels of antibodies in the fetal serum to the transgene (hFIX or β-galactosidase) and to the adenovirus vector. In many cases, fetuses were sampled 2 days after exposure to the antigen. This is not generally thought to be a sufficiently long time interval to allow development of a full immune response and therefore some fetuses injected with adhFIX in early gestation were sampled at later time intervals from exposure (9 and 28 days and birth). Permanent tolerance is believed to take place if antigens are consistently maintained (Ramsdell F and Fowlkes B, 1992). This could occur following gene transfer mediated by integrating vectors such as retrovirus or lentivirus vectors. Long term expression has been seen in mice up to a year after adenovirus injection as fetuses. In sheep however, adenovirus mediated gene transfer is only short term, maximally up to one month after fetal injection. We did not perform experiments therefore to test for immune tolerance in lambs born after fetal injection of adenovirus vectors.

G 1 Fetal sheep receiving umbilical vein injection of adenovirus vectors at 60 days of gestation can mount a humoral immune response to the transgenic protein and adenovirus

We analysed the sera of fetal sheep injected with adlacZ (n = 2) and adhFIX (n = 9, 7 survivors) vectors via the umbilical vein at 60 days of gestation for the presence of antibodies to the β-galactosidase or hFIX transgenic protein and to adenovirus. Fetuses

injected with adhFIX were sampled at several time points following injection to investigate the time course of antibody production.

G 1.1 Antibodies to the transgenic protein

There was a marked variation in the humoral immune response of individual fetuses to the transgene that did not depend on either the time of sampling after injection or the presence of transgene expression (**Table G 1**). By 9 and 29 days after injection, two of three fetuses injected with adhFIX vector had detectable levels of anti-hFIX antibodies, albeit at low levels. In two of these fetuses, hFIX was detectable in their plasma.

Table G 1: Fetal humoral immune response after umbilical vein injection of adenovirus vectors at 60 days of gestation.

Antibody level was determined by comparing the sample ratio with an internal standard; d: days;

Sheep	Vector	Time of sampling (d)	Antibody to transgenic protein (μg/ml)		Expression of transgenic protein (ng)	Antibody to adenovirus (μg/ml)
			hFIX	lacZ		
UV5	adlacZ	2 d	–	0	0	0.34
UV16	adlacZ	2 d	–	0	0	0.68
UV6	adhFIX	2 d	0	–	581	0.91
UV10	adhFIX	2 d	0.13	–	0	0.93
UV7	adhFIX	9 d	0.203	–	199.5	0.85
UV8	adhFIX	9 d	0	–	145	0
UV11	adhFIX	29 d	0.041	–	0	0.42
UV12	adhFIX	birth	0	–	0	0.54
UV15	adhFIX	birth	0	–	0	0.58
UV15	control	birth	0	–	0	0

We analysed the levels of antibody to hFIX transgenic protein over time in the sera of the lambs that came to birth after adhFIX injection (**Figure G 1**). Antibodies to hFIX were detected at birth at low levels and were found at later days of analysis on day 10 in one lamb and repeatedly up to 70 days after delivery in the second lamb, although no antibody was detected in the serum of the control animal.

G 1.2 Antibodies to adenovirus

Analysis of fetal sera at 9 days or later after fetal injection revealed antibodies to the adenovirus were detectable in all but one fetus (**Table G 1**). Similarly antibodies to adenovirus were detected in the sera of both injected lambs but not in the serum from the control lamb (**Figure G 2**).

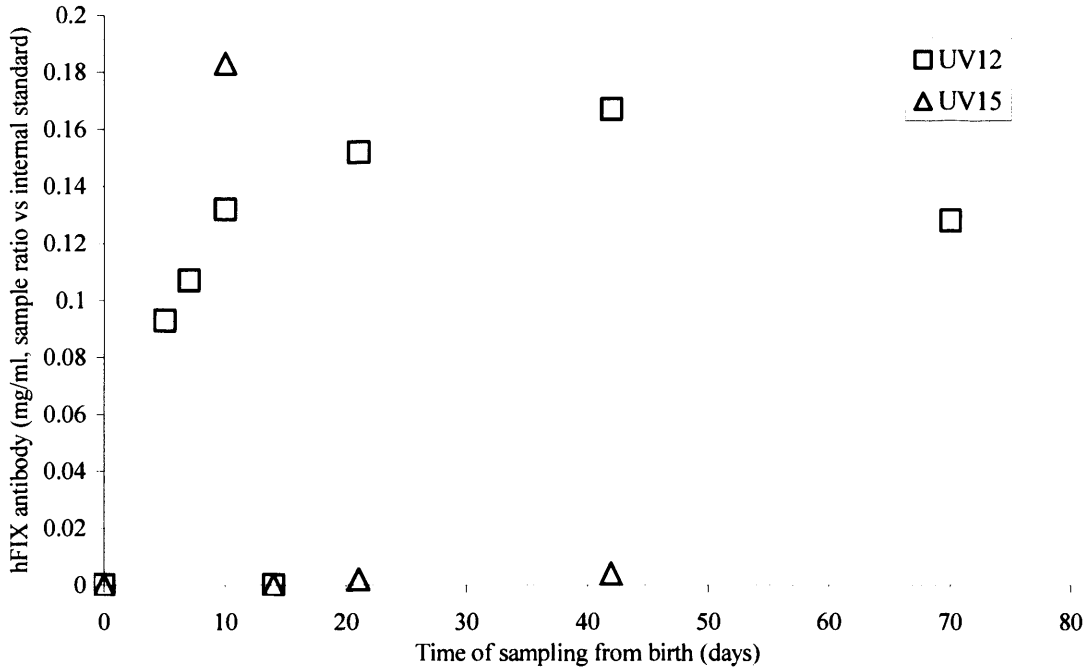


Figure G 1: The humoral immune response to transgenic hFIX protein in lambs born after ultrasound-guided umbilical vein injection at 60 days of gestation.

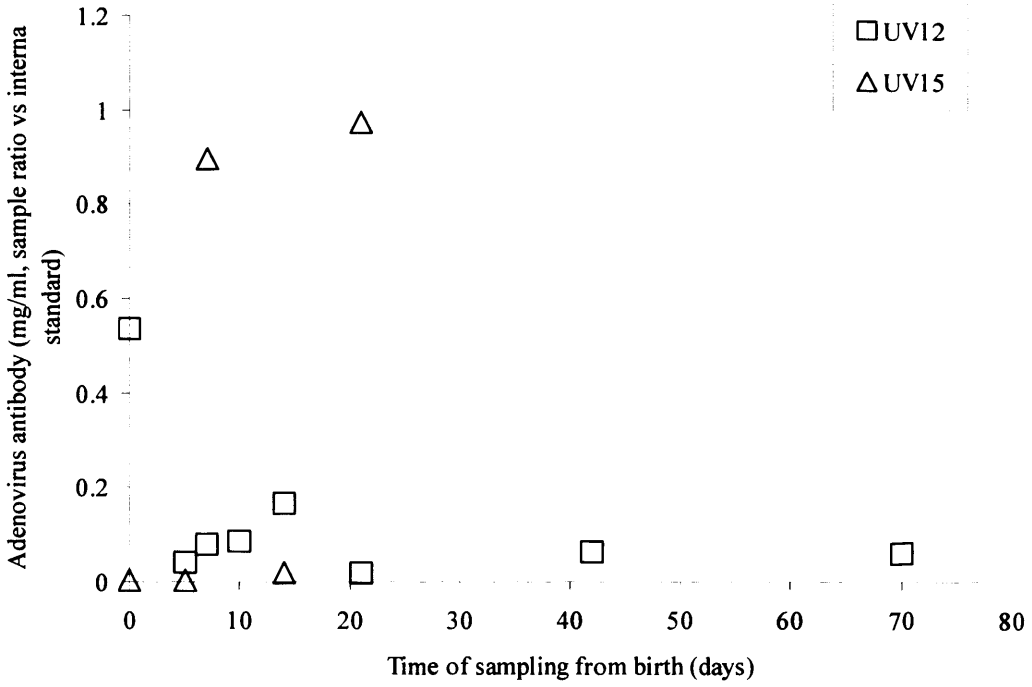


Figure G 2: The humoral immune response to adenovirus vector in lambs born after ultrasound-guided umbilical vein injection at 60 days of gestation

The antibody to the adenovirus was measured at a higher level than the antibody to the transgenic hFIX in sera from fetuses and lambs. In this study we used a standard

calibration curve to determine the antibody level rather than titration methods because of the low levels of antibody we detected. If higher levels of antibody had been detected, titration would have been the more accurate method of quantifying the antibody response.

G 2 The humoral immune response of fetal sheep to umbilical vein injection of adenovirus vectors from 67 days of gestation

We analysed the sera of fetal sheep injected with adlacZ (n = 11, 9 survivors) and adhFIX (n = 1) vectors via the umbilical vein from 67 – 95 days of gestation for the presence of antibodies to the β-galactosidase or hFIX transgenic protein and to adenovirus. All fetuses were sampled 2 days after injection.

G 2.1 Antibodies to the transgenic protein

In three fetuses an antibody response to the transgenic protein was detectable. As can be seen, the presence of antibody to the transgenic protein was not related to detectable transgene expression (**Table G 2**). Antibody detection did appear to be related to the gestational age of the fetus, with antibody only detected in older fetuses.

Table G 2: Fetal humoral immune response after umbilical vein injection of adenovirus vectors from 67 days of gestation.

GA: gestational age at injection; – to +: level of transgene expression; na: not available

Sheep	GA (d)	Vector	Antibody level (μg/ml, sample ratio vs internal standard)			Transgene expression
			hFIX	β-galactosidase	adenovirus	
UV20	67	adlacZ		0	na	(+)
UV17	68	adlacZ		0	0	–
UV18	68	adlacZ		0	0.37	–
UV21	71	adlacZ		0	na	–
UV22	71	adlacZ		0	na	–
UV23	74	adlacZ		0	0.01	(+)
UV14	83	adhFIX	0.158		0.01	–
UV28	87	adlacZ		0	0	–
UV26	95	adlacZ		0.041	0	+
UV27	95	adlacZ		0.026	0	+

G 2.2 Antibodies to adenovirus

There was a similar individual variability in the fetal humoral immune response to the adenovirus (**Table G 2**) although this did not appear to depend on the gestational age of the fetus at which the injection was performed.
Since all fetuses were sampled only 2 days after injection it is difficult to draw conclusions from these results. It is unlikely that the protein detected in the fetal serum 2 days after injection was a true antibody response to the applied antigen since there is usually a lag period of at least 5 days before the first immunoglobulins, in the form of IgM, are detected in the serum. It is more likely that there was some cross-reactivity in the assay or that it detected naturally occurring antibody that we know to be present in fetal sheep (Reynaud CA et al., 1995) and pigs (Cukrowska B et al., 1996) from early gestation.

G 3 Fetal sheep injected with adenovirus vectors before 61 days of gestation mount a humoral immune response to adenovirus but not to the transgenic protein

The sera of fetal sheep receiving ultrasound-guided intraperitoneal, intrahepatic, intramuscular or intra-amniotic injection of adhFIX vector were analysed for anti-adenovirus and anti-hFIX antibodies by ELISA assays. Sera from fetuses injected with adlacZ vector were not analysed (IM5, HE2, HE8, HE11, IP10) since they were sampled at 2 days after injection and we did not expect to observe a humoral immune response so rapidly.

G 3.1 Antibodies to the transgenic protein

No antibodies against hFIX were detected in any of the treated animals at 2, 9 - 11 or 28 - 30 days following injection or at birth. Even when there was strong expression of the transgene, for example following intraperitoneal delivery, no anti-hFIX antibodies were detectable.

G 3.2 Antibodies to adenovirus

Antibodies to the adenovirus vector however, were present following all routes of vector application (**Figure G 3**). Even at two days after injection, low levels of antibody were detected in almost all (6 out of 8) fetuses sampled. Because of the small numbers of fetuses examined at later time points we cannot draw any conclusions as to the effect of gestational age on antibody response. It should be noted however that adenovirus

antibodies were detected in almost all (12 out of 13) fetuses sampled 9 or 28 days after injection.

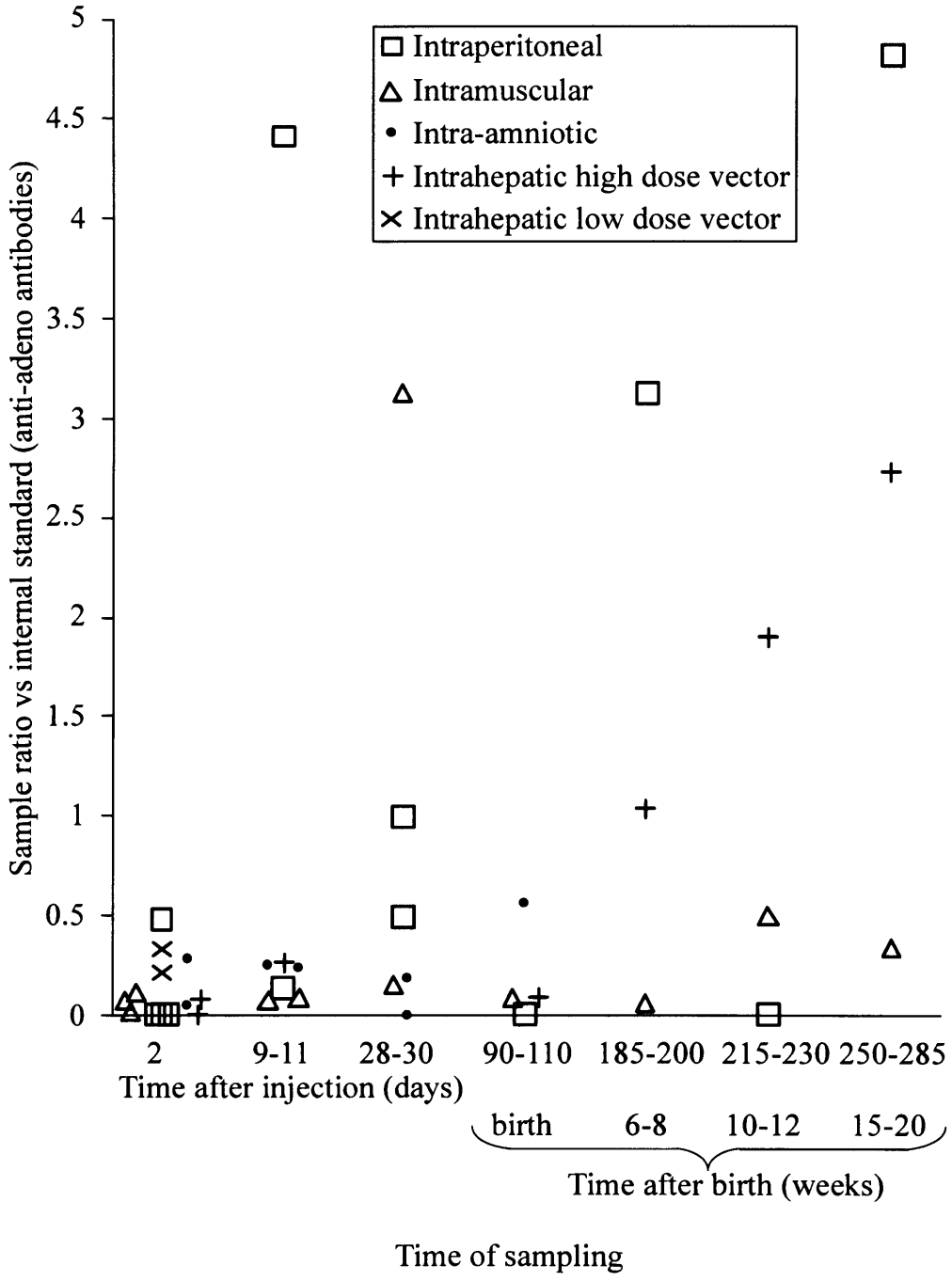


Figure G 3: The humoral immune response to adhFIX vector after ultrasound-guided early gestation delivery.

Five lambs, one for each route of injection (intraperitoneal, intrahepatic, intra-amniotic and intramuscular, were followed and sampled at regular time intervals after birth.

As can be seen, the presence of antibodies to adenovirus was not associated with any particular route of injection and antibody levels varied considerably between fetuses. We did not observe any relation between the extent of procedure related necrosis and inflammation on histological analysis and the level of adenovirus antibody in the fetal serum.

The different findings following umbilical vein injection and other routes of injection in early gestation is interesting. A direct comparison between the routes of injection cannot be made however, since in all but one case (IM4 injected at 61 days of gestation), the intraperitoneal, intrahepatic, intra-amniotic and intramuscular injection procedures were performed on or before 57 days of gestation. It is possible that this earlier time of injection is responsible for the differences in humoral immune responses observed.

G 4 Late gestation fetal sheep receiving intramuscular injection of adenovirus vectors do not mount a detectable humoral immune response to the transgenic protein

The sera of fetal sheep receiving intramuscular injection of adlacZ vector (n = 4) in late gestation (125 –137 days of gestation) were analysed for a humoral immune response. There were no antibodies detectable to the hFIX transgenic protein in any of the animals, even at this late gestation although adenovirus antibodies were detected in all fetuses and lambs (**Table G 3**).

Table G 3: Fetal humoral immune response after intramuscular injection of adenovirus vectors in late-gestation fetal sheep.

GA: gestational age at injection; d: days

Sheep	GA (d)	Time of sampling (d)	Expression of transgenic hFIX protein (ng)	Antibody level (μg/ml, sample ratio vs internal standard)	
				hFIX	adenovirus
IM12	125	2 d	23.5	0	0.54
IM13	125	2 d	16	0	0.21
IM14	137	birth	7	0	0.07
IM15	137	birth	0	0	0.16

G 5 The humoral immune response of mid and late-gestation fetal sheep to intratracheal injection of adenovirus vectors

The sera of fetal sheep receiving ultrasound-guided intratracheal injection of adlacZ (n = 23) or adhCFTR vectors (n = 10) were analysed for antibodies to β-galactosidase and adenovirus by ELISA assays. There is no sheep specific anti-hCFTR antibody currently available to allow calibration of an ELISA assay and we therefore did not analyse fetal sera from sheep injected with adhCFTR vector for anti-hCFTR antibodies.

G 5.1 Antibodies to the lacZ transgene

The majority of fetuses injected with adlacZ vector were sampled 2 days after injection (n = 21) but two were analysed at later time points, at 16 days after injection (IT24) and 6 hours after birth (IT18). Antibodies to β-galactosidase were detectable in the serum of the lamb that delivered but not in the fetus sampled 16 days after injection (**Table G 4**). No antibodies to β-galactosidase were detectable in the sera of fetuses analysed 2 days after injection.

Table G 4: Fetal humoral immune response after intratracheal injection of adenovirus vectors in fetal sheep.

GA: gestational age at injection; d: days; nt: not tested.

GA (d)	No. of fetuses	Vector	Time of sampling (d)	Antibody level (μg/ml, sample ratio vs internal standard)	
				β-galactosidase	adenovirus
137 – 138	3	adlacZ	2 d	0	0
81 – 82	4	adlacZ	2 d	0	0
102 - 116	13	adlacZ	2 d	0	0
116	1	adlacZ	2 d	0	0.24
82	1	adlacZ	16 d	0	0.008
102	1	adlacZ	birth	49	0.563
100 - 102	3	adhCFTR	2 d	nt	0
102 - 113	3	adhCFTR	17 d	nt	0
102 – 110	2	adhCFTR	birth	nt	0
81 - 82	2	adhCFTR	birth	nt	0

A time interval of 2 days from injection to sampling of fetal serum is probably not sufficiently long enough for the fetus to be able to mount a true humoral immune response. The lack of immune response to the transgenic lacZ protein in the fetus sampled 16 days after injection however, is unexpected, especially since there was

evidence of transgene expression in the fetal lung on β-galactosidase ELISA analysis. The lamb that came to birth after tracheal injection was sampled 43 days after injection and showed a substantial anti-adenovirus response. As anticipated from the short-term nature of adenovirus mediated gene transfer, no transgene expression was detectable in the lung of this lamb. It is possible that the delivery of gene therapy vectors to the fetal airways rather than systemically influenced the ability of the sheep fetus to mount an immune response 16 days after injection.

G 5.2 Antibodies to adenovirus

Antibodies to adenovirus were only detectable in 3 out of 23 fetuses that received intratracheal injection of adenovirus vectors, all of which were injected with the adlacZ vector (**Table G 4**). As might be anticipated, the fetuses sampled later than 2 days after injection (16 days after injection or at birth) both had antibodies. In contrast there were seven fetuses injected with the adhCFTR vector that were sampled later than 2 days after injection (17 days after injection or at birth) and none had detectable levels of antibody to the adenovirus. Results of hCFTR transgene expression are not available currently and we cannot confirm therefore that the adhCFTR vector is able to mediate gene transfer in fetal sheep. It is possible that there is a different humoral response of the fetal sheep to the adlacZ and adhCFTR vector although the viral backbone of the vectors was the same. The results could also reflect individual variation in the fetal response to adenovirus.

G 6 The humoral immune response of fetal sheep to intragastric injection of adenovirus vectors at 60-62 days of gestation

The sera of fetal sheep receiving ultrasound-guided intragastric injection of adlacZ vector (n = 11, 9 survivors) were analysed for antibodies to β-galactosidase and adenovirus by ELISA assays. No antibodies were detectable. All fetuses however, were sampled 2 days after injection and it is likely this is not sufficient time from exposure to the antigen for the fetus to mount a humoral immune response.

G 7 Discussion

One of the main caveats in adult gene therapy is the immune response to vector and/or transgene. *In utero* application, on the other hand, aims to circumvent this by treatment before maturity of the functional immune system. The sheep is a particularly good animal model to use for investigation of the fetal immune response to antigen because the synepitheliochorial placenta does not permit passage of gammaglobulin from mother to fetus (Morris B, 1986) and we can therefore be sure that all antibodies in the fetal serum are derived from the fetus and not from the mother. Classic experiments in fetal sheep have demonstrated that the fetal response to antigenic stimulus varies according to the type of antigen and with gestational age (Silverstein AM et al., 1963b,Fahey KJ and Morris B, 1978).

The effect of gestational age on antibody response can be seen in our results after umbilical vein injection. After umbilical vein delivery at 60 days of gestation, antibodies to the hFIX transgenic protein were detected in some but not all of the fetuses that expressed the transgene. The fetus receiving adhFIX at 83 days of injection mounted a robust immune response to the hFIX protein. Antibody to the lacZ protein was not detected until adlacZ injection was performed at 95 days of gestation although this could reflect a lack of protein expression by this route of administration. The previous study with late gestation sheep (day 102-140 of gestation) using the same adenovirus vector construct, showed the presence of antibodies to the adenovirus vector and hFIX protein in all fetuses (Themis M et al., 1999). These findings support other studies showing that most fetuses acquire a capacity to respond to several antigens between 60 to 75 days of gestation although there is considerable variation in the response between individual fetuses (Silverstein AM et al., 1963b,Fahey KJ and Morris B, 1978).

In contrast, fetuses receiving adenovirus vector by each of the intraperitoneal, intramuscular, intrahepatic or intra-amniotic routes, most of which were injected by 57 days of gestation, did not produce measurable levels of antibodies to the hFIX or lacZ protein. This was despite good levels of expression of both proteins that was detectable in the case of hFIX, for longer than two days. Our data would suggest that at the time of injection, these fetuses were pre-immune to the proteins they were exposed to.

It cannot be ruled out that alternative therapeutic proteins may generate an immune response and that these observations were not specific for hFIX or lacZ. In contrast to the adenovirus and lacZ protein, these normal sheep fetuses produce their own factor IX

protein that may be similar to the human protein. It is possible but unlikely that injection by these routes leads to tolerance to the antigen we applied.

Recently, studies have shown that the development of tolerance to self-antigens appears to depend on the presence of T-regulatory cells (Schwartz RH, 2005,Sakaguchi S, 2005). These naturally occurring T cells are specialized for a suppressive function and their depletion can lead to tumour rejection and the development of autoimmunity. Thymic and extra-thymic T regulatory cells are detectable in the human fetus from 13 and 14 weeks of gestation respectively (Darrasse-Jeze G et al., 2005) and function similarly to those found in adults. It is possible that they also may play a role in the development of tolerance to exogenous antigens.

The levels of adenovirus antibodies after early gestation umbilical vein, intraperitoneal, intramuscular and intrahepatic injection varied considerably between fetuses. We investigated whether in these particular animals there was more procedure related necrosis and inflammation which might have an adjuvant effect on the generation and prolongation of the antiviral immune reaction. However there was no indication of particular trauma in those fetuses that did show antibodies. Individual variation in the fetal sheep response to antigenic stimulus is well known and our findings could simply reflect this.

Antibodies to the adenovirus vector were detected following umbilical vein, intraperitoneal, intramuscular and intrahepatic delivery to fetal sheep at any gestational age investigated. By comparison, another group showed a fetal immune response to adenovirus vector at 90 and 125 days of gestation, after umbilical vein injection at 60 days (Yang EY et al., 1999). The different assays used in this study and ours may be responsible for the observations. Yang et al used a neutralizing antibody assay to test for the presence of antibodies that block adenovirus infection of HeLa cells. In contrast our ELISA assay detected all adenovirus specific immunoglobulins except IgE, whether they be neutralizing or non-neutralizing. It is possible that the fetus produces non-neutralizing antibodies that can bind to parts of the virus but that are unable to prevent infection. Certainly in adult humans, antibodies to adenovirus 5 were detectable in all subjects in one study, but only 55% of the antibodies neutralized the virus (Chirmule N et al., 1999).

After intratracheal delivery of adenovirus vector, antibodies to lacZ transgenic protein were detected at 43 days but not at 16 days after injection. It is difficult to draw conclusions with such a few number of experimental animals. However, this could again signify variation in the individual fetal response to antigens, may represent

maturation in the ability of the animal to produce antibody specific to the lacZ protein or reflect the more local delivery achieved by intratracheal delivery of adenovirus. Studies in adult humans have shown that the route of administration of adenovirus vectors alters the humoral (Harvey BG et al., 1999) and innate immune response to the vector, local delivery evoking a smaller response than systemic methods. Vincent et al observed that intratracheal delivery of adenovirus to mid gestation fetal sheep did not elicit a neutralizing antibody response to adenovirus at 3 or 14 days after injection (Vincent MC et al., 1995). After repeated injection of the vector in late gestation however, neutralizing antibodies were detectable, showing that the fetus was not tolerant to the vector (Iwamoto HS et al., 1999).

The immune response to adenovirus following intra-amniotic delivery of vector was interesting, since after injection at 33 days of gestation, anti-adenovirus antibodies were detectable at day 42, within the time period that the sheep fetus is still believed to be pre-immune. Evidence from studies of fetal pigs show that they are able to develop preimmune natural antibodies from 30 days of gestation (term = 114 days) (Sun J et al., 1998) and can mount an immune response to viruses from 38 days of gestation (Butler JE et al., 2001). Our data suggests that a highly immunogenic agent can elicit a humoral immune response and that the hFIX protein is probably less immunogenic. This has important implications for therapeutic gene transfer *in utero* since at present, little is known about the immunogenicity of alternative therapeutic proteins to be used in gene therapy applications.

Late gestation intramuscular injection of adhFIX vector was unable to evoke an antibody response to the hFIX transgenic protein. This was surprising because we anticipated there would be an immune response similar to that observed following late gestation umbilical vein injections (Themis M et al., 1999). In particular, the intramuscular route of injection is known to induce a strong immune response and has been applied in the fetal sheep for immunization against a number of newborn pathogens (Gerdts V et al., 2004). The lack of antibody response to the transgenic protein may reflect the low level transduction we achieved, although similar levels of hFIX expression were detected in one of the lambs born after late gestation umbilical vein injection in which anti-hFIX antibodies were detected.

Our hypothesis that expression of a foreign antigen during early fetal development could result in its recognition as "self" has been based on previous observations by others in a murine system where exposure of the fetus to foreign antigen was maintained (Billingham RE et al., 1953). Although we did not detect antibodies to

transgenic hFIX after early gestation injections, it is not possible to determine tolerance by repeat injection of adhFIX vector due to the presence of anti-adenovirus antibodies and the relatively short-term gene expression provided by this vector. Attempts by others to induce tolerance to adenovirus following fetal delivery via intratracheal (Iwamoto HS et al., 1999), umbilical vein (Yang EY et al., 1999) and intra-amniotic, intrahepatic or intramuscular injection (Jerebtsova M et al., 2002) have been unsuccessful. Nevertheless, evidence to support induced tolerance has been reported from experiments performed in the fetal sheep (Tran ND et al., 2001). Following intraperitoneal injection of retrovirus vectors containing the β-galactosidase gene into the preimmune sheep fetus via laparotomy at 60 days of gestation, there was a reduced cellular and humoral response to postnatal injection of the transgene in lambs when compared with uninjected lambs. We investigated the feasibility of injecting hFIX protein into sheep but the prohibitive costs of this in a large animal precluded its use in this study using adenovirus vector. However we hope that future studies that deliver an integrating vector to the fetal sheep may result in long-term expression of transgenic hFIX and allow us to test for tolerance using exogenous hFIX.

In fetal MF1 mice, yolk sac vessel injection of adhFIX vector lead to the animals being tolerant to exogenous hFIX as adults (Waddington SN et al., 2003b). No tolerance was demonstrated to the adenovirus vector however, and the fetally injected mice developed high levels of antibody to adenovirus after injection of adhFIX as adults. In both these studies, the transgenic protein was maintained, albeit at low levels in the circulation and this may have acted to reinforce tolerance to the antigen. This concurs with the theory that tolerance may be induced by the continual exposure of anergic T cells to antigen (Ramsdell F and Fowlkes B, 1992) or by the maintenance of a T-regulatory population (Schwartz RH, 2005).

Adenovirus is known to be a highly immunogenic virus. Indeed, a systemic inflammatory response to a first generation adenovirus vector resulted in the death of a participant in a phase 1 clinical trial towards gene therapy of the inherited metabolic disorder, ornithine transcarbamylase deficiency (Lehrman S, 1999). Attempts to reduce the immunogenicity and toxicity of the vector and prolong gene expression have led to the generation of the so called 'gutless vectors' in which essentially all adenovirus coding sequences have been eliminated (Chen HH et al., 1997,Schiedner G et al., 1998,Schiedner G et al., 1998). These vectors will be more appropriate for clinical application of prenatal gene therapy.

Fetal application of oncoretrovirus vectors such as lentivirus that integrate into the genome and provide long term transgene expression, may provide tolerance in the adult animal. Certainly, a recent report demonstrated long term hFIX expression and tolerance to exogenous hFIX in adult haemophiliac and immune competent mice that had received yolk sac vessel injection of lentivirus containing the hFIX transgene as fetuses (Waddington SN et al., 2004b). Only by application of integrative vectors that produce good levels of gene transfer in the sheep fetus and testing for tolerance to the transgenic protein after birth of the animal will we be able to determine whether immune tolerance can be reliably achieved in a large animal fetal model.

H The future: experiments with alternative vectors in fetal sheep

Retrovirus and lentivirus vectors offer the advantage of being able to integrate into the genome and therefore provide long-term gene expression. *In utero* application of lentivirus vectors to the mouse showed widespread and sustained gene expression particularly in the liver and musculature (Waddington SN et al., 2003c). Pilot experiments were performed in fetal sheep in early and late gestation using amphotropic retrovirus vectors (Moloney Leukemia Virus, CMV promoter) that have a general tropism for mammalian cells. There are difficulties in preparing sufficient quantities of lentivirus vectors and experiments using these vectors were confined to early gestation fetal sheep.

Recombinant Sendai virus has shown a tropism for respiratory epithelium in a number of small animal models (Yonemitsu Y et al., 2000) and a broad range of tissues were transduced in the fetal mouse including the airway epithelium (Waddington SN et al., 2003a). We therefore performed some pilot experiments in the fetal sheep airways both *ex vivo* and *in vivo*.

H 1 A high rate of cell division is observed in early gestation fetal sheep tissues

Retrovirus vectors only integrate into the genome of actively dividing cells. As a preliminary step to injection of retrovirus vectors into early gestation fetal sheep we wanted to assess cell division in the sheep fetus at this gestational age. Ultrasound guided intraperitoneal injection has been used in mid-gestation fetal sheep to successfully administer the DNA synthesis marker 5-bromo2'-deoxy-uridine (BrDU) (Greenwood PL et al., 1999). To determine the correct dose of DNA label, we extrapolated from this study on the basis of fetal weight, and 250 µl BrDU was delivered into the peritoneal cavity of a fetus at 60 days of gestation by ultrasound-guided injection. Extensive tissue sampling of the fetus was performed at post mortem analysis one hour later and immunohistochemical analysis for BrDU staining showed high cell proliferation in all tissues apart from the adrenal gland (**Figure H 1**). This suggests that retrovirus vectors should be capable of transducing a broad range of early gestation fetal sheep tissues.

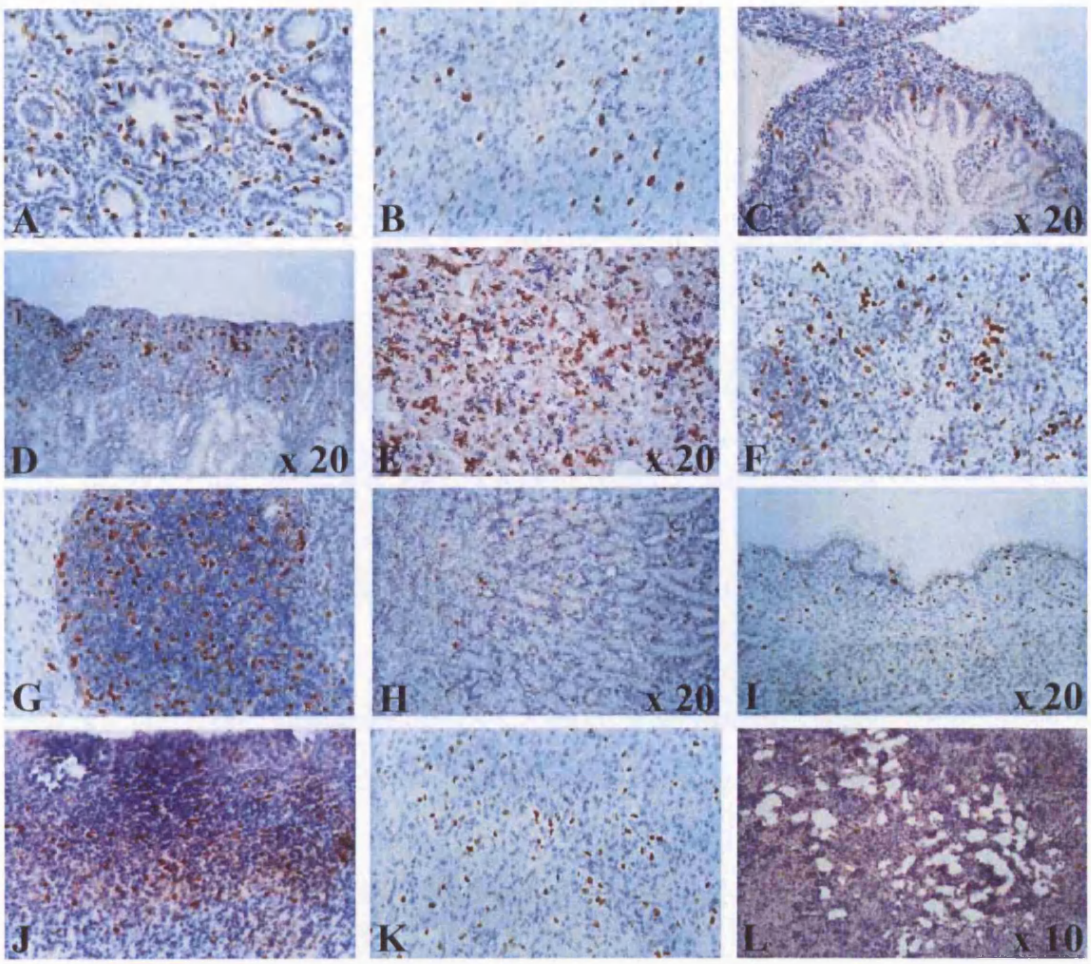


Figure H 1: Cell proliferation in the early gestation fetal sheep.

Immunohistochemistry for BrDU in early gestation fetal sheep tissues after ultrasound-guided intraperitoneal injection of a fetal sheep aged 60 days of gestation. There is strongly positive BrDU staining suggesting a high rate of cell division in the following tissues: (A) lung, (B) myocardium, (C) small bowel, (D) kidney, (E) liver, (F) spleen, (G) thymus, (H) gonad, (I) skin, (J) neurocortex and (K) muscle. In the adrenal (L) however, there is very little positive staining. Original magnifications x 40 except where indicated.

H 2 Retrovirus vector mediated gene transfer to fetal sheep is poor

H 2.1 Fetal sheep liver can be transduced *ex vivo* by retrovirus vectors

Amphotropic retrovirus vectors (Moloney Leukemia Virus, MLV, 1 x 10^8 particles) containing the lacZ gene were applied to the liver of fetal sheep aged 55 and 100 days of gestation *ex vivo*. The vector was applied alone or in complex with DOGS (5μg/ml) or DEAE dextran (5μg/ml). Widespread scanty β-galactosidase expression in

subcapsular cells was observed on X-gal staining 24 hours after transfection, when the vector was complexed with either of the polycations (**Figure H 2**); DEAE dextran enhanced transduction more than DOGS.

Figure H 2: Retrovirus-mediated β-galactosidase transgene expression in the fetal sheep liver.

The liver of a fetal sheep aged 55 days of gestation was incubated *ex vivo* with MLV vector containing lacZ complexed with DEAE dextran (5μg/ml). Positive X-gal staining of subcapsular cells is seen.

H 2.2 Ultrasound-guided intraperitoneal or intrahepatic injection of retrovirus vectors to early gestation fetal sheep does not result in transgene expression

Amphotropic retrovirus vectors (MLV, 2 x 10^7 particles) containing the lacZ gene were delivered to early gestation fetal sheep by ultrasound-guided intrahepatic (n = 2) or intraperitoneal (n = 1) injection (**Table H 1**). Post mortem and histological examination was normal 9 – 10 days after surgery apart from mild vascular congestion in the liver of one fetus (HE16). Transgene expression was not detectable by X-gal staining or β-galactosidase immunohistochemistry. Assuming that this negative result might be due to the immaturity of the fetal liver at this young gestational age, we performed retrovirus injections in later gestation fetal sheep.

Table H 1: Ultrasound-guided intrahepatic or intraperitoneal retrovirus vector injection.

Post mortem and histological findings, and transgene expression after ultrasound-guided intrahepatic (HE) or intraperitoneal (IP) injection of retrovirus vectors (MLV) into early gestation fetal sheep. † indicates the vector was complexed with DEAE dextran (5μg/ml); PM: post mortem; d: days; p/kg: particles per kg.

Sheep	Age (d)	Vector		PM	PM findings	Histological findings	β-galactosidase expression	
		Vol (μl)	Dose (p/kg)				X-gal staining	immuno
HE15†	53	200	4×10^8	9d	normal	normal	–	–
HE16†	53	200	4×10^8	9d	normal	vascular congestion in liver	–	–
IP18†	53	300	6×10^8	10d	normal	normal	–	–

H 2.3 Low level transgene expression is observed after ultrasound-guided umbilical vein injection of retrovirus vectors in mid and late gestation fetal sheep

We delivered retrovirus vectors to mid and late gestation fetal sheep by umbilical vein injection similar to previous experiments using adenovirus vectors (**Table H 2**). Sheep fetuses aged 76 days of gestation (n = 1, 8×10^8 particles) and 137 days of gestation (n = 2, 1×10^8 particles) received MLV vector containing the lacZ gene via the intrahepatic umbilical vein. There was no data available on the toxicity of polycation administration into the circulation of fetal sheep and we therefore complexed the retrovirus with the polycations DOGS (UV24) or DEAE dextran (RV1) in only two of the three experiments in an attempt to improve tissue transduction.

Only low level transgene expression was observed in the fetal tissues from the mid-gestation sheep on X-gal staining with one positively stained area on the umbilical cord, occasional stained villi in the distal small bowel, and pale diffuse staining of the neurocortex (data not shown). X-Gal staining of tissues from the late gestation fetuses showed moderate expression in the fetal heart but only low level expression in the fetal liver and kidney (**Figure H 3**). Vector complexation with DEAE dextran did not significantly improve transgene expression. X-gal staining was very faint on histological section (data not shown) and immunohistochemistry for β-galactosidase expression was negative in all three fetuses.

Table H 2: Ultrasound-guided umbilical vein injection of retrovirus vector.

Post mortem and histological findings, and transgene expression after ultrasound-guided injection of retrovirus (MLV) vectors into the intrahepatic umbilical vein of mid and late gestation fetal sheep. *indicates the vector was complexed with DOGS (5μg/ml); † indicates the vector was complexed with DEAE dextran (5μg/ml); PM: post mortem; d: days; p/kg: particles per kg

Sheep	Age (d)	Vector		PM	Post mortem findings	Histological findings	β-galactosidase expression	
		Vol (ml)	Dose (p/kg)				X-gal staining	immuno
UV24*	76	4	3.2×10^9	7d	small peritoneal adhesion	normal	(+)	–
RV1†	137	20	1.6×10^7	3d	normal	normal	+	–
RV2	137	20	1.7×10^7	3d	normal	normal	+	–

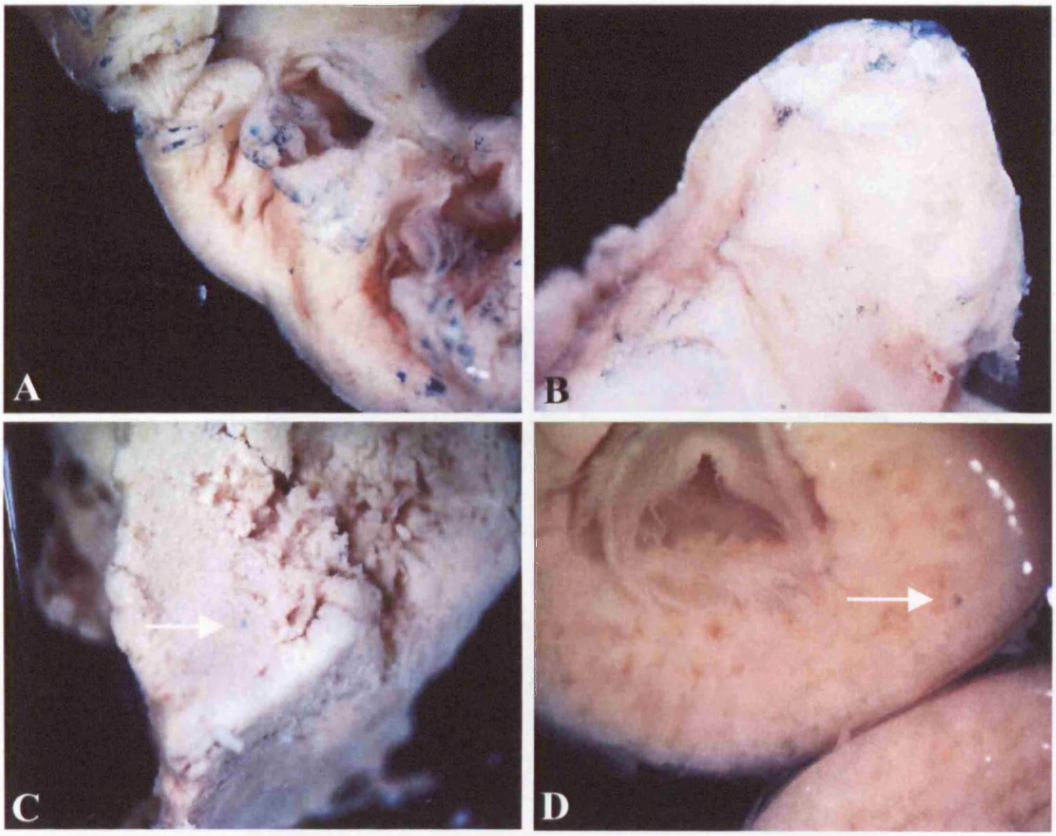


Figure H 3: β-galactosidase transgene expression after ultrasound guided umbilical vein delivery of retrovirus vectors.

Three days after UV injection of MLV vector in late gestation fetal sheep, low-level positive X-gal staining is seen in the fetal heart (A) and (B), liver (C), and kidney (D). All magnifications x 4.

Despite these disappointing results, these preliminary studies have shown that retrovirus transduction of fetal sheep tissues with MLV vectors is possible, albeit at low levels. Increasing titres may therefore allow better transduction.

H 3 Lentivirus vector mediated gene transfer to fetal sheep is poor

H 3.1 Early gestation fetal sheep liver can be transduced *ex vivo* by Equine Immune Anaemia Virus lentivirus vectors

The lentivirus vector, Equine Immune Anaemia Virus, (EIAV, 1 x 10^7 particles) containing the lacZ gene and pseudotyped with VSV-G protein was applied to the liver of a fetal sheep aged 53 days of gestation *ex vivo*. We also investigated the effect of complexing the vector with the polycations DEAE dextran (5μg/ml) or DOGS (5μg/ml) on liver transfection. Widespread scanty β-galactosidase expression was observed in subcapsular hepatocytes on X-gal staining 24 hours later, but only when the vector was complexed with DEAE dextran (**Figure H 4**).

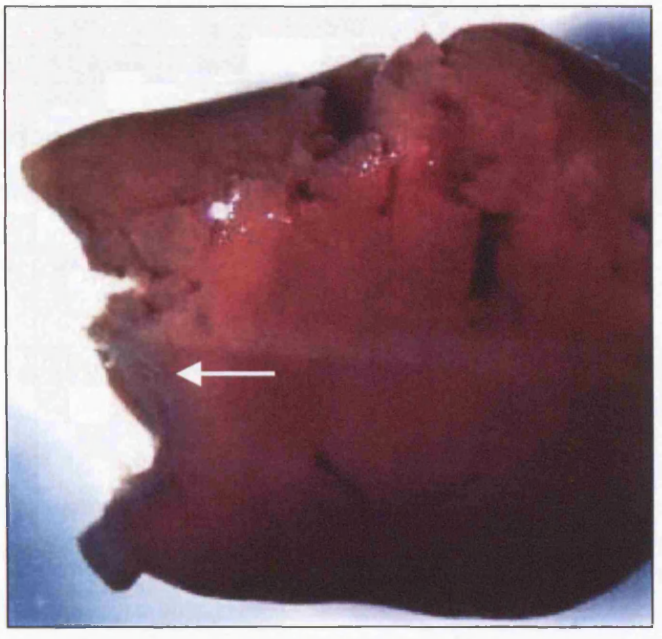


Figure H 4: Lentivirus-mediated β-galactosidase transgene expression in the early gestation fetal sheep liver.

Two days after *ex vivo* infection of a fetal sheep liver (55 days of gestation) with EIAV lacZ vector complexed with DEAE dextran (5μg/ml), positive X-gal staining of subcapsular hepatocytes is seen.

H 3.2 Ultrasound-guided injection of Equine Immune Anaemia and Human Immunodeficiency Virus lentivirus vectors to early gestation fetal sheep did not achieve transgene expression

We applied lentivirus vectors to early gestation fetal sheep by ultrasound guided intrahepatic (n = 2), intraperitoneal (n = 1) or intramuscular injection (n = 4, **Table H 3**).

Table H 3: Ultrasound-guided delivery of lentivirus vectors to early gestation fetal sheep.

HE: intrahepatic, IP: intraperitoneal, IM: intramuscular injection; † indicates the vector was complexed with DEAE dextran (5μg/ml); PM: post mortem; d: days; p/kg: particles per kg; nt: not tested

(A) Experimental details and transgene expression

Sheep	Age (d)	Vector					PM	β-galactosidase expression	
		Type	Pseudo type	Trans gene	Vol (μl)	Dose (p/kg)		X-gal staining	β-gal immuno
HE13†	55	EIAV	VSV-G	lacZ	100	2×10^9	9d	–	–
HE14†	53	EIAV	VSV-G	hFIX	100	2×10^9	3d	–	–
IP19†	53	EIAV	VSV-G	hFIX	200	6×10^9	10d	–	–
IM16	64	HIV	VSV-G	lacZ	100	9×10^7	10d	–	–
IM17	64	EIAV	VSV-G	lacZ	100	2×10^8	1d	nt	nt
IM18	60	EIAV	VSV-G	lacZ	200	1×10^8	11d	–	–
IM19	64	EIAV	Mokola	lacZ	300	4×10^7	13d	–	–

(B) Ultrasound, post mortem and histological findings

Sheep	Vector	PM	USS findings postop	PM findings	Histological findings
HE13†	EIAV	9d	ascites (+) 8d postop	abdominal wall oedema, 10ml ascites, mottled liver	normal
HE14†	EIAV	3d	alive 1d, dead 3d postop	abdominal wall oedema, 2ml ascites, no blood clot; Campylobacter jejuni cultured	fetal liver necrosis, acute inflammation uterus
IP19†	EIAV	10d	ascites +++ 6d postop	abdominal wall oedema, 10ml ascites, mottled liver	normal; normal fetal blood profile; culture negative
IM16	HIV	10d	normal	normal	normal
IM17	EIAV	1d	dead fetus	macerated fetus; Bacillus cereus cultured	necrosis, haemorrhage + bacteria
IM18	EIAV	11d	normal	normal	normal
IM19	EIAV	13d	normal	normal	normal

The Equine Immune Anaemia Virus (EIAV) vector was used in all but one experiment; we applied the Human Immunodeficiency Virus (HIV) vector in this case (IM16). The

vectors were pseudotyped with the VSV-G envelope apart from one experiment (IM19) in which the Mokola envelope was used.

Injection of EIAV VSV-G pseudotyped vector into the liver parenchyma or peritoneal cavity of early gestation fetal sheep resulted in significant fetal morbidity. In one case (HE14) the fetus was alive and well the day following surgery, but on the third postop day the ewe had vaginal bleeding and fetal death was confirmed by ultrasound scanning. This was an unusual finding, since in all other cases of fetal mortality that we observed in this research, death occurred within 24 hours of surgery. At post mortem examination there was skin oedema and a small amount of ascites, but no evidence of intrahepatic or intraperitoneal bleeding. Histological analysis showed acute inflammation of the uterus and Campylobacter jejuni was cultured in some fetal and maternal tissues. We considered this most likely to have been due to ascending infection of the genital tract after fetal death.

In the other two cases, ascites was noted on ultrasound examination 6 days (IP19, **Figure H 5 A**) and 8 days (HE13) after surgery respectively. In both cases the volume of ascites increased over the ensuing days and one experiment was terminated early because of its severity (HE13). Extensive subcutaneous oedema and ascites was found at post mortem examination of both fetuses and the liver was mottled (**Figure H 5 B and C**).

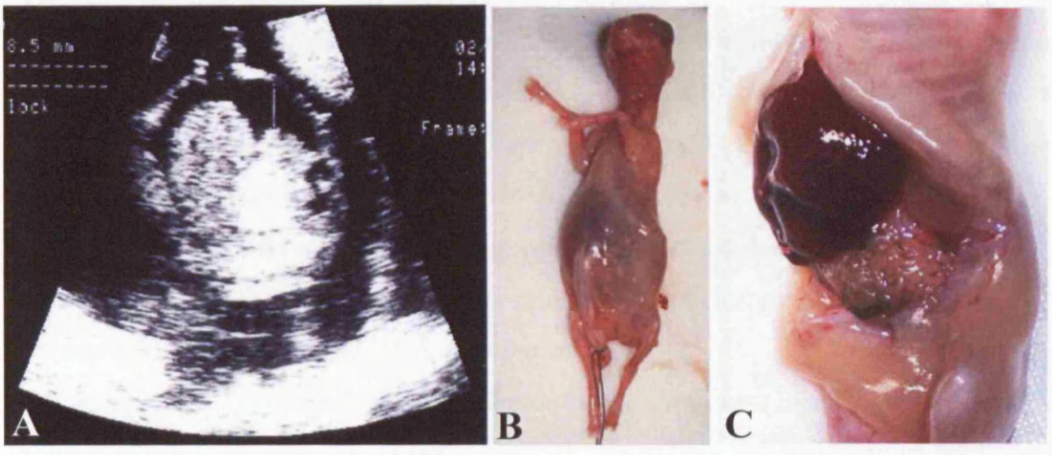


Figure H 5: Fetal morbidity following intraperitoneal or intrahepatic ultrasound guided injection of EIAV vector into early gestation sheep fetuses.

(A) An ultrasonogram of the fetus in transverse section 6 days after intraperitoneal injection (IP19) showing severe ascites, deepest pool of 8.5mm. (B) Subcutaneous oedema of the anterior abdominal wall and abdominal distension with ascites at post mortem examination 10 days after intraperitoneal injection (IP19). (C) Mottled liver and subcutaneous oedema of the anterior abdominal wall at post mortem examination 9 days after intrahepatic injection (HE13).

Histological examination however, showed no abnormality. Fetal blood sent from IP19 showed a normal blood film, blood count, biochemistry and liver function; the ascites was a transudate with low cellularity; culture of fetal blood and ascites was negative. The coagulation profile showed slightly prolonged Prothrombin Time and Activated Partial Thromboplastin Time that can be associated with inflammation. These results suggested that the vector was causing a severe inflammatory reaction that did not result in fetal haemolysis. In view of the delayed onset, the reaction might be immune mediated. No transgene expression was observed in any fetal tissues. This could be due to an inability of the vector to infect fetal cells *in vivo* or perhaps destruction of the vector by an inflammatory process.

In contrast to these findings, intramuscular injection of lentivirus vectors was associated with no fetal morbidity. One fetus (IM17) died within 24 hours of injection due to iatrogenic infection. Post mortem examination showed severe necrosis and haemorrhage of the skin and muscle of the injected left leg that extended in a line up across the abdomen. On histological analysis there was extensive haemorrhage and necrosis in the injected muscle, and bacilli on the surface of the femur. Culture of the fetal tissues showed a pure growth of Bacillus cereus, a known fleece commensal of sheep. This bacteria produces a haemolytic toxin which results in severe necrosis, and clinical infection is associated with immune suppression (Drobniewski FA, 1993).

There were no abnormalities detected on post mortem or histological analysis of the other three fetuses that received intramuscular injection. It is interesting to note that one fetus (IM18) received exactly the same vector as those fetuses that developed oedema and ascites after intrahepatic or intraperitoneal injection. The other two either received a vector with a different backbone (HIV, IM16) or pseudotype (Mokola, IM19). The intramuscular injection route therefore, may not have resulted in the same degree of systemic exposure to the vector, either because the myocytes were not infected or because there was local sequestration of the vector.

Although there was no inflammatory reaction to intramuscular injection of lentivirus vectors, no transgene expression was observed. We did not test infection of fetal myocytes *ex vivo* and it is possible that the vector is unable to infect these cells.

H 4 Sendai vector can mediate gene transfer to the airway epithelium of fetal sheep

H 4.1 Sendai viral vector efficiently transfects the fetal sheep airways *ex vivo*

The trachea from a non-injected sheep fetus aged 83 days of gestation was used to test Sendai vector mediated gene transfer *ex vivo*. Sendai lacZ vector (F-deficient, 5µl containing 5 x 10^5 particles) was applied to the epithelial airway surface of 2cm long sections of fetal trachea in culture medium and incubated for 24 hours. The tracheal sections were fixed in 100% ethanol and stained with X-gal solution for a further 24 hours. There was extensive staining of the epithelial cell surface (**Figure H 6**).

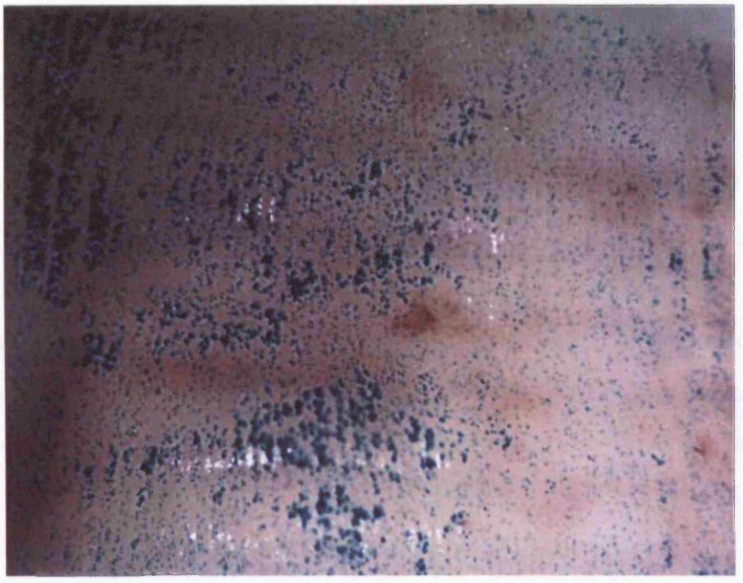


Figure H 6: β-galactosidase transgene expression in the fetal trachea after Sendai virus application *ex vivo*.

X-gal histochemistry 24 hours after application of Sendai lacZ vector *ex vivo* shows strongly positive staining. Original magnification x 6.5.

H 4.2 Sendai virus mediated transgene expression in the fetal sheep airways

We next applied Sendai lacZ vector to the tracheal airway epithelium of fetal sheep at mid-gestation (n = 2) using the ultrasound-guided transthoracic injection technique (**Table H 4**). The injection procedure was straightforward in both cases and details of the procedure are described in Section D 2.8.1.

Table H 4: Ultrasound-guided delivery of Sendai virus to the fetal sheep trachea.

Post mortem and histological analysis of mid-gestation fetal sheep lungs and trachea 2 days after transthoracic injection of Sendai lacZ vector. h: hrs; d:days; PM: time of post mortem after injection; GA: gestational age at injection; p/kg: particles per kg;

Sheep	GA (d)	PM	Vector dose (p/kg)	Post mortem findings	Histology findings
IT36	100	2 d	3×10^6 in 5ml PBS	200µl clear pleural fluid, thickened pericardium	normal lungs, trachea and pericardium; normal fetal blood profile
IT37	104	alive 24 h, dead 2 d	1.5×10^6 in 5ml PBS	no evidence of trauma, haemorrhage; cloudy, smelly amniotic fluid	bronchopneumonia + bacteria; scanty mixed growth on culture

Examination of the first fetus (IT36) two days after injection showed no abnormality apart from a small volume of clear pleural fluid. X-gal staining and immunohistochemical analysis for β-galactosidase showed no positive transgene expression. The batch of vector applied in this fetus had been frozen and thawed twice and its infection efficiency consequently may have reduced.

The experiment was repeated with a fresh batch of virus (IT37). Ultrasound examination of the fetus the day after injection showed no abnormality but at post mortem examination a day later the fetus was dead. There was no evidence that the injection procedure had caused fetal death. The findings of smelly and cloudy amniotic and tracheal fluids suggested infection that was confirmed as mixed growth on culture. Histological analysis of fetal tissues showed bronchopneumonia and numerous bacteria in the airways; only inflammation was present in the alveoli. We concluded that fetal death had probably been due to infection present in the vector or introduced during the procedure. No positive transgene expression was detectable on X-gal staining. It is likely however, that bacterial infection prevented gene transfer or resulted in the death of transduced cells.

H 4.3 Tracheal fluid does not affect *ex vivo* Sendai virus mediated transgene expression in the fetal sheep airways

With a limit on the amount of Sendai lacZ vector left available, we decided to investigate whether fetal tracheal fluid might affect Sendai vector mediated gene transfer to the fetal sheep trachea *ex vivo*. Sendai lacZ vector (F1 deficient, 5×10^5 particles) was incubated overnight at room temperature with tracheal fluid taken from a non-injected sheep fetus aged 83 days of gestation. Sections of the trachea from the

same fetus were then infected with this vector or with the same concentration of unincubated vector for 24 hours. After fixation with 100% ethanol, X-gal solution was applied for a further 24 hours. There was no difference in the level of lacZ expression observed and we concluded that fetal tracheal fluid did not affect *ex vivo* Sendai vector mediated gene transfer.

H 5 Discussion

Retrovirus and lentivirus vectors offer the advantage of being able to integrate into the genome and therefore can provide long-term gene expression. We hoped that application of an integrative vector to the fetal sheep would allow us to show proof of principle for tolerance after prenatal gene transfer. Studies in the fetal mouse and non-human primate have shown widespread and long-term gene transfer after application of both these vector groups. In the fetal sheep however, only studies of retrovirus mediated gene transfer have been reported.

We were unable to demonstrate gene transfer after intrahepatic or intraperitoneal application of amphotropic Moloney Leukemia Virus (MLV) retrovirus vectors to early gestation fetal sheep. Even in mid and late-gestation fetal sheep, umbilical vein delivery resulted in low level transgene expression that was not confirmed by immunohistochemistry. These results should be compared with the strong and widespread expression observed following umbilical vein application of adenovirus vectors (Themis M et al., 1999). Vector complexing with DOGS or DEAE dextran improved gene transfer *in vitro* but not *in vivo*.

Other investigators have similarly observed no evidence of gene transfer after instillation of MLV retrovirus producer cells into the amniotic cavity of early and mid-gestation fetal sheep (Galan HL et al., 2002). Low level gene transfer to the respiratory epithelium has been achieved after direct delivery of amphotrophic MLV vectors to the trachea of fetal sheep (Pitt BR et al., 1995). In addition low level gene transfer into hematopoietic cells was detected in 6 out of 8 early gestation fetal sheep that received an intraperitoneal injection of amphotrophic MLV vector supernatant containing the *neo*^{r} transgene at 57 – 67 days of gestation (Porada CD et al., 1998). The dose of vector that we applied (2×10^7 particles per fetus) was between one to three logs higher than the highest vector dose used in Porada's study and should have lead to widespread gene transfer. Furthermore Porada's group subsequently reported that application of higher doses of their MLV vector supernatant to the same dose as we used, resulted in improved levels of gene expression (Tran ND et al., 2000). Differences in the

experimental technique such as the route of injection or the gestational age at application, might account for the lack of transduction we observed.

Application of EIAV lentivirus vectors to early gestation fetal sheep did not achieve any gene transfer and was associated with significant morbidity and mortality. Post mortem, histological and biochemical analysis of fetuses suggested that the vectors caused a severe inflammatory reaction when they were applied directly to the liver or systemically by intraperitoneal injection. Intramuscular injection did not lead to fetal morbidity but this route of application results in more local delivery to the tissues. The lack of gene transfer we observed *in vivo* probably resulted from destruction of the vector and/or infected cells. Only one experiment was performed using HIV vector and it is therefore difficult to draw any conclusions.

We were surprised by our negative results following EIAV application, since *in vitro* studies in the fetal sheep liver demonstrated gene expression. Furthermore lentivirus application in other fetal animal models has achieved excellent gene tranfer. In the fetal mouse, long term transgene expression is seen up to a year after yolk sac vessel injection of the same EIAV vector we applied to the fetal sheep (Waddington SN et al., 2003c,Waddington SN et al., 2004b,Gregory LG et al., 2004), and intrahepatic or intramuscular injection of HIV vectors efficiently transduces hepatocytes and myocytes respectively (MacKenzie TC et al., 2002). Direct injection of HIV vectors into the lung parenchyma of fetal Rhesus monkeys leads to local gene transfer, with no evidence of toxicity or inflammation (Tarantal AF et al., 2001a). The different results highlight the difficulties in extrapolating between species and from small to larger animal models.

EIAV causes a self-limiting and lifelong but rarely fatal infection of all equidae resulting in plasma viraemia and recurrent thrombocytopenia (Rohll JB et al., 2003). The VSV-G envelope that was used to pseudotype the vector gives it a broad tropism permitting transduction of a wide range of tissue types (Mitrophanous K et al., 1999). The VSV-G protein is toxic to producer cells and it is possible that the toxicity we observed was due to the envelope rather than the vector itself.

Preliminary *ex vivo* results from application of a second-generation Sendai lacZ vector to the fetal sheep trachea were encouraging. Unfortunately this was not reflected by our *in vivo* experimental data, probably resulting from the use of a batch of poor quality vector. In the fetal mouse, this vector has been shown to achieve high levels of gene transfer to the respiratory epithelium after intra-amniotic application, and to the endothelium after intravascular administration (Waddington SN et al., 2004a). Further experiments with newer vector batches may give better results.

I Discussion

I 1 Fetal gene transfer can be achieved using minimally invasive clinically applicable delivery of gene therapy to the sheep fetus

For fetal gene therapy to be applied in clinical practice, delivery of the vector to the fetus will need to be acceptable to both patient and clinician, with a low morbidity and mortality. In this thesis we have used the fetal sheep as an animal model to explore ultrasound-guided delivery of fetal gene therapy. To target therapy to some fetal organ systems, we used existing techniques applied in current fetal medicine practice. For other organ systems, for example to reach the fetal airways, small bowel and brain however, we developed novel routes of application.

For many reasons the fetal sheep is a highly suitable animal model in which to explore ultrasound-guided injection techniques. Unlike in the majority of fetal medicine procedures, the animals were anaesthetized during injections, but it was possible to assess and follow fetal growth longitudinally with the ewes awake and sitting up. In addition, a lighter touch on the probe is required in sheep to prevent compression of the uterus and its contents because they have a thinner anterior abdominal wall musculature than humans. Maternal morbidity and mortality was almost negligible, similar to findings from ultrasound-guided procedures in clinical practice (Elchalal U et al., 2004). Fetal morbidity and mortality was dependent on the injection route applied, for example a higher rate of fetal loss was observed with the more invasive routes such as intraperitoneal and intrahepatic injection (80% and 81% respectively) which are more likely to cause life-threatening fetal trauma, than with less invasive routes such as intra-amniotic and intramuscular injection (86% and 91% fetal loss rate respectively). This is also observed in clinical practice (Alfirevic Z et al., 2003).

The placenta of the sheep and human are very different and this results in a different approach towards ultrasound-guided procedures. In the human fetus which has one discoid placenta, transplacental needling is avoided if at all possible because it is associated with a higher rate of complications, such as haemorrhage and miscarriage (Tabor A et al., 1986). In the fetal sheep, the cotyledons of the synepitheliochorial placenta cover the entire uterine wall so that there is no window through which to avoid transplacental needling. In one study of ultrasound-guided amniocentesis and

allantocentesis in the first trimester fetal goat that has a similar placental structure, penetration of a cotyledon in one of 13 injection procedures resulted in a bloody tap with subsequent fetal abortion (Lovell KL et al., 1995). During our ultrasound-guided injection procedures we avoided passing the needle through a cotyledon if at all possible to prevent associated complications. This was simple to achieve in early gestation when the amount of amniotic fluid relative to the fetus was higher. From 80 days of gestation onwards however, the cotyledons are at their maximum diameter (Kelly RW et al., 1987) and this, combined with the larger fetal size and low amniotic fluid volume resulted in the fetus being positioned directly against the anterior abdominal wall, making it occasionally impossible to avoid transgressing a cotyledon during needling. We did not observe any fetal or maternal complications associated with transplacentomal needling in these cases and concluded that transgressing a cotyledon probably did not significantly affect fetal survival.

Direct translation from the sheep to the human situation should be possible for most of the injection routes that we investigated, and in particular for the intragastric, intramuscular and intrahepatic injection routes that were developed in this thesis. For the transthoracic route into the trachea further data is needed on tracheal views in the human fetus. Obviously long term outcome data is not available for any of the routes that were developed and this is necessary prior to human application. The robustness of the fetal sheep for development of ultrasound guided procedures is supported by the recent translation of the transthoracic tracheal injection technique developed in fetal sheep into the human clinic. This was successfully used to puncture an occlusive balloon in the fetal trachea that had been placed fetoscopically a few weeks earlier for treatment of CDH (Deprest J et al., 2004).

There are few sheep models of human genetic disease and this lessens their usefulness in the development of fetal gene therapy. Efforts to produce transgenic sheep are progressing (Piedrahita JA, 2000). Taking cystic fibrosis as an example, there is close homology between ovine and human CFTR of almost 91%, which is significantly higher than that between murine and human CFTR (Harris A, 1997) and steps have begun to generate a CF sheep (Williams SH et al., 2003). In addition one group in New Zealand is looking for naturally occurring CFTR mutants that exist in heterozygote form for breeding programs and a potential CFTR mutation has been identified (Tebbutt SJ et al., 1996).

I 2 Delivery of gene therapy to the sheep fetus achieves levels of gene transfer that are useful for prenatal treatment of congenital disease

Adenovirus proved to be useful a pathfinder vector in this thesis, having a broad tissue tropism and efficiency of infection. In addition we were able to improve gene transfer to epithelia significantly by sodium caprate pretreatment and DEAE dextran complexation. Although it is unlikely that 1st generation adenovirus will be the vector of choice for clinical application of fetal gene therapy, it is important to know that barriers to gene transfer, for example the tight junctions in the epithelia, can be reversibly and safely overcome by coapplication of other agents. The safety of these agents must also be scrutinized rigorously before clinical application.

A major drawback of the fetal sheep animal model was the toxicity and lack of gene transfer following delivery of integrating vectors. We had hoped that long term transgene expression mediated by retrovirus or lentivirus vectors would allow us to test for immune tolerance to transgenic protein in lambs born after *in utero* injections. The reason for the lack of gene transfer is unclear but is very likely due in part to the toxicity observed. We are conducting further studies looking for evidence of integration of the viral backbone into the DNA from injected fetal sheep tissues.

I 3 Alternative fetal animal models for investigating ultrasound-guided delivery of fetal gene therapy

I 3.1 The fetal nonhuman primate

Nonhuman primates are, strictly speaking, the most appropriate animal model for comparison with the human because of the many similarities in developmental biology. They are close physiologically to humans with menstrual cycles of similar length and hormonal control, comparable cellular and endocrine processes of implantation, and similar timetables of prenatal and immune system development. The placental structure in some nonhuman primates is also the same, for example in the rhesus monkey the placenta is hemomonochorial and bidiscoidal (Benirschke K and Kaufmann P, 1990). For this reason they are used as an animal model in studies of teratology, developmental biology, infertility and contraception (Hendrickx AG and Peterson PE, 1997). Ultrasound guided injection techniques as used in fetal medicine have also been applied extensively in the fetal nonhuman primate with comparable results (Tarantal AF, 1990).

Currently available lentivirus and retrovirus vectors are effective at mediating gene transfer into fetal non-human primates and in this at present they have an advantage over fetal sheep.
However nonhuman primates are significantly more costly than sheep and are difficult to maintain because of the need for appropriate environments and intensive husbandry. There are also no non-human primate models of disease that would be suitable candidates for fetal gene therapy. More importantly perhaps, there are ethical concerns associated with experimentation on nonhuman primates that preclude all but the most necessary investigations. Prenatal gene therapy will require particularly stringent safety assessments. Non-human primates are the most appropriate animal model for this and for the monitoring of long-term efficiency of gene transfer.

I 3.2 The fetal mouse

Transgenic mouse models have proved extremely valuable for studying the efficacy of gene therapy in congenital diseases, for example cystic fibrosis and thalassaemia, where there are no natural animal models. Mice are small and are therefore easy and reasonably cheap to house. They breed quickly with large litters (6-20 pups) and consequently allow study of the effect of prenatal gene therapy on the adult animal, and analysis of trans-generational effects in a reasonably short time-frame. Integrating vectors provide long term gene expression in the fetal mouse and it is for this reason that mice are being used in our laboratory for initial studies into the effectiveness of *in vivo* gene transfer from novel vectors.
There are obvious differences in the size of mice and men that precludes their use for the development of minimally invasive techniques for future clinical application of gene therapy. There are major differences in placentation and in the development of the fetal immune system that in the neonatal mouse is relatively immature as compared with the human neonate. In the case of cystic fibrosis gene therapy, mice are not the ideal animal model. Submucosal glands, which are the site of the highest levels of CFTR expression in the human airways, are only present in very low numbers in the mouse and the main secretory cell type is the Clara cell (Pack RJ et al., 1981). Despite these reservations, fetal mice will continue to be used for first-line *in vivo* gene therapy experiments before moving on to larger animal models that are closer to the human situation.

I 3.3 Other fetal animal models

The rabbit has been studied in some prenatal gene therapy studies. Minimally-invasive

percutaneous ultrasound guided injection and fetoscopic procedures are being developed (Brandt et al., 1997a,Papadopulos NA et al., 1999,Papadopulos NA et al., 1999). Because of the small size of the fetus and litter number however, technically this is only possible from late gestation and the rabbit does not generally provide much advantage over the sheep. The guinea pig has the same placental structure as humans but these animals are not commonly used in prenatal gene therapy studies because of the small fetal size and lack of transgenic models of disease.

The dog is suitable for some minimally invasive delivery techniques and prenatal gene transfer has been attempted using ultrasound guided intraperitoneal or yolk sac injection through the exposed uterus (Lutzko C et al., 1999,Meertens L et al., 2002,Meertens L et al., 2002). In addition there are dog models of a few human congenital diseases including mucopolysaccharidosis type VII, Duchenne muscular dystrophy and haemophilia B, which are useful for investigating the therapeutic effect of gene therapy. Such colonies are costly to maintain however and probably do not provide much advantage over transgenic mice for proof of principle studies.

I 4 Ethical issues and the future of fetal gene therapy

Congenital disease places a huge burden on the community and the health service. A population based study found nearly 12% of pediatric hospitalizations in two US states were related to birth defects and congenital disease. The children stayed longer in hospital, incurred 184% higher charges and had a 4½ times higher rate of mortality than that of children hospitalized for other causes. In a narrower setting a study of pediatric inpatient admissions in 1996 in a US children's hospital found that wholly genetic conditions accounted for one-third of hospital admissions and for 50% of the total hospital charges for that year (McCandless SE et al., 2004). Thus a preventative strategy such as prenatal gene therapy will have an important social and economic impact.

A criticism levelled at fetal gene therapy is that gene transfer to an individual after it is born may be as effective and probably safer than prenatal treatment. Indeed current conventional treatment of some genetic disease is highly effective. One of the most successful examples is that of the metabolic disorder phenylketonuria, in which dietary restriction of phenylalanine prevents the brain damage associated with this condition. However the diet is unpalatable, rigid and must be lifelong, since discontinuation of it in adult life is accompanied by progressive loss of intellectual function and behavioural disturbance (Ding Z et al., 2003). For many genetic diseases treatment is palliative rather than curative, resulting in patients living longer but with a reduced quality of life.

This has been particularly seen in cystic fibrosis, in which life expectancy has risen from school age in 1955 to the mid thirties today (Cystic Fibrosis Foundation). To achieve this however, patients require daily chest physiotherapy, antibiotic treatment, dietary supplementation, insulin for diabetes mellitus and in many cases, lung transplants which require immunosuppressive therapy in addition. Effective treatment *in utero* could cure genetic disease, or at least provide partial correction that may have a huge impact on disease progression.

There are various ethical issues in relation to *in utero* gene therapy that need to be addressed before such therapy could be applied clinically (Fletcher JC and Richter G, 1996,Recombinant DNA Advisory Committee, 2000,Fletcher JC and Richter G, 1996). There is a theoretical risk that the therapeutic gene product or vector that is required at a certain stage during fetal development could cause developmental aberrations to occur. The problem of insertional mutagenesis as a potential risk of retrovirus gene therapy has been debated for some years. This serious adverse event has now been identified in a trial of gene therapy for X-linked severe combined immunodeficiency syndrome in which CD34+ haemopoietic stem cells were transduced *ex vivo* with the γc gene using retrovirus vectors. Two patients out of fifteen treated developed acute lymphoblastic leukemia (ALL) three years after successful gene therapy treatment. Analysis of the lymphocytes showed that the transgene had been inserted adjacent to a potential oncogene, LMO2, the product of which has been implicated in the pathogenesis of ALL (Juengst ET, 2003). Further work is needed to address this issue and to devise strategies to determine and possibly direct integration sites.

While one of the aims of prenatal gene therapy is to achieve immune tolerance to the transgene and delivery system, vectors must be designed to be sufficiently different to the wild type so that the immune system remains able to mount an effective immune response against wildtype virus infection. Germline transmission is another risk that raises ethical concerns and has been addressed previously.

Any fetal therapy or procedure poses risks of infection, immune reactions and the induction of preterm labour for the fetus. An additional challenge for procedures performed on the fetus is that there is the potential to harm the mother. A conflict of interest can arise since treating the fetus may not be in the mother's best interest and in law a fetus has no rights *per se.* Attempts have made to resolve this moral ambiguity using the concept of 'the fetus as a patient'. This aims to provide a moral framework within which the fetus is reliably expected later to achieve the status of becoming a child and eventually a person. 'The fetus as a patient' relies on the mother's decision to

continue with the pregnancy and to present the fetus for medical care (Chervenak FA and McCullough LB, 2002). However it should be emphasised that in many societies, presently the prospective mother has a right to abortion, even of a non-affected fetus, as well as the decision to abort an affected fetus or subject it to fetal gene therapy. The availability of fetal gene therapy should not in any way infringe her autonomy in these decisions. Currently used fetal treatments such as fetal blood transfusion for anaemia are effective and carry a low risk for the mother such that the risk-benefit analysis falls heavily on the side of treatment. For experimental fetal procedures the risk-benefit analysis is uncertain and it is therefore especially important that the mother gives informed consent (Burger IM and Wilfond BS, 2000). This can be difficult since the decision to participate in a fetal gene therapy trial will occur close to the time of prenatal diagnosis of the condition. The professionals involved in counselling the parents must present the information in a non-biased way and ensure that resources are set aside for long-term surveillance of the mother and fetus after birth. The parents must also consider that fetal treatment in this pregnancy may pose risks for a future pregnancy by potentially affecting the mother's health.

Currently many parents when faced with a baby with a genetic disease decide to terminate the pregnancy in a procedure that is very safe for the mother and totally effective. A prenatal gene therapy strategy will have to be extremely safe, reliable and effective at treating the disease (Coutelle C and Rodeck C, 2002). For parents who would not have continued with an affected pregnancy, a partial cure of an affected child resulting in a poor quality of life would be the worst case scenario, and we must not forget the first rule of medicine to 'do no harm'. It is possible that there would be an opportunity to test the fetus after gene therapy treatment to evaluate its effectiveness and to terminate should the prenatal gene therapy have failed. This presents a risk to the pregnancy but allows termination of pregnancy if no effective gene expression can be detected. Such a strategy was used recently in a case report of *in utero* stem cell transplanation for X-linked SCID in which a couple insisted on evaluation of stem cell engraftment following transplantation (Westgren M et al., 2002). Following intraperitoneal injection of fetal liver cells at 14 weeks of gestation, analysis of fetal blood at 24 and 33 weeks of gestation showed 10% and 50% chimerism confirming engraftment.

Assuming that a safe and effective prenatal gene therapy approach were to be possible, how might it work in practice? We could consider a hypothetical syndrome X, an autosomal recessive metabolic condition that results in certain brain damage in an

affected child with death by school age. Without an effective screening strategy with accurate prenatal diagnosis for syndrome X, many families would not know that they were at risk until an affected child was born. For the next pregnancy the parents would have five options: (1) take a 1:4 chance of having an affected baby, (2) prenatal diagnosis and continuation of or (3) termination of an affected child, (4) prenatal gene therapy of an affected child and (5) preimplantation genetic diagnosis (PGD) resulting in a pregnancy with an unaffected child. Option (4) would require diagnosis of syndrome X in the fetus prior to the gestational age for optimum gene therapy treatment probably by chorionic villus sampling. The mother would undergo the invasive procedure to treat the fetus and might have further invasive testing to confirm gene transfer later in the pregnancy. For the more common genetic disorders such as cystic fibrosis an effective and comprehensive prenatal screening policy would need to be implemented. Preimplantation genetic diagnosis (option 5) is often proposed as the most sensible option for parents at risk of having an affected fetus. This would require in vitro fertilization (IVF) to generate a number of embryos to the blastocyst stage when one or two cells (blastomeres) would be removed and tested for syndrome X. Only unaffected embyros would then be transferred back to the mother resulting in a pregnancy in which the fetus is not affected. The main limitations of IVF and PGD are the ovulation induction and invasive procedures that the woman is required to have, that only 20-30% of couples achieve a pregnancy per cycle (Wells D and Delhanty JD, 2001) and that some embryos will be disposed of which for some individuals is of concern (Snowdon C and Green JM, 1997).

One criticism levelled at prenatal gene therapy is a belief that couples pregnant with an affected child would be unlikely to proceed with prenatal gene therapy and would opt for a termination instead. There is almost no research in this area however and the views of the general public and patient groups need to be solicited as this technology comes closer to the clinic. The general public remains concerned that ethical discussion about issues such as gene therapy, cloning and the Human Genome Project are falling behind the technology (Brown P, 2000). It is therefore important to provide adequate information that will allow the public to understand the risks and benefits of these novel techniques and to enable an educated involvement in the decision-making process along with health professionals. This will also help individuals to give informed consent as these procedures become used in clinical practice.

I 5 Future work

This work has clearly demonstrated the value of the fetal sheep model for investigation of ultrasound guided delivery of prenatal gene therapy. We adapted and applied many techniques used in clinical fetal medicine practise, and for these routes such as umbilical vein, intraperitoneal, intrahepatic and intramuscular injection, further experiments will be performed when a suitable long-term integrative vector becomes available.

We also developed new injection methods to target organ systems that had previously not been reached using ultrasound guidance and more evaluation of these procedures is required. For the intraventricular route further work is needed to ascertain the reproducibility of this injection technique as well as to study its long term effect on the fetus and neonate. For the intragastric and intratracheal routes, the long term morbidity and mortality of the injection procedures should be studied, together with the effects of DEAE dextran, sodium caprate and perflubron. The earliest gestational age limit for safe intratracheal injection also needs to be better defined. The ultrasound view of the stomach in the sheep and human fetus is very similar so that it is likely that intragastric injection in the human fetus will be technically comparable. For intratracheal injection however, the shape and structure of the thorax and trachea in the sheep and human fetus differ considerably. Before clinical application can be considered the dimensions of the human fetal trachea and its relations to the great vessels need to be evaluated further in longitudinal section. We are currently undertaking such a study in the Fetal Medicine Unit at University College London and assessing how feasible transthoracic injection of the trachea would be. Having placed a balloon in the fetal trachea, future experiments are planned to ascertain whether occluding the trachea enhances adenovirus gene transfer further.

The lack of stem cell transduction in the intestinal crypts requires further investigation. Firstly to detect vector presence in the crypts and to determine whether the stem cells are transduced but unable to express the transgenic protein, we have sourced an adenovirus vector labelled with a fluorescent protein (EGFP) in its capsid (Le LP et al., 2004). We are also planning some *ex vivo* small bowel experiments to quantify the effect of luminal distension on gene transfer to the intestinal crypts.

Long term gene transfer by application of an integrative vector to the fetal sheep would allow us to show proof of principle for tolerance after prenatal gene transfer. Our results clearly show that EIAV lentivirus vectors pseudotyped with VSV-G protein are unable to mediate gene transfer in the early gestation sheep fetus. We are currently

investigating integration of the viral backbone in fetal tissues to determine whether the lack of gene transfer was related to viral infection of tissues. Pseudotyping with other envelope proteins such as those from the rhabdoviruses rabies and Mokola may be less toxic to the fetal sheep (Rohll JB et al., 2003). Retrovirus vectors have been successfully applied in the fetal sheep (Porada CD et al., 1998) but their use is limited by their inability to transduce non-dividing cells. Alternative lentivirus vectors based on feline, simian or bovine immunodeficiency viruses are available. In addition vectors based on Visna lentivirus (Berkowitz RD et al., 2001) or Jaagsiekte retrovirus (Liu SL et al., 2004) that are both naturally occuring sheep viral infections, are being developed and may be more suitable for use in the fetal sheep, although it should not be forgotten that the overall aim is for long-term integration into human cells for clinical application. Another strategy is to combine the high infectivity of adenovirus with the longer duration of expression from lentivirus in a hybrid vector. The adenovirus particles are used to infect cells and deliver the genes necessary to produce a lentivirus vector containing the required transgene for a short time, that can then transduce the surrounding cells (Kubo S and Mitani K, 2003). Such adeno-lentivirus hybrid vectors are being constructed by our team and may be available for use in the fetal sheep after testing in the fetal mouse.

Appendix 1: General Chemicals and Reagents

acetic acid	BDH Laboratory Supplies, Poole, UK
agarose low melting point (LMP)	Gibco BRL, Life Technologies, Paisley, Scotland
agarose ultrapure electrophoresis grade	Gibco Gibco BRL, Life Technologies, Paisley, Scotland
amoxycillin trihydrate	Norbrook Laboratories Ltd, Newry, NI
benzylalcohol	Sigma Chemical Co, St Louis, MD, USA
benzyl benzoate	Sigma Chemical Co, St Louis, MD, USA
bromophenol blue	Sigma Chemical Co, St Louis, MD, USA
caesium chloride	Sigma Chemical Co, St Louis, MD, USA
calcium chloride	BDH Laboratory Supplies, Poole, UK
chloroform	BDH Laboratory Supplies, Poole, UK
Chronogest® (flugestone acetate)	Intervet UK Ltd, Milton Keynes, UK
dexamethasone	Sigma Chemical Co, St Louis, MD, USA
dimethylsulfoxide (DMSO)	Sigma Chemical Co, St Louis, MD, USA
dithiothreitol (DTT)	Sigma Chemical Co, St Louis, MD, USA
100 bp DNA ladder	Gibco BRL, Life Technologies, Paisley, Scotland
1Kb DNA ladder	Gibco BRL, Life Technologies, Paisley, Scotland
DNase	Gibco BRL, Life Technologies, Paisley, Scotland
ethanol	BDH Laboratory Supplies, Poole, UK
ethidium bromide	Sigma Chemical Co, St Louis, MD, USA
ethylenediaminetetraacetic acid (EDTA)	BDH Laboratory Supplies, Poole, UK
Folligon® (Pregnant Mares Serum Gonadotrophin)	Intervet UK Ltd, Milton Keynes, UK
formaldehyde	BDH Laboratory Supplies, Poole, UK
gelatine	Merck KGaA, Darmstadt, Germany
glutaraldehyde	Sigma Chemical Co, St Louis, MD, USA
glycerol	BDH Laboratory Supplies, Poole, UK
halothane	Merial, Essex UK
Heptavac-P plus®	Hoechst Roussel Vet Limited, Dublin
human Factor IX	Haematologic Technologies Inc, Vermont, USA
hydrochloric acid	BDH Laboratory Supplies, Poole, UK

hydrogen peroxide	Fluka, Sigma Aldrich, Switzerland
insulin	Sigma Chemical Co, St Louis, MD, USA
isopropranol	BDH Laboratory Supplies, Poole, UK
L-glutamine (tracheal culture Lisa)	Gibco BRL, Life Technologies, Paisley, Scotland
magnesium chloride	Sigma Chemical Co, St Louis, MD, USA
methanol	BDH Laboratory Supplies, Poole, UK
mineral oil	Sigma Chemical Co, St Louis, MD, USA
NCTC-135	Sigma Chemical Co, St Louis, MD, USA
dNTPs set (ultrapure)	Amersham Pharmacia Biotech Inc
orange G dye	BDH Laboratory Supplies, Poole, UK
ortho-phenylenediamine	Sigma Chemical Co, St Louis, MD, USA
paraformaldehyde	BDH Laboratory Supplies, Poole, UK
pentobarbitone	Rhône Merieux, Essex UK
PCR primers of the AdRSVßgal virus (first round and nested)	Genosys Biotechnologies Ltd, Pampisford
PCR primers of the human factor IX transgene (first round and nested)	Genosys Biotechnologies Ltd, Pampisford
PCR primers of the endogenous sheep sequence 3`UT of scavenger receptor class B type 1 (SRB1)	originally provided by Dr Jill Maddox, Centre for Animal Biotechnology, University of Melbourne, Australia
phenol (equilibrated to pH 7.9)	Sigma Chemical Co, St Louis, MD, USA
potassium ferricyanide	Sigma Chemical Co, St Louis, MD, USA
potassium ferrocyanide	Sigma Chemical Co, St Louis, MD, USA
povidone iodine antiseptic solution	Grampian Pharmaceuticals Ltd, UK
procaine hydrochloride	Arnolds Veterinary Products Ltd, UK
progesterone sponges	Intervet UK Ltd, Milton Keynes, UK
proteinase K	Gibco BRL, Life Technologies, Paisley, Scotland
Ram Raddle®	Net Tex Agricultural Ltd, Kent, UK
Rnase	Gibco BRL, Life Technologies, Paisley, Scotland
normal saline (0.9% w/v) sodium chloride	Martindale Pharmaceuticals, Essex UK

sodium acetate	BDH Laboratory Supplies, Poole, UK
sodium chloride	BDH Laboratory Supplies, Poole, UK
sodium hydroxide	BDH Laboratory Supplies, Poole, UK
sodium thiopentone	Merial, Essex UK
sodium dodecyl sulphate (SDS)	Sigma Chemical Co, St Louis, MD, USA
sodium phosphate (anhydrous and monohydrate)	Sigma Chemical Co, St Louis, MD, USA
sucrose	BDH Laboratory Supplies, Poole, UK
sucrose buffer	Sigma Chemical Co, St Louis, MD, USA
sulphuric acid	Sigma Chemical Co, St Louis, MD, USA
Super Ewe & Lamb UFAS compound feed	J & W Atlee Ltd, Dorking, Surrey, UK
Taq DNA polymerase	Sigma Chemical Co, St Louis, MD, USA
Transfectam® Reagent (DOGS, dioctadecylamidoglycyl spermine)	Promega Corp., San Luis Obispo, CA, USA
tris (hydroxymethyl) methylamine	BDH Laboratory Supplies, Poole, UK
trypsin	Difco Laboratories, Detroit, USA
ultrasound coupling medium	Electro Medical Supplies Ltd, Oxon UK
X-gal (5-bromo-4-chloro-3-indoyl-β-D-(galactopyranoside)	Melford Laboratories Ltd, Ipswich, UK
xylene	Genta Medical, York, UK

Mammalian cell lines

293 cells	human embryonic kidney
NIH 3T3 cells	mouse fibroblast
HeLa cells	human cervical epithelial
HT 1080 cells	human fibrosarcoma
HBE	human bronchial epithelial

Tissue culture media and equipment

100mm x 20mm cell culture dishes	Corning Inc, New York, USA
Nunclon™ 75 cm3 triple flasks	Nalge Nunc International, Denmark
75 cm^2 cell culture flask	Corning Inc, New York, USA
6 well cell culture cluster plates	Corning Inc, New York, USA
24 well cell culture cluster plate	Corning Inc, New York, USA
bovine serum albumin	Sigma Chemical Co, St Louis, MD, USA
Dulbecco's Modified eagle media (D-Mem) with glutamax-1, glucose and pyridoxine	Gibco, Invitrogen Corporation, Paisley, Scotland
fetal calf serum (FCS)	Gibco, Invitrogen Corporation, Paisley, Scotland
penicillin/streptomycin (10,000 Units/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate in 0.85% saline	Gibco, Invitrogen Corporation, Paisley, Scotland
cell culture dish 100mm x 20mm	Corning Inc, New York, USA
1 x phosphate buffered saline (PBS) without calcium, magnesium and sodium bicarbonate	Gibco, Invitrogen Corporation, Paisley, Scotland
trypsin–EDTA	Gibco, Invitrogen Corporation, Paisley, Scotland

Commercial kits:

β-galactosidase ELISA kit	Roche Diagnostics GmBH, Mannheim, Germany
First-Strand cDNA synthesis kit	Amersham Pharmacia Biotech Inc, Bucks
Asserachrom IX ELISA kit	Diagnostica Stago, Asnières-sur-Seine, France
Wright-Giemsa stain	Shandon Inc, Pittsburgh, PA
bicinchoninic acid (BCA) protein assay kit	Pierce, Rockford, USA

Histology and immunohistochemistry reagents and equipment

mouse anti-βgalactosidase antibody (primary antibody for β-gal immuno)	Promega, Madison, USA
biotinylated goat anti-mouse antibody (secondary antibody for β-gal immuno)	Dako, Cambridge, UK
rabbit anti-human factor IX antibody (primary antibody for human FIX immuno)	Dako, Cambridge, UK
swine anti-rabbit antibody (secondary antibody for human FIX immuno)	Dako, Cambridge, UK
mouse anti-BrdU antibody (primary antibody for BrdU immuno)	Dako, Denmark
rabbit anti-mouse antibody (secondary antibody for BrdU immuno)	Dako, Denmark
5% non-immune serum	Dako, Cambridge, UK
avidin	egg white
biotin	skimmed milk
peroxidase-labelled streptavidin	Rôche, Mannheim, Germany
diaminobenzidine tetrahydrochloride	Sigma Chemical Co, St Louis, MD, USA

Enzyme-linked immunosorbent assay (ELISA) equipment and reagents

cross-reactive goat anti-adenovirus antibody	Skybio, Bedford, UK
horseradish peroxidase labelled rabbit anti-sheep IgG	DAKO, Cambs, UK
cross-reactive goat anti-human factor IX antibody	Skybio, Bedford, UK
cross-reactive sheep anti-β-galactosidase antibody	Biogenesis, Poole, UK

Appendix 2: Solutions

1 litre 0.5 M EDTA (pH 8.0)

186.1 g	186.1g $Na_2EDTA.2H_2O$
approximately 50ml	adjust pH to 8.0 with 10 M sodium hydroxide
make up to 1 litre total volume	distilled water

1 litre 0.1 M Tris HCl (pH 8.0)

12.1g	tris base
58.4ml	hydrochloric acid
make up to 1 litre total volume	distilled water

Tris-EDTA (TE) buffer pH 8.0 (100ml)

200µl	0.5 M EDTA (pH 8.0)
10ml	0.1 M Tris HCl (pH 8.0)
make up to 100ml total volume	distilled water

Proteinase K Buffer (500ml)

25ml	1 M Tris pH 8.0
100ml	5 M EDTA
10ml	5 M NaCl
5ml	10% SDS
360ml	distilled water

500ml 50x Electrophoresis (TAE) buffer pH 8.0

121g	Tris
50ml	0.5M EDTA
28.5ml	acetic acid
make up to 500ml total volume	Distilled water

DNA gel loading buffer

30% v/v	glycerol
0.25% w/v	bromophenol blue

PCR gel loading buffer

30% v/v	glycerol
0.25% w/v	orange G

Fix solution for cell culture

400ml	PBS
10ml	formaldehyde
32ml	glutaraldehyde

Caesium chloride solutions for adenovirus purification

Heavy solution (density = 1.40g/ml)	62 g CsCl in 100ml 10mM Tris pH 8.0
Medium solution (density = 1.34g/ml)	51.20g CsCl in 100ml 10mM Tris pH 8.0
Light solution (density = 1.25g/ml)	36.16g CsCl in 100ml 10mM Tris pH 8.0

2x sucrose buffer (100ml) for adenovirus purification

0.24g	Tris
200µl	1M $MgCl_2$
1.75g	NaCl
68.46g	sucrose

Sucrose buffer (5l) for adenovirus purification

6g	Tris
0.5g	$MgCl_2$
43.8g	NaCl
1711.5g	Sucrose
250mg	10% Tween-80

4% paraformaldehyde in PBS pH 7.4 (500ml)

0.71g	Anhydrous Na_2HPO_4
0.69g	Monohydrate Na_2HPO_4
4.5g	NaCl

Appendix 3: Post mortem form

Date: **Animal No:** **Code:**

Fetal measurements: Fetal Weight:
Crown Rump Length: Femur Length:
Biparietal Diameter: Abdominal Circumference:
Occipito-snout Length:
Post mortem findings:

Photographs taken:

Bloods taken:	Maternal Serum	Maternal Plasma	
	Fetal Serum	Fetal Plasma	
Fetal Blood Count	Fetal Biochemistry/LFT	Fetal Clotting	(RVC)
Fluids taken:	Gastric fluid (**GC**)	Frozen	Fresh
	Tracheal fluid (**TF**)	Frozen	Fresh
	Bronchoalveolar lavage (**BALF**)		Fresh

Other tissues taken:

Tissue samples to be collected from the **MOTHER:**

	code	frozen/formalin
Blood	**E-B**	
Lung	**E-AU**	
Heart	**E-H**	
Ovary	**E-G**	
Brain	**E-NC**	
Liver	**E-L**	
Adrenal	**E-D**	
Kidney	**E-K**	
Spleen	**E-X**	
Thymus	**E-T**	

Tissue samples to be collected from the **FETUS:**

		code	frozen/formalin
0.)	**Placenta**	**P**	
	Umbilical cord	**U**	
	Amniotic Fluid	**F**	
1.)	**Blood (serum & plasma)**	**B**	
	Bone Marrow	**W**	
2.)	**Buccal Cavity/Oesophagus**		
	Soft palate	**O-P**	
	Tongue, lower part	**O-T**	
	Oesophagus, upper part	**O-U**	
	at diaphragm	**O-L**	
3.)	**Airways and Neighbouring Organs**	wt: (trachea & lung)	
	Thyroid wt:	**Y**	
	Thymus wt:	**T**	
	Epiglottis	**A-E**	
	Vocal cords	**A-L**	
	Trachea at 3 levels towards bifurcation	**A-T1**	**A-T2** **A-T3**

	Pleura visceralis		**A-A**
	Bronchial tree, samples from both sides		**A-BR1 A-BL1**
	Blocks from each lobe of the lung		**A-UR1 A-UR2 A-UR3**
	(upper = 1, middle = 2, lower = 3)		**A-UL1 A-UL3**
	Diaphragm		**I**
4.)	**Cardiovascular System**		
	Aorta		**H-A**
	Pericardium		**H-C**
	Pulmonary artery		**H-P1**
	Blocks from each chamber of the heart		**H-LA H-LV**
	(atrium = 1, ventricle = 2)		**H-RA H-RV:**
		wt:	
5.)	**Gastro Intestinal System and Neighbouring Organs**		
	Pancreas		**C-P**
	Stomach, Rumen		**C-A**
	Reticulum		**C-B1**
	Abomasum		**C-B2**
	Omasum		**C-B3**
	Duodenum		**C-D**
	Jejunum		**C-J**
	Small Bowel at 3 levels		**CS-1 CS-2 CS-3**
	Caecum		**C-C**
	Colon transversum		**C-T**
	Rectum		**C-R**
	Spleen	wt:	**X**
6.)	**Liver**	wt:	
	Portal system		**L-P**
	Area where hepatic vein joins vena cava		**L-H**
	Gall Bladder	wt:	**L-G**
	Blocks from each lobe of the liver		**L-R L-C L-L**
7.)	**Gonads**	wt:	**G Female Male**
8.)	**Urinary Tract & Neighbouring Organs**		
	Adrenal glands	wt:	**D-L D-R**
	Kidney	wt:	**K**
	Bladder		**Z**
9.)	Muscle		**M**
	<u>or</u> where muscle taken in parts		**Mu-R1 Mu-R2**
			Mu-L1 Mu-L2
10.)	Skin		**S**
	Abdominal wall		**AW**
11.)	**Brain & Neighbouring Organs**		
	Cortex		**N-C**
	Medulla oblongata		**N-M**
	Basal ganglia		**N-B**
	Pituitary gland		**N-P**
	Nasal sinuses		**A-N**
	Skull		**S-K**

Appendix 4: Publications

Original Papers

Peebles D, Gregory LG, David A, Themis M, Waddington SN, Knapton HJ, Miah M, Cook T, Lawrence L, Nivsarkar M, Rodeck C, Coutelle C
Widespread and efficient marker gene expression in the airway epithelia of fetal sheep after minimally invasive tracheal application of recombinant adenovirus *in utero*.
Gene Therapy 2004;**11**:70-8

David AL, Peebles DM, Gregory L, Themis M, Cook T, Coutelle C, Rodeck CH
Percutaneous ultrasound-guided injection of the trachea in fetal sheep: a novel technique to target the fetal airways.
Fetal Diagnosis and Therapy 2003;**18**:385-390

David A, Cook T, Waddington S, Peebles D, Nivsarkar M, Knapton H, Miah M, Dahse T, Noakes D, Schneider H, Rodeck C, Coutelle C, Themis M
Ultrasound guided percutaneous delivery of adenovirus vectors encoding the β-galactosidase and human factor IX genes to early gestation fetal sheep *in utero*.
Human Gene Therapy 2003;**14**:353-364

David AL, Peebles DM, Gregory L, Waddington SN, Themis M, Weisz B, Ruthe A, Perocheau D, Lawrence L, Cook T, Rodeck CH and Coutelle C Reporter gene delivery to the fetal gut by ultrasound-guided gastric injection towards prenatal prevention of cystic fibrosis intestinal pathology.
Submitted

David AL, Weisz B, Roubilova X, Perocheau D, Themis M, Cook T, Coutelle C, Deprest J, Rodeck CH and Peebles DM
Ultrasound-guided tracheal occlusion using a detachable inflatable balloon in the fetal sheep model.
Submitted

Review Articles

David AL, Themis, Rodeck C

Fetal gene therapy: the present and the prospects.

Yearbook of Obstetrics and Gynaecology 2004, RCOG Press, London

David AL, Themis M, Waddington SN, Gregory L, Buckley SMK, Nivsarkar M, Cook T, Peebles D, Rodeck CH, Coutelle C

The current status and future direction of fetal gene therapy.

Gene Therapy and Molecular Biology 2003;**7**:181-209

Coutelle C, Themis M, Waddington S, Gregory L, Nivsarkar M, Buckley S, Cook T, Rodeck C, Peebles D, David A

The hopes and fears of in utero gene therapy for genetic diseases – a review.

Placenta 2003;**24**:S114-S121

David AL, Themis M, Cook T, Coutelle C, Rodeck C

Fetal gene therapy.

The Ultrasound Review of Obstetrics & Gynaecology 2001;**1**:14-27

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