Novel Cell Transplantation Strategies for the Treatment of Degenerative Retinal Disease

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ABSTRACT

Retinal degenerations, both hereditary and acquired, are major causes of visual morbidity world-wide. Two of the principal disease categories are age-related macular degeneration (AMD) and retinitis pigmentosa (RP). AMD is the major cause of blind registrations in the over 65 age group in the western world and retinitis pigmentosa has an incidence of 1:3500 but, significantly, affects much younger patients. As yet there are no treatments available for these diseases that are either definitively preventative or curative.

Retinal cell transplantation is one treatment strategy that has been proposed for such patients: retinal pigment epithelium (RPE) grafts for AMD and neural retina for retinal dystrophies. The genetic heterogeneity of retinitis pigmentosa and the lack of understanding of the key pathobiological mechanisms in AMD make treating the final common pathway with retinal cell transplantation an attractive option. To date results in humans have not been encouraging, highlighting the need for further studies in animal models in terms of visual function, the basic cellular and molecular mechanisms involved and exploration of other potential cell types for transplantation.

This thesis examines the potential of several different donor cell types, both immortalised allogeneic (LD7.4) and xenogeneic (human: h1RPE7 & ARPE19) RPE cell lines for treating retinal degeneration in the dystrophic Royal College of Surgeons (RCS) rat. The dystrophic RCS rat has a primary RPE defect resulting in failure of rod outer segment ingestion and subsequent secondary photoreceptor degeneration. Additionally, syngeneic neonatal Schwann cells were transplanted into the subretinal space of the rhodopsin knockout (Rho -/-) mouse, a knockout model that never elaborates outer segments.

Dystrophic RCS rats (21 days old) received subretinal grafts of immortalised allogeneic (LD7.4) RPE cell lines in non-immunosuppressed rats or immortalised xenogeneic (h1RPE-7 or ARPE19) cell lines in immunosuppressed rats. Appropriate sham control injections were delivered to age-matched groups of rats. The animals' visual function was assessed longitudinally using a head-tracking system at 16, 20, and 26 weeks of age. Retinal sensitivity maps were produced for selected animals from each group at 20 weeks (LD 7.4 only) and 26 weeks (h1RPE7 and LD7.4 cell). Further groups of animals received grafts of ARPE19 or LD7.4 cells pre-labelled with bromodeoxyuridine (BrdU) in order to identify donor cells *in vivo*. Another labelling technique, transfecting LD7.4 cells with Human Foamy Virus (HFV) expressing green fluorescent protein (GFP), was also tested.

Syngeneic neonatal Schwann cells were grafted to the Rho -/- mouse at 5 weeks of age, prior to total photoreceptor loss and analysed for functional (electroretinogram, ERG) and histological rescue at 10 weeks.

Each cell type demonstrated behavioural, functional and morphological rescue in the RCS rat model. In the human cell-BrdU labelled group (i.e. with immunosuppression), grafted cells were seen integrated between host RPE cells on Bruch's membrane in association with areas of photoreceptor rescue up to five months post-grafting. In contrast, animals that received allografted cells prelabelled with BrdU (without immunopuppression) were not detectable on the host Bruch's membrane immunohistochemically by 4 weeks post-transplantation. Even though no donor cells were detectable, host visual function and photoreceptors were preserved. This result was repeated in the LD7.4/GFP labelled cell group.

The Schwann cell grafted Rho -/- mice showed significant photoreceptor preservation over sham-injected animals at 10 weeks of age.

These results demonstrate a number of key points regarding cell transplantation strategies for retinal degeneration. In some circumstances it is possible to achieve integration of grafted RPE cells on host Bruch's membrane with preservation of photoreceptors and visual function in the RCS rat. Allografts of immortalised RPE cells do not appear to survive in the subretinal space of the RCS rat beyond 4 weeks. Schwann cell transplantation to two different rodent models (RCS rat and Rho -/- mouse) preserved visual function in the former and morphology in both groups.

Cell transplantation for retinal degeneration is an exciting prospect for treating these diseases, RPE transplantation for AMD or primary RPE disease and Schwann cell transplantation for hereditary retinal disease (RP). Before these strategies become clinically useful a number of issues need to be addressed including integration of the grafted material with the host, appropriate functioning of the grafted tissue in the subretinal space (SRS) and long-term survival of the transplants.

Chapter 1

INTRODUCTION

Blinding disorders that stem from the degeneration of photoreceptor cells in the retina affect at least 50 million individuals worldwide. There is currently no effective treatment for most of these conditions, including age-related macular degeneration (AMD) or retinitis pigmentosa (RP). One approach that has been explored is to transplant cells to replace defective ones. The excitement that accompanied the first studies showing the potential of retinal cell transplantation to alleviate the progress of blindness in such diseases as AMD and RP has diminished as attempts to apply research to the clinic have failed, so far, to maintain or restore vision. What these studies have shown, however, is not that the approach is flawed, but rather that the steps that need to be taken to achieve a viable clinical treatment are many. This thesis details a number of experiments conducted to address some of the deficiencies and to introduce alternative cell transplants as potential therapies for hereditary degenerations.

The first section of the introduction describes retinal anatomy; the second outlines some of the principal retinal degenerative diseases and the third discusses some of the animal models of retinal degeneration. Section four describes established and experimental therapies for AMD and for some of the inherited degenerative diseases such as RP. Lastly, the immune status of the subretinal space is discussed.

The ultimate goal of the work is to improve graft survival and function and to develop transplant protocols that are effective in the clinical setting.

1.1 Retinal Anatomy

1.1.1 Retinal Pigment Epithelium

The retinal pigment epithelium (RPE) is a monolayer of post-mitotic cells (Fig 1.1A) that play an important role in vision and in the maintenance of ocular immune

Figure 1.1: Retinal Pigment Epithelium

A) Schematically illustrates the retinal pigment epithelium. The RPE cells lie between the neural retina and Bruch's membrane. These cells have apical microvilli (7-10 μm) that engulf shed photoreceptor outer segments. As the RPE cells form a crucial part of the outer blood retinal barrier there are numerous junctions including tight junctions (shown: apical zonula occludens), adherens junctions (zonula adherentes) and gap junctions (on the baso-lateral lateral membrane). The short (1μm) basal infoldings are responsible for attachments to Bruch's membrane through integrin expression. B) Intracellular reactions of retinoid metabolism in the RPE cells. The RPE cell recycles rhodopsin. The initial conversion (following photon stimulation) from 11-*cis* to all-*trans* retinal triggers this recycling process. The molecules are transported from the photoreceptor outer segment to the RPE cell as all-*trans* retinol via the inter-photoreceptor matrix binding protein (IRBP). Through a series of enzymatic processes within the RPE cell, 11-*cis* retinal is shuttled back to the outer segment to be combined with an opsin protein to reform rhodopsin.

1.1.2 Neural Retina

The neural retina, shown schematically in Fig 1.2B and in transverse section in Fig 1.2 C, D, is an extension of the central nervous system. It converts photic energy into neural activity (phototransduction) as the initial step in image formation. This process occurs in the outer retina in photoreceptors (cones and rods) with recycling of the photopigment by retinal pigment epithelium (Bok, 1993). The inner retina is responsible for initial visual processing involving bipolar cells, amacrine cells, horizontal cells and ganglion cells prior to transmission through the optic nerve to visual centres in the brain.

The gross retinal structure (Fig 1.2C) consists of three nuclear layers: outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL). Synapses are made between and within these layers in the plexiform layers (inner, IPL and outer, OPL) allowing vertical and horizontal transmission of signals in the retina (Fig 1.2B)

Photoreceptors are composed of two cell types, rods (highly sensitive, low resolution) and cones (low sensitivity, high resolution, variable photopigment, and greater wavelength range). Both cell types have an outer and inner segment and their nuclei are located in the outer nuclear layer (Fig 1.2C,D). The outer segment is the site of phototransduction and photoreactive proteins (opsins) are embedded in lipid membranes (discs), which are separate in rods and invaginated in cones. There is one rod photopigment (rhodopsin) made up of retinal (a vitamin A derivative) and an opsin. Cone photopigments are one of three distinct types depending on the attached opsin protein (blue, green or yellow) with maximal sensitivity respectively for those three wavelengths. These discs are renewed following diurnal shedding into the subretinal space (Hogan *et al.*, 1972); maximal rod disc shedding occurs approximately one hour after waking.

Late histological changes in CNV include sub-RPE fibrosis, RPE hyperplasia and basal laminar deposits. The scar is located in the SRS and intra-Bruch's region (e.g. see 1.5D). The scars may be vascularised from the choroid and occasionally from retinal vessels (Green and Enger, 1993). These scars lead to clinical problems as a result of exudative leakage with serous retinal detachment and/or haemorrhage with subsequent destruction of the photoreceptors.

1.2.2 Hereditary Dystrophies and Related Diseases

Retinitis pigmentosa (Fig 1.10A) describes a group of heterogenous inherited diseases; the majority of mutations target various molecular entities with photoreceptors, of which the following list represents common examples.

- Rhodopsin
- Peripherin/rds
- ROM-1
- α-Phosphodiesterase
- β- Phosphodiesterase
- α-subunit of rod outer segment gated channel
- Myosin VII A (Usher 1B)
- GTP-ase regulator (RP3)
- α-toco-pherol-transfer protein (secondary vitamin E deficiency leading to photoreceptor degeneration)

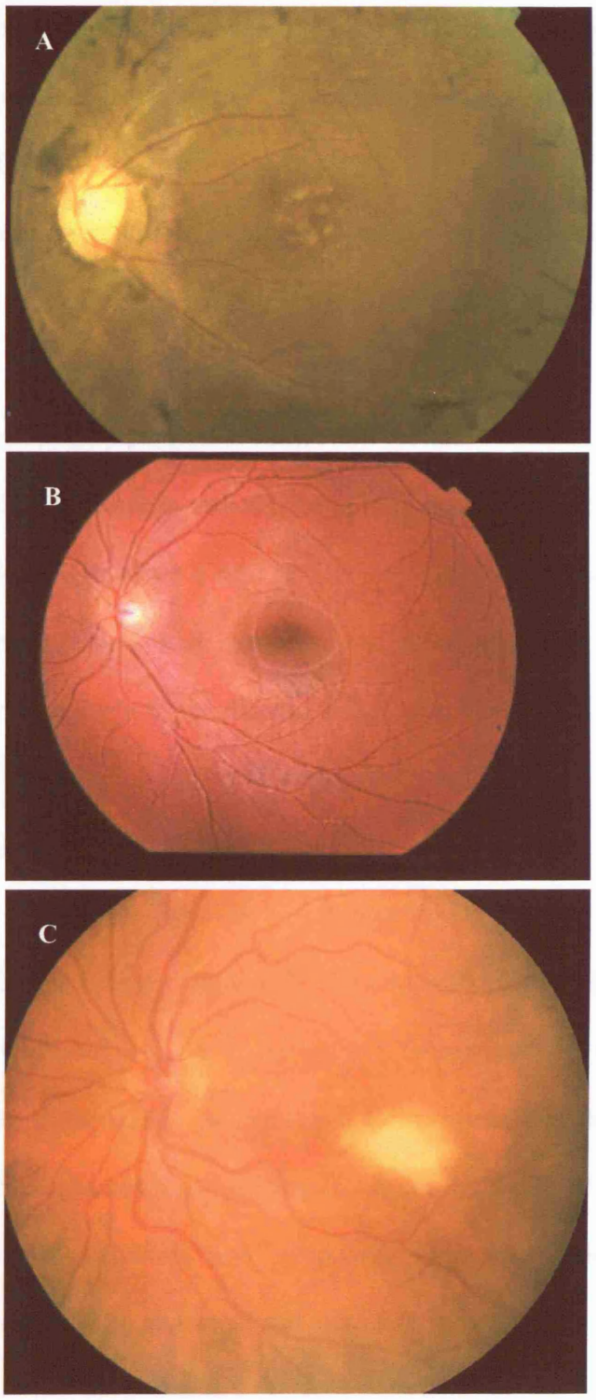
Although some affect RPE cells directly (Fig 1.10B and Gu *et al.*, 1997; Marlhens *et al.*, 1997; Maw *et al.*, 1997; Petrukhin *et al.*, 1998). Mutations in over 140 genes have been identified which cause photoreceptor loss and many have already been cloned (Daiger, RETNET, 1996-2004; Fig 1.11). Their effects are widely distributed

Figure 1.10: Hereditary Retinal Disease

This panel shows some examples of diseases that are inherited. A) is a retinal photograph of a patient with retinitis pigmentosa. It shows the classic features of this condition, with "bone spicule" pigmentation, retinal vessel attenuation and optic disc pallor. B) Shows a patient with early features of Stargardt's disease. There is an abnormal reflex from the fovea. This disease is due to mutations in the ABCA4 gene, expressed in the RPE. Other Stargardt's phenotypes exist with numerous pale sub-retinal flecks (fundus flavimaculatus). C) This retinal photograph demonstrates the classic features of a patient with Best's disease (an RPE dystrophy). There is a classic pale, yellowish lesion at the macula. This lesion goes through a series of changes (Stage 1-5: stage 3 shown). See 1.2.2

Figure 1.10

Hereditary Retinal Dystrophies



involving such processes as movement of materials in cells, movement of molecules between photoreceptors and RPE cells (by targeting transport proteins) and phototransduction (van Soest *et al.*, 1999).

Clinically, patients are typically affected by night blindness in their second to third decade with a gradual reduction in peripheral visual field. Typically they lose central vision later in life, in the fifth to seventh decades. The mechanism of photoreceptor cell death is by apoptosis (Milam *et al.,* 1998) as is also seen in animal models of RP (Portera-Cailliau C 1994).

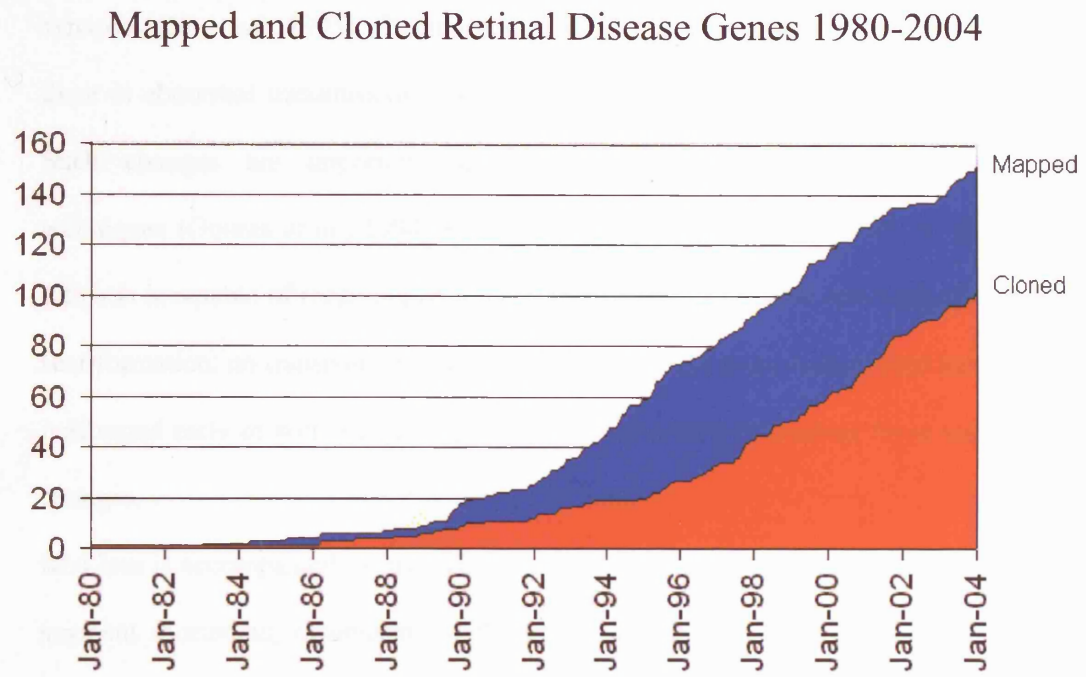
Much work has been done on the histological changes in the retinae of RP patients. A summary of these changes has been published in an excellent review by Milam *et al.,* 1998.

Rod loss begins in the mid-peripheral retina and gradually spreads to involve central and peripheral rods. This pattern varies between the genotypes of RP. Animal studies suggest that incident light plays a role in this degenerative condition because dark-reared animals have less severe degeneration than light-reared animals. Outer segments are shortened early in the disease and in rhodopsin mutations, for example, the mutant protein production may add to problems with normal cellular processing and accumulation of abnormal opsins. In turn this can lead to inner segment inclusions and/or failure to make outer segment discs, causing phototransduction abnormalities. The connecting cilium in RP may occasionally be abnormal leading to transport defects. Cilium defects are a feature of Ushers syndrome (Type 2, Hunter *et al.*, 1986). As with other retinal diseases and following retinal detachment there is evidence of abnormal rod neurite sprouting (Li *et al.*, 1995). In RP this phenomenon is limited to the mid-peripheral and peripheral retina and cone neurites do not demonstrate this feature. The abnormal rod neurites bypass the horizontal cells and

Figure 1.11: Graph of Mapped and Cloned Retinal Dystrophy Genes 1980-2004

This graph highlights the vast number of genes involved in retinal disease and the rate of discovery, especially over the past 10 years.

Figure 1.11



From RETNET Sept 2004

rod bipolar cells and are closely associated with hypertrophied Müller cell processes. This feature has been reproduced *in vitro* with bFGF suggesting a crucial interplay between Müller cells and rod neurites. They do not appear, however, to form true synapses (Li *et al.*, 1995). Electroretinogram (ERG) recordings from patients show there is abnormal transmission from outer to inner retina (Hood and Birch, 1996). Such changes are important when considering photoreceptor transplantation techniques (Gouras *et al.*, 1994; Kwan *et al.,* 1999, Jones *et al.*, 2003). If the inner retina is incapable of receiving an input signal because of retinal remodelling or glial scar formation, no transplant would work. Therefore transplantation would have to be performed early or with a co-therapy aimed at preventing or treating these secondary changes.

Rod loss is accompanied by secondary cone changes and loss. Changes include outer segment shortening, cytoplasmic inclusions, axonal elongation and death (Milam *et al.*, 1998). The reason for this secondary loss is presently unclear and numerous hypotheses exist, including: a build up of toxic products, loss of structure (e.g. the IPM), abnormal supporting cells, altered connectivity or a loss of trophic factors from the rods. The latter explanation is increasingly likely now given the recent work by Sahel's group in Strasbourg (Mohand-Said *et al.*, 1997; Mohand-Said *et al.*, 1998 Mohand-Said *et al.*, 2000; Leveillard *et al.*, 2004). They have identified a factor (Rod Dependent Cone Viability Factor I, RdCVF-I: Fig 1.16) that may play a role in maintaining cones in rod-based degeneration. Cone loss is the key event in RP leading to marked visual morbidity. When all rods are lost the macula maintains a monolayer of cones with shortened outer segments (Bunt-Milam *et al.*, 1983). Interestingly however, Sieving's group have described the histology from a patient with autosomal dominant RP (P23H) with a flat ERG and a monolayer of cones who maintained 6/12

vision until her death (Sieving 1999). This has key implications for treatment, since researchers may initially aim to preserve just two to three layers of photoreceptors to maintain functional vision before attempting heroic treatments such as photoreceptor transplantation.

The subretinal space in RP is depleted in volume and characterised by the loss of IPM (LaVail *et al.*, 1985) with total loss of photoreceptors. The collapse of the SRS and alterations in the constituents of the SRS, such as depleted inter-photoreceptor retinal binding protein (IRBP) may compromise any attempted therapy (Milam *et al.*, 1996). Reduced IRBP may effectively lead to a local vitamin A deficiency and explain the efficacy of vitamin A therapy in some patients with RP (Berson *et al.*, 1993).

Retinal pigment epithelial cells in RP detach from Bruch's membrane and migrate to blood vessels giving the classical bone-spicule pigmentation seen in these patients. It is not known why the cells detach and migrate but it is thought to reflect the RPE affinity for basal lamina (Li *et al.*, 1995). The choriocapillaris underlying regions of degeneration also tends to atrophy (Li *et al.*, 1995).

Müller cells undergo considerable change in RP, including cellular hypertrophy, migration to the outer retina and increased glial fibrillary acidic protein (GFAP) content in reactive cells. Müller cell reactivity and the invariable glial scar formation have marked implications for photoreceptor transplantation and integration. As previously mentioned grafted photoreceptors may only form neurites rather than true synapses, and these neurites may not be able to extend through the glial scar.

Intraretinal microglia play an important role in removing apoptotic neurons and there is evidence of increased numbers of activated microglia in animal models of degeneration such as the RCS rat (Thanos and Richter 1993).

Inner retinal neurons have been felt to remain largely unaffected in hereditary retinal degeneration (Milam *et al.*, 1998). However, recent studies on animal models by Villegas-Peréz *et al.*, (1998) and Jones *et al.*, (2003) have demonstrated marked inner retinal remodelling with amacrine, bipolar and ganglion cell loss, Müller cell hyperplasia and ganglion cell axon looping all centred on glial cell columns.

Ganglion cell loss is a feature of RP and generally parallels photoreceptor loss (Stone *et al.*, 1992). Vascular changes also occur, for instance, vessels with RPE attached acquire an extracellular matrix between the vessel wall and RPE cell (Li *et al.*, 1995) and eventually this matrix may occlude the vessels, seen clinically as attenuated vessels (Fig 1.10A). Ultrastructural studies have identified fenestrations between the endothelial cells (Li *et al.*, 1995) explaining the leakage of dye seen during fluorescein angiography (Krill *et al.*, 1970).

It is clear that retinitis pigmentosa does not just affect the outer retina, and future therapies must take these secondary changes into consideration. Transplantation strategies may need to be applied early in the disease or a preventative/maintenance therapy, such as growth factor or gene delivery, may have to be used.

In contrast to AMD, retinal dystrophy research has been aided by the availability of a range of animal models (either naturally occurring or engineered) that compare variably with human disorders. This is particularly true for RP, where there are a range of homologous disorders in mice, dogs and cats. More recently, the range of models has been augmented by transgenic animals, including rats and pigs, expressing human gene defects for particular forms of RP.

1.3 Animal Models of Retinal Degeneration

1.3.1 The Royal College of Surgeons Rat

The dystrophic Royal College of Surgeons rat has a defect of the retinal pigment epithelium characterised by an inability to phagocytose rod outer segments. The defect has been localised to a gene encoding the receptor tyrosine kinase *Mertk* (D'Cruz *et al.*, 2000). As a result there is a gradual degeneration of photoreceptors commencing at 3 weeks of age with near complete loss by 12 weeks (Fig 1.12B) compared with the congenic retina (Fig 1.12A; Dowling and Sidman, 1962; Sauvé et al., 2001). The initial changes are seen in the rod outer segments (Davidorf *et al.*, 1991). The model shares homology with a few rare phenotypes of human retinitis pigmentosa (Gal *et al.*, 2000) but may also be relevant to AMD. The exact aetiology of AMD is presently unclear but the RCS rat, although not directly homologous with any known human form of AMD, does compare in having an RPE dysfunction leading to photoreceptor loss. It thus provides a useful bioassay for examining the potential for RPE replacement in alleviating retinal disease secondary to RPE dysfunction.

In addition to photoreceptor degeneration there are secondary inner retinal vascular changes which result in the loss of ganglion cells (Villegas-Peréz et al., 1996, 1998; Jones et al., 2003). Similar changes are observed in human retinitis pigmentosa (see 1.2.2). Thus the features of the RCS rat lend it to studies on both AMD and RP. Treatment strategies will be presented in this thesis.

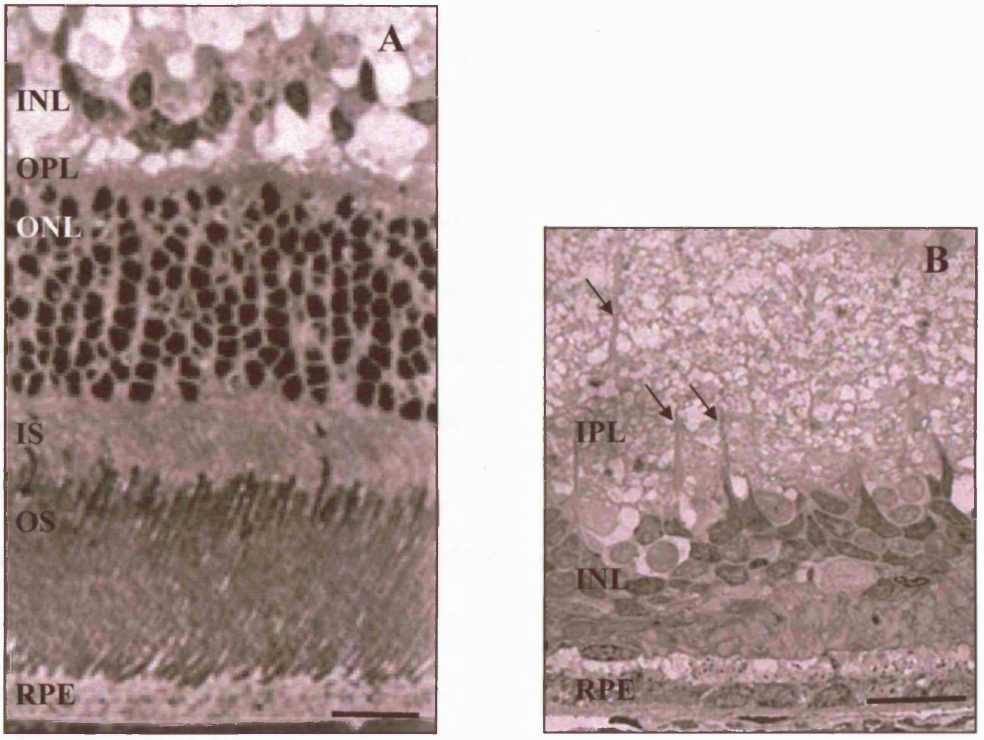
Numerous transplant studies in the RCS rat have demonstrated a protective effect of allogeneic and xenogeneic retinal pigment epithelial transplants in preserving photoreceptors (Li and Turner, 1988; Lopez *et al.*, 1989; Yamamoto *et al.*,1993; Litchfield *et al.*, 1997; Castillo *et al.*, 1997) and their function (Jiang *et al.*, 1994, Sauvé *et al.*, 1998). Other donor cell types shown to be of benefit include Schwann cells (Lawrence *et al.*, 2000) and iris pigment epithelial cells (Rezai *et al.*, 1997).

Figure 1.12: Retinal Histology of Royal College of Surgeons (RCS) Rat

A) Light micrograph is from a 6 month old congenic RCS rat. It illustrates the normal retinal architecture in these animals. There is a full complement of nuclei in the outer nuclear layer (ONL). The inner (IS) and outer (OS) segments are regularly arrayed adjacent to the retinal pigment epithelium (RPE). Inner nuclear layer (INL); Outer plexiform layer (OPL). Scale bar: 40μm. B) Light micrograph of a retina from a 6 month old dystrophic RCS rat. There is total loss of photoreceptors (the ONL is absent) and a disorganised inner nuclear layer (INL). There is also an increase in Müller cell fibrils (arrowed). Scale bar: 30μm.

Figure 1.12

Retinal Histology of the Royal College of Surgeons Rat



1.3.2 The Rhodopsin Knockout Mouse

The rhodopsin knockout (rho-/-) mouse has a targeted disruption of the rhodopsin gene such that rod outer segments are not generated (Humphries *et al.*, 1997). Photoreceptors are lost from four weeks to 12 weeks of age by apoptosis (Fig 1.13 C,D). A normal mouse retina is shown in Fig 1.13 A and B. Animals as young as eight weeks of age have lost photopic ERG responses. There is never a scotopic response as would be expected in an animal model that fails to elaborate rod outer segments (Humphries *et al.*, 2001).

Since many of the human forms of retinitis pigmentosa are caused by disruptions in the rhodopsin gene, studies on this animal model are important and relevant. In contrast to the RCS rat, the pathology and progression of disease is more analogous to human disease states. As in human retinitis pigmentosa rods are lost first and there is a secondary degeneration of cones.

Further studies on the knockout mouse developed by Humphries and co-workers have looked at differential degeneration in two strains of rhodopsin knock-out mice on different genetic backgrounds: C57BL/6J and Sv129 (Fig 1.13E). Photoreceptor degeneration commences at 1 month with near total loss by 4 months. The rate of loss in the Rho-/- Sv129 is more rapid than in the Rho-/- C57BL suggesting a differential apoptotic process in cones between the two species (Humphries *et al.*, 2001). Thus differences in genetic background may play a role in modifying apoptosis, although specific genetic differences are yet to be identified.

Studies on another rhodopsin knockout mouse (Lem *et al.*, 1999), using the C57BL/J background, have demonstrated the potential protective benefit of a gene therapy approach delivering secretable CNTF (sCNTF) to the retina (Liang *et al.*, 2001).

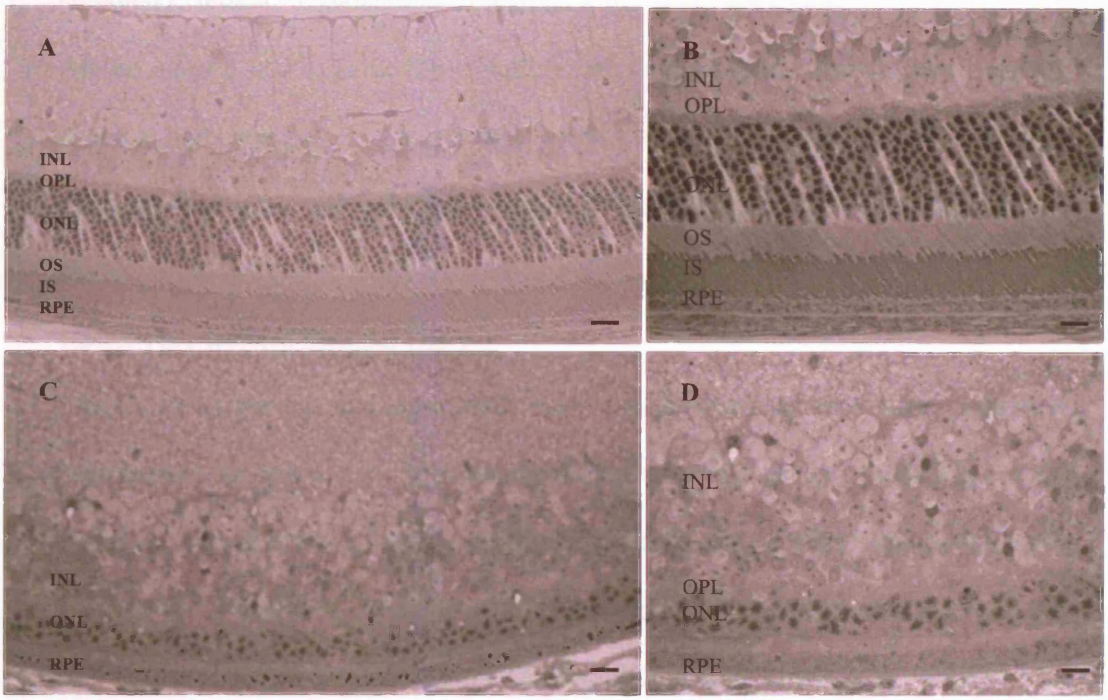
The studies presented in this thesis (Chapter 6) use the Rho-/- Sv129 strain.

Figure 1.13: Retinal Histology of Rhodopsin Knockout (Rho-/-) Mouse

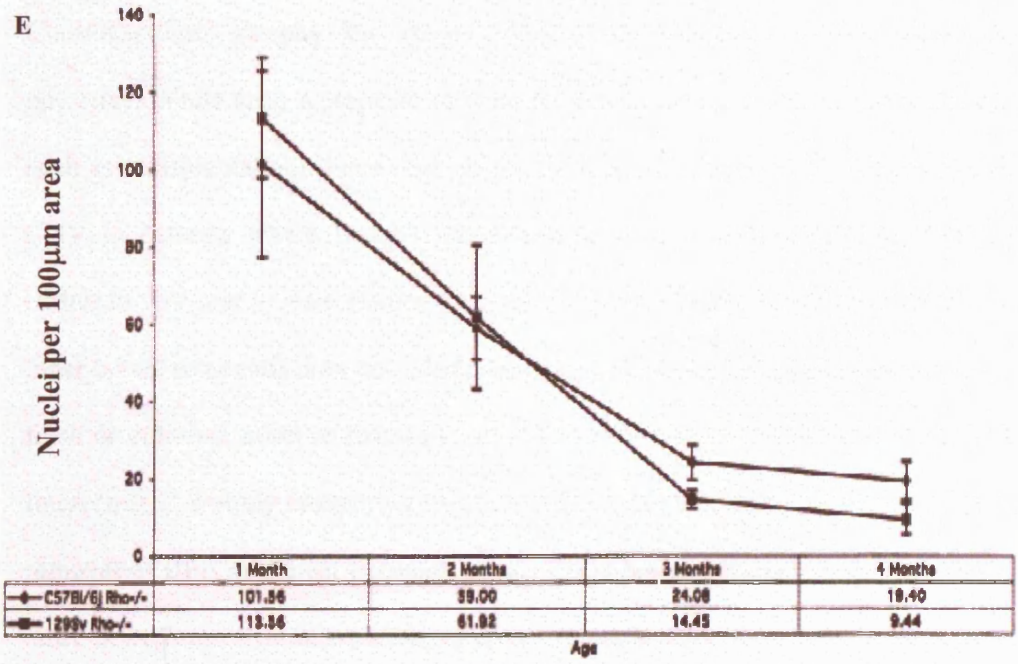
A) Low power view of a normal Sv129 mouse at 10 weeks of age illustrating a full complement of nuclei in the outer nuclear layer (ONL) and a regular arrangement of inner (IS) and outer (OS) segments. Scale bar: 40μm. B) Shows a higher power view of the same retina. Scale bar: 20μm. C) Low power light micrograph of a 10-week-old dystrophic Sv129 mouse with the rhodopsin gene deleted (Rho-/-). There is a marked reduction in nuclei in the outer nuclear layer. There are no outer segments. Scale bar: 40μm. D) Higher power view illustrates this point more clearly, the nuclei are only separated from the RPE by a layer of Müller cell processes. Scale bar: 20μm. E) Graph demonstrating the reduction in nuclei in the outer nuclear layer of two strains on rhodopsin knockout mice (on an Sv129 or a C57 background). Retinae at 1, 2, 3, and 4 months postnatal. The tabulated numbers of ONL nuclei represent the mean of at least 10 eyes. The rate of decline is clearly seen and is more pronounced in the Sv129 strain. From Humphries *et al.*, (2001).

Figure 1.13

Retinal Histology of Rhodopsin Knockout (Rho -/-) Mouse



Reduction in Numbers of Nuclei in ONL of Two Strains of Rho-/- Mice



1.3.3 Large Animal Models

As mentioned above, there are no good animal models of AMD available for pre-clinical study. By comparison there are a number of well-characterised models for hereditary retinal degeneration research with more analogous human correlates (Hafezi *et al.*, 2000; Li 2001) including cat (Barnett and Curtis, 1985), dog (β-phosphodiesterase defect: Aquirre et al., 1978; RPE65: Narfstrom *et al.*, 1989; rdy: Kommonen *et al.*, 1994) and pig (rhodopsin: Petters *et al.*, 1997). See 1.3.3.2 for more details.

1.3.3.1 Age-Related Macular Disease

Large animal models of age-related macular degeneration have been limited to debridement models and laser induction of choroidal neovascular membranes. Until the exact pathogenesis of AMD is discovered this will probably remain the case. Debridement models do, however, allow investigation of the effects of RPE cell loss on the neural retina and underlying choriocapillaris. Del Priore *et al.*, (1996) have examined these effects *in vivo* in a porcine model, using mitomycin C and edetic acid. Choriocapillaris atrophy and retinal atrophy was observed at four weeks post-operative. While such a preparation is useful for the study of replacement strategies, such as transplantation, it does not mirror the clinical situation following removal of CNV in patients where Bruch's membrane is invariably damaged. As mentioned earlier *in vivo and in vitro* studies have shown that damaged Bruch's membrane (with inner layers removed) does not adequately support seeded RPE cells whether they be fresh or cultured, adult or foetal (Tezel *et al.*, 1999a and b; Tsukahara *et al.*, 2002). Interestingly, a study comparing two methods of debridement, abrasive and hyraulic removal of RPE in the cat (Leonard *et al.*, 1997) has shown that abrasive removal is more deleterious. Secondary changes are more marked and there is no RPE regrowth;

this more closely resembles the human situation following CNV removal. The hydraulic removal method does, however, provide a good model for treating geographic atrophy.

Laser induction of CNV is another technique used to create animal models of AMD. Eyes are exposed to a long duration intense argon laser burn (Ohnishi *et al.*, 1987). These models have been used to assess treatments for CNV, such as argon laser and photodynamic therapy (Obana *et al.*, 2000) and anti-angiogenic drugs (Hikichi *et al.*, 2001). While these models have some validity they do not allow researchers to develop preventative therapies.

There is a naturally occurring model expressing the early changes seen in AMD. A colony of semi-free-ranging rhesus monkeys has been located in the Caribbean and they have been intermittently monitored for 16 years. These monkeys are considered a model for AMD, since they show drusen, pigmentary changes and, in late stage disease, hyperfluorescent patches (related to atrophy and not CNV). Electrophysiology showed loss of central visual function. All of these changes were age-related and the incidence was higher in specific maternal lineages (Dawson *et al.*, 1989; Hope *et al.*, 1992). To date, these animals have not been used in therapeutic studies and have been reserved for the analysis of the natural history of the disease.

1.3.3.2 Hereditary Retinal Disease

There are many rodent models of hereditary retinal disease (reviewed in Li 2001). In recent years large animal models have been developed and/or discovered. Large models allow investigation of aspects of potential treatments that cannot be assessed in smaller models such as surgical technique, reliability and secondary retinal effects. The Abyssinian cat (Barnett and Curtis, 1985) is a model of retinitis pigmentosa with a defect in the rdy gene characterised by degeneration commencing at 22 months and

complete by 27 months. The degeneration appears to specifically affect the outer retina with relative sparing of the inner retina (Narfstrom *et al.*, 2001).

The Briard dog (Narfstrom *et al.*, 1989) has a defect in RPE65 gene, an essential component of the functioning RPE cell (Bok, 1993), and is responsible for approximately 20% of human congenital stationary night blindness (CSNB).

Fulton Wong's group has developed a transgenic pig (Petters *et al.*, 1997) with an autosomal dominant rhodopsin mutation (Pro347Leu substitution) characterised by a bimodal retinal degeneration. Rod degeneration commences by week 2 with complete loss by 9 months. Cone loss follows rod loss, commencing at 4 weeks of age with early immunohistochemical changes, and structural degeneration is seen from 9 to 20 months (Tso *et al.*, 1997; Li *et al.*, 1998).

1.4 Established Therapies and Potential Treatments for AMD

1.4.1 Introduction

Presently no effective treatments are available for this disease but there are a number of lines of investigation, which suggest potential approaches.

Treatments for AMD fall into three categories: i) controlling the secondary changes in late AMD, notably the development of choroidal neovascular membranes (CNVM), ii) preventing RPE degeneration and iii) replacing damaged or lost RPE cells by translocation or transplantation. The ideal treatment would prevent RPE cell loss, but until the exact pathogenesis of AMD is known such an approach is not feasible. The next best approach is to control the early events in the disease process such as drusen deposition and RPE cell loss. This could be achieved by transplanting healthy RPE cells.

1.4.1.1. Established Therapies for AMD

1.4.1.1.1 Controlling secondary degenerative events

In humans, most attention has concentrated on controlling the secondary histopathological events in AMD, particularly by treating choroidal vessels that have broken through the weakened Bruch's membrane.

Current proven treatments aimed at limiting the progression of disease and minimising trauma to the neural retina include argon laser photocoagulation (Macular Photocoagulation Study Guidelines: MPS, 1991a,b,c; 1993a,b; 1994) and photodynamic therapy (PDT, Schmidt-Erfurth *et al.*, 1999). These treatments are destructive and are only appropriate for a sub-group of patients with AMD i.e. those with predominantly classic type membranes. The MPS guidelines recommend the treatment for those with classic extra- and subfoveal membranes. Subfoveal membrane treatment results in total loss of central vision and the relative benefit (greater in the marked visual loss group: visual acuity (VA) < 6/36) is only seen two years following treatment (MPS, 1994). Extrafoveal treatments delay central visual loss but there is a 10% per year recurrence risk and ultimately central vision tends to be lost.

Photodynamic therapy is a relatively new treatment that has been proposed for treatment of choroidal neovascular membranes, specifically subfoveal neovascular membranes. The technique, a two-step outpatient procedure, involves an injection of a photosensitive dye, verteporfrin (Visudyne™), followed by delivery of a "cold" light spot to the membrane (Miller *et al.*, 1999). This results in the local production of oxygen free radicals with focal tissue damage and destruction of the neovascular membrane complex. Optimum performance of the drug is determined by selectivity, absorption spectrum, photosensitising potency and pharmacokinetic properties. The ability of verteporfin to clear rapidly from the outer retina (within 2 hours) and from the body (plasma half life of 2-5 hours) and responsivity to long wavelengths (689nm)

means that it lends itself to ocular use. Once injected, verteporfrin binds to plasma LDL (low density lipoprotein) and enters the cells via LDL receptors which are preferentially found on endothelial cells. Pre-clinical studies and early phase I/II trials determined the effective and safe dose of veteporfin to be used. Systemically the dose used was safe; however, leakage of fluorescein recurred at 12 weeks post-treatment. A laser energy of $50J/cm^2$ was used because it was associated with the greatest degree of absence of leakage at 4 weeks, and least progression at 12 weeks. With these details decided, a phase III trial was designed. The main outcomes of this study were a) the proportion of eyes with moderate (> 3 lines) and severe (>6 lines) vision loss was greater in the placebo group; b) verteporfin-treated eyes tended to have better vision at 12 months (better than 20/200) and c) there was overall better preservation of contrast sensitivity in the verteporfin group. Further analysis showed that the group with wholly classic or predominately classic (>50%) CNV did particularly well with PDT treatment. Occult and minimally (<50%) classic CNV lesions did not do statistically better with PDT treatment.

While photodynamic therapy produces improved results by comparison with argon laser photocoagulation treatment, it does not address the causes of the neovascular process or the events leading to RPE failure.

1.4.1.2. Non-surgical Experimental Treatments for AMD

1.4.1.2.1. Non-Surgical and drug treatments

There are many potential treatments under investigation including i) radiotherapy (Marcus *et al.*, 2001), although several studies have shown no benefit for preserving visual function in patients with choroidal neovascular membranes secondary to age-related macular degeneration; ii) transpupillary thermotherapy (TTT) has shown some benefit in preliminary studies for the treatment of CNV (Newsom *et al.*, 2001) and a

randomised trial is underway (TTT4CNV study); iii) anti-angiogenic strategies have also been applied to this disease including interferon 2-α but many studies (e.g. Pharmacological Therapy for Macular Degeneration Study Group, 1997) have determined that not only is interferon not beneficial in this disease it is also potentially detrimental to the general health of the retina. Lastly, Thalidomide treatment has been shown to have some potential benefits (e.g. Ciulla *et al.*, 1998).

Recent exciting agents that have undergone early clinical trials include Macugen (an anti-VEGF165 aptamer) and RhuFab (an anti-VEGF antibody). These agents have managed to suppress CNV growth in patients with both classic and occult membranes.

1.4.1.2.2. Dietary supplements

In AMD, there is contradictory evidence for the beneficial effects of antioxidants in the diet. Seddon *et al.*, (1994) suggested that increasing the consumption of foods rich in certain carotenoids, in particular dark green, leafy vegetables, might decrease the risk of developing advanced or exudative AMD, but both Mares-Perlman *et al.*, (1996) and Smith (1999) found no beneficial effect of dietary antioxidants on AMD.

Recently the results of a large multi-centred, randomised controlled clinical trial were published, examining the benefits of high-dose supplementation with vitamins C and E, β-carotene and zinc in AMD (AREDS Report, 2001). This study demonstrated the benefit of dietary supplementation with zinc and antioxidants for patients with intermediate changes (drusen size >64μm: category 3) or fellow eye, late stage AMD (category 4). The risk of developing marked visual loss and development of CNV or geographic atrophy was significantly reduced in the group that took antioxidant and zinc supplementation. This is a significant result since it is the first clear

demonstration of a protective effect using dietary supplements for the treatment of AMD.

1.4.1.3 Experimental Surgical Techniques for AMD

1.4.1.3.1 Surgical removal of choroidal neovascular membranes

Surgical strategies have been explored for treating age-related macular degeneration. These include removal of subretinal neovascular membranes (Submacular Surgery Trial I& II, 2000a and b). This trial indicated no benefit of submacular surgery and removal of CNVM compared with argon laser photocoagulation. Over 80% of treated eyes had a final vision <6/36 following surgery (Thomas *et al.*, 1994). Most of these failures related to the histopathological observation, already discussed, that most membranes in AMD are sub-RPE and their removal will also remove the RPE and photoreceptors. Other complications include CNV regrowth (46%), entry site breaks, retinal detachment and cataract. For this technique to work there must be regrowth of RPE across the defect or donor cells must be grafted in their place. Numerous animal studies have demonstrated that RPE cells do not grow well on damaged Bruch's membrane (e.g. Leonard *et al.*, 1997) which is invariably the position following membrane removal.

1.4.1.3.2 Macular translocation

Another approach that has been advocated is moving the macula away from the subfoveal scar to a new location with relatively healthy underlying retinal pigment epithelium. Macular translocation has been performed using two different methods: i) a 360 degree retinotomy followed by a total retinal detachment and relocation of the retina either superior or inferior to the neovascular membrane (Eckardt *et al.*, 1999) or ii) limited translocation, termed redistribution of the neural retina (NRN, DeJuan and Vander 1999; Wong *et al.*, 2000) with subsequent argon laser treatment for the

CNVM. These techniques have been uniformly classified recently (Au Eong *et al.*, 2001). There has been a limited success using this treatment with up to 40 per cent of patients describing some improvement in vision (Eckardt *et al.*, 1999). The first technique (total retinal detachment with 360° translocation) has, however, been complicated by a number of sequelae, notably proliferative vitreoretinopathy (PVR), retinal detachment and strabismus. These complications have also been described in limited macular translocation (Fujii *et al.*, 2000). With all these considerations the American Academy has decided to withhold ratification of translocation as a mainstream practice pending a controlled trial (AAO Committee on Ophthalmic Procedures Assessment Retina Panel, 2000). While the overall results have not been very encouraging, there has been no randomised clinical trial and the few successes described may give hope that early intervention could be beneficial. Transplantation could play an important role in replacing scarred, diseased RPE with fresh cells, although survival and integration of donor tissue remains a concern.

1.4.1.3.3 RPE translocation

In an approach that is similar in concept to macular translocation, some groups have proposed RPE translocation, either as a patch including Bruch's membrane (Stanga *et al.*, 2002; van Meurs *et al* 2004) or by harvesting autologous RPE cells (autotransplantation, Binder *et al.*, 2002). The latter technique involved removing cells from underneath the nasal retina, centrifuging them to a pellet and resuspending them in a small volume of fluid. This generated approximately 5,000-50,000 cells/ml for transplantation. Cells were then injected into the subretinal space following removal of subfoveal neovascular membranes. A number of patients (57.1%) also had cataract surgery at the same time. Results showed an improvement of visual acuity of up to 6 Snellen lines (1 patient) but scotometry and fixation tests failed to identify

foveal fixation (none at all in 50% and extrafoveal in 50%). Although some of the visual results are encouraging, the lack of surgical controls and performance of cataract surgery at the same time in a significant proportion of this group limits the conclusions that can be drawn from the results. While this technique might overcome difficulties of immune rejection, it fails to address the other crucial issues necessary for successful transplantation, namely cell viability and cell integration. The donor cells are subjected to a high degree of stress and they are then required to settle onto damaged Bruch's membrane. Zarbin and co-workers (Tsukahara *et al.,* 2002) have identified key features for *in vitro* cell integration on Bruch's membrane. Foetal cells integrate more reliably than adult cells, yet cultured cells integrated best of all. All groups integrated more completely when Bruch's membrane was intact, preservation of the RPE basement membrane or at least the inner collagenous layer of Bruch's being necessary. This work confirms previous *in vitro* studies conducted by Del Priore and co-workers (Tezel *et al.*, 1999a and b). Zarbin *et al.*, (2002) suggest that cultured foetal cells may integrate more efficiently because they express more key adhesion or integrin proteins. Thus, there are many reasons why these autografts may not succeed. There may be insufficient donor cells to patch the damage, they may not express key integrins and Bruch's membrane may be damaged by the surgery.

1.4.2 Transplantation of RPE cells

1.4.2.1 Cell transplantation to the RCS rat retina

RPE transplantation has the potential to replace the defective cell type in age-related macular disease, unlike the treatments previously described.

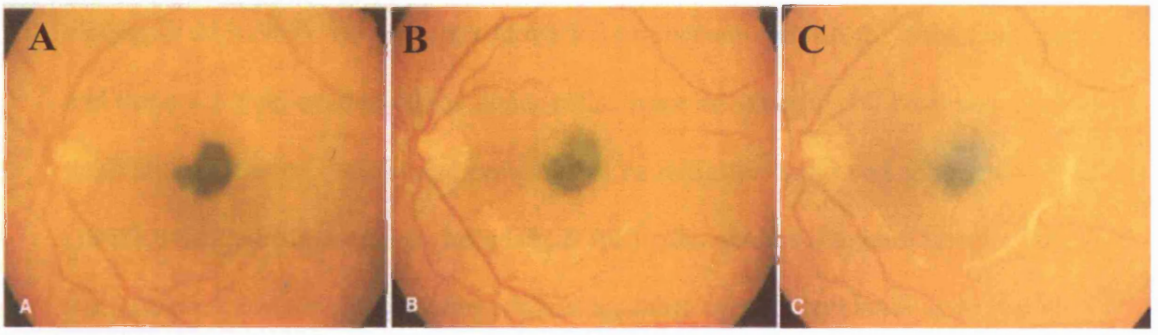
Introduction of RPE cells into the subretinal space, before substantial photoreceptor loss has occurred, can limit the disease process and functional deterioration in the RCS rat. Donor cells used to date include fresh (Li and Turner, 1988; Li *et al.*, 1991;

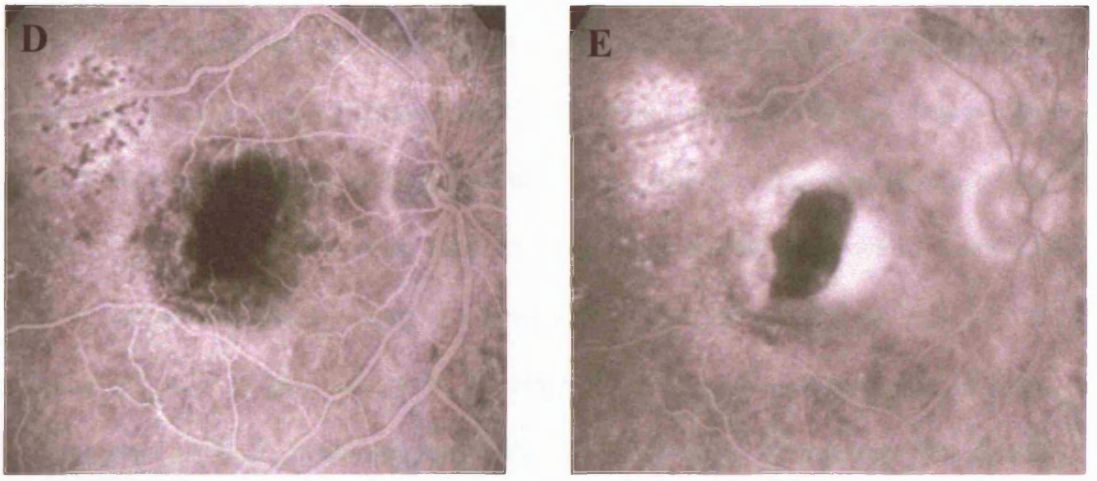
Figure 1.14: Transplantation of Foetal RPE Cells to Patient with Exudative AMD.

A) Fundus photograph from patient one day after RPE transplantation. The graft is seen as a pigmented patch at the fovea. B) and C) show the same patient at 1 and 3 months respectively: there is increasing macular oedema (see E) and subretinal fibrosis (whitening above the graft in C). The fluorescein angiogram at one month is shown: early (D) and late (E) views of oedema in the region of the graft. (From Algvere *et al.*, 1994).

Figure 1.14

Foetal RPE Transplant to a Patient with Exudative AMD





From Algvere P, Berglin L, et al. (1994)

Lopez *et al.*, 1989, Whiteley *et al.*, 1996), cryopreserved (Durlu and Tamai, 1997), cultured primary (Lopez *et al.*, 1987; Sheedlo *et al.*, 1989), or immortalised RPE cells of animal or human origin (Keegan *et al.*, 2000; Coffey *et al.*, 2000a and b) and iris pigment epithelial cells (Rezai *et al.*, 1997; Schraermeyer *et al.*, 1999, 2000). Early studies concentrated on the RCS rat (as it has a primary RPE defect: see 1.3.1). Neonatal RPE cells, freshly harvested from pigmented rats, and grafted into the subretinal space of albino RCS rats preserve photoreceptors (Turner and Blair, 1986; Gouras *et al.*, 1989). Both groups identified pigmented cells in the subretinal space and these were believed to be the donor cells. Since no specific cell label was used in these studies, the cell origin and type cannot be definitively proved. Zhang and Bok (1998) extended these studies in the RCS rat to include an immunological analysis (see 1.4.1.3.5: Immunology section). While again no specific cell label was used, they were able to detect donor cell specific major histocompatibility Type II (MHCII) antigens at 5 months post-grafting. In reality however, until recently a direct relationship between donor cell survival and host photoreceptor rescue had not been described (see Lund *et al.*, 2001a or b; Coffey *et al.*, 2002; and Chapter 3). Also, as will be discussed later and detailed in Chapter 5, almost any treatment preserves photoreceptors in the dystrophic RCS rat retina, including sham surgery alone (Silverman and Hughes, 1989), growth factor injections (eg. Faktorovich *et al.*, 1990) and cell grafts of various types. Thus, it would seem that the presence of donor cells may not always be necessary to preserve photoreceptors. Indeed, iris pigment epithelial cells transplanted to the choroid of RCS rats also preserve photoreceptors (Schraermeyer *et al.*, 2000) suggesting that a trophic effect is the primary mechanism by which grafts preserve photoreceptors in this model. The relationship between graft cell survival and function is not known and may be a more important index to be

assessed c.f. photoreceptor preservation. Recently, RPE cells have been transplanted to the retina of the RPE65 (-/-) knockout mouse (Gouras *et al*, 2002). The results suggested a morphological and a functional (electroretinogram) benefit. However, again the cells were not labelled and the ERG findings may be equivocal.

In animal studies, photoreceptor loss has secondary consequences, involving both cell loss in the inner retina and vascular pathology (Villegas-Peréz et al., 1998, Jones *et al* 2003). These issues have received much less attention in experimental interventions although it has been suggested that RPE transplants can reduce some secondary vascular events (Seaton and Turner, 1992; Seaton *et al.*, 1994).

1.4.2.2 RPE cell transplantation to other animal models

Other transplant studies have transplanted RPE cells from a range of sources (fresh, cultured and with/without membrane support) to other animal models such as rabbits (Shiragami *et al.,* 1998), and pigs (Kiilgaard *et al* 2002: Del Priore *et al* 2003 a and b; 2004). Xenografts into rabbits survive using immunosuppression (Craaford *et al.*, 2000). Interestingly this group could not repeat these results using allografts with or without immunosuppression (Crafoord *et al.*, 1999, 2000). Recently, autologous RPE cells transplanted into rabbits have attenuated choriocapillaris atrophy and reduced photoreceptor loss (Phillips *et al.*, 2003). Although morphological rescue of photoreceptors was demonstrated in these studies, there was no functional assay. Nevertheless, each study adds to the knowledge base and eventually a clear direction for clinical therapies will emerge.

1.4.2.3 RPE transplantation to patients with AMD

A number of studies have been published describing the results of RPE transplantation in patients with AMD (Algvere *et al.*, 1994, 1997, 1999; Kaplan *et al.,* 1998; Weisz *et al.*, 1999; Del Priore *et al.*, 2001). In the first group of experiments

(Algvere *et al.*, 1994) patients with neovascular AMD (n=5) received transplants of patches of RPE cells freshly harvested from aborted foetuses. These patients had a reduction in visual acuity initially at 2 weeks but it recovered to pre-operative levels within one month. However, the three patients with foveal transplants experienced further reduction in vision to below pre-operative levels (Algvere *et al.*, 1994, 1997). The foveal transplants were associated with macular oedema, which was interpreted as evidence of graft rejection and failure (Fig 1.14). This conclusion may not be entirely correct since one might expect that graft loss would mean there was no obstruction to fluid outflow and the retina would be atrophic rather than oedematous. Similar results were seen in a second group of patients, with geographic atrophy, who also received grafts (Algvere *et al.*, 1997, 1999). To date, clinico-histopathological correlates (Del Priore *et al.*, 2001) are only available for one patient who was on triple immunosuppression (prednisolone, cyclosporine and azathiaprine) following RPE patch transplantation. A multilayered patch of pigmented cells was assumed to be the donor cells. Some cells appeared to be integrated on host Bruch's membrane and the retina overlying these patches was better preserved. Areas with mounds of cells were not so well preserved and there was invasion of the inner retinal layers by these pigmented cells. Macrophage-like cells were seen in the subretinal space and occasional lymphocytes were seen in the choroid, in spite of immunosuppression. The visual function in this patient, and 12 others treated similarly, was unaffected by the treatment. There were, however, no ophthalmic signs of graft rejection. The multilayered pigmented cells are a feature seen in some animal studies of RPE transplantation (see chapter 3 and 4; Del Priore *et al.*, 2003a and b). Further analysis of these cell groups showed that many macrophages were present in the subretinal space and even on Bruch's membrane. Multilayering may also be due to folding of the

graft or the failure of the cells to attach to Bruch's membrane. Care should be taken in the interpretation of clinical and histological results since cells identified as grafted RPE cells could, in reality, be host macrophages loaded with donor cell debris. Some 100 patients have received RPE cell grafts and as such, transplantation is the only interventional experimental technique to have been applied clinically. Results so far have not been encouraging but since most recipients were at an advanced stage of functional loss, little improvement could be expected. Benson *et al.*, (1998) noted some limitation of further deterioration, using a series of psychophysical tests: otherwise objective functional assessment after transplantation has not been employed. It must also be added that the human studies so far have included only some of the pre- and post-operative assessments that would be necessary for drawing reasonable and substantive conclusions, see Chapter 7, section 7.10 (Algvere *et al.*, 1997; Binder *et al.*, 2002). With the technology that is now available the work-up should be as thorough as the patients can tolerate, including high-resolution confocal scanning laser ophthalmoscopy (cSLO) (microperimetry), focal ERGs and autofluorescence. The SLO is especially important in the current cohort of patients being recruited to trials since they have poor central vision pre-operatively and good vision is unlikely to be restored by a supportive RPE graft. The SLO would be able to pick up visual improvements as a result of grafting at the edges of the lesion, as opposed to the fovea. Trials could then proceed to less severely affected patients whose central vision is only moderately affected and where it might be expected that transplantation strategies could maintain or even improve vision. Careful selection of a distinct patient phenotype for testing will also increase the success rate.

In spite of the fact that clinical experiments are in progress, basic research remains essential and to some extent, these have been prompted by the clinical results obtained

so far. New treatments need to be tested, effective screening 'bioassays' must be developed, immune problems must be overcome, alternatives to fresh cell transplantation sought, and a comprehensive assessment of the degree of functional recovery must be made. The poor results achieved so far do not necessarily mean that the transplant approach is without merit.

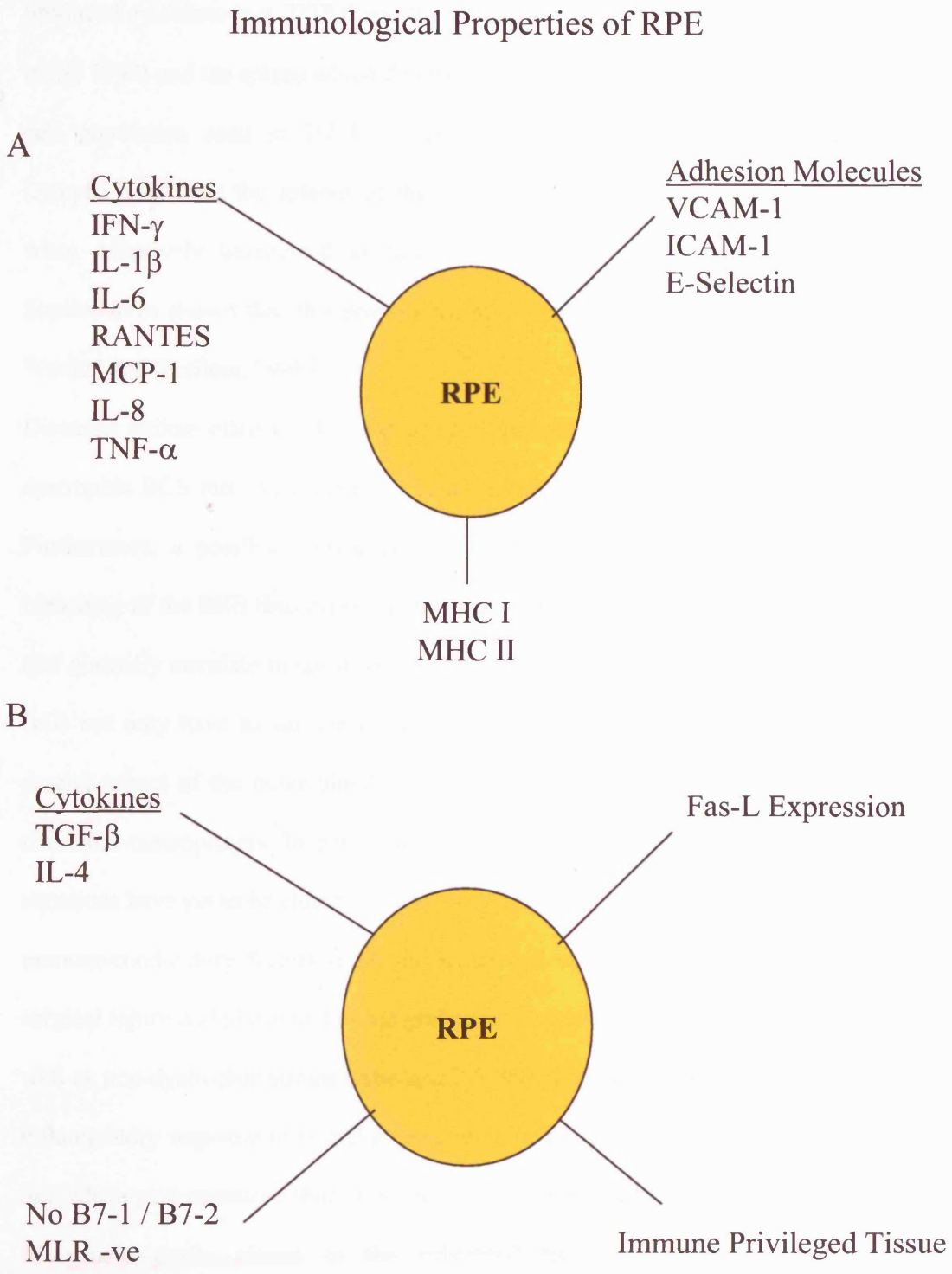
1.4.2.4 Problems to be resolved in transplantation studies: Immunology

Apart from all the issues raised in the above section, it is obvious that for successful transplantation, potential immune or inflammatory reactions should be avoided or controlled. Allogenicity of donor and recipient and mismatch of major histocompatibility complex (MHC) haplotypes (especially class I, Zhang and Bok, 1998) can compromise the graft, as does the up-regulation of MHC class II expression in donor cells (Kohen *et al.*, 1997). There have been reports of RPE cell rejection (Jiang *et al.*, 1994, 1995; Algvere *et al.*, 1994, 1997; Zhang and Bok. 1998; Gabrielian *et al.*, 1999), even though the undisturbed subretinal space has been regarded as an immune-privileged site. While the subretinal space may be considered a partially immunologically privileged site, foreign tissue is not necessarily protected from immune attack. Its immune status is atypical (Jiang et al, 1995; Grisanti *et al.*, 1997) and like the anterior chamber, exhibits a phenomenon now referred to as Anterior Chamber Associated Immune Deviation (ACAID, Streilein *et al.*, 1980; Streilein and Niederkorn, 1985; Ksander and Streilein, 1989). This response to antigen placement in the subretinal space is an active systemic process leading to down-regulation of the normal delayed-type hypersensitivity (DTH) response seen

Figure 1.15: Immunological Properties of RPE Cells

A) Illustrates the pro-immunogenic factors of RPE cells. These properties may predispose RPE cells to graft rejection (see section 1.4.2.4). B) Demonstrates the anti-immunogenic properties of RPE cells that may aid graft tolerance following transplantation (see 1.4.2.4).

Figure 1.15


Immunological Properties of RPE
A
Cytokines
IFN-γ
IL-1β
IL-6
RANTES
MCP-1
IL-8
TNF-α
Adhesion Molecules
VCAM-1
ICAM-1
E-Selectin
RPE
MHC I
MHC II
B
Cytokines
TGF-β
IL-4
Fas-L Expression
RPE
No B7-1 / B7-2
MLR -ve
Immune Privileged Tissue

after transplantation of tissue to non-immune privileged sites (Hall, 1991; Piccotti *et al.*, 1997). The mediators of this predictable response are thought to be locally produced cytokines (e.g. TGF-β and IL-10; D'Orazio and Niederkorn, 1998; Takeuchi *et al.*, 1998) and the spleen which diverts the immune response from the Th-1 type T-cell population seen in DTH to the Th-2 type (Streilein 1990, 1995, 1999). Lymphocytes from the spleens of these animals were capable of suppressing DTH when adoptively transferred to naïve individuals (Wenkel and Streilein, 1998). Studies have shown that this process requires an intact blood-retinal barrier (BRB, Wenkel and Streilein, 1998).

Diseased retinae often display leakage and abrogation of the BRB as do those of dystrophic RCS rats (Villegas-Peréz *et al.*, 1998) and rd mice (Wang *et al.*, 2000). Furthermore, a possible consequence of transplantation to the retina will be the breaching of the BRB thus exposing the graft to immune surveillance by lymphocytes that normally circulate in the blood (Wekerle *et al.*, 1987). Once in place the grafted cells not only have to survive immediate immunological attack but must reform a crucial aspect of the outer blood retinal barrier and will be exposed to circulating choroidal macrophages. In both instances the exact mechanisms occurring in these situations have yet to be elucidated. The type, pattern and timing of inflammatory and immuno-modulatory factors (both molecular and cellular) produced in response to surgical injury and placement of the grafted cells must be determined in dystrophic as well as non-dystrophic strains (Abe *et al.*, 1999). To date no detailed analysis of the inflammatory response to retinal degeneration or to retinal cell transplantation in the immediate post-operative phase has been made in a dystrophic model.

Allogeneic grafts placed in the subretinal space can still be lost despite immunosuppressive therapy (Cyclosporine-A, Craaford *et al.*, 2000), again indicating

possible non-T-cell dependant graft destruction. Fas (CD-95)/Fas-ligand (CD-95 L) interactions are important in immune modulation as this interaction leads to the apoptosis of Fas-expressing activated T-cells (Griffith *et al*, 1995; Jorgensen *et al.*, 1998). It has been shown that the RPE cells express Fas-L (Weller *et al.*, 1996; Jorgensen *et al.*, 1998; Winton *et al.*, 1999), and this feature of host RPE cells may be important in down-regulating activation of T-cells in the region (Jorgenssen *et al.*, 1998). Moreover, it has been shown that RPE cells constitutively express Fas but are resistant to Fas / Fas-L dependent apoptosis under normal conditions. However, in inflammatory conditions such as proliferative vitreo-retinopathy (PVR) this protection is lost (possibly by inhibition of RNA or protein synthesis) and these cells can be apoptosed via the Fas/Fas-L pathway (Weller *et al.*, 1996). This may one mechanism of graft cell loss post-transplantation. It is obvious that the RPE cell possesses both pro- and anti-immunogenic properties and as such it is difficult to predict the exact processes that will dominate in the subretinal space following transplantation (Fig 1.15).

Additionally, it has been shown that RPE cells must attach to undamaged Bruch's membrane. Any impediment to grafted RPE cells accessing Bruch's membrane would prejudice against survival and lead to apoptosis. In contemplating such transplantation in humans the graft bed will have to be carefully prepared so that Bruch's membrane is stripped of host cells but remain undamaged.

Another factor to affect the stability of any immune privilege is the loss of vascular integrity since disruption of the blood-retinal barrier will expose the retina to immune surveillance (Wenkel and Streilein, 1998). This may complicate the use of the RCS rat in transplantation studies unless the grafts are syngeneic because its retinal vessels become fenestrated and 'leaky' as they near the choroid (due to the loss of

photoreceptors and RPE migration into the retina). Since vascular integrity is lost in some human retinal diseases, notably RP and neovascular AMD, the potential problems of transplantation into the subretinal space with a persistently compromised BRB cannot be ignored. The duration of the disease process could also affect immune privilege since it is lost in older rd mice (Welge-Lussen *et al.*, 1999). It is clear from the above evidence and earlier studies in the brain that the question of immune privilege and transplant rejection is a particularly important one for clinical use. To date, results from the RCS rat have been difficult to interpret in the context of graft rejection because donor cells have generally not been labelled and studies to assess the number of RPE cells that may have been lost because of failure to adhere to Bruch's membrane have also not been carried out.

In summary, it is clear that mismatched cells introduced into the subretinal space are potentially vulnerable to immune surveillance and rejection. Since diseased retinae often have a compromised BRB, the risk of rejection is increased. However, an understanding the basic mechanisms underlying the concept of 'immune privilege' in the retina is essential for the management of mismatched grafts.

1.5 Potential Treatments for Hereditary Retinal Dystrophies

1.5.1 Introduction

A number of potential treatments are being considered. They include the administration of growth factors, gene therapy, dietary and drug treatments and transplantation of retinal tissue or microelectrode prostheses.

1.5.2 Preventative Treatments

1.5.2.1 Administration of growth factors

A number of growth factors (including basic fibroblast growth factor, bFGF; ciliary neurotrophic factor, CNTF; glial-derived growth factor, GDNF) and, to a lesser

extent, their receptors have been identified in the retina or in retinal cultures (e.g. Rakoczy *et al.*, 1993; Gao and Hollyfield, 1995; Jing *et al.*, 1996; Hallböök *et al.*, 1996; Bugra and Hicks, 1997; Carwile *et al.*, 1998; Jomary *et al.*, 1999). Although there is some variability in the results from these studies, this is almost certainly due in part to the use of different developmental stages, use of different animals and use of techniques with different sensitivities when studying factor and receptor localisation.

All the above growth factors have been shown to slow photoreceptor loss in a range of rodent models, including light damage (Faktorovich *et al.*, 1992; LaVail *et al.*, 1992; Masuda *et al.*, 1995), the RCS rat (Faktorovich *et al.*, 1990; Perry *et al.*, 1995), the retinal degenerate, rd (LaVail *et al.*, 1998; Frasson *et al.*,1999a) and the retinal degenerate slow (rds) mouse (LaVail *et al.*, 1998) as well as in transgenic rats (LaVail *et al.*,1998). Similar studies are being conducted in larger animals (e.g. CNTF in the Abyssinian cat, Chong *et al.*, 1999). A simplified mechanism of action is shown in Fig 1.16.

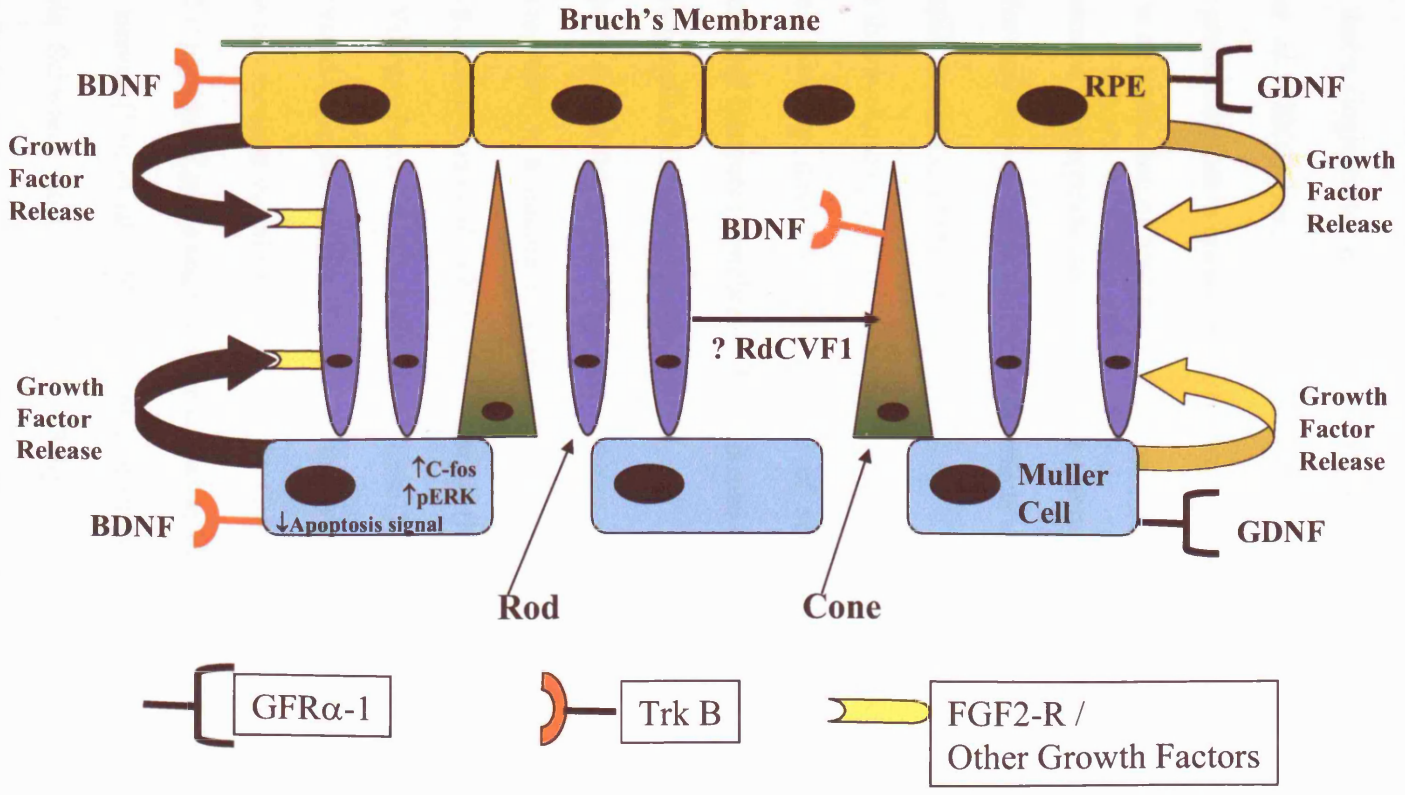
To achieve rescue, these factors have been injected into the vitreous cavity with a single or multiple injection regimen. Since it is apparent that some factors at least (e.g. PEDF, Cayouette *et al.*, 1999) remain in the vitreous for as little as 24 hours, other methods of factor delivery need to be devised for the long-term maintenance of photoreceptors. One such approach is the development of encapsulated cell technology (REFS) to deliver a sustained amount of growth factor (CNTF) to treat retinal degenerations in rodent and large animal models. Another approach has been to introduce message for growth factors using gene transfection (see 1.5.1.2.3), and the results obtained validate the principle of growth factor delivery as a treatment of potential importance. Functional assessment in growth factor delivery experiments has so far been restricted to electroretinogram (ERG) recordings, which indicate

Figure 1.16: Possible Growth Factor Mechanisms in the Retina

This figure illustrates a simplified scheme of growth factor production within the pigmented and neural retina with the sites of action shown (receptors). Note that Müller cells appear to play a key orchestrating role, mediating growth factor activity in the retina. Brain-derived neurotrophic factor (BDNF), Ciliary neurotrophic factor (CNTF), Glia-derived neurotrophic factor (GDNF), Rod-derived cone viability factor (RdCVF1), FGF2 (Fibroblast growth factor-2, also known as bFGF, basic fibroblast growth factor), Growth factor receptor-1 (GFR-1), Tyrosine receptor kinase receptor (TrkB). See 1.2.2 and 1.5.2.1.

Figure 1.16

Proposed Growth Factor Mechanisms in Retina



functional responses in some models (CNTF in rds, Cayouette *et al.*, 1998) but not in others (bFGF in mi/vitiligo mouse, Smith *et al.*, 1996). This variability suggests, not surprisingly, that individual growth factors may be differentially effective in different forms of RP and that a single factor may not be a cure for all forms of the disease. Indeed, Ogilvie *et al.*, (2000) have shown that a combination of growth factors rescued rd mouse photoreceptors in organ culture but each factor presented singly did not. As yet there is no indication of how much form and pattern vision remains after growth factor treatment. Appropriate dosage protocols (amount and frequency) need to be established for each growth factor, and any side effects assessed.

1.5.2.2 Schwann cells as sources of growth factors

Schwann cells are the myelinating cells of the peripheral nervous system. They extend multiple processes, forming internodal myelinations of several axons. They are surrounded by their own basement membrane. There is also a subpopulation of non-myelinating Schwann cells (Mirsky and Jessen, 1983).

Previous studies have shown that grafts of peripheral nerve segments support neuron survival and axon regrowth in a number of central nervous system locations including the spinal cord (e.g. Richardson *et al.*, 1980), and thalamus (Morrow *et al.*, 1993). In the retina, (e.g. Villegas-Peréz *et al.*, 1988) they have been shown to promote regeneration of severed optic axons from the back of the eye to the CNS where they form synapses and can mediate visual responses to light. Greater retinal ganglion cell rescue is achieved if peripheral nerve segments are placed in the vitreous of eyes with axotomised optic nerves (Lau *et al.*, 1994). These properties are dependent on the presence of viable Schwann cells in the graft (Berry *et al.*, 1988; Smith and Stevenson, 1988). Purified populations of Schwann cells transplanted on artificial substrates (Xu *et al.*, 1997) or grafted as cell suspensions (e.g. Brook *et al.*, 1993;

Raisman *et al.*, 1993; Brook *et al.*, 1994; Stichel *et al.*, 1996,) have also been shown to support cell survival and axon regrowth in the brain and spinal cord. Schwann cells injected into the vitreous of the eye promote RGC survival after optic nerve section (Maffei *et al.*, 1990). When grafted into the visual cortex of dark-reared rats, Schwann cells contributed to the normal development of visual response properties (Fagiolini *et al.*, 1997). Schwann cells play a very important supportive role to neurons, producing neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF, Meyer *et al.*, 1990), neurotrophin-3 (NT-3) and neurotrophins-4/5 (NT-4/5) (von Bartheld, 1998). Other trophic factors produced are CNTF (Sendtner *et al.*, 1992), bFGF (Fujimoto *et al.*, 1997; Neuberger and de Vries, 1993) and GDNF (Hammarberg *et al.*, 1996). Many of these factors have been shown to have retinal activity (e.g. LaVail *et al.*, 1992, 1998) and receptors for them are present on photoreceptors and RPE cells (See section 1.5.2.1 and Fig 1.16).

All this evidence raises the possibility that Schwann cells, which can be delivered as an autologous graft, might be effective not only in supporting ganglion cell survival but also of protecting photoreceptors from death. Recent studies by Lawrence *et al.*, (2000 and 2004) show this to be the case and a Schwann cell line engineered to overexpress GDNF or BDNF maintains visual function as well as photoreceptor morphology.

1.5.2.3 Gene therapy

Several studies have indicated that a) cells in the retina can be transfected using an adenovirus vector (e.g. Li *et al.*, 1994) and b) photoreceptor loss may be slowed with appropriate wild type transfects (Bennett, 1996; Ali *et al.*, 1996,1997, 2000; Jomary *et al.*, 1997). Growth factor transfects can survive in the retina and reduce photoreceptor loss. Thus, rd mice had more rows of surviving photoreceptors 18 days after Ad-

CNTF injection (Cayouette and Gravel, 1997) and improved survival was also seen in the rds mouse for as long as 52 days (Cayouette *et al.*, 1998). Injecting adenovirus-carrying bFGF has improved photoreceptor survival in the RCS rat for 56d compared with untreated rats (Akimoto *et al.*, 1999). A recent study has demonstrated significant photoreceptor preservation by transfecting the Rho -/- mouse retina with an attenuated adenovirus (AAV) vector containing a CNTF construct (Liang *et al.*, 2001).

Very exciting results are arising from the work of Bennett and co-workers on gene therapy and the Briard dog (an animal model of Leber's Amaurosis characterised by a gene defect affecting RPE65 protein). They have successfully treated a dog and have evidence of restored visual function (Acland *et al.*, 2001) Other workers, also using a RPE65 mutant dog, have demonstrated morphological evidence of rescue (Narfstrom *et al.*, 2003).

In terms of safety, several studies reported no detection of virus in the blood or in any other organs, suggesting that infectivity had not spread. However, Dudus *et al.*, (1999) found evidence of a persistent transgene product in the CNS after introduction into the eye, raising the possibility that virus might spread beyond the site of injection. Recent fatalities in human gene therapy experiments (Lehrman, 1999; Marshall, 1999; Sibbald 2001) and the development of leukaemia in two gene therapy subjects (for x-SCID, Williams and Baum 2003) argue for caution in the use of the current vector approaches and for the development of new vectors. It is important, however, to emphasise that the latter two patients received systemic therapy (of adenovirus and a retrovirus respectively), and once investigated there may be no causal link between the treatment and the deaths. Locally delivered genes may not affect patients in the same way.

As with the growth factor studies, most of the replication defective adenovirus transfer studies in animals reported a loss of label and loss of photoreceptors after relatively short periods of time. The reasons for this are not known: possibly the transgene is unstable, exposure to the virus is eventually toxic, or alternatively, the transgenes may persist but become ineffective because other regulatory elements are lost. A further explanation is that the cells themselves are eventually removed by immunological mechanisms (Reichel *et al.*, 1998).

However, until safe vectors, producing stable, long-term expression of factors can be identified this is unlikely to be a suitable treatment, especially since for maximum effectiveness the treatment has to be given early in life. In addition, the complex genetics of RP makes it difficult to establish commercially viable treatments when homologous genes are introduced, especially since each patient group will often be quite small (the most common subtype in the USA and Europe, P23H, represents about 11% of autosomal dominant RP (Sohocki *et al.*, 2001). For the larger AMD group, since the genetic correlates are presently undefined, a gene therapy treatment would be difficult to develop at this point other than one involving a relatively non-specific approach such as growth factor delivery or anti-angiogenic factors as described above.

1.5.2.4 Dietary and drug treatments

Modification of diet may be useful in some specific forms of retinal dystrophy. Examples include Refsum disease (a phytanic acid deficiency); vitamin A supplementation for a-beta-lipoproteinaemia (Gouras *et al.*, 1971) and vitamin B6 for gyrate atrophy (Kennaway, *et al.*, 1989) but not all patients benefit from the treatment, suggesting disease heterogeneity. Such heterogeneity may also have been a factor in a randomised, controlled, double-blind clinical trial on the use of vitamin A

and vitamin E in the treatment of RP (Berson *et al.*, 1993), where only a subset of patients appeared to benefit from vitamin A supplementation. Moreover the beneficial effect was only demonstrated in electroretinograms; there was no treatment effect on visual acuity. Recently D-cis-diltiazem, a calcium-channel blocker that also acts on light-sensitive cGMP-gated channels, has been shown to rescue photoreceptors and preserve visual function in the rd mouse (Frasson *et al.*, 1999b). Controversially, this finding was not replicated by a separate group of investigators (Pawlyk *et al.*, 2001) and further studies will be required to assess whether this drug will have a real application in retinal disease therapy.

1.5.3 Treatments Directed at Replacing Lost Photoreceptors

1.5.3.1 Introduction

Once the photoreceptors are lost, the only option available is to reconstruct the outer retina. This is not an insignificant task, requiring reconstruction of information flow circuitry. Presently two approaches are being explored i)transplantation of new photoreceptors either as cell dissociates or as a retinal sheet and ii) the implantation of a microchip that can take over the phototransduction role of the photoreceptors and relay information to the CNS sufficient for sensory discrimination.

1.5.3.2 Cell transplantation

Early experiments transplanting photoreceptors used embryonic or perinatal tissue grafted into adult rat eyes (Turner and Blair, 1986). Graft success appeared to be age-dependent, with a marked drop in viability after embryonic day 21 (Aramant et *al.*, 1988). Instead of showing normal tissue orientation, the grafts formed rosettes. Silverman *et al.*, (1989) harvested and transplanted photoreceptors as a sheet, a procedure that improved cell orientation. There was also evidence from these pioneer

studies that photoreceptors might integrate with host neural structures (del Cerro *et al.*, 1989; Silverman *et al.*, 1992; Gouras *et al.*, 1994; Adolph *et al.*, 1994).

More recently, sheets of embryonic retina have been introduced into the subretinal space using a carrier device that allows them to unfold flat (Bergstrom 1994; Sharma *et al.*, 1997; Aramant *et al.*, 1999; Ghosh and Ehinger, 2000). These sheets subsequently survived and maintained orientation in the subretinal space. In another approach, Kwan *et al.*, (1999) transplanted partially dissociated post-natal mouse retina into the subretinal space of rd mice. These grafts reformed into a well-organised photoreceptor layer, which developed a synaptic interface with host retina. They also showed improved visual function in photophobia tests.

Clinical retinal transplantation experiments, apart from showing that such grafts appear not to cause additional damage, have produced either no, or ambiguous, evidence of functional improvement (Kaplan *et al.*, 1997; Das *et al.*, 1999; Berger *et al* 2003). For systematic application to humans, not only is it necessary to identify a source of easily available, tissue-matched, disease-free cells of appropriate age, but also neuronal pathways must be reconstructed with sufficient precision to provide an effective substrate for vision. In a pilot attempt to transplant retinal sheets, two blind patients with RP reported some visual sensation (Radtke *et al.*, 1999). Whether such responses were mediated through the transplant or were due to effects on the remaining retina is not presently clear.

Like RPE grafts, some neural retinal grafts have been shown to survive for prolonged periods following transplantation to the subretinal space (Gouras *et al.*, 1994) and Larsson *et al.*, (1999) showed that allografts, albeit of foetal tissue, had comparable survival times to syngeneic grafts. Neural retinal grafts placed in immune-privileged sites (eg. the anterior chamber) display features of ACAID, with a down-regulation of

normal delayed-type hypersensitivity (Jiang *et al.*, 1993), but they eventually succumb to atypical graft rejection (Jiang *et al.*, 1995).

To provoke a more typical form of graft rejection, antigen must be presented to host T-cells in association with MHC II molecules on antigen-presenting cells (Van Buskirk *et al.*, 1997, and see Fig 7.1) and some up-regulation of MHC II expression in subretinal grafts has been described (Ghosh *et al.*, 2000; Larsson *et al.*, 1999). Unlike RPE cell grafts, neural retinal grafts contain a heterogeneous cell population and, although the identity of the antigen-presenting cells in the neural retina is not entirely clear, donor microglia (termed passenger leukocytes by Ma and Streilein, 1998) are likely candidates. The observed increase in MHC II expression may be on these microglia. Thus, while the neural retina itself may not be immuno-provocative, other associated donor cells could be. Their management might provide a focus for the control of graft rejection in the future.

1.5.3.3 Microelectrode prostheses

An alternative approach to replacing photoreceptors with cells is to use implanted microphotodiodes to carry a visual signal to the cells of the inner retina (Humayun and de Juan 1996; Zrenner *et al.*, 1997; Chow and Peachey, 1998, 1999; e.g. see Majji *et al.*, 1999; Normann *et al.*, 1999; Rizzo *et al.*, 2001). Using various animal models, photodiode arrays have been placed either subretinally (Peyman *et al.*, 1998) where they would be expected to stimulate bipolar cells or adjacent to the vitreal surface to stimulate cells of the ganglion cell layer. These artificial implants appear to remain stable and are biocompatible (Peyman *et al.*, 1998; Zrenner *et al.*, 1997; Majji *et al.*, 1999).

In humans, direct pattern stimulation of the surface of the retina can elicit simple percepts (Humayun *et al.*, 1996), but long-term implantation of functioning photodiode/electrode arrays has yet to be reported.

Much still has to be done to ensure that a significant signal can be presented to cells of the inner retina and that the progressive deterioration of the inner retina (which normally follows outer retinal cell degeneration) does not compromise efficacy. It has yet to be seen how far the CNS can interpret signals delivered through such prostheses to elaborate visual images. Presently the best that might be expected from such endeavours would likely be a fairly crude image, but this may be sufficient to permit navigation around obstacles.

Nevertheless, this type of therapy is potentially very exciting and advances are being made in chip technology. The first human chip implantation was recently performed in the USA (Margalit *et al* 2003). They implanted a chip on the patient's macula, securing it with retinal tacks. The chip was linked to an external camera, which received, encoded and then transmitted to the chip. The implant decoded the signal and generated another signal to stimulate the array to facilitate neuronal transmission. Zrenner and colleagues have concentrated on developing a biocompatible system (Zrenner 2002) that the retina tolerates, and which allows neuron growth but the chip erodes by 6 months. This may be a future therapy, although many technical problems need to be overcome, but one of the greatest difficulties would be the secondary inner retinal remodelling that occurs in retinal disease (Jones *et al.,* 2003). The retinal ganglion cell pathway could be so compromised that no interpretable image could be sent to the visual centres of the brain.

1.6 Summary

Some of these therapies have great potential but so far no treatment is problem free. The approaches to AMD and RP will essentially be different. Therapy for AMD will rely on preventative dietary supplementation and vascular containment initially. Until the pathogenesis of AMD understood, it will be necessary to concentrate on treating the final effects of the disease i.e. RPE failure with CNV and GA development. To this end replacement of the diseased RPE is an attractive option but the treatment would have to be performed prior to extensive photoreceptor loss.

I believe that initial trials in inherited retinal disease should concentrate on maintenance strategies, such as the use of growth factors or cells that release growth factors. Since genetic research has given us the opportunity to identify specific groups of patients pre-clinically it will be possible to identify suitable cohorts of patients who would benefit most from any given treatment. While the ultimate goal of this research would be the restoration of as near-normal a retina as possible, simply maintaining some degree of functional vision in patients (who would otherwise be blind) would be highly desirable, both for the patient and the economy. It is through carefully designed animal studies that such clinical goals can be achieved.

1.7 Specific Aims of the Thesis

Development of Alternative Cell Transplantation Paradigms for Retinal Disorders

A) Investigate the potential of immortalised RPE cell lines (both human and rat) for preventing photoreceptor loss in the dystrophic RCS rat.

B) Determine the behavioural and functional outcomes of these procedures, as well as morphology.

C) Examine the relationship between function and morphology following RPE transplantation to the retina of the RCS rat.

D) Reliably locate the grafted cells following transplantation.

E) Investigate the potential of syngeneic Schwann cells to rescue photoreceptor morphology in the rhodopsin knockout mouse.

Chapter 2 will describe the materials and methods common to all the experiments. Chapters 3-6 describe the experimental design and the results with a specific discussion relevant to the aims of the experiments. Chapter 7 discusses general themes important for the application of cellular transplantation as a clinical therapy.

Chapter 2

MATERIALS AND METHODS

2.1 Introduction

The following chapter describes 1) the generation and characterisation of all the donor cell types used in the experiments; 2) the techniques common to most of the projects such as transplantation surgery and visual function tests and 3) the experiments themselves.

Tissue culture, transplantation surgery, behavioural studies, immunohistochemistry and histological analysis have all been carried out by myself. The electrophysiology and the development of the complex mathematical formulae have been carried out by my collaborators. Details of these have been included in the thesis because they are important and integral to the work. I am, however, familiar with these procedures.

2.2 Generation and Characterisation of Potential Donor Cells

2.2.1 Immortalised Retinal Pigment Epithelial Cells

2.2.1.1 LD7.4, an immortalised rat pigment epithelial cell line

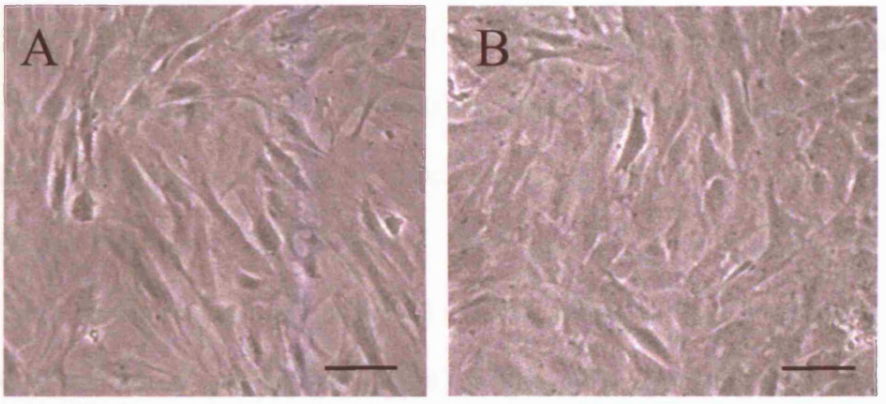
A retinal pigment epithelial cell line (named LD7.4) was used for allogeneic transplant studies. It was derived from the PVG rat (Greenwood *et al.*, 1996) at the Institute of Ophthalmology, London. Briefly, primary cultures of RPE cells were derived from 6-8 day old PVG rats (Fig 2.1A). A replication deficient SV40 retrovirus (encoding a temperature sensitive large T-antigen (tsa58) and a neomycin resistant gene) was used to infect cultures. Cells were subsequently selected with 800μg/ml G418 (Life Technologies, Paisley, UK) and appropriate clones were grown in selective media. A single clone with stable RPE phenotype and morphology was selected (LD7.4) and expanded for use in transplantation studies (Fig 2.1B). Cells were grown in Ham's F-10 (Gibco/ Life Technologies, Paisley, UK) supplemented

Figure 2.1: Primary and Immortalised Rat RPE Cells

A) Phase contrast light micrograph of primary rat retinal pigment epithelial (RPE) cells in culture (passage 3). B) Phase contrast light micrograph illustrating an immortalised RPE cell line following infection with a replication deficient retrovirus encoding a temperature sensitive large T-antigen (tsa58). Illustration shows cells at passage 22. Note the preserved morphology. Scale bar = 20μm.

Figure 2.1

Primary and Immortalised Rat RPE Cells



with 20% foetal calf serum (FCS, Life Technologies, Paisley, UK), 20mM HEPES, 7.5% sodium bicarbonate, 2 mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin (Sigma, Poole, Dorset).

2.2.1.2 LD7.4 Cells Labelled with Green Fluorescent Protein

2.2.1.2.1 Generation of Human Foamy Virus:

The replication defective Human Foamy Virus (HFV) was generated as described in Bieniasz *et al.*, (1997). In brief, a plasmid pRFF62 was constructed containing HFV LTRs, gag, pol and env genes with bel-1 and bel-2 reading frames destroyed. The green fluorescence protein (GFP) gene was inserted into the bel genomic region under the transcriptional control of an SV40 promotor.

2.2.1.2.2 Transfection of LD7.4 Cells:

RPE cells were seeded at $5x10^3$ cells/well in a 24 well plate. Twenty-four hours later the cells were transfected with $5x10^4$ virus particles. After six hours, the medium was changed and fresh medium containing $5x10^4$ virus particles added to the cells. The medium was changed on the following day. When the cells were 90% confluent (2 days later) the cells were detached from the 24 well plate using trypsin and grown in 2 Falcon flasks (Nunc, Gibco, UK) for another 3 days. After trypsinisation the cells underwent flow cytometry, which showed a transfection rate of more than 60%. The GFP-transfected rat RPE cells were separated from the non-transfected cells by cell sorting (B & D cell sorting, BD Biosciences, UK). Once 10,000 positive cells had been collected, they were grown again in a Falcon flask. Even after 4 weeks, flow cytometry showed that more than 90% of cells were positive.

2.2.1.3 h1RPE-7, an Immortalised Human Retinal Pigment Epithelial Cell Line

A human RPE cell line, h1RPE-7, also derived at the Institute of Ophthalmology, was used for xenogeneic transplant studies. Briefly, RPE cells were cultured from an eye

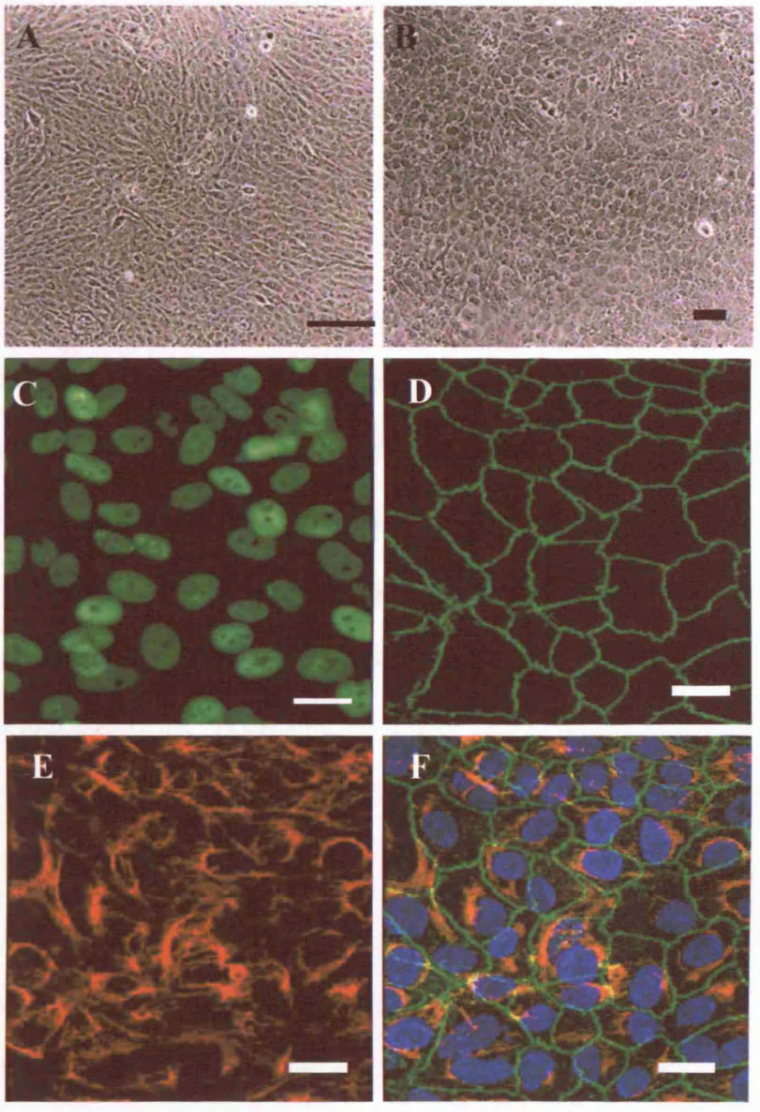
obtained with informed consent from a 50 year-old female Caucasian donor under local ethics committee approval. The RPE was isolated, the cells extracted and seeded onto tissue culture flasks and culture medium added. The culture medium consisted of Ham's F-10, 20% heat-inactivated foetal calf serum, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin (Gibco / Life Technologies, Paisley, UK) and 1μg/ml amphotericin B (Boehringer Mannheim, Germany). Medium was changed every 2-3 days and all cells were cultured at 37°C in 5% CO_2. Primary cultures were genetically modified to extend their *in vitro* life span by transfection with RSV puro (Neurotech S.A., Evry, France; which encodes a puromycin-selectable marker) and a construct encoding SV40 large T-antigen that was deleted for the small T-antigen (pVim TΔt). The construct was generously provided by Denise Paulin, University of Paris, Paris, France. Lines were established by selection in 1μg/ml puromycin. A number of genetically modified human RPE cell lines were screened for the expression of SV40 large T-antigen, cytokeratins (5, 8 and 18) and for the junctional protein zonula occludens-1 (ZO-1). Cell monolayers were fixed with either 3.7% paraformaldehyde (for detection of ZO-1 and SV40 large T-antigen) or cold acetone: methanol (1:1 for cytokeratins). Cells were permeabilised with 0.25% Triton X-100 (BDH/Merck, Poole, Dorset) and phosphate-buffered saline (PBS) and blocked for 15 mins with 10% goat serum and PBS. Primary antibodies were diluted in blocking solution and placed on the cells for 1h at room temperature and detected using appropriate FITC- or TRITC-conjugated secondary antibodies (1: 50) for 1h in the dark. Omission of the primary or secondary antibody provided negative controls. Primary antibodies for immunohistochemistry were hamster SV40 large T-antigen (ascites; a gift from Dr M. Viguier, Institut Cochin de Génétique Moléculaire, Paris);

Figure 2.2: Primary RPE Cells and Characterisation of Immortalised Human h1RPE7 Cells

A) Phase contrast micrograph of contact-inhibited monolayer of primary cultured human donor RPE cells 10 days after seeding and B) of immortalised human clone h1RPE-7 cells derived from the culture depicted in A (scale bar: 100μm). Both cultures exhibit the cobblestone morphology characteristic of RPE cells. C) Immunocytochemical detection by epifluorescence microscopy of SV40 large T antigen showing correct nuclear expression (scale bar: 20μm). D) Immunocytochemical detection by confocal scanning laser microscopy (projected images) of junctional protein ZO-1 showing an almost continuous pattern of peripheral staining (scale bar: 20μm). E) Immunocytochemical detection of the RPE cytokeratins 5 & 8. F) Overlay of images depicted in immunomicrographs D) and E) plus bisbenzimide (Hoechst) DNA stain (blue) to highlight the cell nuclei (scale bar: 20μm).

Figure 2.2

Primary Human RPE Cells and Characterisation of Immortalised Human RPE (h1RPE7) Cells



rabbit polyclonal ZO-1 (Zymed, Cambridge Bioscience, Cambridge); monoclonal antibodies RGE-53, specific for cytokeratin 18, RCK-102 specific for cytokeratins 5 and 8 (Eurodiagnostica, Arnhem, The Netherlands) and NCL-5D3, specific for cytokeratins 8 and 18 (Novocastra Laboratories, Newcastle-upon-Tyne). The goat anti-mouse and goat anti-rabbit secondary antibodies were supplied by Jackson ImmunoResearch Laboratories (Westgrove, PA). Cells were viewed on a Zeiss epifluorescence or confocal laser-scanning microscope. From this immunocytochemical assay, a cell line, designated h1RPE7, was selected (Fig 2.2) (see Winton *et al.*, 2000), which was positive for SV40 large T-antigen, ZO-1 and cytokeratins 5, 8 and 18.

ARPE 19, a spontaneously derived RPE cell line obtained from the American Type Culture Collection (ATCC; CRL-2302; Rockville, MD) and exhibiting an extended *in vitro* life span was also examined. These cells were cultured in medium that consisted of Dulbecco's modified Eagle's medium (DMEM): Nutrient Mixture F12, 1:1 mixture, with Hepes buffer (Gibco, UK) containing 10% fetal bovine serum , 56 mM final bicarbonate concentration, 1% (by volume) 200 mm L-glutamine, 0.1 mg ml^{-1} streptomycin and 100 U ml^{-1} penicillin (all Gibco / Life Technologies, Paisley, UK). The medium was replaced weekly, as previously described, (Dunn et al., 1996). Cells were also positive for ZO-1 and the cytokeratins.

2.2.1.3.1. Mixed lymphocyte reaction to evaluate the potential of h1RPE7 and ARPE19 cell lines to induce T-cell proliferation.

Ideally donor cells should not provoke a host lymphocyte response. The two human RPE cell lines were tested to assess their capacity to induce T-cell proliferation *in vitro* as follows:

Human peripheral blood mononuclear cells (PBMCs) were harvested from a healthy human donor by density-gradient centrifugation on Ficoll-Paque™ (Pharmacia Biotech AB, Uppsala, Sweden). The PBMCs were then plated onto plastic Petri dishes for 45 min at 37°C/5% CO_2 to allow monocytes to adhere. Non-adherent cells were removed and the enriched population of T-lymphocytes was co-cultured (2×10^5 cells/well) with either stimulated (IFN-γ, R&D Systems, Abingdon, UK, 100U/ml for 48 h) or unstimulated human RPE cell lines that had previously been irradiated (240Gy). The RPE cell lines, h1RPE7 and ARPE19, were plated out at various concentrations: 2×10^4, 1×10^4, 0.5×10^4 and 0.25×10^4 cells/well in triplicate in 96 well plates in 200μl of RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 10% FCS, penicillin 100 U/ml and streptomycin 100 μg/ml. Two irradiated (120Gy), allogeneic human B-cell lines (MOU, 9,050; SA, 9,001; from the Xth International Histocompatibility Workshop, New York, 1987) were used as positive controls at the same concentrations as the RPE cells. The allogeneic T-cells were harvested and proliferation was assessed on day 6 following an eighteen-hour pulse with [^{3}H]-thymidine (0.5μCi/well) by measuring incorporated [^{3}H]-thymidine by liquid scintillation spectroscopy. Background uptake of [^{3}H]-thymidine into the irradiated cells alone was subtracted from the harvested T-cell data.

2.2.2 Generation of Neonatal Mouse Schwann Cells

Mouse Schwann cells were generated from S129 mice using sciatic nerves dissected from postnatal day 5-7 pups (n=17) and transferred to Leibowitz's L15 medium (Life Technologies, Paisley, UK). After removal of contaminating tissues, the nerves were chopped into 100μm pieces using a McIlwain Tissue Chopper and digested in a collagenase/trypsin mixture (Dong *et al.*, 1997) in Dulbecco's Modified Eagles Medium (DMEM, Life technologies, Paisley, UK) plus HEPES and FCS for 75 mins

at 37°C. The digestion was stopped using DMEM plus 10% foetal calf serum (Harlan Seralab, Crawley Down, UK). After spinning at 1000rpm for 5mins the cells were resuspended in medium and triturated through a glass capillary and a 25-gauge needle. Cells were plated onto poly-l-lysine (Sigma-Aldrich Co., Irvine, UK) coated dishes in DMEMF, plus glutamine, pyruvate and penicillin/streptomycin (Brook *et al.*, 1993) and incubated at 37°C / 5% CO_2. After 24h the medium was changed to remove unattached cells and debris. One week later, cells were removed from the dish with trypsin/EDTA and suspended in DMEM. Contaminating fibroblasts were removed using a panning method (Dong *et al.*, 1997). Briefly, cells were incubated at 37°C in a 100mm plastic dish (not tissue culture plastic) previously coated with rabbit anti-rat IgG (Dako, Ely, UK) and Thy 1.2 IgM (the latter being a supernatant from a mouse 129 hybridoma cell line, generous gift of Dr. Roger Morris, Kings College, London). Most fibroblasts had attached to the dish after 10mins, and floating cells were removed, centrifuged and resuspended in DMEMF prior to replating onto poly-l-lysine coated dishes (Fig 2.3). Cells were ready for transplantation within 24-48h. After assessing for the percentage of contaminating fibroblasts (less than 10%), cells were removed from the dish and centrifuged as before. Cells were counted and transplanted in suspension to Rho-/- mice in DMEM plus DNase (DNase 1, 5µl/ml, Sigma UK). DNase reduced cell aggregation in suspension by digesting the 'sticky' DNA released by dead cells.

2.2.2.1 Growth factor production by mouse Schwann cells *in vitro*

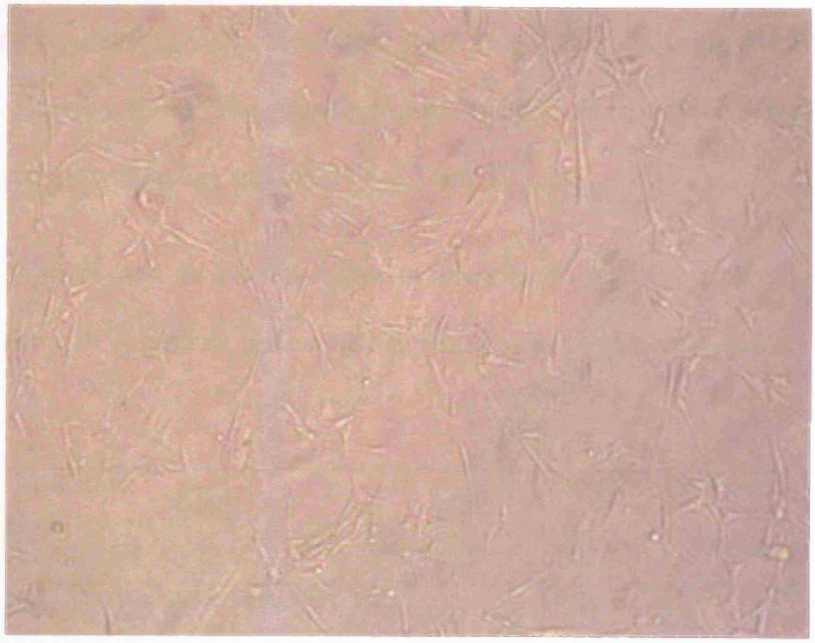
To examine growth factor production by mouse Schwann cells, a batch of cells was prepared as for transplantation but left in culture for a further 7 days after purification. Cells were trypsinised from the dish, and the enzyme activity quenched with DMEMF. The cells were centrifuged at 1000 rpm for 5 minutes, the pellet was

Figure 2.3: Neonatal Mouse (S129) Schwann Cells invitro.

The spindle-shaped appearance of the mouse (129) Schwann cells is shown. The isolation technique involves purifying the culture to remove the majority of contaminating fibroblasts. Primary cultures do not divide rapidly hence the relatively sparse numbers.

Figure 2.3

Neonatal Mouse (S129) Schwann Cells *In Vitro*



resuspended in a large volume of DMEM and the cells were spun again. The supernatant was removed and the cells were suspended in a small volume of DMEM and transferred to an eppendorf tube. The cells were centrifuged once more and as much DMEM as possible was removed. They were frozen rapidly prior to analysis by reverse transcriptase PCR (RT-PCR) following the manufacturer's protocol (Life Technologies, Paisley, UK). Messenger RNA was extracted using the RNeasy kit (Qiagen, Crawley, UK). The growth factors assayed were BDNF, CNTF, GDNF, NGF (nerve growth factor) and bFGF. See Table 1.

Table 1: Primers used for the target growth factor genes with the product sizes of the corresponding PCR product, annealing temperatures and number of cycles.

Gene	Sequence	Product Size (bp)	Number of cycles	Annealing Temp (^{0}C)
1. NGF	5'-ATGTGGTTCCTGATCCTG-3' 5'-TGTGATACTCAGGGG-3'	796	35	48.2
2. bFGF	5'-GCCACTTCAAGGATCCC-3' 5'-GGAAGAAACAGTATGGCCTTC-3'	378	35	57
3. CNTF	5'-GTAGCCGTTCTATCTGG-3' 5'-GAAGGTCATGGATAGACC-3'	479	35	48.2
4. GDNF	5'-AAGCACAGCTACGGGATG-3' 5'-GGGAGGAGCAGCCATTG-3'	707	35	57
5. BDNF	5'-ACGTCCACGGACAAGG-3' 5'-CACTTGGTCTCGTAG-3'	489	35	48.2

2.2.2.2 Fibroblasts

Since it was not possible to completely eliminate fibroblasts from the mouse Schwann cell cultures and in order to control for any potential rescue effect conferred by them,

fibroblasts were also transplanted. These donor cells were obtained from the cells left attached to the Thy1.2 coated dishes after panning (2.2.2). They were trypsinised off the dish and transferred to flasks containing DMEMF (10% FCS). The fibroblast cultures were left to expand almost to confluency. Prior to transplantation cells were trypsinised off the flasks, counted and suspended in carrier medium (DMEM) with DNase, as for the mouse Schwann cell cultures.

2.3 Subretinal Transplantation Surgery

All animal care was in accordance with Institutional and Home Office (UK) regulations and the UK Animals (Scientific Procedures) Act, 1986.

For the transplantation procedure (Fig 2.4), rats and mice were anaesthetised with intraperitoneal injections of ketamine (80 mg/kg)/xylazine (20 mg/kg), and the pupil was dilated using Tropicamide (1% Mydriacyl®, Alcon Labs., Hemel Hempstead, UK). Surgery was visualised using a Leitz operating microscope. The eye was proptosed with a 6/0 silk suture and stabilised with a fine forceps. The conjunctiva and Tenon's capsule were gently torn open with a bent 27G needle. The sclera was breached using a 30G needle, extending to the subretinal space. Cultures of LD7.4, h1RPE7, ARPE19 or neonatal mouse Schwann cells and fibroblasts were trypsinised, washed and cells in suspension were delivered trans-sclerally to the dorso-temporal subretinal space of anaesthetised host dystrophic RCS rats (P21-23) or rho-/- mice (P35). Cells were suspended in 2μl of Hams F-10 (Gibco) and injected through a fine glass pipette (internal diameter 75-150μm) attached to a 10μl Hamilton syringe. All transplants were to one eye only. Unoperated eyes served as controls. Sham-injected rats received the same volume of carrier medium alone (or carrier medium plus DNase in the Schwann cell and fibroblast groups). The degree of retinal detachment was assessed at the time of surgery (Fig 2.4B), using a scale from 1 to 10 where 10

Figure 2.4: Transplantation Procedure

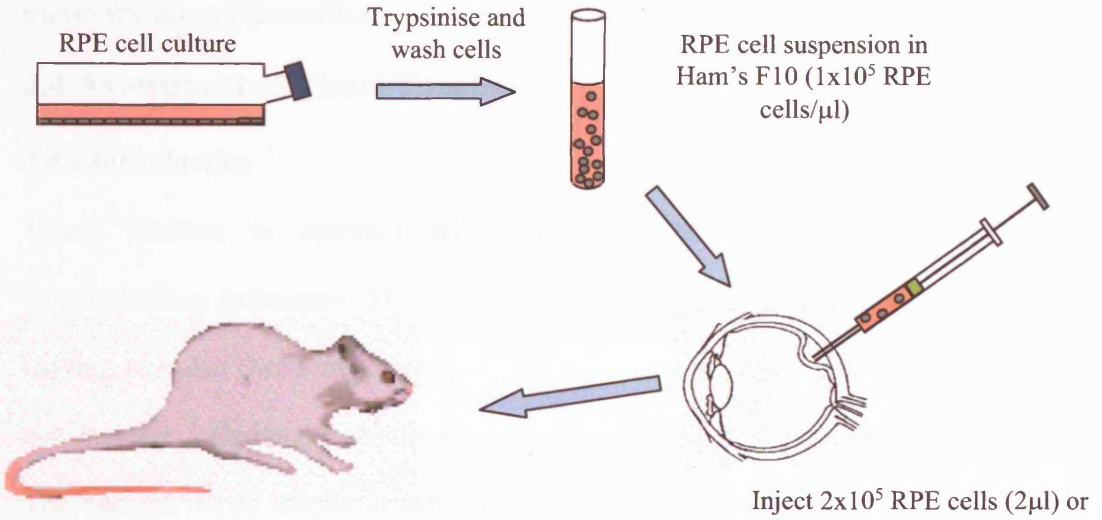
A) Schematic diagram of the process of cell culture from cell dissociation to transplantation. Cells, in suspension, are drawn up into a microsyringe and injected into the subretinal space of the host animal's eye at a concentration of $1x10^5$ RPE cells/ml (2μl) or $1x10^4$ Schwann cells (2 μl). The animals are then assessed behaviourally and the eyes sectioned for histology.

B) Illustrates the transplant procedure photographically: left panel shows the animal's posterior pole (note the centrally placed optic disc: arrowed), middle panel demonstrates the sclerostomy (arrowed) and the right hand panel shows the induced peripheral detachment (arrowed).

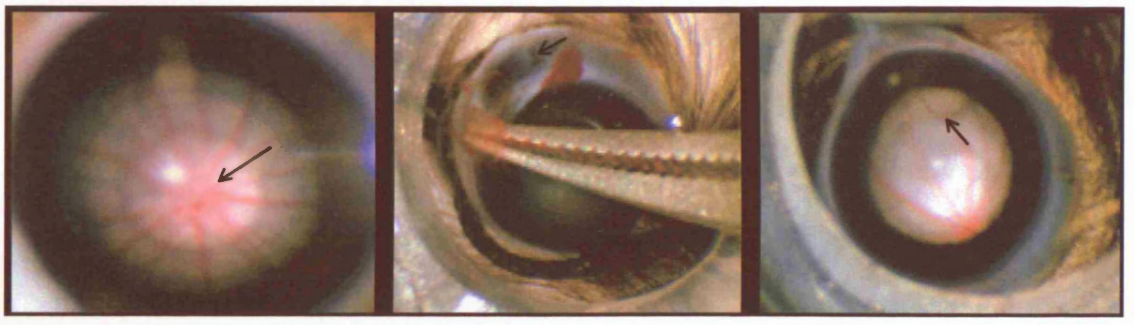
Figure 2.4

Transplantation Procedure

A



B



represented an excellent detachment. Animals were removed from the experiment if grafted cells penetrated through the retina to the vitreous. Animals receiving xenografts (ie. cells of human origin) and the sham controls in xenograft experiments were placed on *ad libitum* oral cyclosporine A (concentrate for infusion, Sandimmun, Sandoz, Camberley, UK; 210mg/l of drinking water) from 2 days before transplantation until sacrifice.

2.4 Assessment of Visual Function

2.4.1 Introduction

Visual function in operated and unoperated rats was assessed using two complementary techniques. The first involves testing the animal's ability to track a moving stimulus (head-tracking) and is dependent on an intact retina. Since it is a non-invasive technique it enables longitudinal determination of visual performance. The second, more discriminatory, test assesses retinal function by determining threshold sensitivity to a variable light stimulus, recording responses in the superior colliculus (SC).

2.4.2 Head-tracking

This procedure is based on an optokinetic test devised by Cowey and Franzini, (1979) (Fig 2.5A). At regular intervals (usually 4 weeks) animals were placed individually into a stationary enclosed perspex container surrounded by a motorised drum (Fig 2.5B) that revolved at a constant velocity (12^{o}/sec). Interchangeable panels (mean luminosity 240 lux) of vertical black and white stripes with spatial frequencies of 0.125, 0.25 and 0.5 cycles/degree were placed on the wall of the outer drum. Each was presented, in a random order, to the animal over three consecutive days. A test period consisted of 4 one-minute sessions of rotation interspersed with 30sec rest intervals. The direction of rotation was alternated so that at the end of the test period

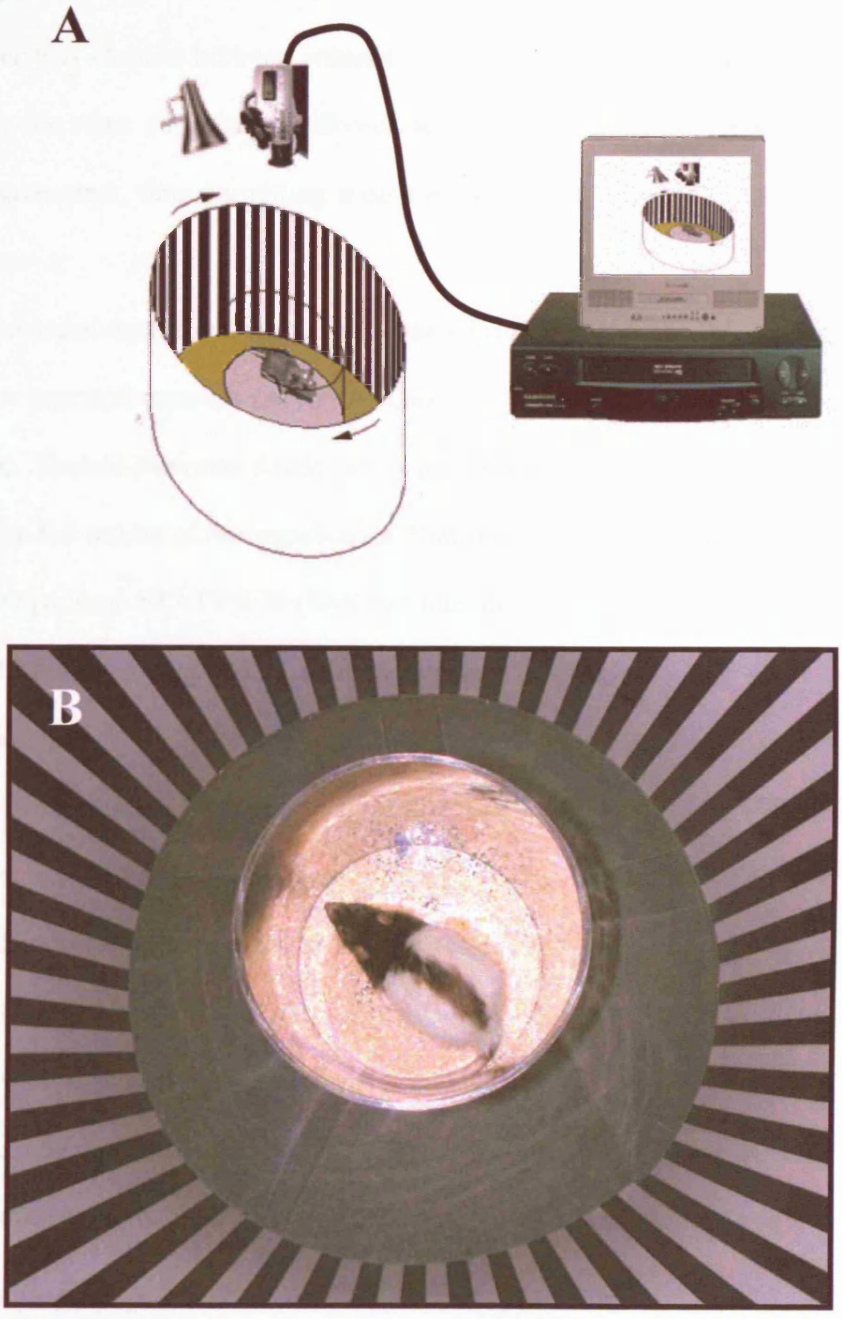
Figure 2.5: Head-Tracking to High Contrast Square-Wave Gratings.

A) Diagram of head-tracking apparatus. This figure shows the set-up employed to record the head tracking performance. The subject rat is placed in a Perspex holding chamber and varying sized gratings are rotated around them. A video-camera mounted above the animal records its movement. The investigator, monitor and video-recorder are placed in an adjacent room so that there are no extraneous stimuli. Time spent tracking the moving stripes is documented by the observer.

B) Photograph of a RCS rat in the holding chamber surrounded by the rotating drum lined with square-wave grating.

Figure 2.5

Head-Tracking to High Contrast Square-Wave Gratings



rats had experienced 2x1min clockwise rotation and 2x1min anticlockwise rotation. Presentation of the moving vertical stripes stimulated a turning reflex such that a seeing animal involuntarily moved its head, tracking the lines. Animals were timed only for periods during which head turning corresponded with the speed of rotation of the stripes: habitual and other randomised movements were excluded. The perspex container was cleaned between animals. At each time point, testing was carried out blind by the same observer. A video-camera mounted above the apparatus recorded head movements, thus permitting measurements to be checked by an independent observer.

All behavioural data at each time point was subjected to a 3-way analysis of variance with one repeated measure (ANOVA: Group x Eye x Grating Frequency-repeated). Post-hoc Student-Newman-Keuls were performed on any significant factors to determine the source of the significance. Statistical analysis was performed using the statistical package STATVIEW (SAS Institute Inc.).

2.4.3 Electrophysiology (Threshold Collicular Mapping)

Following the final head-tracking tests electrophysiological assessments of the area of visual field rescue and changes in threshold sensitivity were made (Fig 2.6). Under urethane anesthesia (1.25g/kg) the test eye was immobilised using 6-0 sutures attached to the frame, and the pupil was dilated with topical Tropicamide (Alcon Labs., Hemel Hempstead, UK). Corneal clouding was prevented using a non-corrective contact lens. Animals were dark-adapted for one hour at $0.02cd/m^2$ (low mesopic range) prior to recording from the superficial layers of the superior colliculus (SC) contralateral to the experimental eye. Multi-unit activity was recorded across the surface of the SC along a rectilinear grid of 200μm periodicity: each step corresponded to approximately 10°-15° displacements in the visual field. This

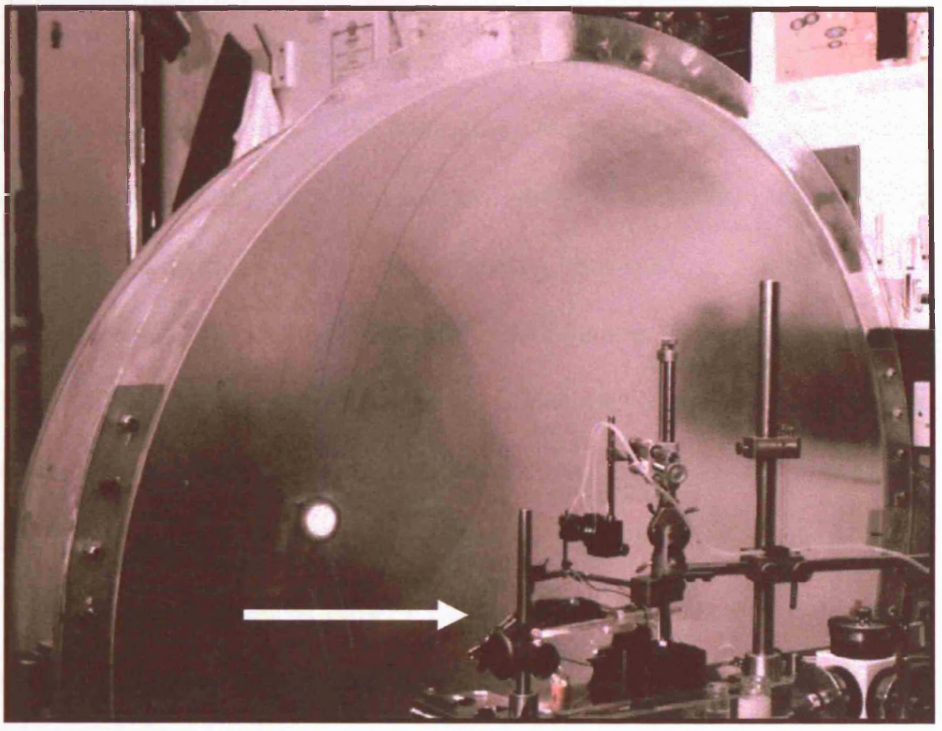
Figure 2.6: Electrophysiological Collicular Field Mapping

A) Photograph of the apparatus used for electrophysiological collicular mapping. The large Ganzfield bowl is set up such that the entire visual field of the animal is covered. Light stimuli of varying intensities are presented to the animal while they are on the platform (arrowed). B) Illustrates this process diagrammatically. A light stimulus is presented and nearly all retinal input is transmitted to the contralateral superior colliculus. An electrode within the colliculus detects unfiltered transmission from the retina. Threshold responses necessary to ellicit a response are recorded at 76 points across the colliculus and each is recorded to generate a threshold response map of the entire retina, this two dimensional map is shown in the bottom right corner.

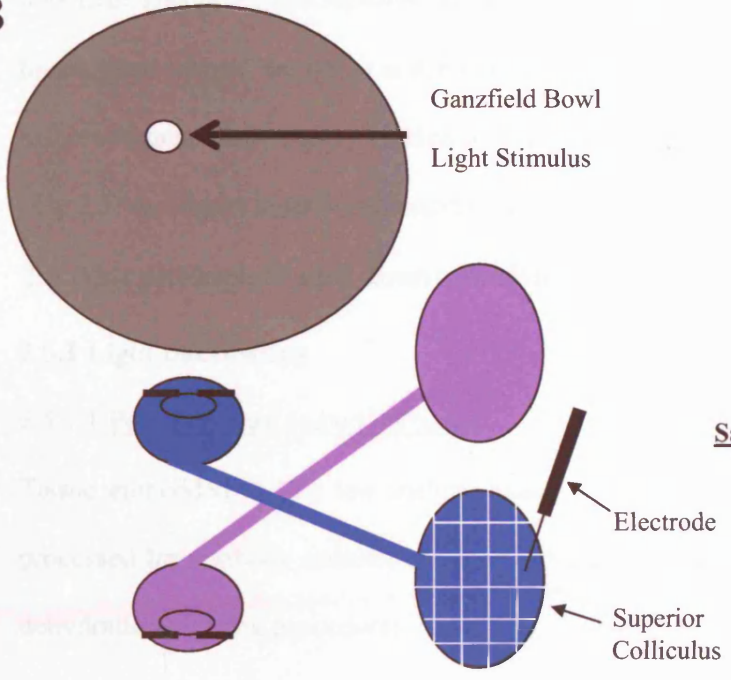
Figure 2.6

Electrophysiological Collicular Field Mapping

A



B



Sample Threshold Response Map

4.0 3.2 2.3 3.5
4.0 3.8 3.6 1.8 1.4
4.0 3.7 3.5 1.5 0.9 1.1
3.8 3.5 2.8 2.6 1.7 2.3
3.8 3.6 3.8 2.9 2.8
3.6 2.8 3.5 3.6

procedure was repeated for 76 independent points. In some animals recordings were made at 400μm intervals, in which case intervening points were interpolated by taking average visual threshold recordings from all the immediately adjacent points. The validity of this approach was confirmed by random recording of such points. Visual thresholds were measured as the increase in intensity over background (maintained at 0.02 candela/m^2 to minimise rod saturation) required for activating units in the superficial 200μm of the SC with a spot of light 3° in diameter. Data gathered from each point was assembled to give a global image of the eye.

After head-tracking or electrophysiological recording eyes were processed for light or electron microscopy as described in 2.5 below and retinal sensitivity/histology correlation maps were generated. Sample sections are taken at regular intervals (700μm) through the retina (the central ¾ of the retina is taken, as the outer regions are cut too tangentially to be reliably used). Each photoreceptor nucleus is counted across the section in 700μm bins. Thus a numerical gradient across the retina is obtained. This process is repeated for sections at 700μm intervals. A two dimensional histological map of the retina is achieved. Using a bespoke mathematical programme a three-dimensional map is created and compared with the retinal sensitivity map. (Fig 2.7: see figure legend and chapter 3.2 for details).

2.5 Morphological and Immunohistochemical Studies

2.5.1 Light microscopy

2.5.1.1 Polyester wax embedded tissue

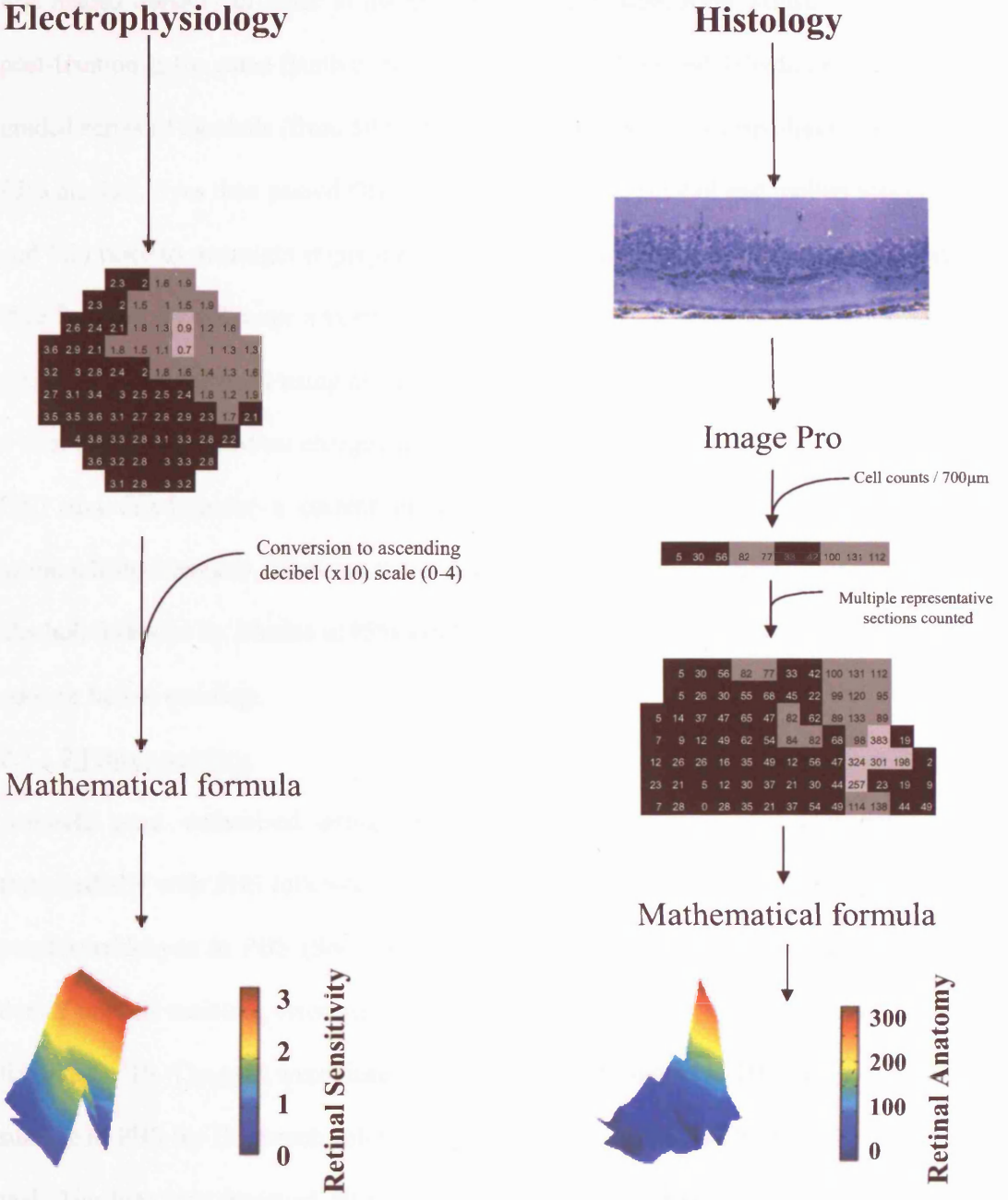
Tissue embedded in this low melting point wax (circa 40°C, BDH) can be readily processed for antibody staining because 100% alcohol and xylene are not used in the dehydration/clearing procedures.

Figure 2.7: Generation of the Retinal Sensitivity/Histology Correlation Maps

This figure details the process involved in generating the correlation maps. Electrophysiological recordings are obtained as described (2.4.3 and Fig 2.6). These logarithmic scales are converted to a decibel scale (ascending, thus greater sensitivity is shown as a peak). Through a bespoke mathematical programme the two-dimensional maps obtained are converted into a three dimensional map for comparison. On the right hand side of the figure the process for generating the histological map is shown. See chapter 3.

Figure 2.7

Generation of The Retinal Sensitivity/Histology Correlation Maps



Animals were euthanised using Euthatal (Rhone Merieux, UK) and perfused transcardially with PBS followed by periodate-lysine-paraformaldehyde (PLP, McLean and Nakane, 1974, see Fixatives, Appendix I). Prior to eye excision a suture was placed dorsally in order to maintain tissue orientation. After excision and brief post-fixation in the same fixative, eyes were washed in PBS and dehydrated through a graded series of alcohols (from 50 to 95%). The cornea and lens were dissected out in 95% alcohol. Eyes then passed through mixtures of 95% alcohol and molten wax (2:1 and 1:2) prior to overnight impregnation with pure wax, and embedding the next day (See Protocol for polyester wax embedding, Appendix I).

Sections (8μm) were cut using an American Optical bench microtome (maintained in a cool room), mounted on charged glass slides (Superfrost Plus, BDH/Merck, Poole, UK) and dried under a current of air before storing at 4°C. Prior to use for immunohistochemistry, sections were dewaxed by briefly immersing in absolute alcohol, followed by 10mins in 95% alcohol. Sections were dried again in a current of cool air before staining.

2.5.1.2 Frozen sections

Animals were euthanised using Euthatal (Rhone Merieux, UK) and perfused transcardially with PBS followed by periodate-lysine-paraformaldehyde (PLP) or 4% paraformaldehyde in PBS (See Fixatives, Appendix I). A suture was placed in the dorsal pole to maintain orientation and the eye excised and post-fixed in the same fixative for 1h. The eyes were rinsed in PBS and cryoprotected in 10%, 20% and 30% sucrose in PBS for 1h in each solution or until the tissue had sunk to the bottom of the vial. The lens was removed through a cut in the cornea and the eye embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Miles, Raymond Lamb, London, UK). The tissue was frozen in a freezing mixture of dry ice and acetone and

stored at -70°C. Sections were cut at 14μm using a Leica CM3050 cryostat and mounted on charged glass slides in 5 consecutive series. The first series was stained with cresyl violet (0.5%, BDH/Gurr, Poole, UK) or haematoxylin and eosinyellowish (BDH/Gurr, Poole, UK: see Histological stains, Appendix I) for examination of retinal histology including the degree of photoreceptor survival and any evidence of pathological events. The remaining series were stored at -70°C and used for immunohistochemical studies. Prior to staining sections were brought to room temperature and dried in a current of air for one hour.

2.5.1.3 Immunohistochemistry

Sections were placed in PBS and then blocked with 5% defatted milk in PBS or serum appropriate to the animal the secondary antibody was raised in. Milk could not be used with secondary antibodies supplied by Jackson Laboratories. After blocking sections were placed in the primary antibody, diluted in antibody diluent and blocking serum. Sections were usually incubated overnight at 4°C in a humidified chamber. After washing in PBS, sections were reacted with diluted biotinylated secondary antibody for 30 mins followed by a streptavidin complex tagged with horseradish peroxidase (ABC Elite; Vector Laboratories, Peterborough, UK), or by streptavidin complexed with alkaline phosphatase (Dako, Ely, UK). Horseradish peroxidase was visualized using 1% nickel chloride and 3-3' diaminobenzidine (DAB, Sigma, Poole, UK) and alkaline phosphatase (AP) was visualised with Vector blue (Vector Laboratories, Peterborough, UK), using levamisole (Dako, Ely, UK) to inhibit endogenous alkaline phosphatases. Sections visualised with DAB were dehydrated through a graded series of alcohols and xylene and mounted (DePeX; Merck/BDH, Poole, UK). AP sections were dehydrated through alcohols to Neo-Clear (Merck/BDH, Poole, UK) and mounted in Vector Mount (Vector Laboratories,

Peterborough, UK). Negative control sections were incubated in defatted milk or serum overnight without primary antibodies before proceeding with the secondary antibodies as described. For details see Appendix I.

The antibodies used will be listed with each experiment.

2.5.2. Electron microscopy

Animals were overdosed with Euthatal (Rhone Merieux, UK), perfused intracardially with PBS and fixed with 2.5% paraformaldehyde (TAAB, Aldermaston, UK), 2% glutaraldehyde (TAAB, Aldermaston, UK) and 0.01% picric acid in 0.1M phosphate buffer (See Fixatives, Appendix I). A suture was placed opposite the site of transplantation before the eye was excised into fresh fixative in order to maintain tissue orientation. After overnight immersion, eyes were transferred to 0.1M cacodylate buffer (TAAB, Aldermaston, UK) and the lenses removed through an incision in the cornea. Eyes were post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer, followed by dehydration through a graded series of alcohols and epoxypropane before embedding in Agar resin (Agar Scientific, Stanstead, UK). For details see: Processing tissue for electron microscopy, Appendix 1. Semithin sections were cut on a Leica ultramicrotome and stained with toluidine blue (Sigma, Poole, UK). Ultrathin sections were cut from selected areas, mounted on copper grids (Agar Scientific, Stanstead, UK) and stained with alcoholic uranyl acetate and aqueous lead citrate (see Appendix I). Sections were viewed using a Jeol 1010 electron microscope.

2.5.3. RPE Donor Cell Identification

2.5.3.1. Introduction

Accurate identification of grafted donor cells in the host animal is exceptionally important in transplantation studies. A considerable amount of time has been devoted to developing reliable and reproducible labelling techniques for RPE cell lines.

2.5.3.2 Identification of bromodeoxyuridine labelled cells

Cells for grafting were pre-labelled *in vitro* with 20μM bromodeoxyuridine (BrdU, Sigma, Poole, UK) 48 hours prior to transplantation.

Frozen or polyester wax sections from the BrdU-labelled eyes were reacted with mouse anti-BrdU antibody (1:1000; Sigma, Dorset, UK). The secondary antibody was a preadsorbed biotinylated goat anti-mouse (1:200; Jackson ImmunoResearch Laboratories, Inc, USA). Streptavidin, complexed with alkaline phosphatase, was visualised with Vector Blue (Vector Laboratories, Peterborough, UK). For details see: BrdU visualisation protocol in Appendix I.

2.5.3.3 Identification of HFV/GFP labelled LD7.4 cells

Green fluorescent protein-labelled cells were identified in frozen section using anti-GFP antibody (Chemicon, Harrow, UK, 1:750) visualised with alkaline phosphatase and Vector Blue as in 2.5.3.2.

2.6. Experimental Design

A number of experiments were designed to test the capacity of the various donor cell types to maintain photoreceptors and visual function in the dystrophic RCS rat and the rhodopsin knockout mouse. These are described in detail in chapters 3-6. A scheme of each experiment is illustrated in Figures 3.1, 4.1, 5.1 and 6.1.

Chapter 3

3. TRANSPLANTATION OF IMMORTALISED ALLOGENEIC RPE (LD7.4) CELLS TO THE SUBRETINAL SPACE OF DYSTROPHIC RCS RATS

3.1 Introduction

Freshly harvested RPE cells have been shown to delay both functional and anatomical retinal degeneration in the RCS rat (1.3.1). However, while effective, they are likely to have limited application clinically given the large number of patients that may benefit. In particular, it would be difficult to provide sufficient numbers of healthy and phenotypically robust cells that are free from potentially harmful pathogens. Furthermore, ethical issues regarding the use of foetal tissue must also be considered (Gieser, 2001). This has led to the development of the genetically engineered cell lines, LD 7.4 (Greenwood *et al.*, 1996) and h1RPE-7, (Kanuga *et al.*, 2002; Lund *et al.*, 2001a) to be tested in this thesis. Preliminary work in the laboratory initially confirmed the efficacy of the LD 7.4 cell line compared with primary rat RPE cultured cells to preserve photoreceptors.

3.2 Experimental design

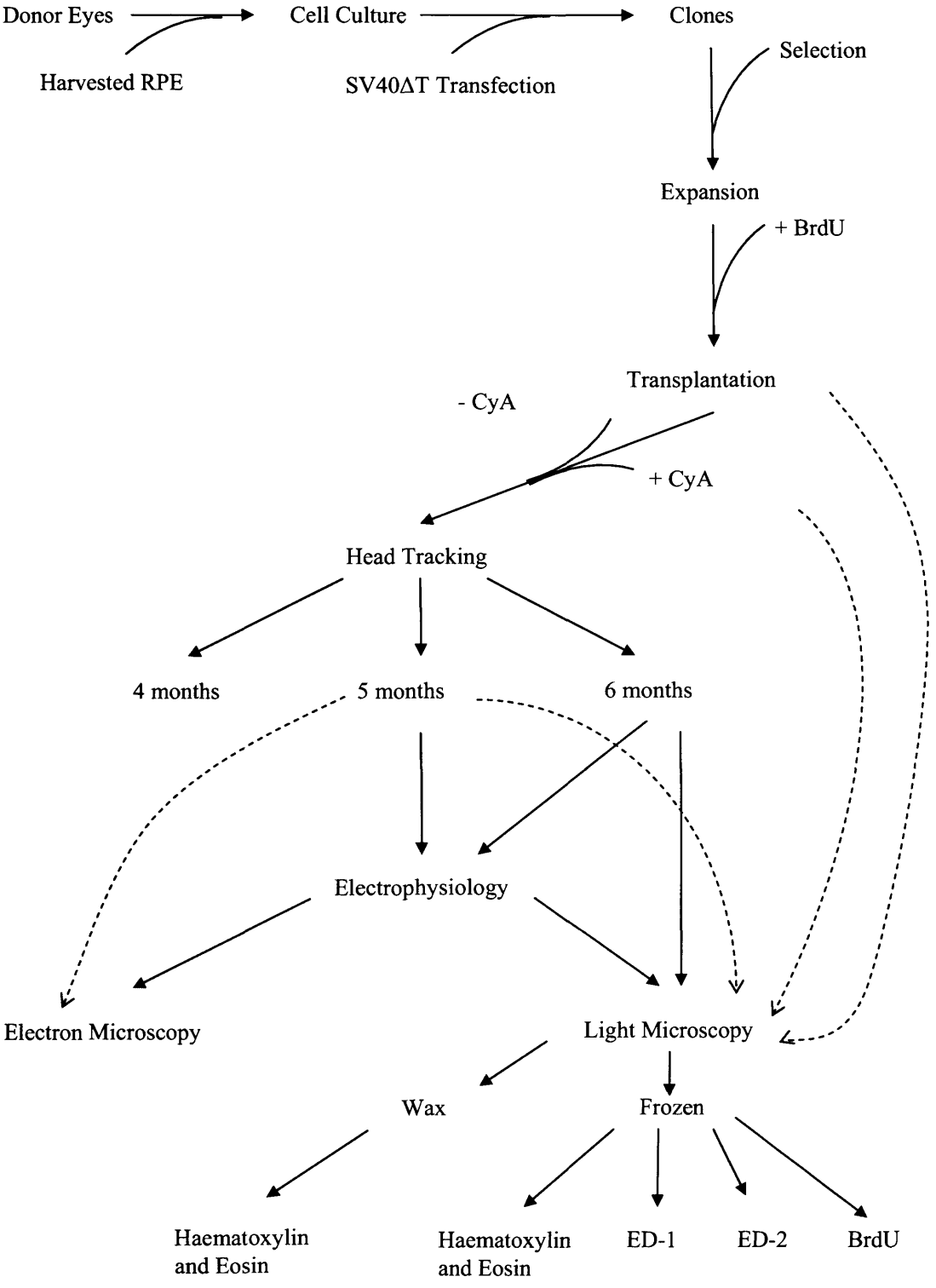
The purpose of this experiment is to examine the degree to which visual function can be preserved by allografted cell lines and the level of functional rescue that can be sustained over time without the use of immunosuppression. The experimental design is shown schematically in Fig 3.1. A total of 49 rats received unilateral subretinal grafts ($2x10^5$ cells per injection) of LD7.4 cells and 30 had sham injections. Nine grafted rats were allowed to survive for 5 months and 10 were sacrificed at 6 months with the remainder being sacrificed at earlier time points for anatomical assessment of

Figure 3.1: Flow Diagram of Transplantation Studies using Immortalised LD7.4 Allogeneic RPE Cells

See section 3.2

Figure 3.1

Flow Diagram of Transplantation Studies Using Immortalised Allogeneic RPE Cells



short-term changes associated with transplantation. Age-matched non-dystrophic RCS rats provided an additional control group. Viability of residual RPE cells remained at >90% as assessed by trypan blue exclusion at the end of a transplant session. No immunosuppression was used in this group. A separate group of animals (n=11), for histological analysis alone, received grafts of LD7.4 cells and were placed on *ad libitum* oral immunosuppression (cyclosporine: 210mg/l of drinking water). Lastly, in order to establish whether the fall off in photoreceptor preservation and visual function over time was due to the loss of grafted cells, 25 dystrophic RCS rats were grafted with LD7.4 cells prelabelled with bromodeoxyuridine.

Rats were head-tracked at 4 months (10 unoperated non-dystrophic animals, 28 grafted dystrophic animals and 16 sham-operated dystrophics), 5 months (n=8, 22 and 11, respectively) and 6 months (n=4, 6 and 5 respectively) of age. The times spent head-tracking were summed, averaged and the groups compared. Transplanted animals were divided into two groups for analysis, those that fell within two standard deviations of the sham performance (unsuccessful transplants) and those performing above this level (successful transplants): validity was confirmed on histology of "poor tracking" transplanted animals versus "good tracking" animals. Behavioural data was stored using Excel (Microsoft, USA). Results were analysed using a two-way analysis of variance (ANOVA), and post-hoc analysis on factors was performed on factors shown to be significant, using the Student-Newman Keuls test.

Electrophysiological recordings were made from 34 animals, (transplanted animals at 5 months, n=5; and 6 months, n=6; non-dystrophic controls at 5 (n=3) and 6 months (n=3); unoperated dystrophic controls at 5 (n=3) and 6 months (n=4) and sham-operated dystrophics at 5 (n =5) and 6 months (n=5). Results from individual points of up to 6 animals per group were averaged for the respective 76 points in the superior

colliculus and subjected to statistical analysis using both a randomisation test and a t-test to compare the thresholds from animals receiving LD7.4 cells compared with shams. A group of grafted (n=3) and sham-operated (n=2) animals were compared with histological maps (see below: Fig 2.7) to test for any correlation.

At the end of the experiment, at five or six months of age, eyes were retrieved, fixed and embedded in polyester wax (transplanted eyes n = 5 at 5 months and 3 at 6 months; sham-operated eyes n = 3 and 3 respectively) or resin (transplanted eyes n = 4 and 3 respectively; sham-operated eyes n = 1 and 2 respectively). Three of the retinae (from animals at 6 months) and two sham-operated retinae were analysed for detailed histological assessment using cresyl violet (CV) or haematoxylin and eosin (H&E). Photoreceptor counts were made across representative sections (10-12 per eye) to give a detailed histological picture using an image analysis system (Image Pro Plus, Media Cybernetics, USA). The wax embedded eyes were cut at 8μm intervals in three series. Sections located 700μm apart were selected for analysis. Individual sections were divided into 700μm blocks (10-13 per section) and each photoreceptor cell counted in the block. The data from multiple sections were grouped into a histological map that was comparable to the retinal sensitivity maps generated from SC mapping. This histological map was then compared with retinal sensitivity maps and any correlation observed (Fig 2.7).

At various survivals, sectioned eyes were reacted for BrdU, macrophage markers (ED-1, 1:200 and ED-2, 1:400: Chemicon, Harrow, UK) and examined for apoptosis with NeuroTACS™ II *in situ* apoptosis detection kit, (R&D Systems Europe, Abingdon, Oxon, UK), see Appendix I.

3.3 Results

3.3.1 Head-Tracking

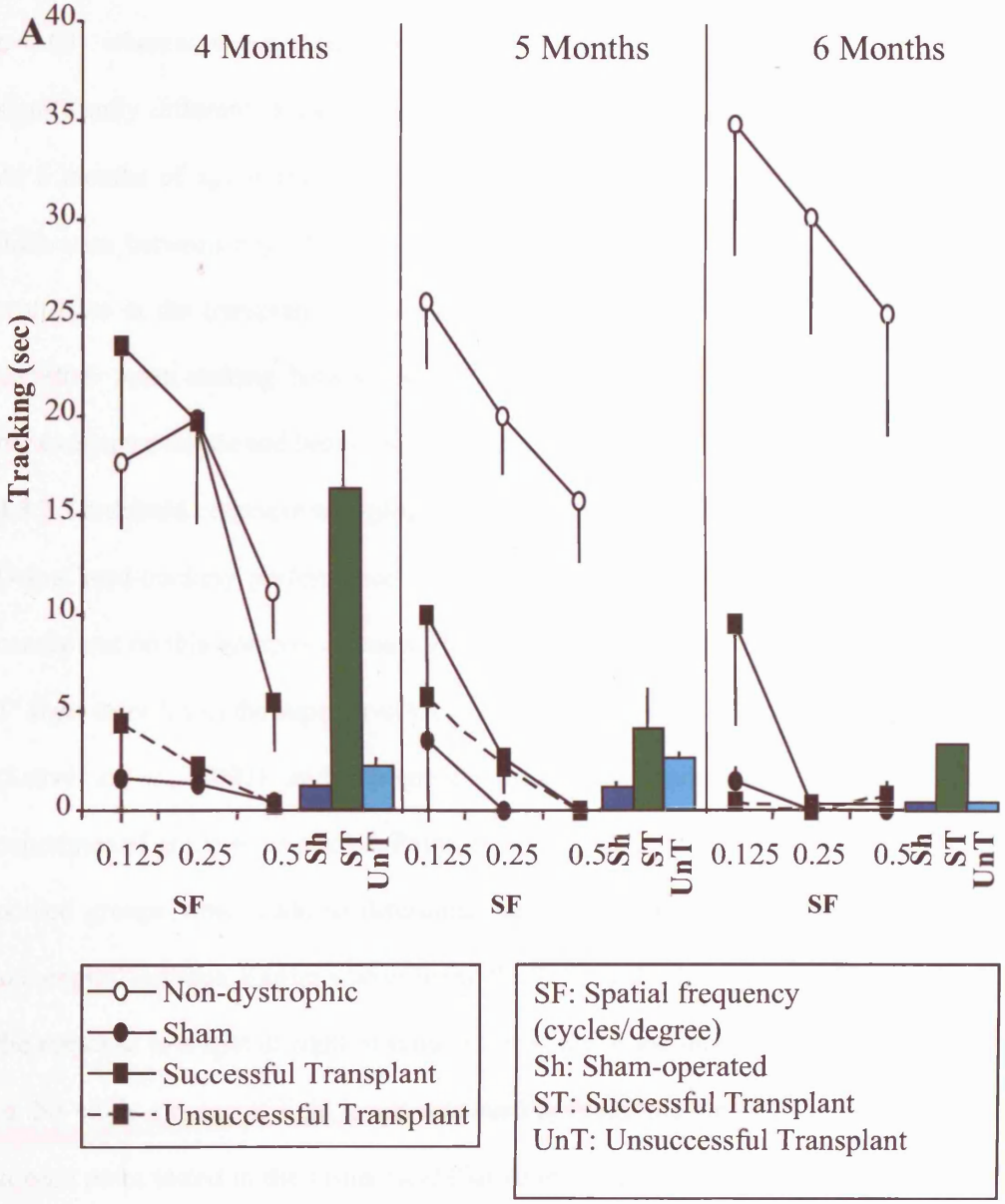
Previous studies have described the pattern of head-tracking in dystrophic versus non-dystrophic pigmented RCS rats (Lund *et al.*, 2001b; Lawrence *et al.*, 2000) and demonstrated that by 2 months of age dystrophic animals had lost the ability to track gratings of 0.125, 0.25 and 0.5 cycles/degree (c/deg). The performance of both control animals and those that had received subretinal grafts of LD7.4 cells at 3 weeks of age was evaluated using the same head-tracking procedure (2.4.2). By 4 months of age, two distinct sub-groups had emerged in the transplanted animal group: those that tracked well and those that did not (Fig 3.2A). This separation was based on the performance of transplanted animals at 4 months. Those animals with a head-tracking performance within two standard deviations of the mean sham performance were judged to be in the poor transplant group while those with performances better than two standard deviations were placed in the good transplant group. The differences in head-tracking performance between the good and poor transplanted groups at the 4 month time point could not be explained by the perceived success of the grafting procedure at the time of surgery (section 2.3) but did exhibit a close post-hoc correlate with anatomical preservation of the retina as illustrated in Fig 3.2B,C which shows the anatomy of a representative good (B) and a representative bad (C) transplant at the same time point. Thus, a grafted animal that tracked well at 4 and 5 months (12.82 and 9.08 seconds respectively) had better preservation of its outer nuclear layer compared with a grafted animal that tracked poorly (3.84 and 4.06 seconds respectively) at the same time points (Figure 3.2D). Analysis of variance (ANOVA) of the head-tracking data at 4 months of age revealed significant differences between the different experimental groups (F=13.408, df=3,49 p<0.0001). Post-hoc Student-Newman-Keuls showed no significant difference between the congenic non-dystrophic group (n=10) and the transplanted animals that tracked well (n=8).

Figure 3.2: Head-tracking responses of rats with grafts of LD 7.4 cells and sham-operated rats at 4, 5 and 6 months and comparative histology.

A) Head-tracking performance of transplanted animals (successful and unsuccessful transplants) compared with normal (non-dystrophic) and sham-operated (sham) animals at 4, 5 and 6 months. Mean head-tracking times (all gratings) for successful transplants versus sham and unsuccessful transplants at 4, 5 and 6 months are shown as histograms on the right of each graph. Validity of this separation was confirmed on sample animals with histology (B,C). Data for dystrophic controls is not shown, as the mean was less than one second at all time points. There is some variation in head-tracking for normal congenic animals but they perform significantly better than unsuccessful transplant and sham groups ($p < 0.05$), and at 4 months there is no significant difference between successful transplant and non-dystrophic ($p > 0.05$) groups. At 4 months successful transplants are significantly better than sham and unsuccessful transplants ($p < 0.05$). B) A representative retina from an animal that performed well on head-tracking with a marked preservation of the outer nuclear layer (ONL) and outer segment material (OSM). C) Illustrates a representative section of retina from a poorly performing animal, analogous to the area shown in B, i.e. within 200μm of the injection site: there is no ONL survival. Ganglion Cell Layer (GCL), Inner Plexiform Layer (IPL), Inner Nuclear Layer (INL), Outer Plexiform Layer (OPL), Outer Nuclear Layer (ONL), Outer Segment Material (OSM), Retinal Pigment Epithelium (RPE). Scale bar: 20μm. D) Histogram comparing the average transplanted and sham-operated animals head-tracking ability with the representative "good" and "poor" performers at 5 months, histologically represented in B and C respectively. Transplanted group (LD), Sham-operated group (Sh), Successful grafted animal (S) and Unsuccessful grafted animal (Un).

Figure 3.2

Head-tracking Responses of Rats With Grafts of LD7.4 Cells and Sham-operated Rats at 4, 5 and 6 Months and Comparative Histology



However, non-dystrophic animals and animals with good transplants were significantly better than poor transplants (n=13) and sham-operated (n=5) animals ($p < 0.05$). There was no significant difference between sham and poorly performing transplant animals ($p > 0.05$). Furthermore, increasing the spatial frequency of the grating resulted in a significant decrease in the amount of tracking in all groups (F=9.019, df=2,98 p<0.001). At 5 months of age non-dystrophic animals still tracked all the gratings (F=13.24, df=3,36 p<0.0001; post-hoc Student-Newman-Keuls, $p<0.05$) whereas the performance of the good head trackers fell to a level not significantly different to those of poor trackers and sham-operated animals ($p>0.05$). At 6 months of age overall performance was reduced and there was no significant difference between any of the dystrophic groups. This reduction in visual function with time in the transplanted animals, as assessed by head-tracking behaviour, was therefore most striking between 4 and 5 months of age. At 6 months, however, photoreceptor rescue and head-tracking performance no longer correlated.

3.3.2 Threshold response mapping

Using head-tracking performance to identify good grafts, further studies were then carried out on this group of animals. Perimetric evaluation of threshold responses to a 3° light stimulus in the superior colliculus (SC) was assessed as previously described (Sauvé *et al.*, 2001) and topographic data from individual animals of each experimental group were pooled. Point-to-point comparisons across the visual field of pooled groups were made to determine statistical differences between transplanted and control animals. Earlier studies using this technique (Sauvé *et al.*, 1998) measured the response to a spot of light of standard intensity, 4 log units above background but in this study a range of light levels was used to determine the relative threshold level at each point tested in the visual field (Sauvé et al., 2001). Such an approach allowed

a more careful analysis of sensitivity across the retina. Transplanted animals were tested at both 5 (n=5) and 6 (n=6) months of age together with unoperated dystrophic (n=4) and sham-operated (n=4) animals. The specific time points of five and six months of age were chosen in order to determine whether there was deterioration in graft efficacy with time. At earlier time points, the background and sham effects are still prominent making it difficult to separate deterioration of the graft-induced effect from background events. Furthermore, these ages were ones at which donor cells could no longer be detected histologically, allowing the opportunity to see whether function persisted beyond graft survival. The relative pooled threshold results at 6 months of age are shown in Fig 3.3A-D where the threshold scores (log c/m^2) have been inverted (inverted value: 0 - 4.5) to represent greater sensitivities as a peak. In non-dystrophic animals, a threshold level of between 3.8 – 4.2 was typical (Fig 3.3A) whereas in dystrophic non-transplanted animals, retinal sensitivity was extremely poor at 6 months of age, with levels typically of 0 – 1(Fig 3.3B). Sham-operated animals demonstrated a focal area of improved retinal sensitivity in the immediate region of the injection site (Fig 3.3D) with individual points as good as 2.0. In the transplanted animals, an area covering as much as 30% of the retina had sensitivities better than 2.5 (Fig 3.3C) extending well beyond the area of injection. Some points, however, had sensitivity thresholds as good as 3.9.

Additionally, a point-to-point comparison was made across the visual map of the colliculus in animals at six months. The analysis showed that in grafted animals, there was an area with statistically significant higher sensitivity levels which corresponded with the dorso-temporal region. This matched the region where the graft was placed (Fig 3.4A). Cross-group comparison revealed that the extent of significantly preserved threshold sensitivity was far greater in the transplanted retinae compared with both

Figure 3.3: Retinal sensitivity mapping of non-dystrophic, dystrophic, grafted and sham-operated rats with comparison of grafted and sham data.

This figure maps the collated and averaged threshold sensitivities of animals at 6 months of age. Threshold (log) scores (A-D) have been inverted (0 – 4.5) to represent greater sensitivity as peak values, see 3.3.2. A) Non-dystrophic (n=3) B) Dystrophic unoperated (n=4) C) Dystrophic transplanted (n=5) and D) Dystrophic sham-operated (n=5). Non-dystrophic animals have uniformly good retinal sensitivity levels (>3.9) across the retina. Dystrophic animals have uniformly poor sensitivity levels at this age (<0.8). The transplanted animals have preserved sensitivity in the dorso-temporal region (injection site). Sham-operated animals demonstrated threshold levels in the dorso-temporal retina better than unoperated dystrophic retinae but significantly worse than transplanted animals, (D).

Figure 3.3

Retinal Sensitivity Mapping

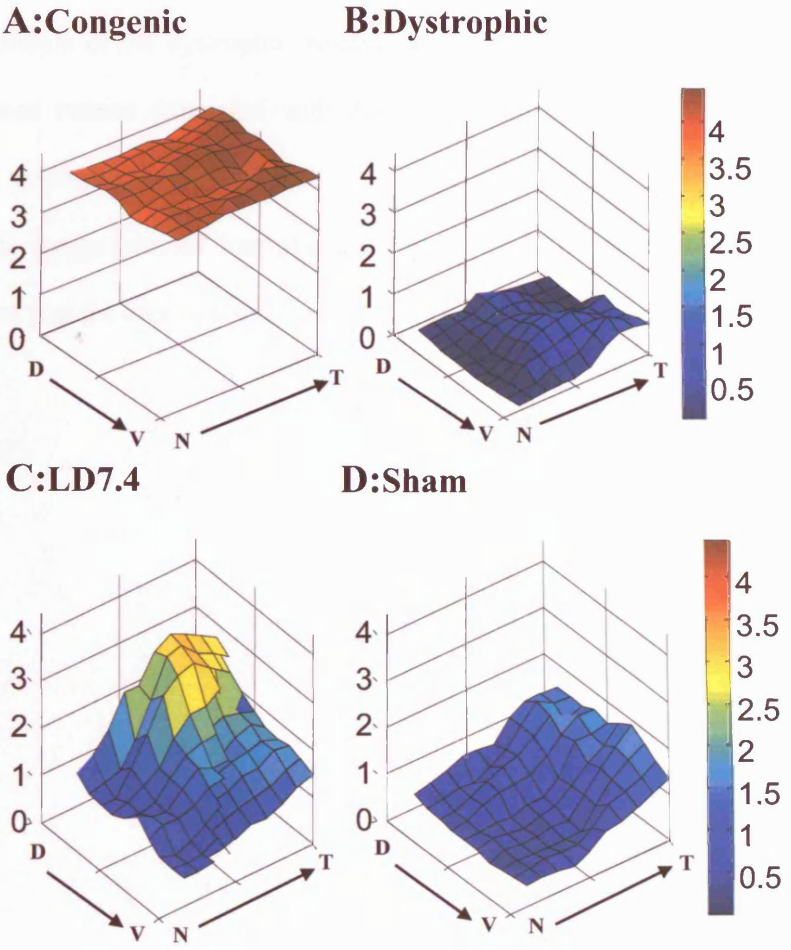
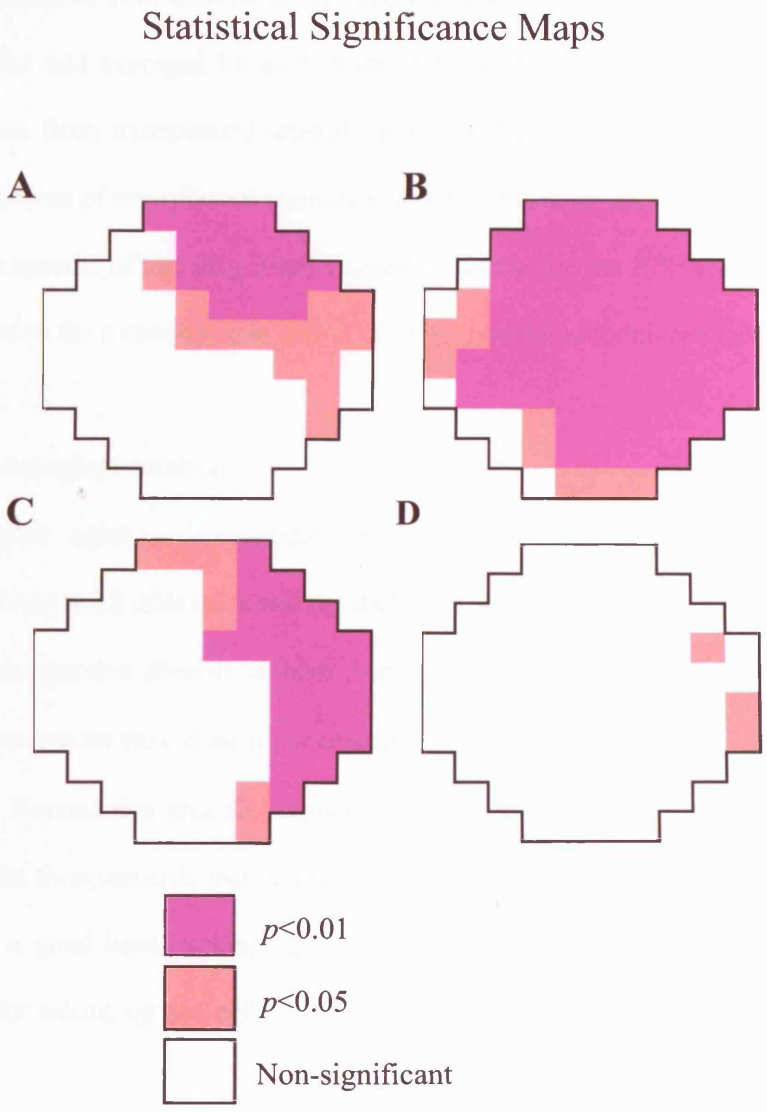


Figure 3.4: Statistical significance maps: comparing grafted, sham and unoperated dystrophic rats at 6 months and comparing grafted rats at 5 and 6 months.

This figure illustrates significance on a direct point-to-point comparison, i.e. analagous threshold points on the superior colliculus are summed and compared with these points in the other groups, for transplanted retinae compared with A) sham-operated animals or B) dystrophic animals at six months. A statistical analysis of sham-operated retinae compared with dystrophic retinae is shown in C. Specific differences are shown.

A statistical comparison of transplanted animals at 5 (n=5) and 6 (n=6) months demonstrates that the area of preserved sensitivity is greater in the younger group (D).

Figure 3.4


Statistical Significance Maps
A
B
C
D
p<0.01
p<0.05
Non-significant

sham-operated retinae (Fig 3.4A) and unoperated dystrophic (Fig 3.4B). Sham-operated retinae also demonstrated significantly better threshold levels compared with unoperated dystrophics (Fig 3.4C).

The threshold data from individual transplanted animals at five months of age (n=5) were grouped and averaged for each point in the plot and compared with similarly grouped data from transplanted animals at 6 months of age (n=6). The threshold response patterns of transplanted animals at five months were significantly better than those at six months of age (Fig 3.4D) suggesting that while the RPE grafts preserved retinal function for a considerable period of time, the effect diminished between these time points.

3.3.3 Morphological Analysis

Non-dystrophic animals demonstrated normal retinal architecture with an outer nuclear layer up to 12 cells thick and regularly arranged inner and outer segments (Fig 3.5A). Sham-operated animals at both 5 and 6 months of age had a focal area of photoreceptor rescue very close to, or extending 300 - 400 μm from, the injection site (Fig 3.5D). Beyond this area no photoreceptors could be identified (Fig 3.5E). At 5 and 6 months those animals that received successful LD 7.4 cell grafts, as previously defined by a good head-tracking response, demonstrated a considerable amount of photoreceptor rescue, up to 6 cells thick, with marked outer segment preservation (Fig 3.5B,C).

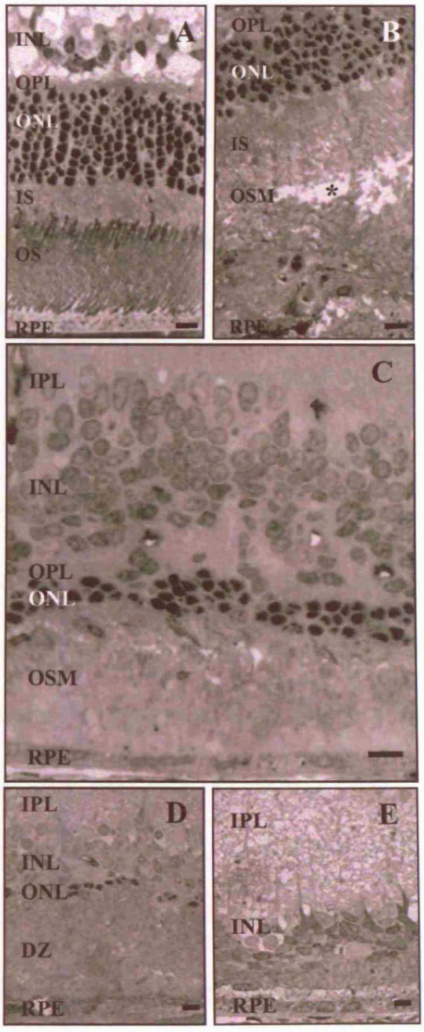
The area of rescue extended far beyond the injection site (up to 3000μm): this is illustrated in Fig 3.6A. Three grafted and two sham-operated animals at 5 months were subjected to a more detailed histological examination. The data from multiple sections were grouped into a histological (multipoint) map that was comparable to the retinal sensitivity maps generated from SC recordings. This analysis demonstrated

Figure 3.5: Semi-thin histology of grafted compared with sham-operated rats at 6 months.

A) Non-dystrophic RCS rat retina at 6 months of age. B) Grafted dystrophic retina at 6 months of age (5 months post-transplant). There is a large amount of photoreceptor rescue (up to six cells thick), inner segments (IS) are retained with disorganized outer segment material (OSM). There is a small artifactual detachment (asterix) at the site of the outer limiting membrane. C) Also shows rescue at 6 months of age in a transplanted animal: this is a more typical picture of preservation with ONL 3-4 cells thick. D) Shows a sham-operated animal at 6 months of age immediately adjacent to the injection site. There are some photoreceptors (ONL) and a persistent debris zone (DZ). E) Distant from the injection site there are very few photoreceptors and a sparse debris zone, similar in appearance to an unoperated dystrophic RCS rat retina of the same age. Scale bar: 20μm.

Figure 3.5

Semi-thin Histology of Grafted Compared with Control and Sham-Operated Rats at 6 Months



that the area of photoreceptor rescue varied between 2 and 6 cells thick (90-380 cells/700μm) and covered between 10% and 30% of the total retinal area. In regions distant from the area of graft injection, occasional photoreceptors were encountered in a density similar to that found in unoperated or sham-injected rats. The peak cell counts correlated to the region of highest retinal sensitivity. Correlation of these maps demonstrated the close relation between the amount of photoreceptor rescue and retinal sensitivity (Fig 3.6A,B).

3.3.4 Survival of RPE Cell Grafts

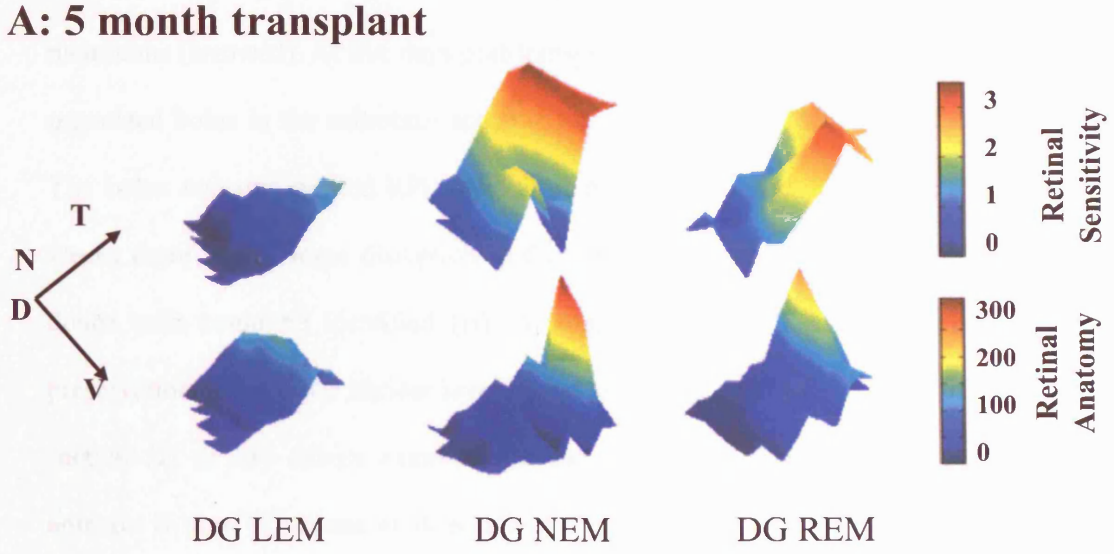
Cells labelled with BrdU were seen in the subretinal space of eyes retrieved at 1 and 5 days and two weeks following transplantation (Fig 3.7A-H). Eyes examined at 4 weeks following transplantation failed to demonstrate the presence of grafted cells by BrdU immunohistochemistry (Fig 3.7J). Despite the apparent loss of grafted cells (Fig 3.7L,N) the retina adjacent to the injection site showed preservation of photoreceptors at 3 and 5 months post-transplantation (Fig 3.7K,M). It was also apparent that the area of maximal photoreceptor cell rescue declined with time (Fig 3.7I,K,M). The grafted cells did not appear to integrate with host RPE on Bruch's membrane (Fig 3.7D,F,H). To confirm that rapid graft cell loss was occurring, PCR amplification studies were performed to identify the presence of SV40 large T-antigen signal at day 5 in 3 animals studied (Fig 3.8). By day 28 and at 3 months survival, genomic material was detected infrequently and by 5 months survival (6 months of age) no signal was detectable in any sample (n=7). Single cells (added exogenously) were detected in control sections. In conclusion, at 5 months post-graft the absence of signal correlates with the absence of grafted cells as determined by immunohistochemistry.

Figure 3.6: Comparison of retinal sensitivity maps with histological maps of individual representative animals at 5 months.

A) Electrophysiological (upper) and histological (lower) maps represented in 3-D. Dorsal (D), Ventral (V), Temporal (T) and Nasal (N). In the dorso-temporal region of the retina there is greater photoreceptor cell preservation that correlates with the maximal area of retinal sensitivity (DGNEM and DGREM). The one transplanted animal represented here with poorer retinal sensitivity (DGLEM) also has fewer photoreceptors. B) Sham-operated animals generated flatter maps, however the local area of photoreceptor rescue is clearly seen and is represented as a spike rising to 90-cells/700μm block. These maps were produced by a custom-generated computer package in MatLab, version 5.1, using bespoke programmes. The maps confirm the relationship between photoreceptor cell preservation and retinal sensitivity. Legends under the maps refer to individual animals.

Figure 3.6

Comparison of Retinal Sensitivity Maps with Histological Maps at 5 Months



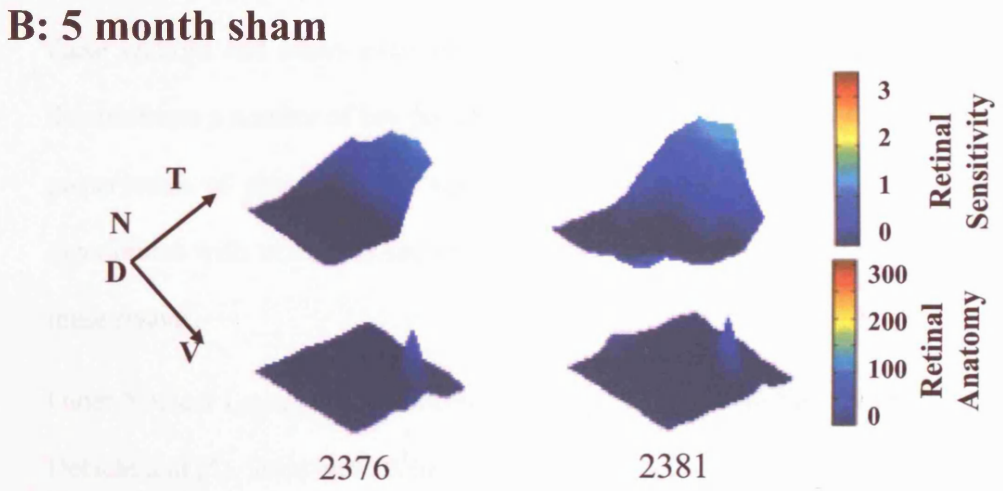


Figure 3.7: Localisation of grafts

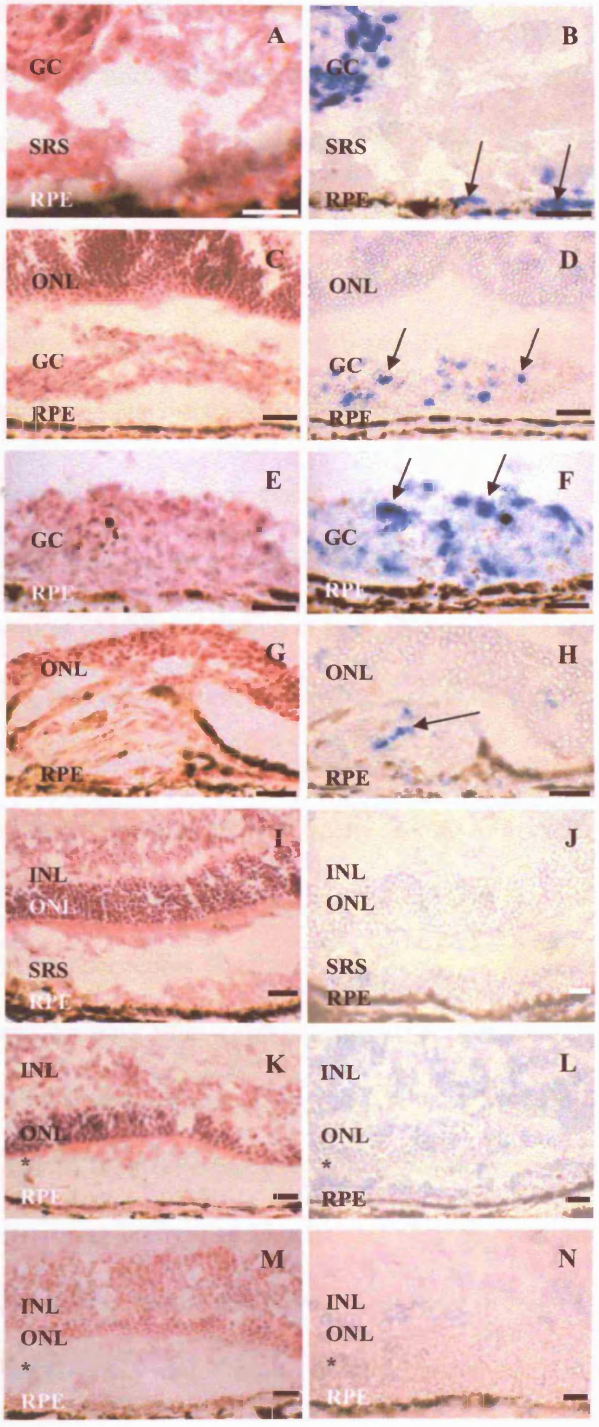
Haematoxylin and Eosin (left column) and Bromodeoxyuridine labelling (right column) of retinae taken from animals at 1 (A and B) and 5 (C-F) days, 2 (G and H) and 4 (I and J) weeks and 3 (K and L) and 5 (M and N) months post-transplantation.

At day one the grafted cell bolus is clearly seen (A) and confirmed with BrdU labelling (B). There is some disorganization in the subretinal space post-graft. In addition to the grafted bolus some labelled cells are seen adjacent to Bruch's membrane (arrowed). At five days post-transplantation, the grafted cells are seen in an organized bolus in the subretinal space (C) and at slightly higher magnification (E). The bolus contains grafted RPE cells as illustrated in D and F (arrowed). By two weeks there is still some disruption in the subretinal space (G) but only occasional donor cells could be identified (H). At four weeks (7-8 weeks of age) there is preservation of the outer nuclear layer (I) yet no grafted cells can be identified in this section (J) or any others examined (n=6). Photoreceptor preservation in grafted animals is seen in retinae at three (K) and five (M) months post-grafting. BrdU-labelling at three (L) and five (N) months shows the absence of positive labelling in these sections and others examined (n=8 and 7 respectively). This panel of images demonstrates a number of key points: the loss of grafted cells over time, the persistent preservation of photoreceptors up to five months post-graft and that preservation deteriorates with time. The sections illustrated represent maximal rescue in each of these retinae.

Outer Nuclear Layer (ONL), Grafted Cells (GC), Subretinal Space (SRS), Artifactual Detachment (*). Scale bars: 20μm.

Figure 3.7

Location of Grafts



3.4 Discussion

This study shows that transplantation of a genetically engineered immortalised allogeneic RPE cell line, in the absence of immunosuppression, can delay photoreceptor degeneration and preserve visual function for up to six months in the RCS rat. Preservation of photoreceptors and visual function at 6 months of age, as assessed by retinal sensitivity mapping in the SC, was significantly better than in sham-operated animals and the two parameters showed a close correlation. However, despite significant attenuation in disease progression following cell transplantation, there was no evidence of graft cell survival beyond 4 months post-grafting, a time point when head-tracking behaviour had reduced to background levels.

Following transplantation of immortalised allogeneic RPE cells, morphological examination of good head-tracking animals tested at early survival times revealed substantial preservation of photoreceptors at both five and six months of age (4 and 5 months post-grafting respectively) even though head-tracking no longer discriminated cell grafted and sham-injected rats. At 6 months it was not uncommon to find retention of two or more rows of photoreceptor nuclei (Fig 3.5C) over as much as 30% of the retinal area and in some cases the rescue was as much as 7 cells thick (i.e. 60% of the normal photoreceptor thickness: Fig 3.5B). Associated with this area of rescue was clear evidence of outer segment material. This histology was in marked contrast to unoperated animals of the same age where only occasional photoreceptors could be identified or to sham-injected animals where there was only a small amount of rescue (1-2 cells thick) immediately adjacent to the injection site. The level of rescue obtained after cell transplantation was similar to previously published results with fresh RPE allografts (Li and Turner, 1988; Lopez *et al.*, 1989; Whiteley *et al.*, 1996).

In previous studies, a direct correlation between photoreceptor rescue and a functional parameter has not been made. This study demonstrates that the region of photoreceptor preservation clearly correlates with low threshold response levels recorded electrophysiologically in the SC. Thus, the region of preserved retina correlates with the region of preserved visual function (Fig 3.6A,B). This observation mirrors the situation in some patients with retinal disease such as retinitis pigmentosa, where there may be areas of uneven function and retinal preservation (Sieving *et al* 1999). Continued function with modest rescue, also noted in a previous study using human immortalised RPE cell lines (Coffey *et al.*, 2002), also has a correlate with human studies where despite having only a few remaining photoreceptors, patients maintain some functional central vision, up to a visual acuity of 20/30 (Sieving *et al*, 1999). This has implications for clinical studies since it may be sufficient to preserve only a few rows of photoreceptors to permit retention of useful vision. The ability to predict retinal sensitivity from anatomical data will have a positive impact on the interpretation of other experimental treatment strategies.

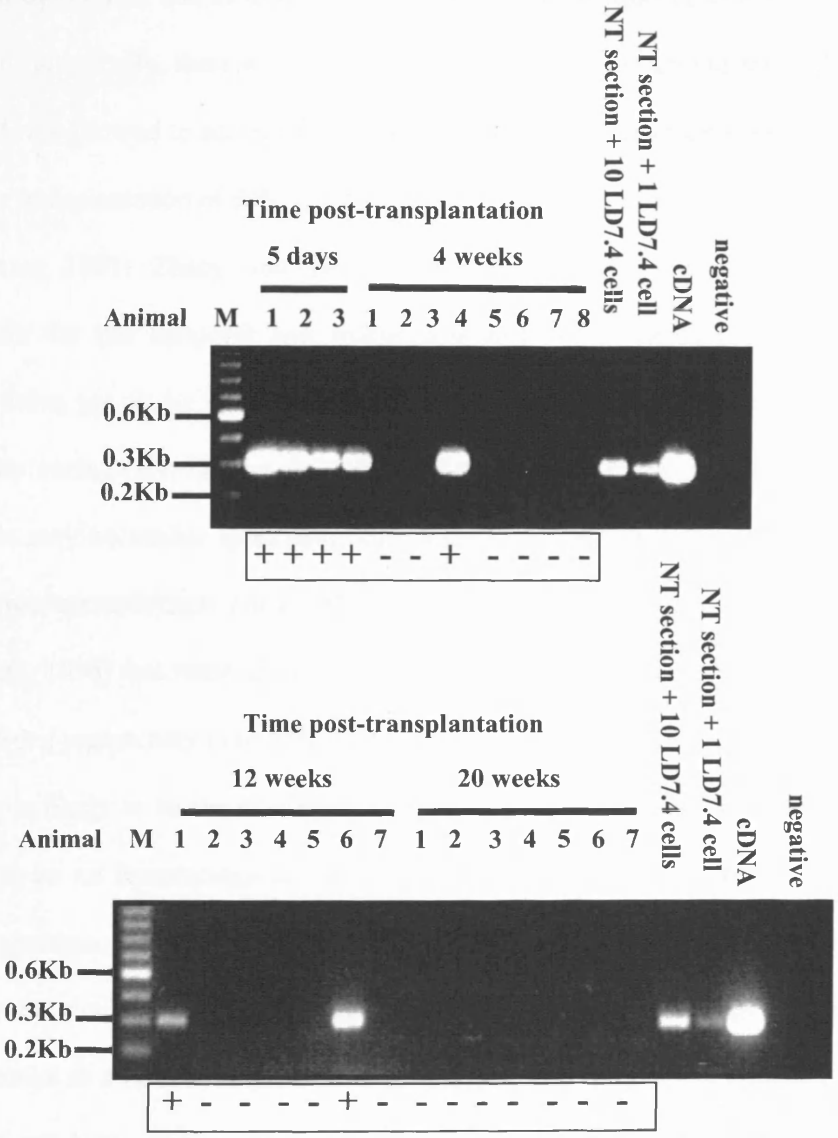
The ability of head-tracking to provide a good non-invasive technique for measuring transplant efficacy at early survival times makes it a very valuable tool for initial screening to discriminate between good and poor transplants (Fig 3.2B-D). The deterioration of head-tracking responses with time has been found in previous studies (Lawrence *et al.*, 2000) although the reasons for this are presently unclear. Head-tracking was originally developed for assessing retinal function in rats without retinal pathology (Cowey and Franzini, 1979), but has not been evaluated when used, as here, in a situation in which only part of the retina is functional. It is possible that good head-tracking performance requires preservation of a sufficiently large area of retina, even perhaps a particular region of retina. Thus, even though retinal rescue was

Figure 3.8: PCR detection of SV40 large T-antigen DNA in retinal sections of LD7.4 transplanted RCS rats.

Amplification of SV40 large T-antigen from genomic DNA isolated from five 8μm retinal sections taken from the graft site of animals previously transplanted with bromodeoxyuridine labelled LD7.4 cells. SV40 large T-antigen is amplified from some animals at 12 weeks post-transplantation but is not present in any of the animals tested at 20 weeks post-transplantation. Sensitivity of the PCR reaction has been established as a single copy of the SV40 large T-antigen sequence (1 cell). Also shown are positive control amplifications from SV40 large T-antigen cDNA and from retinal sections spiked with a single and 10 LD7.4 cells. Negative PCR controls have the DNA template omitted. The table details the number of animals assayed and corresponding positive results at each time point.

Figure 3.8

PCR Detection of SV40 Large T-Antigen DNA in Sections from LD7.4 Transplanted Rats



Time post-graft	Number of animals	Number of Positive Animals
5 Days	3	3
4 Weeks	8	1
12 Weeks	7	2
20 Weeks	7	0

demonstrated for several months, the area of preservation may decrease with time to a level below the threshold required to initiate a head-tracking response or possibly eliminating the crucial region for initiation of the response. This explanation is supported by the fact that although preservation of retinal function could be detected electrophysiologically, there was a significant reduction in the area of rescue between groups of rats allowed to survive for 5 and 6 months. The loss of photoreceptors with time after transplantation of RPE cells to RCS rats has a precedent in other studies (Li and Turner, 1991; Zhang and Bok, 1998). However, the determining factors responsible for the temporal and spatial loss both of photoreceptors and visual function have yet to be fully resolved. Various processes may be operating, not necessarily exclusively, that result in retinal deterioration. For example, transplanted RPE cells may be unable to sustain other necessary functional attributes with time such as phototransduction (Bok, 1993). Furthermore, secondary events (Villegas-Peréz *et al.*, 1998) that result in vascular remodelling in the RCS rat retina adjacent to the preserved region may gradually encroach on the preserved retina. A major factor, however, is likely to be the host immune response. Although the subretinal space is thought to be an immunologically privileged site (Jiang and Hamasaki, 1994) and RPE an immunologically privileged tissue (Wenkel and Streilein, 2000), these properties are not absolute and may be further compromised by leakage of the blood-retinal barrier as a consequence of both the surgery and the disease pathology: inner retinal vessels in the RCS rat become fenestrated and leaky as photoreceptors are lost and the vessels come to lie adjacent to the choroid (Villegas-Peréz *et al.*, 1998) In this study, allogeneic cells were grafted and the loss of the blood-retinal barrier may lead to some form of immune response and the ultimate destruction of the grafted cells. It is clear from preliminary data that a classical immune response is not occurring since

there is no leucocyte cuffing of blood vessels or infiltration of lymphocytes but it may be driven by a mechanism involving macrophages and activated retinal microglia. Immunosuppression with cyclosporine does not alter the kinetics of allograft cell loss (unpublished observations) confirming a recent report that cyclosporine does not protect against allograft failure in a rabbit model (Craaford *et al.*, 2000). This suggests that a non T-cell dependant mechanism is responsible for the loss of grafted cells. However, in a complementary xenograft study in which human RPE cell lines were grafted into the RCS rat, cyclosporine immunosuppression led to preservation of photoreceptors and visual function along with persistence of grafted cells up to 8 months post-transplantation (see Chapter 5; Lund *et al.*, 2001a; Coffey *et al.*, 2002). This study shows preservation of photoreceptors also at 6 months of age, yet, in the absence of grafted cells. Similar results have also been reported following human RPE cell transplantation in the rabbit (Lai *et al.*, 2000).

LD7.4 cells prelabelled with BrdU were used to establish whether the loss of retinal photoreceptors and visual function following transplantation was due to poor graft survival in the host retina (see also Ye *et al.*, 1993; Lund *et al.*, 2001a; Coffey *et al.*, 2002). Although grafted cells were found to survive in the early post-transplant period, no cells could be identified at later time points despite the retina in the region of the injection site showing significant preservation (Fig 3.7). In order to confirm this observation, PCR was used to detect the presence of the SV40 large T-antigen gene in sections of grafted retina up to 6 months of age (5 months post-graft: Fig 3.8). Data from these studies demonstrated the likely presence of grafted cells in some samples up to 4 months of age but no positive detection thereafter. The sampled regions were small, so it was not possible to be sure that all the donor cells were lost. Nevertheless, these results demonstrate that there is a substantial reduction in graft cell population

with time. Graft cell loss is unlikely to be a consequence of the stability of the grafted cells as the SV40 large T immortalised LD 7.4 RPE cell line is phenotypically stable through multiple passages *in vitro*. Moreover, ongoing studies have shown that these cells display normal immunological properties such as MHC and cytokine expression. They may fail due to an inability to integrate with the resident host RPE and anchor themselves on Bruch's membrane. One important conclusion from this work is that continued presence of large numbers of donor RPE cells is not a necessary prerequisite for photoreceptor survival, but rather that survival continues beyond the loss of donor cells by two months or more, yet RPE cell loss will ultimately lead to functional failure. This novel observation emphasises the need to employ reliable cell tracking techniques in experimental transplantation to determine the relationship between preserved retina and grafted cells.

To summarise, transplantation of an immortalised allograft RPE cell line delays the deterioration of both retinal histology and function. Of particular interest is the finding that donor cells do not survive for more than a few months, but that rescue of photoreceptors and of visual function continues for a further two or more months. The model provides an opportunity to probe the mechanisms that contribute to this rescue process, its deterioration over time and the development of strategies to improve the efficacy and survival of such grafts.

Chapter 4

4. TRANSPLANTATION OF GREEN FLUORESCENT PROTEIN LABELLED ALLOGENEIC RPE (LD7.4) CELLS TO THE SUBRETINAL SPACE OF NON-IMMUNOSUPPRESSED DYSTROPHIC RCS RATS

4.1 Introduction

In the previous chapter, it was found that photoreceptors and visual function were preserved for longer periods than the donor cells themselves.

In other RPE transplant studies various labelling techniques have been utilised such as β-galactosidase (Osusky *et al.*, 1995), bromodeoxyuridine (Ye *et al.*, 1993; Lund *et al.*, 2001a), Green Fluorescent Protein (GFP, Lai *et al.*, 2000) and *in situ* hybridisation of the Y-chromosome in male to female grafts (Wang *et al.*, 2001). Other studies have included Del Priore (2003 a and b, 2004)). To date no group has successfully labelled grafted cells for *in vivo* imaging in the RCS rat model; this would provide an important addition to the study of progress of the transplanted cells. The ability to compare the location of grafted cells relative to preserved photoreceptors and retinal function is essential in determining the relationship of that intervention with outcome. The large animal models (e.g. rabbit: Lai *et al.,* 2000) used in previous studies with GFP-labelled donor cells were without an inherent degeneration and, aside from describing donor cell location, little else could be determined.

In addition to serving as a replacement for defective host RPE cells, the possibility is being explored for using the cell as a delivery system for targeted gene therapy e.g. antiangiogenics (Otani *et al* 2002) or growth factors (Tao *et al.,* 2002). That possibility prompted this particular experiment.

In this chapter a novel vector, the Human Foamy Virus (HFV) expressing green fluorescent protein, will be used to transfect LD7.4 cells. The Human Foamy Virus is a novel retrovirus that does not cause pathology in humans (Ali *et al* 1996) and has been shown to transfect cells *in vitro* (Bieniasz et al 1997) effectively.

4.2 Experimental Design

The aim of the study is to use GFP labelled LD7.4 cells as donor cells and trace their survival through time *in vivo* using a confocal scanning laser ophthalmoscope. Figure 4.1 illustrates the experimental design schematically.

Twenty four dystrophic RCS rats (PN21) received subretinal grafts of LD7.4 cells transfected with HFV expressing GFP (Fig. 4.2). Two microlitres of LD7.4/GFP cells in suspension were injected (1x 10^5 cells/μl) into the dorso-temporal retina. Twelve dystrophic RCS rats (PN21) received sham surgery (medium alone).

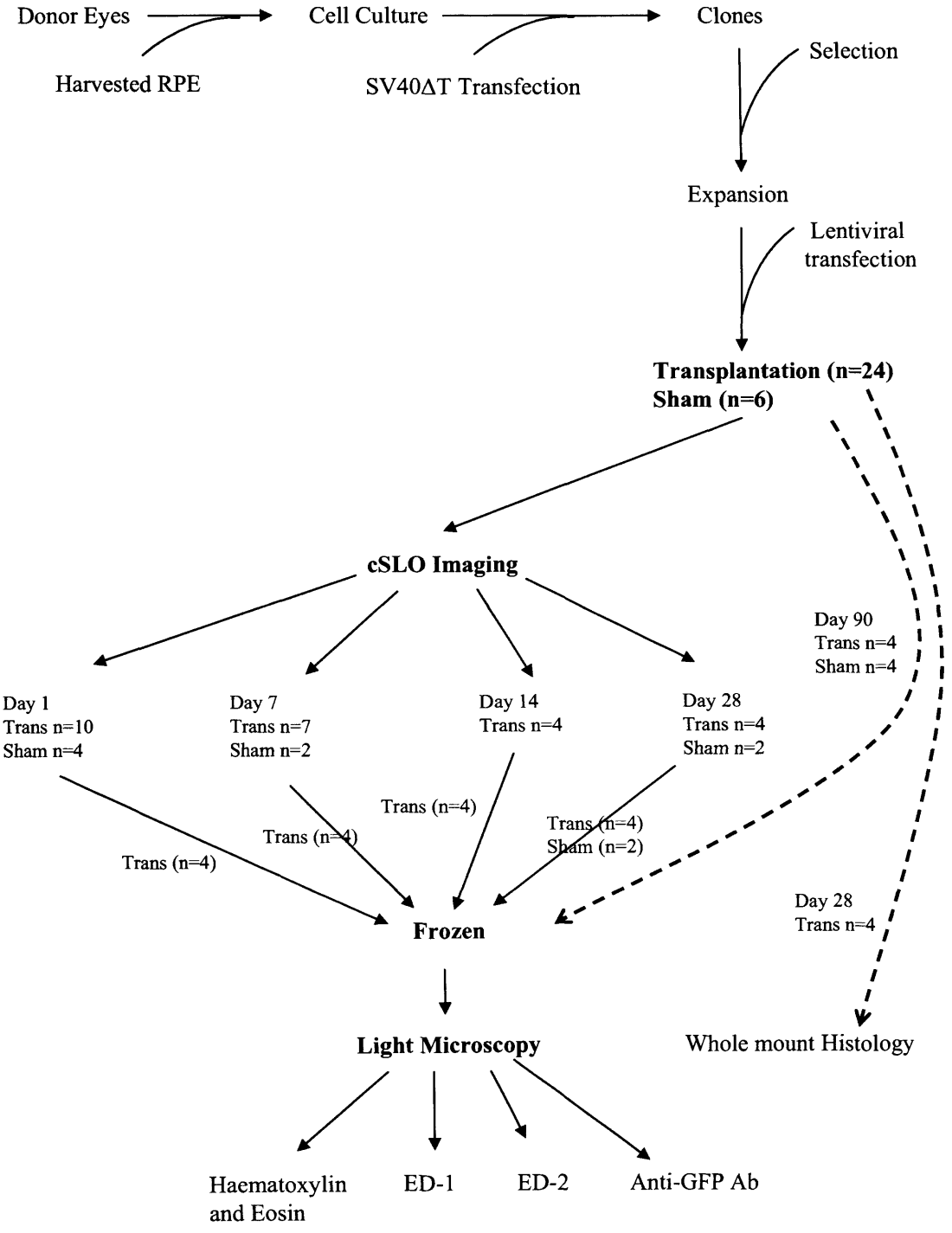
Sham-operated and animals that received LD7.4/GFP cells underwent serial fluorescent imaging using a Zeiss confocal scanning laser ophthalmoscope (cSLO) at 1 day, 1, 2, and 4 weeks post-transplantation. Rats were anaesthetised with a ketamine/xylazine mix (100mg/20mg) and pupils were dilated using Tropicamide (Alcon, Hemel Hempstead, UK). An argon laser light (488 nm) was used for illumination, and a wide band pass filter with short wavelength cut off (521 nm) was inserted in front of the detector to record fundus autofluorescence. The argon laser was measured at regular intervals and found to have a constant power output of 250 μW at the cornea. The automatic gain control was switched off and the instrument gain was kept constant for all measurements of both transplanted and sham-opertaed animals. A series of images of the eye was recorded at standard video scanning rates on VHS video tape. Later, 32 consecutive frames were digitised, aligned, and averaged to obtain each image.

Figure 4.1: Flow Diagram of Transplantation Studies using Immortalised RPE/GFP Cells

See section 4.2

Figure 4.1

Flow Diagram of Transplantation Studies Using Immortalised RPE/GFP Cells



Eyes from all treatment groups were harvested at 1, 7, 14, 28, 84 and 120 days post-surgery and processed for histology to identify donor cells and the host macrophage response. The rats were perfused trans-cardially with PBS and 4% paraformaldehyde. The eyes were removed, cryoprotected with 10%, 20% and 30% sucrose in PBS embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Miles, Raymond Lamb, London, UK) and frozen. A number of series of sections were cut at 12μm and stained with cresyl violet or haematoxylin and eosin. Adjacent serial sections were reacted with antibodies for GFP (Chemicon, Harrow, UK 1:750) and macrophages (anti-ED1; 1:200 from Chemicon, Harrow, UK and anti-ED2, 1:400, from Chemicon, Harrow, UK).

Following transplantation, apoptotic cells were identified in the subretinal space using a NeuroTACS™ II *in situ* apoptosis detection kit (R&D Systems Europe, Abingdon, Oxon, UK).

4.3 Results

4.3.1 Cell Labelling

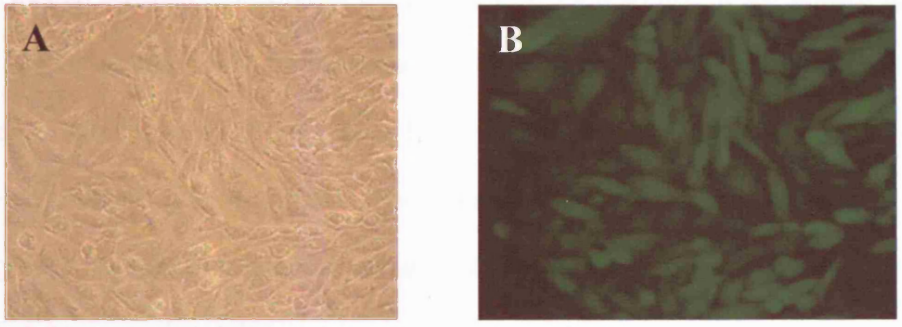
The generation of the vector is detailed in section 2.2.1.2.1. The green fluorescence protein (GFP) gene was inserted into the bel genomic region under the transcriptional control of an SV40 promotor. RPE cells were seeded at $5x10^3$ cells/well in a 24 well plate. The cells were then transfected with $5x10^4$ virus particles. Cells transfected with the HFV demonstrated GFP expression, by flow cytometry, in 60% of cells (Fig 4.2). These were sorted and expanded and when analysed by fluorescence-activated cell sorting (FACS) more than 90% of the cells were GFP positive. This was maintained through serial passages. This population of high yield GFP positive cells were used for transplantation to the dystrophic RCS rat retina.

Figure 4.2: Retinal Pigment Epithelial Cells (LD7.4) Expressing GFP *in vitro*

A) Phase contrast and B) fluorescent photomicrographs of cells prior to confluence. There is a high expression rate (>90%) of GFP which remains stable through multiple passages.

Figure 4.2

LD7.4 Cells Expressing GFP *In Vitro*



4.3.2 Confocal Scanning Laser Imaging

Figure 4.3A shows a cSLO image of a suspension of GFP labelled LD7.4 cells prior to transplantation. Fourteen rats were imaged at day one. No fluorescent patch was evident in the sham-operated animals (Fig 4.3B,C) whereas all grafted animals demonstrated a patch of fluorescence in the dorsal retina consistent with the site of the transplant (Fig 4.3D,E). As cell suspensions were used the observed area was diffuse with an indistinct edge. All retinal detachments had resolved by day one. By week one there was an obvious reduction in the strength of fluorescence in the grafted retinae (Fig 4.3F,G) and there was further reduction by week 2 (Fig 4.3H,I). By week 4 there was no difference in the fluorescence level in the grafted (Fig 4.3J,K) versus sham operated animals (Fig 4.3 L,M). The fluorescence seen at this time point is a background fluorescence seen in all animals as they age due to RPE/debris zone autofluorescence. The GFP signal seen at earlier times is a brighter fluorescence which eventually disappears. The time course of disappearance of *in vivo* fluorescence is mirrored in by the histological results using fluorescence microscopy and the anti-GFP antibody (Figs 4.4-4.7).

4.3.3 Histology

To determine whether the reduction in fluorescence demonstrated by cSLO imaging was due to a loss of donor cells or due to a reduction in GFP expression, graft cell survival was examined in sectioned material. Figure 4.4A,B shows the gross morphology of the grafted retina at the site of injection. Fluorescent images from adjacent sections show numerous fluorescent cells (GFP positive) in the subretinal space at one day post-transplantation (Fig 4.4 C,D). However, using macrophage antibodies and the Neurotacs™ apoptosis kit, it was found that the cell bolus contained a mixed cell population of donor cells, both viable (Fig 4.4E,F) and

Figure 4.3: Confocal Scanning Laser Ophthalmoscope (cSLO) Images of Cells *in vitro* and *in vivo*

A) Demonstrates the fluorescent cSLO appearance of a suspension of GFP-expressing LD7.4 cells prior to transplantation. Line shows limit of cell suspension. B–M) illustrate reflectance (upper) and fluorescent (lower) confocal *in vivo* imaging of RCS rat retinae following sham injection or transplantation. B and C) illustrate images from a sham-operated animal one day post-transplantation, there is no fluorescence. D and E) are images from a grafted animal at day one. Vessels are arrowed (relate to analogous landmark vessels in reflectance and fluorescent images). There is marked fluorescence at one day, presumably emanating from grafted cells. F and G) illustrate that this fluorescence is reduced at week one and further at week two (H and I). At 4 weeks transplanted (J,K) retinae are indistinguishable from sham-operated retinae (L,M) Imaging at week four demonstrates the inability to distinguish between transplanted (J and K) and sham (L and M) due to a) the loss of defined fluorescence in the graft and b) background autofluorescence in the RCS rat model.

Figure 4.3

Confocal Scanning Laser Ophthalmoscope Images of Cells *In Vitro* and *In Vivo*

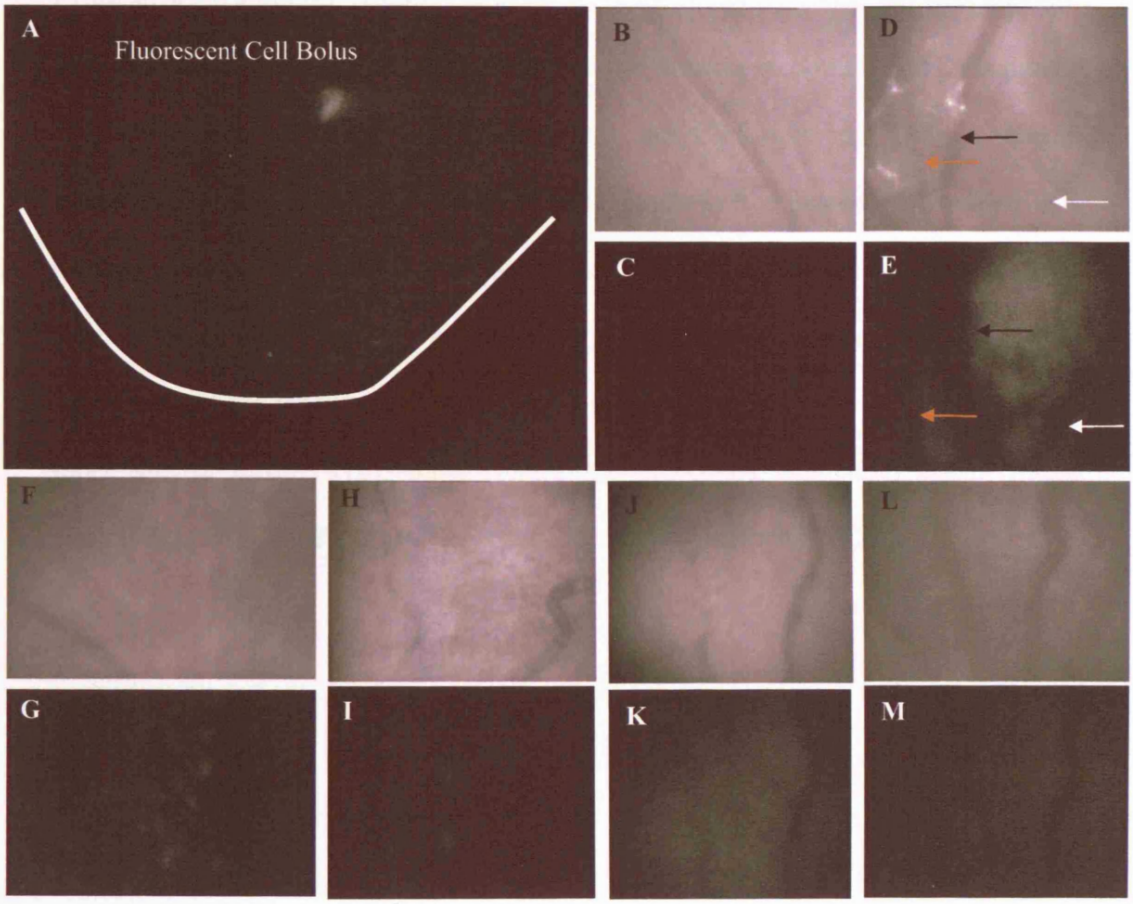


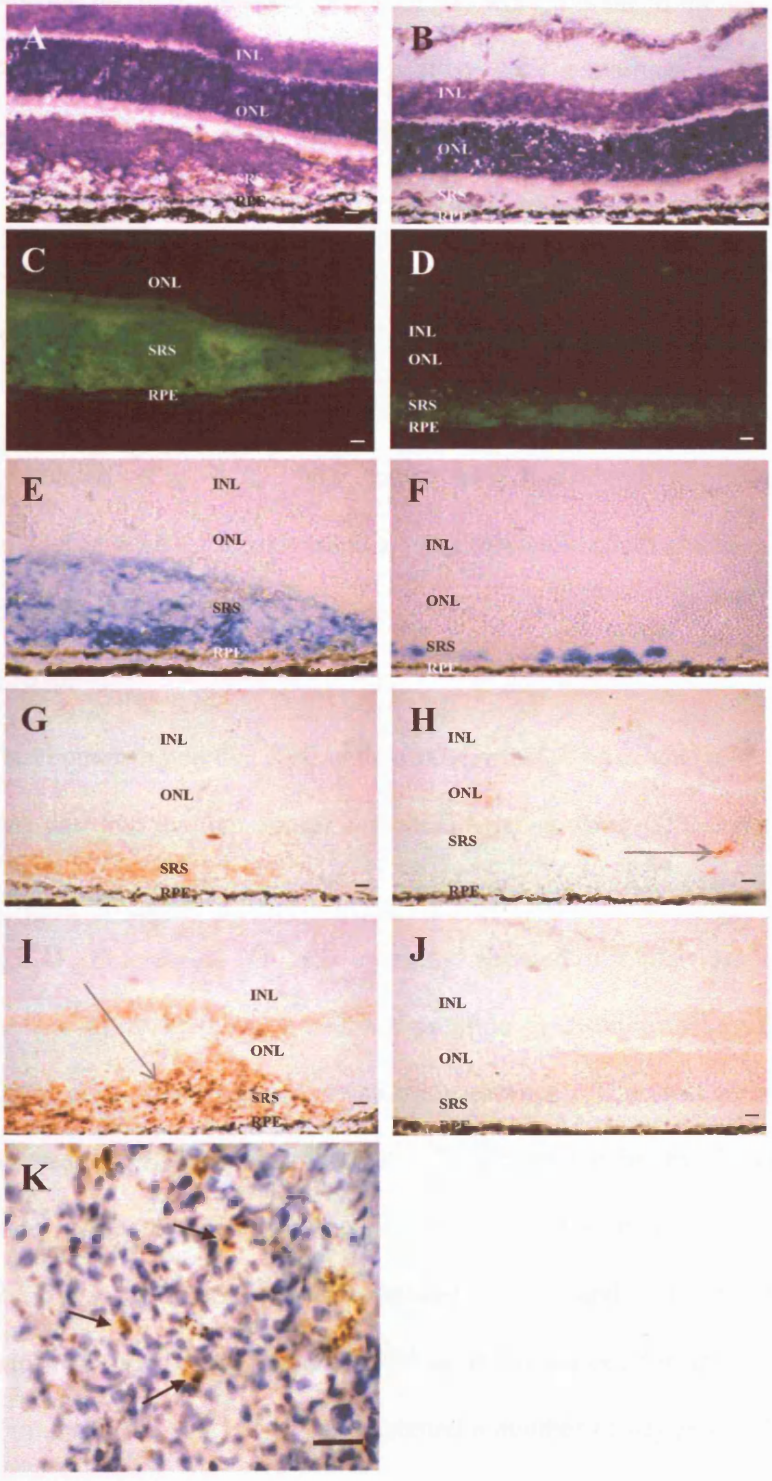
Figure 4.4: Histology and Immunohistochemistry of Retina One Day after Grafting LD7.4/GFP Labelled Cells

A) Light micrograph showing gross morphology of the retina, stained with cresyl violet, at the injection site and B) adjacent to it. Numerous subretinal cells can be identified. C and D) are fluorescent images from sections adjacent to A and B. E and F) are adjacent anti-GFP antibody labelled sections (antibody visualised with alkaline phosphatase and Vector blue) confirming that the fluorescence in C) and D) is due to GFP. G) and H) show sections labelled with anti-ED1 antibody. Only a weakly labelled microglial cell can be identified in H. I) shows the presence of many ED2-positive cells in the subretinal cell population seen in A. There are no ED-2 positive cells identified away from the graft site (J). K) illustrates the presense of apoptotic cells (arrowed and labelled with DAB) in the graft cell bolus.

Inner Nuclear Layer (INL), Outer Nuclear Layer (ONL), Subretinal Space (SRS), Retinal Pigment Epithelium (RPE), Neural Retina (NR). Scale bars 20μm.

Figure 4.4

Histology and Immunohistochemistry of Retina One Day After Grafting LD7.4/GFP Labelled Cells



apoptotic (Fig 4.4K) and ED-2 positive cells (host macrophages and microglia: Fig 4.4I). Less reactivity was seen with the ED1 antibody (Fig 4.4G,H) at one week post-transplantation the number of labelled cells appeared to have diminished (Fig 4.5A-E) and cells positive for ED1 and ED2 (Fig 4.4F-H) were present in the vicinity of the graft. By two weeks there were no GFP-positive cells remaining in the subretinal space (Fig 4.6E) although ED-1 and ED-2 positive cells remained. There is a population of fluorescent cells but these exhibit broadband fluorescence (480 – 590μm), i.e. they do not represent cells that are specifically expressing GFP (maximal emission at 510μm) as the signal intensity on the confocal microscope is no different at variable wavelengths (Fig 4.6C,D). Four months after surgery no grafted cells could be detected (Fig 4.7C) yet there was considerable preservation of photoreceptors (Fig 4.7A). Occasional ED-1 positive cells could still be observed in the inner retina (Fig 4.7D).

4.4 Discussion

This study has demonstrated that RPE cells can be transfected *ex vivo* with the Human Foamy Virus, and that the expression is stable. After grafting GFP-expressing RPE cells to the subretinal space of the RCS rat, the fluorescent marker was easily imaged with the cSLO. Fluorescent *in vivo* imaging showed a rapid loss of specific fluorescence and by four weeks it was not possible to distinguish any fluorescent-labelled grafted cells above the normal autofluorescence of the host RPE cells. The histological study confirmed the loss of GFP-positive cells by 2 weeks post-transplantation. Numerous apoptotic cells were seen and this loss of cells was accompanied by an infiltration of macrophages (ED-1 and ED-2 positive cells). Despite the loss of donor cells, photoreceptors were maintained for up to four months post-transplantation. This study has demonstrated a number of key points. Firstly, the

Figure 4.5: Histology and Immunohistochemistry of Retina One Week after grafting LD7.4/GFP Labelled Cells

The panel illustrates marked retinal disruption with a mixed cell population in the subretinal space (SRS); A and B) are cresyl violet stained sections of adjacent areas in the retina; C and D) are the corresponding fluorescent images. E) Section visualised with anti-GFP antibody, alkaline phosphatase and vector blue showing few GFP labelled cells remaining (arrow), F) ED2-positive cells and G,H) many ED1-positive cells (arrows).

Inner Nuclear Layer (INL), Outer Nuclear Layer (ONL), Retinal Pigment Epithelium (RPE), Neural Retina (NR). Scale bars = 20μm

Figure 4.5

Histology and Immunohistochemistry of Retina One Week After Grafting LD7.4/GFP Labelled Cells

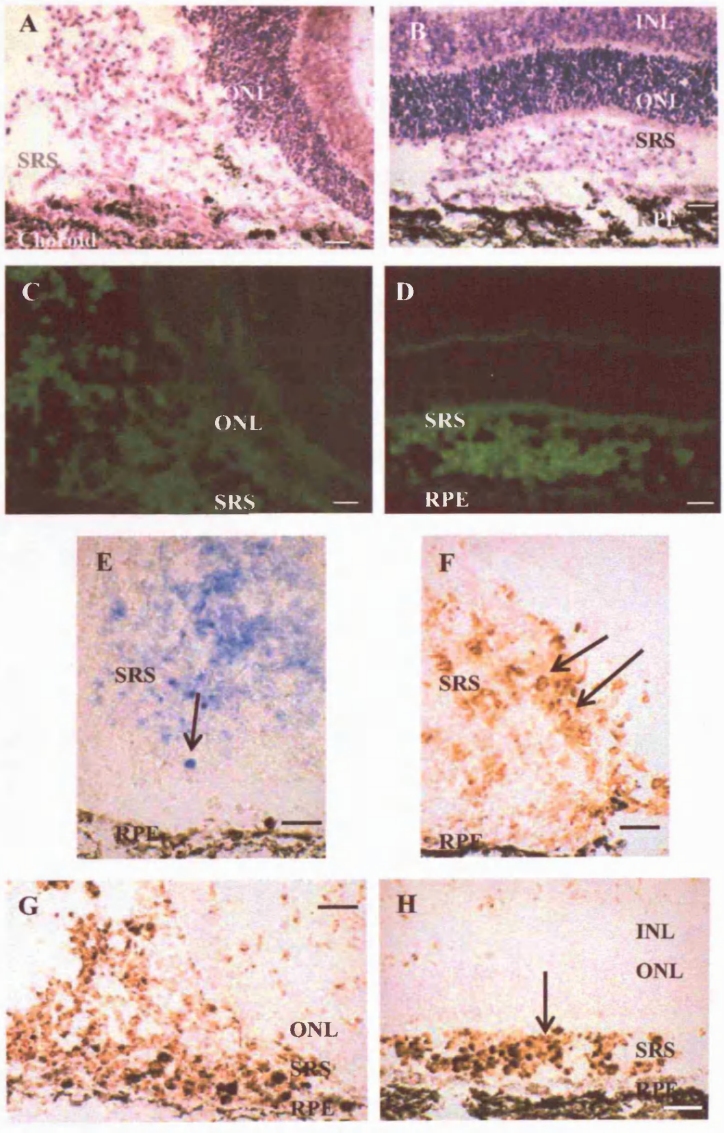


Figure 4.6: Histology and Immunohistochemistry of Retina Two Weeks Post-transplantation

A) Cresyl violet stained section showing marked retinal disruption. B) Fluorescence micrograph of the area in (A) shows many fluorescent cells. C) and D) illustrate that these subretinal fluorescent cells emit a broad-band fluorescent signal (i.e. the strength of the signal at the red (580μm) and green (510μm) wavelengths is equal) and appear to be macrophages (see H). E) These cells are not GFP positive but are in association with G) many ED1 and H) ED2 positive cells.

Inner Nuclear Layer (INL), Outer Nuclear Layer (ONL), Subretinal Space (SRS), Retinal Pigment Epithelium (RPE), Neural Retina (NR). Scale bars = 20μm

Figures 4.6

Histology and Immunohistochemistry of Retina Two Week After Grafting LD7.4/GFP Labelled Cells

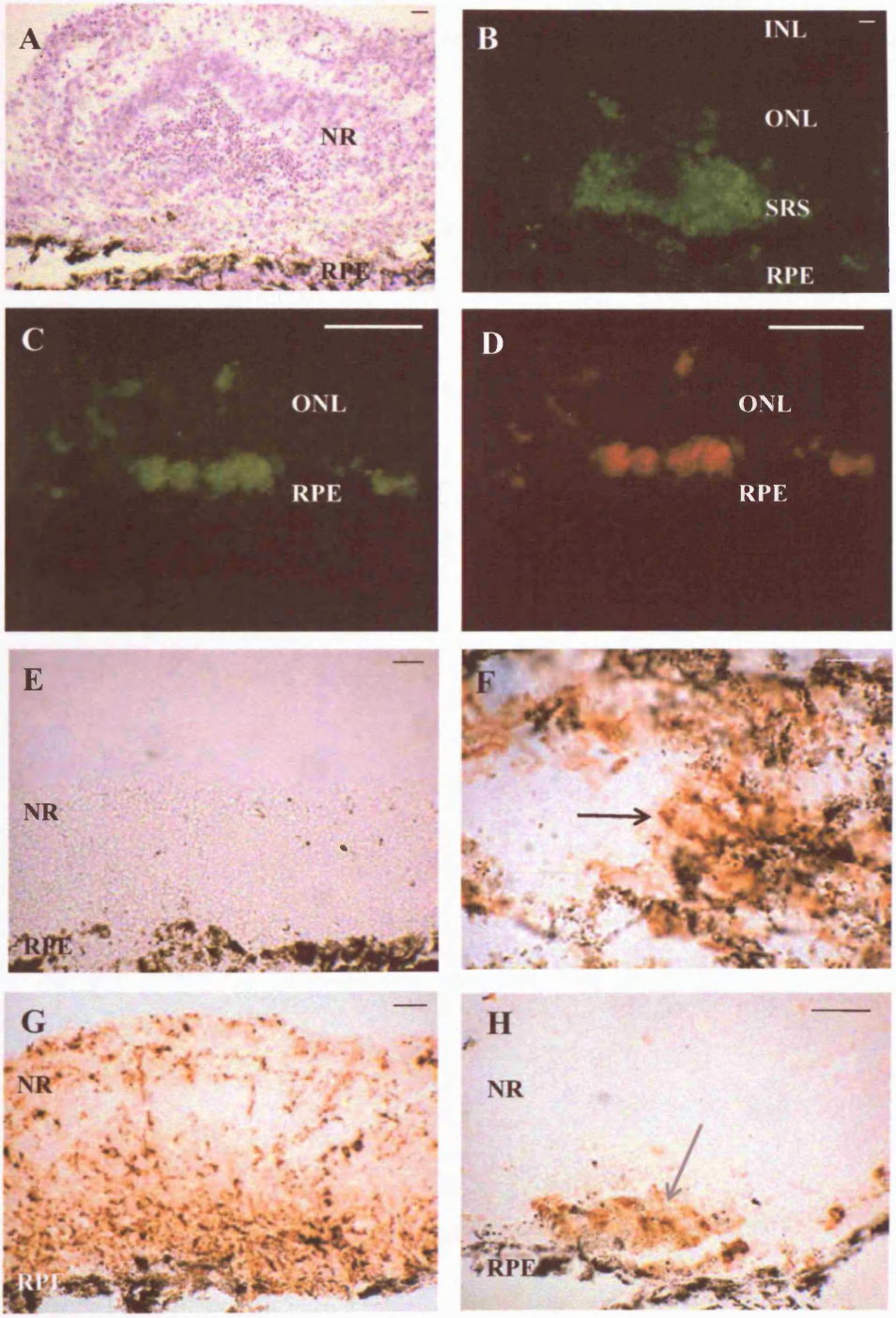


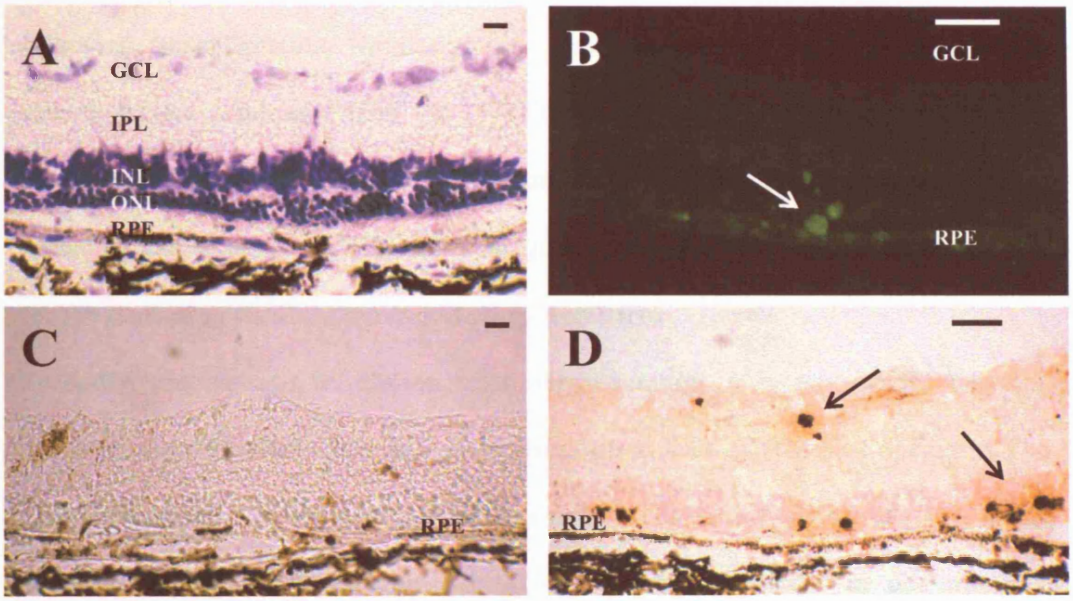
Figure 4.7: Histology of Retina at 4 Months Post-transplantation

A) A cresyl violet stained section of retina with preserved photoreceptor nuclei (ONL) adjacent to the injection site. B) Fluorescent micrograph of an area analogous to A) showing fluorescent cells (arrow). C) The fluorescent cells are not GFP-expressing cells confirmed by the absence of GFP anti-body labelling.. D) shows a number of ED1 positive cells remain in the area.

Inner Nuclear Layer (INL), Outer Nuclear Layer (ONL), Subretinal Space (SRS), Retinal Pigment Epithelium (RPE). Scale bars = 20μm

Figure 4.7

Histology and Immunohistochemistry of Retina Four Months After Grafting LD7.4/GFP Labelled Cells



Human Foamy Virus can act as a very efficient vector for transfection of RPE cells and that expression is maintained through numerous passages. This has important implications for the use of the RPE cell as a delivery system for molecules that could benefit the retina in degenerative conditions such as AMD or retinitis pigmentosa. One such strategy might be to transfect RPE cells with novel anti-angiogenic agents such as Truncated Tryp (Otani *et al.*, 2003), reducing the risk of CNV recurrence following transplantation. Similarly they could be used as a delivery system for growth factors (such as GDNF or CNTF) that are known to be beneficial for the neural retina (LaVail *et al.*, 1992; Lawrence *et al.*, 2004). Thus, in addition to replacing lost or removed RPE cells, the grafts could provide supplementary support for the remaining photoreceptors and choriocapillaris.

Secondly, macrophage infiltration after transplantation is an early event but they persist in the subretinal space and inner retina up to four months post-operative. The continued presence of macrophages in the subretinal space may reduce the toxic effect of accumulating outer segment debris and thereby contribute to photoreceptor survival. In experimental autoimmune uveitis the macrophages in early and late disease are different and produce altered levels of nitric oxide (NO), and what is observed in this study may mirror that phenomenon.

Thirdly, this technique has the potential to be used in longitudinal studies on the same series of animals and the results from this and from the sectioned material confirm the findings in Chapter 3, that photoreceptor survival after transplantation in the RCS rat is not dependant on the prolonged presence of grafted RPE cells (Keegan *et al.*, 2002). Although the principal role of macrophages is to remove dead cells, it is possible that paradoxically they may release factors that support photoreceptor survival after donor cell death. For instance, IL-1β or bFGF secreted by host

macrophages or microglia could contribute to photoreceptor survival. The beneficial effects of these agents in the RCS rat have been demonstrated in other studies (Akimoto *et al.*, 1999; Whiteley *et al.*, 2001). The finding that photoreceptors persist in the RCS rat long after donor cells are lost emphasises the importance of adequate donor cell labelling in order to make an accurate assessment of the beneficial effect of any potential treatment. In summary, the Human Foamy Virus efficiently and stably transfects RPE cells and this could have a therapeutic application. In agreement with the findings in Chapter 3, LD 7.4 RPE cells transplanted to the retina preserve photoreceptor morphology and function in the RCS rat for several months, yet most of the graft itself appears to be lost by about 2 weeks.

Chapter 5

5. TRANSPLANTATION OF IMMORTALISED XENOGENEIC HUMAN RPE (h1RPE-7 AND ARPE 19) CELL LINES TO THE SUBRETINAL SPACE OF IMMUNOSUPPRESSED DYSTROPHIC RCS RATS

5.1 Introduction

As described in Chapters 1 and 3, major restrictions for RPE transplantation are imposed by the limited supply of human donor eyes and the finite numbers of phenotypically stable and functional RPE cells that can be harvested from primary culture. Furthermore, the impracticality of performing full safety testing on primary cultures from every donor further limits their wide-scale clinical use. Relatively little attention has been given to functional parameters in the systematic assessment of graft efficacy (Little *et al.*, 1998). Neither levels of photoreceptor rescue (Gouras *et al.*, 1989; Li *et al.*, 1991; Little *et al.*, 1996; Lopez *et al.*, 1989) nor ERG measurements (Jiang *et al.*, 1994) are indicators of how much vision is restored following intervention.

The human cell line, h1RPE-7, was developed in the Institute of Ophthalmology. It has been genetically modified to extend its *in vitro* life span and it will be tested alongside a spontaneously derived human RPE line (ARPE19, Dunn *et al.*, 1996). These lines can undergo an extensive number of cell doublings and can be subjected to rigorous testing to produce cell banks of pathogen-free and phenotypically identical cells for use in transplantation. The purpose of this chapter is to describe the characterisation of h1RPE-7, and to assess the effects of transplanting both cell lines into immunosuppressed dystrophic RCS rats. Head-tracking behaviour and threshold

sensitivity reponses (recorded from the SC) will be used to assess the degree of preserved visual function.

5.2 Experimental design

This experiment is designed to compare xenograft cell and photoreceptor survival and to assess maintenance of visual function.

Thirty-three rats (h1RPE7, n=20 and ARPE19, n=13) received subretinal grafts ($2x10^5$ cells per injection) and 10 had sham injections. All animals were maintained on oral cyclosporine A (210mg/l of drinking water) from 2 days before transplantation until sacrifice. Grafted rats (h1RPE7, n=5; ARPE19, n=6) were allowed to survive for 5 months post-operatively with the remainder being sacrificed at earlier time points for histological assessment of short-term changes associated with transplantation. A further 6 non-operated age-matched dystrophic and 3 non-dystrophic RCS rats provided cyclosporine treated controls. To examine donor cell survival, a separate group of rats (h1RPE-7, n=5 and ARPE19, n=7) received cell grafts that had been pre-labelled *in vitro* with 20μM bromodeoxyuridine (BrdU, Sigma, Poole, UK) 48 hours prior to transplantation.

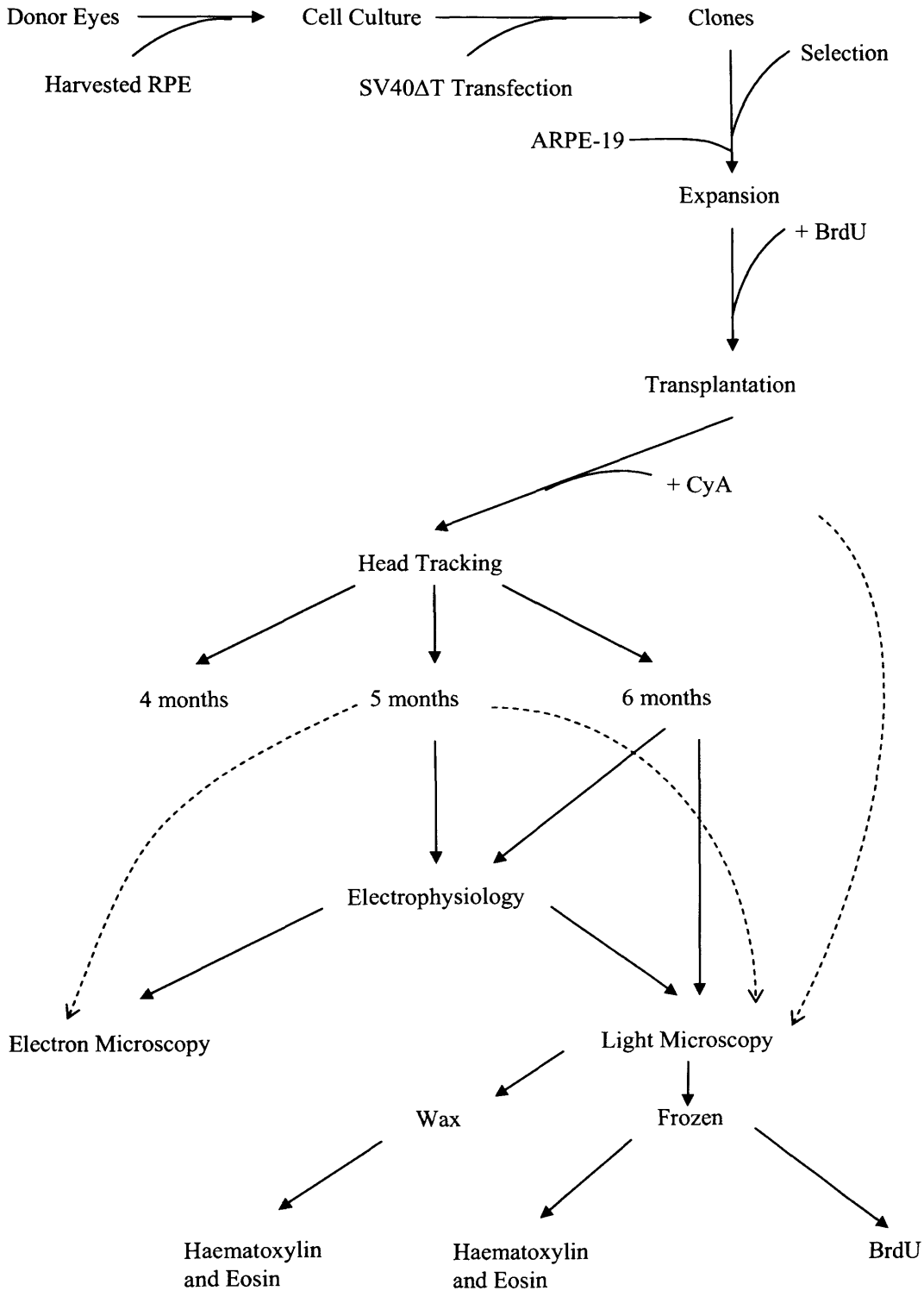
Animals were head-tracked at 10 weeks (h1RPE-7 only) and 20 weeks (both h1RPE-7 and ARPE19 groups) postoperatively. The BrdU group was tested at 20 weeks post-operatively. Behavioural data was analysed as described in 2.4.2. Electrophysiological data (see 2.4.3) collected from individual points of up to 5 of the above animals were averaged and subjected to statistical analysis using both a randomisation test and a t-test to compare h1RPE-7 thresholds against sham thresholds. The randomisation test used was based on the analysis of variance F statistic. Both statistical procedures produced identical results. The graphical representation of the statistical analysis was derived from the randomisation results.

Figure 5.1: Flow Diagram of Transplantation Studies using Immortalised Human (h1RPE-7 and ARPE19) RPE Cells

See section 5.2

Figure 5.1

Flow Diagram of Transplantation Studies Using Immortalised Allogeneic RPE Cells



At the end of the experiment, at five or six months of age, eyes were retrieved for polyester wax embedding. Photoreceptor counts and histological maps were generated on three grafted eyes (h1RPE-7) and two sham-operated eyes, as described in 3.2, at six months of age. The experiment is shown schematically in Fig 5.1.

Additionally, PCR amplification of SV40 large T-antigen was performed on h1RPE7 cells and sections of unoperated dystrophic and h1RPE7 transplanted eyes after various survival times. Globes from h1RPE7 grafted animals were removed and incubated in digestion buffer (50mM Tris-HCl pH 8.0 100mM EDTA, 100mM NaCl, 1% SDS) overnight at 55°C. Digested globes were subsequently extracted twice with phenol:chloroform (25:24:1). Genomic DNA was precipitated in 96% ethanol and removed with a sterile loop. DNA pellets were washed in 70% ethanol, air dried and resuspended in 10mM Tris-HCl, 1mM EDTA. PCR amplification of the SV40 large T-antigen was performed using 1U Qiagen HotStar Taq system and 10pmoles of primers (forward 5' TGTATAGTGCCTTGACTAGAG 3' and reverse 5' GCTGTCAGCAAATATAGCAGC 3') in a 50µl reaction generating a 296bp fragment of the SV40 large T-antigen gene sequence. Reactions were subjected to an initial denaturation step of 95°C for 15 min followed by 45 cycles of 95°C for 30s, 57°C for 30s, 72°C for 30s. Reactions were maintained at 72 °C for 10 minutes prior to analysis on ethidium bromide stained agarose TAE gels. Genomic DNA was isolated from cell cultures using the GenElute Mammalian Genomic DNA Kit (Sigma, Dorset, UK).

5.3 Results

5.3.1 Cell line characterisation and tumour-forming properties

After extensive selection and characterisation of the SV40 large T-antigen transfected cells, a single line designated h1RPE7 was selected for subretinal transplantation into

RCS rats (see 2.3.1.3) since it exhibited a non-transformed phenotype and expressed RPE markers including ZO-1, and cytokeratins 5, 8 and 18. Expression of ZO-1 and cytokeratins 5 and 8 in h1RPE-7 cells is illustrated (see Fig 2.2). Independent assessment of phenotypic cell transformation was performed following subcutaneous injection of 10 million h1RPE-7 cells in 200μl of PBS plus 10mM sucrose into 10 irradiated (5 grays) athymic Swiss mice at an age of 8 weeks. After 3 and 15 weeks h1RPE7 cells failed to produce tumours in all examined mice unlike U87 human glioblastoma cells that were injected as a positive control. h1RPE7 cells also failed to form aggregates or foci when plated into soft agar. ARPE19 cells were similarly characterised and shown to have generally similar expression patterns.

5.3.2 T-cell proliferation assay

To evaluate the potential of the RPE cell lines to induce T cell proliferation, a mixed lymphocyte reaction assay was performed. The two RPE cell lines, h1RPE7, and ARPE19, did not induce significant T cell proliferation (Fig 5.2) irrespective of IFN-γ activation or the number of RPE cells used. As expected, the two B cell lines used as positive controls were able to induce significant T cell proliferation in the absence of other co-stimulatory factors, particularly at a concentration of $2x10^4$ cells/well (Fig 5.2).

5.3.3 Functional Assessment

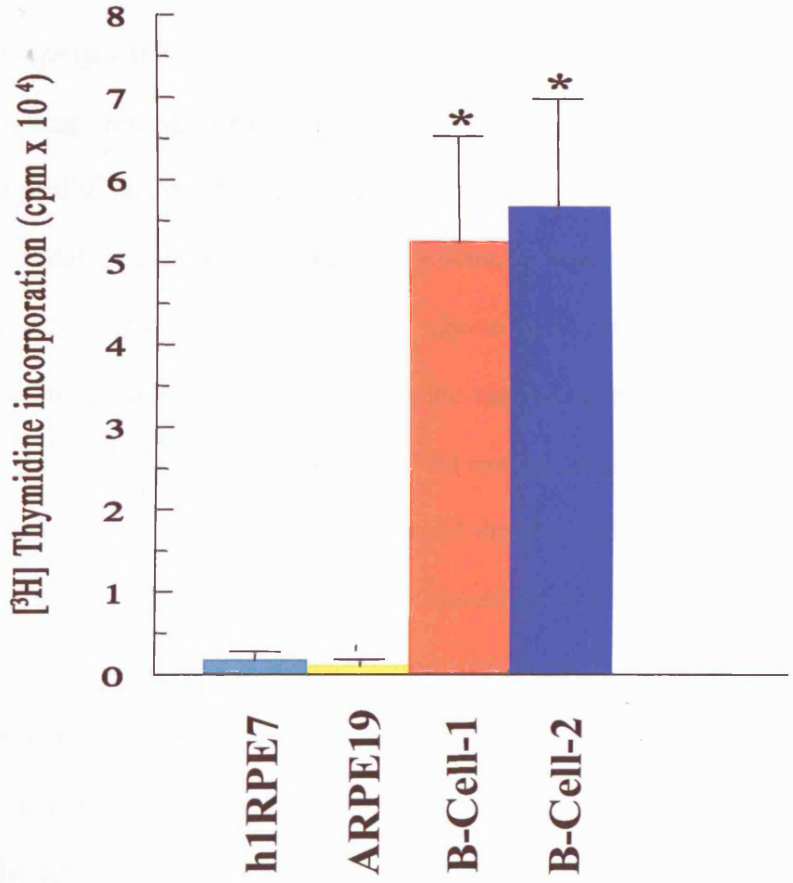
The effectiveness of the two types of cell transplants in preventing visual loss was monitored by head-tracking performance (see 2.4.2 and Fig 5.3A), and this revealed a distinction between graft and sham-injected animals by 10 weeks postoperatively (Fig 5.3B). Both non-dystrophic animals and dystrophic animals that received a transplant of h1RPE7 cells were able to track all grating stimuli and were significantly better than sham-operated and non-operated dystrophic control animals (Fig 5.3B). By 20

Figure 5.2: Mixed Lymphocyte Response of Human RPE Cell Lines

Example of a mixed lymphocyte reaction. h1RPE7, ARPE19 or the B cell lines 7 and 29 ($2x10^4$ cells per well) were cultured in the presence of human T cells ($2x10^5$ cells per well). RPE cells did not induce a significant T cell proliferation (no significant difference between h1RPE7 or ARPE19) whereas a significant proliferation was observed when cocultured with the B7 and B29 B cells. Significant difference from RPE cell lines * $p<0.0005$.

Figure 5.2

Mixed Lymphocyte Response of Human RPE Cell Lines



weeks postoperatively (Fig 5.3C,D), sham-operated controls were unable to track a visual stimulus while h1RPE7 and ARPE19 transplanted animals performed as well as non-dystrophic animals when tracking a visual stimulus of 0.125 cycles/degree. At a grating of 0.25 cycles/degree grafted animals tracked significantly better than both sham-operated and dystrophic animals although at 0.5 cycles/degree this ability was lost. Thus, in contrast to sham-operated and dystrophic animals, h1RPE7 transplanted animals were able to track visual stimuli at both 10 and 20 weeks postoperatively. Animals in the BrdU group demonstrated a similar behaviour profile to the main group. The unlabelled and labelled groups performed significantly better than sham at 20 weeks ($p < 0.01$) on post-hoc analysis. There was no difference between ARPE19 and h1RPE7 groups ($p > 0.05$) at 20 weeks.

After head-tracking testing was completed, visual performance was studied electrophysiologically in the h1RPE7 group. The threshold sensitivity response to light was used to define the area of visual field rescue. In non-dystrophic rats visual thresholds never exceeded 0.7 log candela/m^2 above background (Fig 5.4A). By contrast, the thresholds of non-operated dystrophic rats were in the order of 4 log units higher (Fig 5.4B). Sham-injected rats showed some highly localised functional rescue in the temporal retina with lowest averaged thresholds of 2.6 log units (Fig 5.4D). However, the h1RPE7 transplanted rats exhibited substantially greater levels of visual preservation with thresholds averaged from data from 5 individual rats of as low as 1.6 log units and individual points as low as 0.7 log units (Fig 5.4C). The precise region of maximal sensitivity varied slightly among animals, due to small variations in the siting of the graft, leading to higher values when superimposing individual maps to obtain averages. When the grafted retinae were averaged, 52% of the area was associated with thresholds lower than 3.0 log, and 16% with thresholds

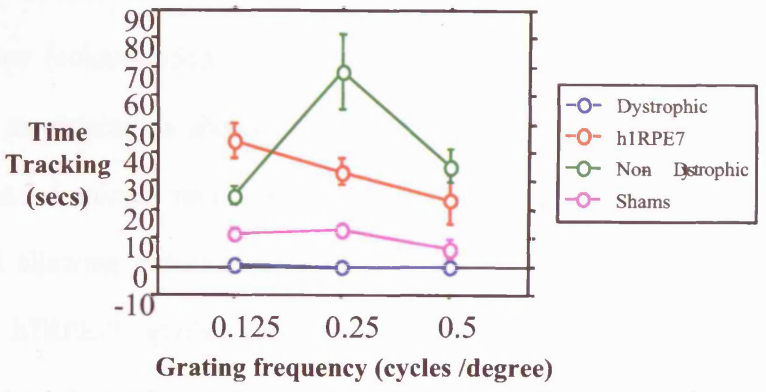
Figure 5.3: Head-Tracking to High Contrast Square-Wave Gratings.

A) Ten weeks post-transplantation, total amount of time h1RPE7 grafted animals spent tracking a moving square-wave grating, in seconds, over a period of 4 minutes. B) Head-tracking 20 weeks post-h1RPE7 transplantation. Error bars represent standard error of the mean. *$p<0.01$ represents a significant difference as compared to both Sham and Dystrophic groups. $^{+}p<0.05$ represents a significant difference as compared to the h1RPE7 group. C) Comparative analysis of mean tracking over all frequencies for ARPE19, h1RPE7 and sham showed a significant difference ($F = 7.05$, $df = 2,23$, $p < 0.01$ Student-Neuman-Keuls post hoc analysis) between sham and both transplant groups but no significant difference between transplant groups.

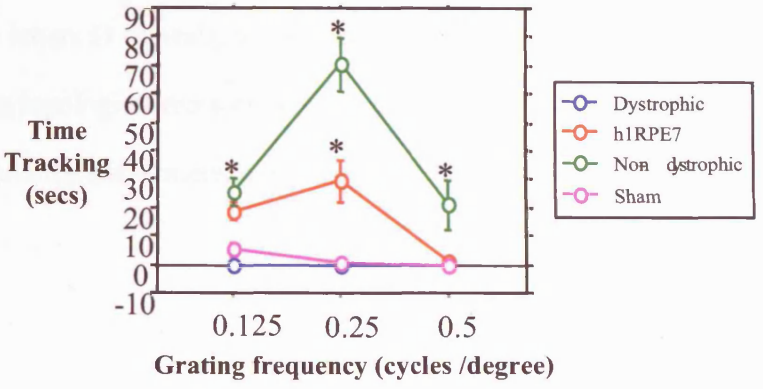
Figure 5.3

Head-Tracking to High Contrast Square-Wave Gratings

A: Head-tracking 10 weeks postoperative.



B: Head-tracking 20 weeks postoperative.



C: Comparative head-tracking (Summed Time) 20 weeks postoperative.

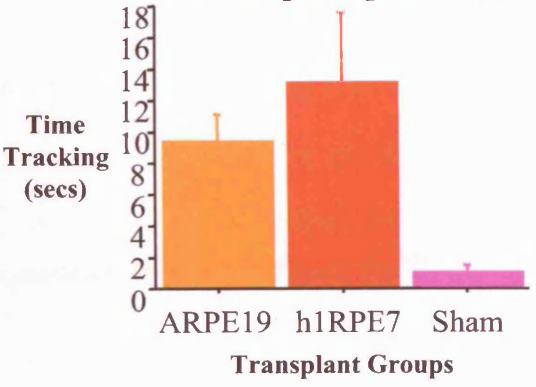
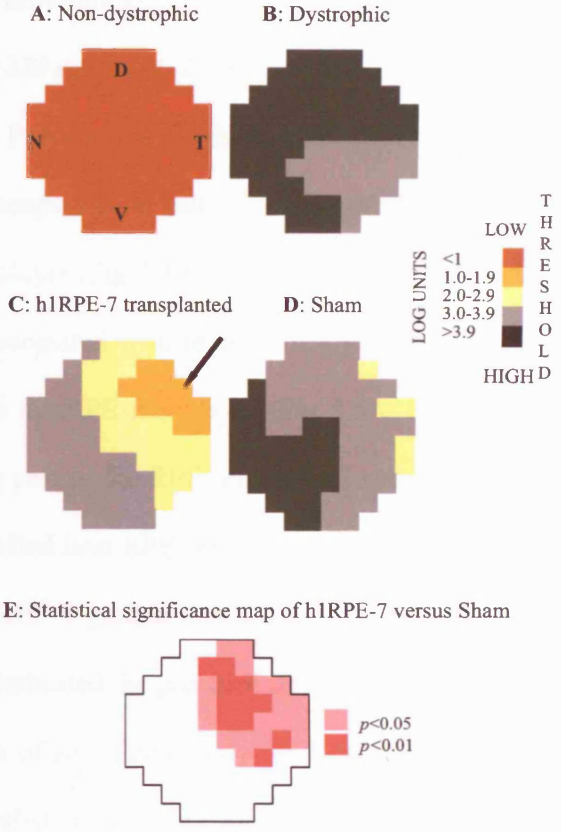


Figure 5.4: Threshold Light Sensitivity Maps at 6 Months of Age

RCS rats were divided into 4 groups: A) normal (3 non-dystrophic rats); B) no treatment (6 dystrophic rats); C) h1RPE-7 cells injected into one eye (5 dystrophic rats); and D) sham-injected (5 dystrophic rats). Schematic representation of a dorsal view of the superior colliculus showing respective thresholds for 76 individual recording sites (colour-coded squares) at 6 months. A log scale of thresholds measured in candela/m^2 is shown. Each point shows the mean response for that position in the 3-6 animals used in each experimental group. Variance was calculated at each point allowing statistical evaluation of differences between groups. To test efficacy of h1RPE-7 grafts versus sham-injected animals, significance was determined at each of the 76 points using a randomisation test. E) An area of significantly preserved visual function was recorded for the h1RPE7-transplanted animals. The letters D (dorsal), V (ventral), N (nasal), and T (temporal) indicate the corresponding topological representation of the retina onto the superior colliculus and all figures retain the same orientation. The arrow represents the grafted quadrant.

Figure 5.4

Threshold Light Sensitivity Maps at 6 Months of Age



lower than 2.0 log units, while in sham-injected retinae these proportions were 7% and 0% respectively. When significance between the sham and h1RPE-7 injected rats was determined, 29 of the 76 points analysed showed a significant improvement in visual function ($p<0.05$) (Fig 5.4E). The area of maximal rescue was invariably confined to the region of cell injection.

The mean blood cyclosporine level in these animals as assessed at the termination of the experiments was 321.6μg/l (+/- 21.9).

5.3.4 Histology and Polymerase Chain Reaction (PCR) Results

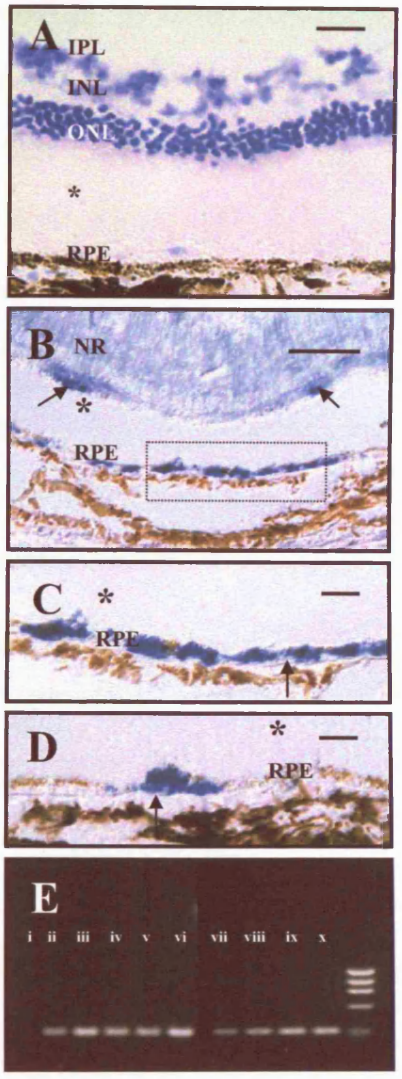
At 5 months post-transplantation cells labelled with BrdU occupied sites on Bruch's membrane as a monolayer (Fig 5.5B,C) or interspersed with unlabelled host cells (Fig 5.5D). They were associated with regions of photoreceptor rescue and on occasion could be seen above the RPE monolayer (Fig 5.5A, B). Pigment distribution in the donor cells (forming part of the RPE monolayer) was polarised to the apical domain as in adjacent unlabelled host RPE cells. PCR amplification of SV40 large T-antigen in eyecups from h1RPE-7 grafted animals at 1, 2 and 7 weeks and 5 months post-transplantation also indicated the presence of SV40 large-T (Fig 5.5E). There was no histological evidence of an inflammatory reaction and infiltrating immune cells were not observed in Nissl-stained (cresyl violet) sections. Histological analysis of non-dystrophic rat retinae showed an outer nuclear layer (ONL) about 8-10 cells thick (Fig 5.6A), while in non-operated dystrophic rats, this was reduced to an occasional cell lying in the outer border of the inner nuclear layer. In sham-operated dystrophic rats (Fig 5.6C) it was usual to find a few photoreceptors around the injection site in the region of the disciform scar. By contrast, retinae that had received h1RPE-7 cell transplants, showed larger areas of photoreceptor survival, as much as 6 cells thick in the general area of the injection site typically thinning to 2 cells further from

Figure 5.5: Localisation of ARPE 19 Cells at 6 Months Following Transplantation

A representative cresyl violet stained frozen section imaged under Nomarski optics A) demonstrating rescue of photoreceptors in an eye at 6 months that received a graft of BrdU pre-labelled ARPE19 cells. Scale bar: 50μm. B) and C) There is extensive rescue of photoreceptor cells that lie over the BrdU-labelled donor cells The grafted cells are mainly located on Bruch's membrane (serial section to A above); Scale bar: 50μm. Immunoreactive cells are also occasionally seen above the RPE monolayer (arrowed) separated in this image by an artifactual detachment (*). A higher power view of the cells seen in B are shown forming a monolayer on Bruch's membrane (arrowed) in C; Scale bar: 10μm. D) is an image of an area of retina distant from A-C) above in which labelled donor cells (arrowed) are integrated between non-labelled host RPE on Bruch's membrane. Pigment is distributed apically as in surrounding host RPE. Scale bar: 10μm. Inner Plexiform Layer (IPL), Inner Nuclear Layer (INL), Outer Plexiform Layer (OPL), Outer Nuclear Layer (ONL), Retinal Pigment Epithelium (RPE), Neural Retina (NR). E) PCR amplification of SV40 large T-antigen DNA. Genomic DNA isolated from non-transplanted eyes (i), h1RPE7 cells (ii), or eyes transplanted with h1RPE-7 cells at 1 (iii & iv), 2 (v & vi), and 7 (vii & viii) weeks and 5 months (ix & x) post-transplantation were subject to PCR amplification. There is persistence of SV40 large T-antigen at 5 months post-grafting suggesting graft cell survival.

Figure 5.5

Localisation of ARPE19 Cells at 6 Months of Age Following Transplantation



the graft focus (Fig 5.6B). Further analysis showed that an area of retina with a preserved layer of photoreceptors two or more cells thick corresponded quite closely with an area of visual field with threshold sensitivities 2 log units or better over baseline.

5.4 Discussion

These results show that genetically modified human cell lines, which *in vitro* exhibit long-term stability and phenotypic and immunological characteristics of RPE cells, support photoreceptor survival and limit the deterioration of visual function for up to 5 months after transplantation into the subretinal space of RCS rats. In these cyclosporine-treated animals the grafted cells showed no evidence of inducing an overt inflammatory response. In addition, despite long-term immunosuppression, there was no evidence of uncontrolled growth of the grafted cells.

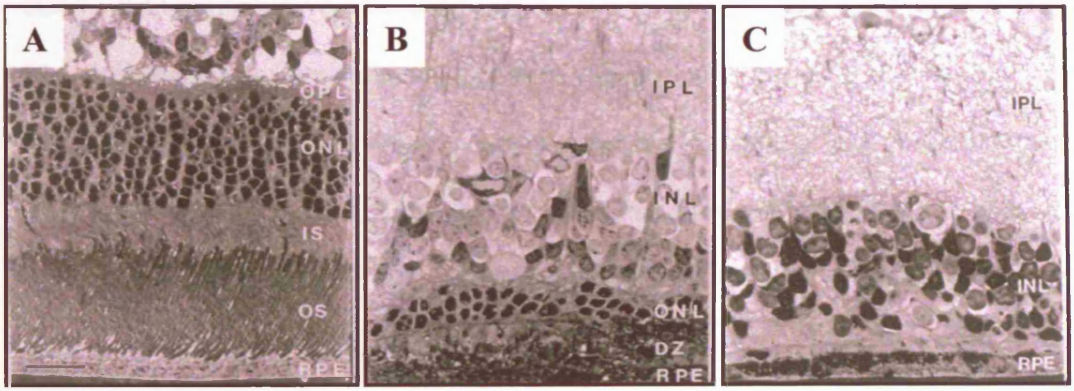
In cell grafting strategies for clinical application, cell lines have the advantage that they can be expanded as required and readily tested for safety, including chromosome abnormalities, tumourogenicity and potential pathogens. These important characteristics make them preferable to primary or short-term cultured cells for transplantation, which enter crisis after limited cell doublings. A number of other RPE cell lines have been described, although they have not been characterised as fully as the present cell lines nor have their efficacy in transplantation been examined. Other 'immortalisation' techniques are emerging, which may be preferable to the use of SV40-T antigen or spontaneously derived lines. The question of immune rejection of grafted human cell lines has yet to be addressed. From the present studies, it might be possible to establish a collection of cell lines with different histocompatibilities or alternatively to further genetically modify the cells to express down-regulatory

Figure 5.6: Anatomical Rescue of Photoreceptors at 6 Months of Age Following Transplantation of h1RPE7 Cells to RCS Rats

Semi-thin sections of retinae taken from animals used in the electrophysiological studies: A) 6-month-old non-dystrophic RCS rat showing full outer nuclear layer thickness. B) h1RPE7 transplanted RCS rat 5 months post-grafting demonstrating significant preservation of the outer nuclear layer: this level of rescue corresponds to a threshold sensitivity 2 log units above background. Area shown was 1400μm from injection site. C) Sham-operated RCS rat, 5 months post-operative, showing complete loss of the outer nuclear layer: there is also marked disruption of the inner nuclear layer. All sections were stained with toluidine blue. GC: ganglion cell layer. IPL: inner plexiform layer. INL: inner nuclear layer. OPL: outer plexiform layer. ONL: outer nuclear layer. IS: inner segments. OS: outer segments. RPE: retinal pigment epithelial cell layer. Scale bar: 20μm.

Figure 5.6

Anatomical Rescue of Photoreceptors at 6 Months of Age
Following Transplantation of h1RPE7 Cells



molecules, such as the cytokine IL-10, capable of inducing local immunosuppression (Croxford *et al.*, 2001).

Sham injections delay functional deterioration, perhaps by flushing debris from the subretinal space or by activating microglia or macrophages and by provoking growth factor production, but by 10 weeks post-transplantation the effect of the graft is clearly distinguishable from the sham effect.

The measurement of collicular threshold sensitivity used in this study provides an indication of relative preservation of visual field. Its importance lies in the fact that there is both evidence of some degree of correlation between the amounts of functional rescue and anatomical preservation and that the data collected compares with visual field perimetry testing in humans (Beck *et al.*, 1985). A reliance on functional assessment is clearly preferable when providing a background for clinical treatments, since it is not clear what relation commonly used measures (such as thickness of photoreceptor layer) have to degree of functional rescue. ERG has been used extensively to assess visual function after photoreceptor loss, but while it gives information as to changed electrical activity in the retina, it does not provide direct information about the image forming capability. Furthermore, ERG measures are known to correlate poorly with visual capacity in retinitis pigmentosa patients (Szlyk *et al.*, 1997). This study shows clear evidence of functional efficacy of grafting human RPE cell lines into the subretinal space and that rescue occurs in the general region in which the grafted cells are located. It is still not clear whether these cells function by phagocytosing outer segments or by producing trophic factors, which act on host RPE or on cells of the outer retina. *In vitro* studies have shown that the donor cell line used here does phagocytose outer segment fragments and in addition produce a range of factors such as BDNF and bFGF known to support photoreceptor survival. It should

be noted that in parallel studies (Lawrence *et al.*, 2000; 2004), Schwann cells or Schwann cells engineered to over express BDNF and GDNF grafted into the subretinal space of RCS rats also supported prolonged photoreceptor survival. However, they did not appear to phagocytose outer segments themselves nor did they influence phagocytosis by host RPE.

In summary, the ability of human RPE cell lines to rescue visual function has been demonstrated in the RCS rat using physiological and behavioural tests that are analogous to those used to assess visual function in humans. The use of cell lines could provide a potential and very valuable approach to the prevention of retinal degeneration in humans.

Chapter 6

6. TRANSPLANTATION OF NEONATAL SYNGENEIC SCHWANN CELLS TO THE SUBRETINAL SPACE OF RHODOPSIN KNOCKOUT MICE

6.1 Introduction

Retinitis pigmentosa comprises a group of inherited retinal degenerative diseases characterised by photoreceptor loss, vascular changes, invasion of the inner retina by retinal pigment epithelial cells (RPE) and blindness. Photoreceptor loss can be limited in experimental models of RP by delivery of growth factors either by direct injection into the eye or by gene therapy approaches (as outlined in Chapter 1).

Previous work (Lawrence *et al.*, 2000) showed that Schwann cells grafted subretinally into the dystrophic RCS rat supported photoreceptor survival for up to 9 months postoperatively. The rationale for using these cells was that they produce such retinally active growth factors as CNTF (Sendtner *et al.*, 1992), BDNF (Meyer *et al.*, 1992), GDNF (Hammerberg *et al.*, 1996) and bFGF (Neuberger *et al.*, 1993). After transplantation to the central nervous system (CNS), they can promote neural regeneration (Neuberger *et al.*, 1993). As such, they potentially present an additional trophic factor delivery approach for the eye.

A further study, Lawrence *et al.*, (2004) showed that a Schwann cell line, engineered to over express GDNF or BDNF, transplanted into the SRS of dystrophic RCS rats produced good photoreceptors rescue and maintenance of visual function. The rhodopsin knockout mouse was used to assess whether the rescue potential of Schwann cells may extend beyond the RCS rat to include RP models in which there is an intrinsic photoreceptor defect, rather than an RPE defect. The retina of the rhodopsin knockout mouse has a normal complement of photoreceptors at birth although rod outer segments never develop (Humphries *et al.*, 1997). By 8 weeks an

ERG is no longer detectable (Toda *et al.*, 1999) and by 3 months nearly all photoreceptors are lost except for some residual cones. By transplanting Schwann cells to the subretinal space at an age prior to the loss of significant numbers of photoreceptors, it is possible to explore whether rescue of remaining cells can be achieved.

6.2 Experimental Design

Rhodopsin knockout (Rho-/-) mice (n=15) received unilateral subretinal transplants of neonatal Schwann cells (1 x 10^4 cells in 2µl of DMEM plus DNase) or sham injections (2µl of DMEM + DNase: n=15) at PN35. Unoperated eyes served as controls. Since a small population of contaminating fibroblasts remained in the Schwann cell preparations, a further group of Rho-/- animals (n=5) received subretinal injections of fibroblasts to both eyes (See Fig 6.1).

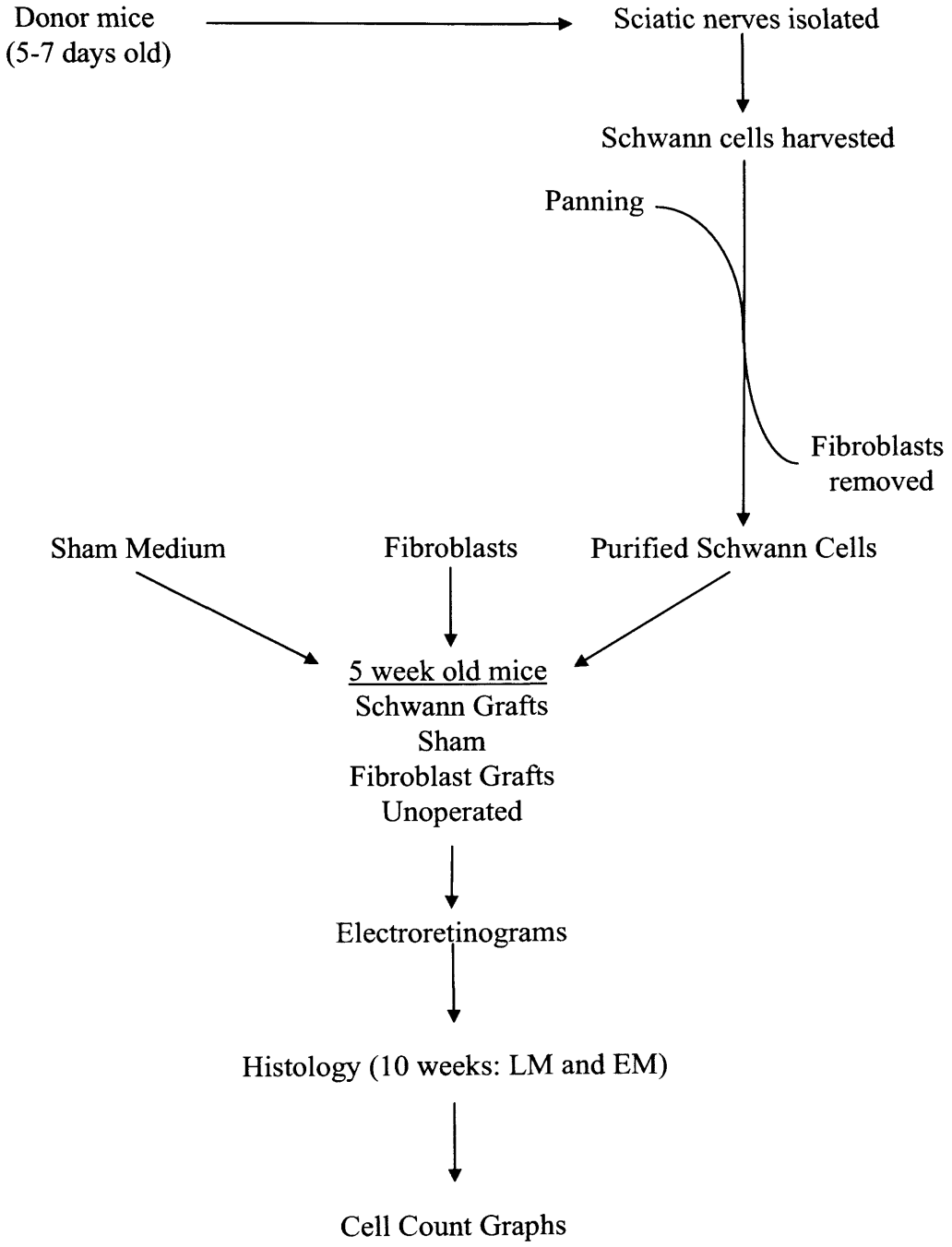
At PN70 animals (Schwann cell transplanted Rho-/-, n=10; fibroblast transplanted (n=5); sham-operated, n=10 and unoperated controls, n=6) were overdosed with Euthatal (Rhone Merieux, UK) and the tissue processed for electron microscopy (see Appendix I). In order to quantitate the number of surviving photoreceptors in each group of treated animals, semithin sections were cut through the region of the transplant and stained with toluidine blue (Sigma, Poole, UK). A temporal to nasal orientation was maintained throughout using a suture placed in the ventral corneo-scleral junction as a marker. Representative sections (adjacent to or within 200 µm of the injection site) from each retina were photographed and the photographs montaged. The total number of photoreceptors was counted blind, by the same person, for each sample in 100 µm bins (approximately 50 per eye). Maximal cell counts for each bin location in each experimental group and mean cell counts for each bin location in each experimental group across the retinae (temporal to nasal) were generated and

Figure 6.1: Flow Diagram of Transplantation Studies to Rho -/- Mouse using Mouse Schwann Cells

See section 6.2.

Figure 6.1

Flow Diagram of Transplantation Studies Using Mouse Schwann Cell Grafted to Rho-/- Mouse



compared. Analyses were performed using StatView (SAS Corporation, USA) for PC. All group data were subjected to a one-way analysis of variance in the temporal region of the retina (highlighted). Post-hoc analysis was carried out on significant group findings. Additionally some retinae from each group were sectioned for ultrastructural studies.

6.3 Results

6.3.1 Donor cells

Schwann cell cultures of high purity (more than 95%) were obtained (Fig 2.3). The Schwann cells had a typical spindle-shape morphology, which was readily distinguishable from contaminating fibroblasts which had large nuclei and extensive flattened cytoplasm.

6.3.2 RT-PCR

The Schwann cells expressed mRNA for BDNF, CNTF and GDNF (Fig 6.2). There was no evidence of NGF or bFGF message.

6.3.3 Rhodopsin Knockout Mouse: Retinal histology

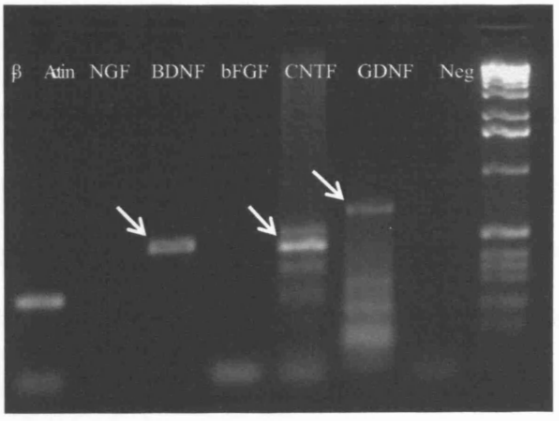
Histological analysis of grafted animals (n=10) at PN70 showed a distinct region of photoreceptor preservation in the temporal retina (Fig 6.3 J,K,L and Fig 6.5), 6-7 cells deep and approximately 800μm wide. This was in contrast to fibroblast-grafted (Fig 6.3 G,H,I), sham-operated (Fig 6.3D,E,F) and unoperated animals (Fig 6.3 A,B,C) where generally only one to two layers of photoreceptors, mostly cones, remained. Although more photoreceptors survived in the Schwann cell transplanted retinae, typical outer segments never formed. Cone inner segment morphology was distinctly preserved (Fig 6.3 K,L). Schwann cells were identified in the general region of the area of rescue (Fig 6.4A, B), where as described previously (Lawrence *et al.*, 2000) they could be identified by their indented nuclei, which were larger

Figure 6.2: RT-PCR Showing Growth Factor Profile of Mouse Schwann Cells in vitro.

RT-PCR profile of primary Schwann cells harvested from 129 (PN5-7) pups for growth factor mRNA. The specific factors screened for here include: Nerve Growth Factor (NGF), Ciliary Neurotrophic Factor (CNTF), Brain-Derived Neurotrophic Factor (BDNF), basic Fibroblast Growth Factor (bFGF) and Glia-Derived Neurotrophic Factor (GDNF). There is strong expression of BDNF and CNTF and weak expression of GDNF. Messenger RNA is undetectable for NGF and bFGF.

Figure 6.2

Growth Factor Profile (rt-PCR) of Neonatal Mouse Schwann Cells
In Vitro



than the elongated nuclei of fibroblasts, distributed among the Schwann cells (Fig 6.4B). Although the retina immediately overlying the graft was disrupted there was marked preservation of photoreceptors. Some of the fibroblasts seen in the vicinity of the graft may have been transplanted with the Schwann cells and some may be host fibroblasts introduced from the sclera at the time of surgery. Because animals that received grafts of pure fibroblasts showed no improved photoreceptor rescue and no cone inner segment preservation, the rescue seen in the Schwann cell graft recipients (with some fibroblast contamination) is likely due to the Schwann cells themselves. Maximal photoreceptor counts for each group, along the length of the retina temporal to nasal, are presented in Fig 6.5A. The outlined area demonstrates a region of approximately 2300μm in the vicinity of the temporal injection site, where there was a marked increase in photoreceptor survival. This region was subjected to statistical analysis. The Schwann cell transplant group had significantly more photoreceptors than the fibroblast graft, sham-operated and unoperated groups (F=148.01, df=3,32, p<0.001). There was approximately a 55% increase in photoreceptor survival in the Schwann cell transplant group compared with the sham-operated group and a 225% increase in the Schwann cell transplant group as compared with the fibroblast transplant group and the unoperated group. Furthermore, the sham-operated group had approximately twice as many photoreceptors as the fibroblast and unoperated group, which was significant (sham versus fibroblast transplant group: t=13.06, df=16, p<0.01; sham versus the unoperated control: t=17.13, df=16, p <0.01). A similar area, 2300μm, (indicated by the outline box in Fig 6.5B) was analysed for the mean data from each group. Once again, a region in the temporal retina showed a significant rescue effect in the Schwann cell transplant group over fibroblast transplant, sham and unoperated control groups (F=33.15, df=3,38, p<0.001). There was approximately a

Figure 6.3: Comparative Anatomy of Control (A, B&C), Sham-operated (D, E&F), Fibroblast Grafted (G, H&I) and Schwann Cell Grafted (J, K&L) Rho -/- Retinae at PN70.

A) Semi-thin section of a control retina at PN70. There is near complete loss of the outer nuclear layer (ONL).

B) A higher power view of the boxed area in A demonstrates these features more clearly. There is total absence of outer segment structure and a grossly compromised inner segment (IS) layer. (*) outer limiting membrane (OLM).

C) An electron micrograph showing stunted photoreceptor inner segments (IS) and OLM (*).

D) Semi-thin section of a sham-operated retina. It shares some features of a control retina, i.e. thinning of the ONL and loss of inner segment morphology.

E) A higher power view of the boxed area in D.

F) An electron micrograph of the same sham-operated eye (seen in D and E) shows that inner segments are rare and there are no outer segments. Hypertrophied Müller cell processes (mp) surround remaining photoreceptors. OLM (*).

G) Semi-thin section of a retina that received a fibroblast transplant. The ONL is reduced and inner segments are much reduced.

H) A higher power view of the boxed area in G.

I) An electron micrograph of a fibroblast grafted retina. There is a total absence of outer segment structure and a grossly compromised IS layer. Hypertrophied Müller cell processes (mp) surround remaining photoreceptors.

J) Semi-thin section of a retina that received a Schwann cell transplant. There is considerable preservation of the outer nuclear layer (ONL) compared with the

unoperated control, sham-operated and fibroblast grafted retinae (A, D and G respectively).

K) A higher power view of the area boxed in J. There are no outer segments, but there is preservation of cone inner segment morphology (arrowed) at PN70 compared with fibroblast, sham and control retinae.

L) An electron micrograph from the area of retina illustrated in J and K that had received a graft. Note the more distinct cone inner segment morphology (arrowed) compared with that seen in C, F and I. As in those panels there are no demonstrable rod or cone outer segments. Note also that there is some hypertrophy of Müller glial cell processes (mp).

Scale bars A, B, D, E, G, H, J and K: 25μm; C, F, I and L: 2μm.

Figure 6.3

Comparative Anatomy of Control, Sham-Operated, Fibroblast Grafted and Schwann Cell Grafted Retinae at PN70

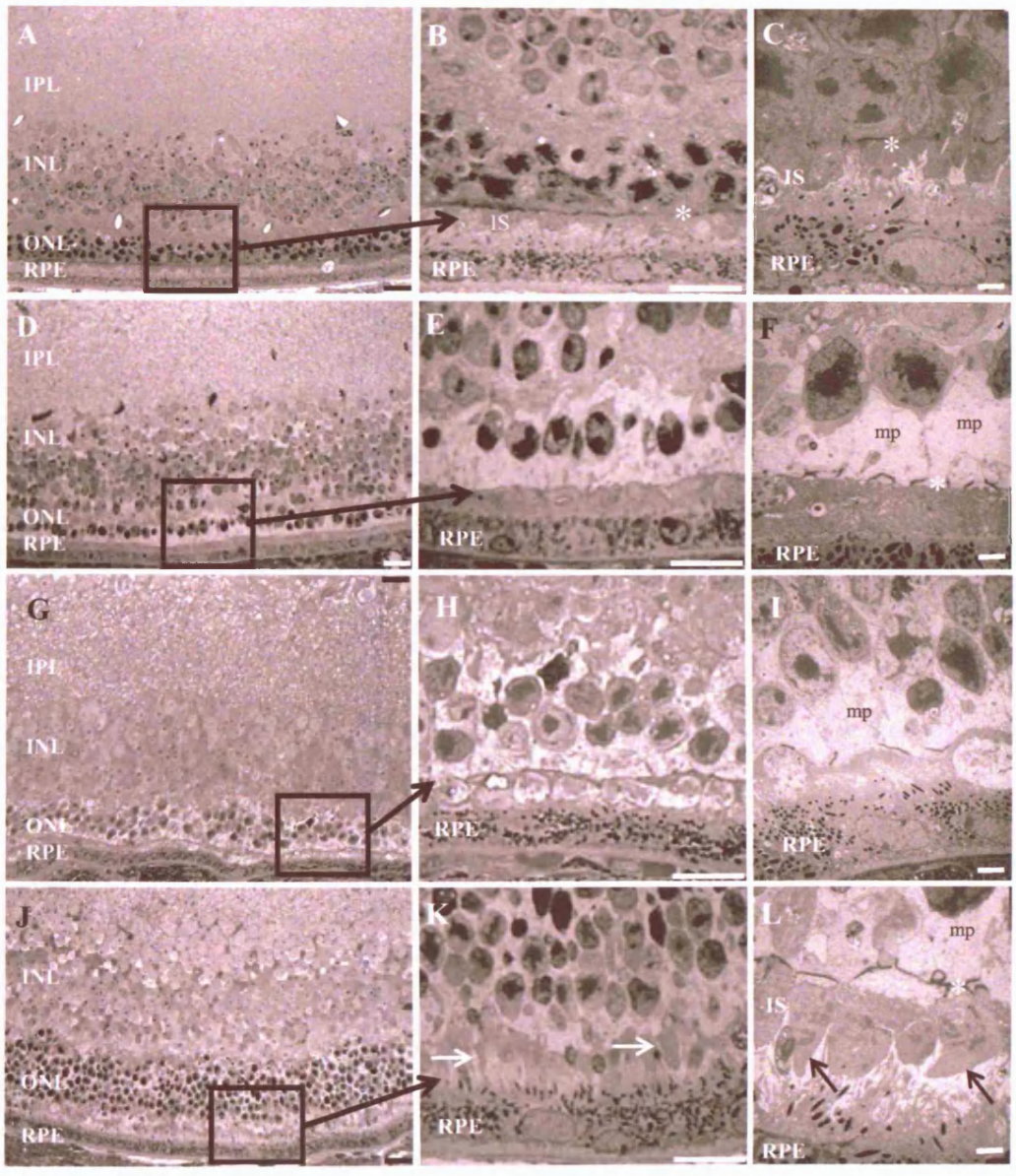


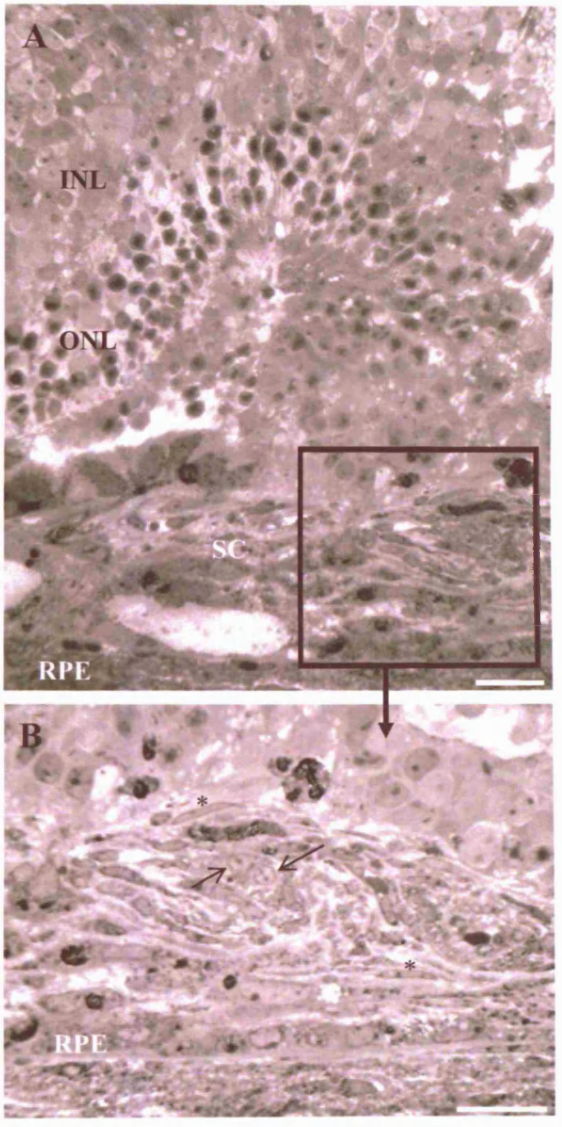
Figure 6.4: Schwann Cells in the Subretinal Space of PN70 Rho-/- Mouse Grafted at PN35.

A) Semi-thin section showing Schwann cells grafted into the subretinal space at PN35 and harvested at PN70. The cells are layered over the host retinal pigment epithelium (RPE). The overlying retina is disrupted but the photoreceptor layer (ONL) is preserved. Inner nuclear layer (INL). Scale bar: 50μm.

B) A higher power view of the area boxed in A. The Schwann cells (SC) are seen interspersed with some fibroblasts. The Schwann cells have larger, indented nuclei (arrowed) compared with fibroblast nuclei, which are elongated (*). The fibroblasts may have been introduced as a contaminant at the time of grafting or pushed in from the host sclera at the time of surgery. The mixed cells lie in a group on host RPE. Scale bar: 50μm.

Figure 6.4

Schwann Cells in the Subretinal Space of PN70 Mouse Grafted at PN35



30% increase in photoreceptor survival in the Schwann cell transplant group compared with the sham-operated group and a 66% increase in the Schwann cell transplant group compared with the fibroblast transplant and unoperated group. As before, a significant increase in photoreceptors was observed in the sham-operated groups compared with the fibroblast transplant group and the unoperated control group (sham versus fibroblast transplant group: t= 5.96, df= 44, $p< 0.01$; sham versus unoperated knockout control: t= 3.43, df= 44, $p<0.01$). Thus, there was a focal rescue effect adjacent to the site of injection and in this particular area the effect was significant.

6.4 Discussion

The results show that Schwann cells grafted into the rho-/- mouse can preserve a focal region of photoreceptors up to at least PN70. The results are of interest as they show that Schwann cells are able to preserve photoreceptors in an animal model where the defect lies in the photoreceptors themselves (Fig 6.3 and 6.5) in contrast to previous work showing rescue in RCS rats where the defect lies in the RPE. In the eyes with evident grafts the cells appear normal, and integrate into the subretinal space (albeit with occasional fibroblasts: Fig 6.4B). Fibroblasts appear to produce little or no rescue, and the rescue effect observed is most likely due to the Schwann cells alone. None of the cells appear to provoke evident inflammatory reactions, although there is a Müller cell response. However, because glial hypertrophy occurred after sham-surgery and in both transplant groups, this is likely to be a respose to surgical intervention. In parallel studies in RCS rats (Lawrence *et al.*, 2000), outer segments were preserved in the vicinity of the Schwann cell grafts for quite extensive periods of time in contrast to fibroblast and sham-injected eyes. In the present model, rod outer segments never develop in the rho-/- mouse, and nor are they seen after Schwann cell

grafting or AAV-CNTF treatment (Liang *et al.*, 2001). However, after Schwann cell grafting, inner segment cone morphology appears to be better preserved. It is presumed that the Schwann cells preserve photoreceptors by delivering growth factors into the subretinal space, because *in vitro* at least, they are a source of several factors known to preserve photoreceptors from degeneration. The cones could be directly rescued or secondarily through rod rescue in a manner similar to that described in other studies (Mohand-Said *et al.*, 1997). The primary target of the growth factors is still unclear, but the finding in C57BL/6J mice that intravitreal injections of CNTF, BDNF and FGF2 (bFGF) activate intracellular signalling mechanisms in Müller glia (and other inner retinal cells) but not photoreceptors (Wahlin *et al.*, 2000) suggests that the photoreceptor rescue may be via a second order pathway. The degree of rescue was less extensive than that shown by Liang *et al.*, (2001) who delivered secretable CNTF mediated by adeno-associated virus (AAV) at a much earlier time-point (PN2-5). Delivery at this earlier time may be important since the main peak of apoptosis in the outer nuclear layer of the rho-/- mouse (129Sv background) is at about PN35 (Humphries *et al.*, 2001) proceeding rapidly to approximately PN63, and in the present study grafting did not take place until PN35. This later time was chosen, as it would be more analogous with a potential treatment point in humans. However, it is possible that by transplanting late in the process of degeneration, the cell death cycle is already in progress and can only be delayed. In addition, donor cells are likely to be more locally distributed than an AAV-mediated product, so reducing their overall efficacy. Liang *et al.*, (2001) showed that cells engineered with non-secretable CNTF could not promote photoreceptor survival. Because Schwann cell CNTF may not be readily available in a secretable form and other growth factors produced by them could give rise to the observed effect. Although photoreceptors

Figure 6.5: Analyses of Photoreceptor Numbers in Schwann Cell Grafted versus Fibroblast Grafted, Sham-operated and Control Retinae.

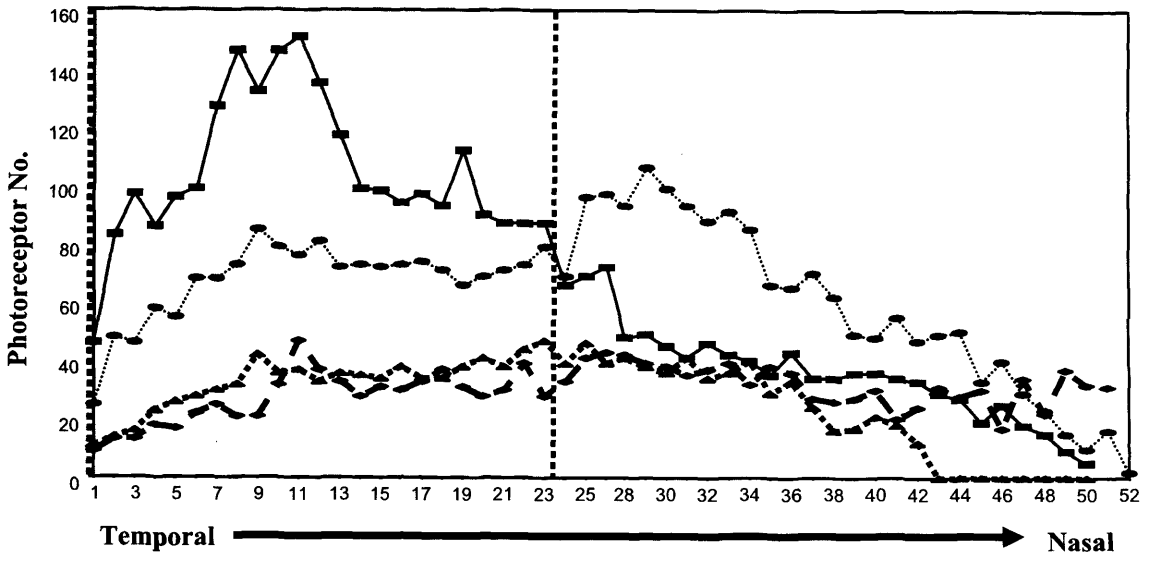
A) This graph illustrates photoreceptor counts across the retina from temporal to nasal (left to right). Maximal counts for each group at each bin location are shown. The Schwann cell transplant group tends to have higher cell counts in the temporal (surgical region) than sham-operated, fibroblast grafted or unoperated control retinae. This difference is significant ($p<0.001$).

B) Mean photoreceptor counts for all the groups (n=5 in each group). Again the Schwann cell transplant effect is demonstrated in the temporal retinal region. The difference between Schwann cell grafted retinae and sham-operated, fibroblast grafted and unoperated control retinae over this temporal region (outlined) is again significant ($p<0.001$).

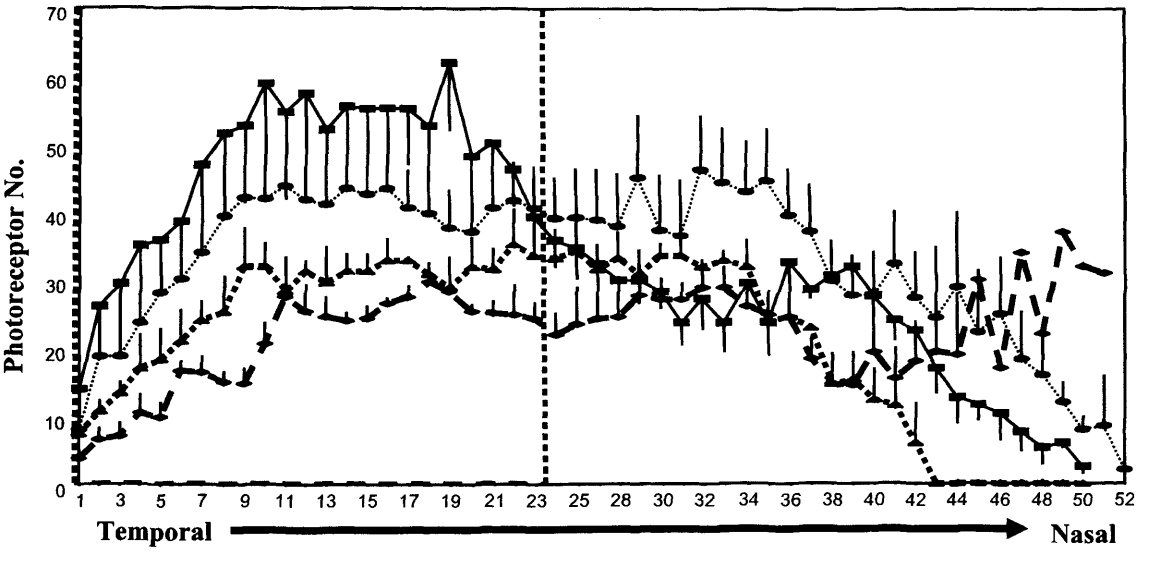
Figure 6.5

Analyses of Photoreceptor Numbers in Grafted Versus Sham-operated and Control Retinae

A: Maximal Photoreceptor Cell Counts



B: Mean Photoreceptor Cell Counts



——— Schwann Cell Transplant

..... Sham

▪▪▪▪▪▪▪ Unoperated Dystrophic

– – – Fibroblast Cell Transplant

have been preserved in this study, no outer segments were generated, and this was also observed after AAV-CNTF delivery (Liang *et al.*, 2001). As indicated in that study an ERG response was not demonstrated. Unoperated controls are also without outer segments and never show evidence of an ERG (Toda *et al.*, 1999). Furthermore, Sieving's group (Machida *et al.*, 2000) have determined the electrophysiological relationship between outer segment length and photoreceptor nuclei number and showed that, without outer segments, it is not possible to generate an a-wave. Thus, the absence of outer segments would indicate that an intact ERG response is unlikely. In agreement with these findings a preliminary ERG investigation of the present group of transplanted animals prior to fixation failed to demonstrate any significant response. Viral (rAAV or lentivirus) secretable CNTF injected into the eyes of other rodent models of RP (Liang *et al.*, 2001; Timmers *et al.*, 2002) provided long-term photoreceptor preservation but had no or negative effects on ERG amplitudes. However, patients with RP may also fail to show full-field ERGs and have greatly reduced numbers of photoreceptors with shortened or absent inner and outer segments, yet have some functional vision remaining (Sieving *et al.*, 1999). Therefore, an absent ERG does not necessarily indicate that there is no visual processing. Rod rescue *per se* would not be expected to rescue visual function in this animal. However, other investigators (Mohand-Said *et al.*, 2000) have proposed that by rescuing rods, cones that may otherwise die will be preserved. The present preparation provides a special situation in which to examine this possibility.

In summary, Schwann cell grafts limit photoreceptor loss. The most parsimonious explanation for this phenomenon is that they function by delivering a range of growth factors. The potential of a steady continuous release of a mixture of factors from transplanted cells affords some advantage over intravitreal injection. Local release at

the site of need avoids the inherent problem of delivering factors at non-physiological levels to other tissues of the eye or body. The use of syngeneic grafts also obviates the need for immune suppression, which can be a serious problem in transplantation studies. The degree of functional vision preserved by such grafts remains to be established.

Chapter 7

7. DISCUSSION

7.1 Summary

Cell transplantation strategies for the treatment of retinal disease are an exciting and novel prospect for the future. While the findings presented in this thesis are very encouraging, there is much to do before clinicians will be confident enough to offer this technique routinely to their patients. The results do, however, offer clues for future developments.

The potential of expanded, extended life cell lines for treating conditions characterised by retinal pigment epithelium failure (such as AMD) is considerable. To date most human RPE transplants have been derived from foetal tissue, which is neither ethically very desirable nor practical, considering the amount of material that would be required. The use of cell lines permits extensive safety checks to be carried out prior to clinical trials which is very necessary since conditions like AMD are non-fatal.

Schwann cell transplantation represents an attractive option for the treatment of retinal dystrophies. It is a simple concept that should gain acceptance more readily in the clinical community. The potential to perform autografts should allay many fears since problems of immunological rejection and the presence of foreign infective agents in donor tissue would be removed. Growth factor delivery appears to be a viable treatment option in these diseases, yet to establish a continuous delivery of a factor at a physiological dose remains elusive: Schwann cells are biologically active cells producing physiological amounts of neurotrophins that can be placed at the desired site of action.

There is still much to be done to refine these treatment strategies in the laboratory, yet the potential exists for these therapies to enter the clinical realm in an experimental capacity within a reasonable timeframe.

7.2 Mechanisms of photoreceptor rescue by donor cells

7.2.1. LD7.4 RPE allografted into the SRS of non-immunosuppressed dystrophic RCS rats

The studies have demonstrated that allografts of LD7.4 cells, placed in the subretinal space of non-immunosuppressed dystrophic RCS rats, produce superior rescue at both functional and anatomical levels when compared with sham-operated rats. The benefit persisted up to six months of age (five months post-transplantation). Closer analysis of the data revealed deterioration in function with time, suggesting the existence of cellular events that compromise rescue. Grafted cells could not be identified by immunohistochemistry beyond four weeks post-transplantation (Fig 3.7) yet PCR studies have demonstrated the presence of DNA unique to the grafted cells up to 3 months following transplantation (Fig 3.8). There are, however, numerous ED-1 positive cells post-transplantation (Fig 4.5G,H) that persist in the retina to 5 months (Fig 4.7D). These results suggest that photoreceptor morphology and function can be preserved in this model of retinal degeneration for a period of time in the presence of grafted LD7.4 cells, yet function and morphology appear to persist beyond the survival time of the donor cells. There are a number of possible explanations for this. Firstly, many factors have been shown to preserve photoreceptors in the dystrophic RCS rat e.g. bFGF (Faktorovich *et al.*, 1990) or IL-1β (Whiteley *et al.,* 2001) albeit for short periods of time. Additionally, IL-1β protects photoreceptors in the light-damaged rat model (LaVail *et al.*, 1992) but importantly it is also associated with the recruitment/ proliferation of macrophages (Bamforth *et al.*, 1997). Macrophages

infiltrate LD7.4 cell grafts in non-immunosuppressed rats and resident host microglia will also be activated. It is possible that photoreceptor rescue may be mediated by prolonged production of pro-inflammatory cytokines by microglia (Ng and Streilein, 2001). Alternatively, the macrophages and activated microglia may phagocytose photoreceptor outer segment debris and reduce the build-up of toxic products in the SRS. A phagocytic role for microglia was seen when retinae were grafted intracerebrally (Banerjee and Lund, 1992). However, when Li and Turner (1991) transplanted macrophages to the subretinal space they could not identify any protective effect.

Another possibility is that neurotrophins released by RPE cells (Kanuga *et al.*, 2002) and other host cells (such as Müller glia) in the transplanted retina may reduce the number of photoreceptors that undergo apoptosis during the early stages of retinal degeneration (Tso *et al.*, 1994). This could be why grafts fail to support photoreceptors in older animals (Li *et al.*, 1991), when maximal apoptosis has already occurred. Lastly, it is possible that a few donor cells do survive for longer intervals but they have somehow escaped detection in the material sampled. If this is the case, then very few donor RPE cells must be supporting numerous photoreceptors, which again suggests there is diffusion of some supportive growth factor.

Subsequent studies (e.g. See 7.3) will concentrate on identifying more precisely the mechanisms that are involved in photoreceptor rescue. For instance, LD7.4 cells will be transplanted into macrophage-depleted rats to determine whether donor cells and photoreceptors survive for shorter or longer periods in the absence of macrophages.

7.2.2 h1RPE7 and ARPE19 cells xenografted into the SRS of immunosuppressed dystrophic RCS rats

These human cell lines have been shown to survive for up to 5 months following transplantation in the studies reported here and for even longer in other studies (Coffey *et al.*, 2002). The grafted cells appear to maintain their polarity on Bruch's membrane suggesting that they could be functional (Fig 5.5B, C and D). The presence of polarised molecules (e.g. apical Na^+-K^+ ATPase) on the donor RPE cells has yet to be confirmed *in vivo* with immunohistochemistry however it has been shown *in vitro* (Turowski *et al.*, 2004). The fact that donor cells are found adjacent to areas of maximal photoreceptor rescue implies a direct supporting role for these cells. As with the LD7.4 cells (7.2.1), these cells may support photoreceptors by release of the growth factors they produce (Kanuga *et al.*, 2002) or they may indirectly support photoreceptors by stimulating other retinal cells to release supportive factors. There is no direct evidence that donor RPE cells can phagocytose outer segment debris, although the debris zone is much reduced in regions of good rescue. However, resident microglia or macrophages could be responsible for debris removal. Given the information derived from the LD7.4 studies, it cannot automatically be assumed that the grafted human cells function by simply replacing defective RPE cells.

7.2.3 Syngeneic Schwann cells transplanted into the subretinal space of rhodopsin knockout mice

The mechanism by which Schwann cells maintain photoreceptors is presumed to be due to the local delivery of growth factors. Cells used for mouse-to-mouse grafts express message for GDNF, CNTF and BDNF (Fig 6.2) although an *in vivo* growth factor profile has yet to be determined.

LaVail and colleagues (LaVail *et al.*, 1998) have determined the rescue profile of injected growth factors in various mouse models of retinal disease e.g. the P23H, rd, rds and the Q344Ter mouse. While individual trophic factors were found to be

beneficial in many cases, no single factor maintained photoreceptors in all the degenerative models tested (LaVail *et al.*, 1998). Differences have also been noted in the efficacy of different factors in different species, e.g. bFGF appears to be beneficial in rat models but not in mouse models of retinal degeneration (LaVail *et al.*, 1998; Green *et al.*, 2001). However, treatment with a combination of growth factors has the potential to work better than individual factors alone, as demonstrated *in vivo* (LaVail et al 1998) *in vitro* (Ogilvie *et al.*, 2000; Caffé *et al.*, 2001) and is suggested by studies using rod transplants to preserve cones (Mohand-Said *et al.*, 1998, 2000). The rationale for delivering multiple growth factors in physiological doses is a sensible one and Schwann cell transplants may provide that opportunity.

7.3 Relationship of Studies to Treatments for Clinical Disease

7.3.1. RPE transplantation for AMD

The aim of the work presented here is to begin to provide a solid scientific platform from which to develop realistic and suitable treatments for patients with retinal degeneration. Previous clinical studies were built on a less than comprehensive base (Algvere *et al.*, 1994, 1997 & 1999), and if nothing else, pointed to the real problems that need to be addressed before serious clinical application can be contemplated. The present work begins to address the gaps in previous knowledge in this field. It has demonstrated an anatomical benefit of RPE cell line transplants (both human and rat) to the RCS rat and has shown preservation of visual function by simple behaviour (head-tracking) and more complex retinal sensitivity (collicular threshold mapping) methods.

The application of these functional tests has revealed an important feature of allografts in the RCS rat paradigm, namely the deterioration of function over time (chapter 3). Before embarking on clinical trials it will be essential to explore further

the mechanisms that underlie this deterioration, some of which have been discussed in 7.2.1 and 7.2.2. Before clinical studies commence ways must be found of prolonging graft survival in the subretinal space by either immunosuppression or pre-transplant manipulation of the donor cells to down-regulate their MHC II expression or delete co-stimulatory molecules. There are a number of possible areas in the immunological system that could be manipulated (see Fig 7.1)

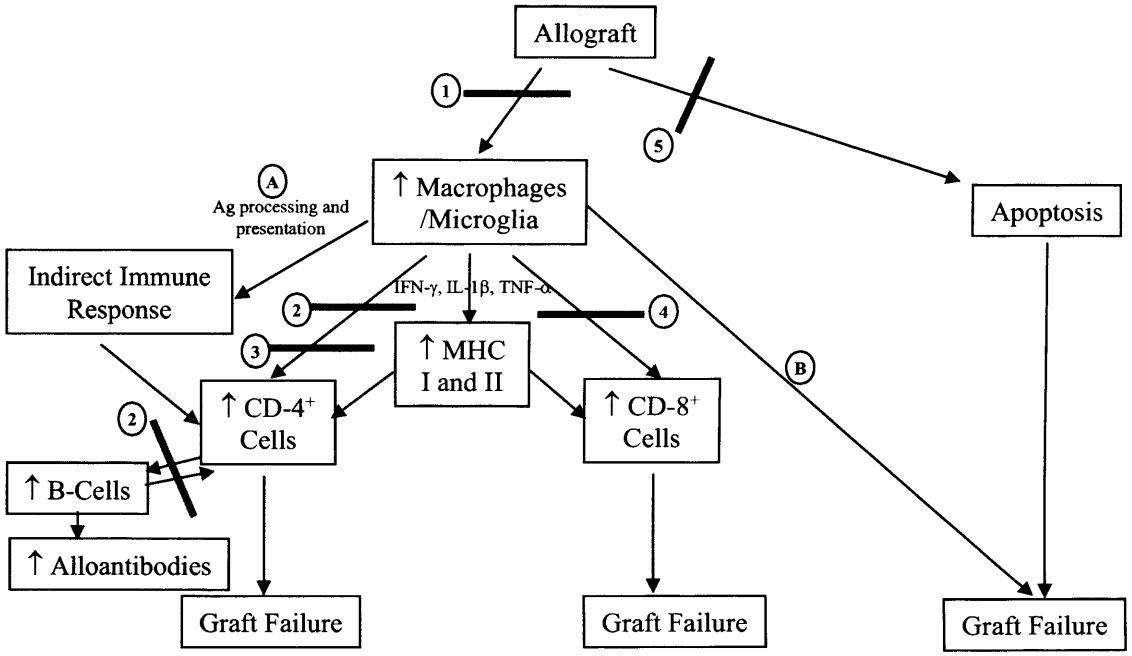
Studies are underway to examine the mechanisms involved in cell loss using depletion strategies. Other transplant paradigms may provide clues to interpreting and improving RPE cell transplantation. Pancreatic islet cell transplantation, a treatment strategy for type I diabetes, involves grafting islet cells rich in β-cells (secreting insulin) to various sites in the abdomen including liver and renal capsule. Early loss of these cells is associated with macrophage infiltration at the graft site similar to that seen here with RPE allografts. Intra-hepatic islet cell graft survival can be significantly improved by depleting host macrophages with dichloromethylene diphosphate (clodronate) liposomes (Bottino *et al.*, 1998). Macrophage depletion may reduce the capacity for antigen presentation thereby suppressing the activation of alloreactive CD4+ T-cells. Clodronate, used in corneal transplant strategies, has a similar effect. Subconjunctival administration of clodronate-loaded liposomes with a high-risk graft leads to long-term survival of the transplants (van der Veen *et al.*, 1994). By depleting macrophages there is a reduction in cytotoxic activity, antibody production (Slegers *et al.*, 2000) and impaired complement-dependant antibody activity (van der Veen *et al.*, 1994) following transplantation.

Reduction of the CD4+ cell population in these transplant strategies has also led to improved transplant survival and indicates the down-stream mechanisms involved. Anti-CD4+ antibodies have been used successfully to prolong allograft survival in rat

Figure 7.1: Potential Sites of Intervention to Limit Graft Cell Loss as a Result of Host Inflammatory or Immune Responses

Figure 7.1

Potential Sites of Intervention to Limit Graft Cell Loss as a Result of Host Inflammatory or Immune Responses



Simplified hypothesis of graft/macrophage interaction and effects **A)** Afferent effects **B)** Efferent effects. Potential interference targets:- **1)** Clodronate **2)** anti CD-40L Ab, **3)** anti-CD-4$^+$ Ab **4)** anti CD-8$^+$ Ab, **3** and **4** anti-CD3$^+$ and **5)** anti-apoptotic agents (e.g. caspase inhibitors).

experimental transplant models including the heart and kidney (Yin *et al.*, 1998) both with and without dual therapy with anti CD8+.

Blocking the CD40-CD40L co-stimulatory pathway has also been shown to prolong graft survival in islet cell transplant paradigms (Kover *et al.*, 2000) and corneal transplants (Qian *et al.*, 2001). By blocking a key co-stimulatory pathway (CD40-CD40-L) which is involved in T-cell activation (CD40 on T-cells and CD40L on antigen-presenting cells, APCs), down-stream upregulation of co-stimulatory (B7-1 and B7-2) is prevented.

Development of an immortalised syngeneic RPE cell line from the congenic RCS rat strain is underway at the Institute of Ophthalmology. This new cell line, together with primary cultures derived from non-dystrophic RCS and PVG rats, will be used in key baseline immunological experiments. The experiments described above should provide essential information about the importance of the alloresponse and whether the immortalisation procedure confers additional immunogenicity. Further studies using the allogeneic cell line (LD7.4) will aim to identify the post-transplantation host immune responses in the subretinal space, local lymph nodes, spleen and humoral system. Using a simplified model of the sequence of events that may follow transplantation (Figure 7.1), strategies are planned that will interfere with the different arms of the immune response. It is envisaged that the results will provide information leading to the identification of those host responses that are responsible for poor graft survival. This will then allow future strategies to be devised that will lead to improved cell graft survival. The ability to genetically manipulate a clonal cell line, which can subsequently be transplanted, will greatly aid these aims.

Grafted human RPE cell lines also rescue photoreceptor function and morphology (Chapter 5) in the dystrophic RCS rat. Using immunosuppression, the donor cells

survive up to five months post-operative. Future work will concentrate on identifying functional proteins at the cellular level (e.g. apical Na^+-K^+ ATPase or CRALBP) allowing the identification *in vivo* of the factors considered essential for the proper functioning of these cells (Bok *et al.*, 1993).

The ability to reliably label donor cells, track them with time and utilise a simple functional outcome (head-tracking) will prove very important tools in experimental RPE transplantation. Once optimum grafting conditions have been determined using BrdU pre-labelled cells, cells labelled with the Human Foamy Virus/GFP will be used again to image the graft longitudinally *in vivo* using confocal SLO. It may be possible to assess the effect on donor cell survival of immunosuppression and its withdrawal, allowing extrapolation to the clinical setting. It may be that immunosuppression is only necessary around the critical time when the blood-retinal barrier is opened at the time of transplant surgery. All these studies will aim to a) identify the mechanisms involved in cell loss and b) develop the therapies that will limit that loss.

7.3.2. Schwann cell transplantation for Retinitis Pigmentosa

Studies have shown the efficacy of Schwann cell transplantation in two disparate animal models of hereditary retinal disease, the RCS rat (Lawrence *et al.*, 2000) and the rhodopsin knockout mouse (chapter 6). The limitations of these models are discussed in 7.4.1 and 7.4.2. The results show transplantation of a cell type that can act as local factory for neurotrophin production can delay retinal degeneration.

The finding that a non-specific treatment, such as Schwann cell transplantation, can work in hereditary retinal dystrophies is very exciting. The potential exists to transplant a patient's own Schwann cells, which can be harvested, for example, from their sural nerve (in the leg), grown and purified in culture and grafted into the

paramacular region to preserve remaining cones. Any potential immune or infective problems should have been overcome.

The issues remaining to be addressed experimentally with this treatment are: i) the application of Schwann cell grafting (transvitreal) to a larger eye (e.g. the cat), this is the approach likely to be adopted for human transplant surgery and ii) assessing the impact of local growth factor release on first to third order neurons in a well-characterised model (Lewis *et al.*, 2003): this work is in progress and preliminary studies are being conducted at the Institute of Ophthalmology. iii) The *in vivo* growth factor profile of donor Schwann cells should be measured using *in situ* hybridisation. iv) Different transplant times should be tested in the rho-/- mouse to determine whether a more optimal intervention time exists. These experiments should provide information about any detrimental effects to the host system of prolonged neurotrophin release, the growth factor expression profile of the grafted Schwann cells through time and the optimum time to intervene surgically in a patient's management.

With microchip technology still a distant prospect it is important to concentrate on methods by which cone function can be maintained in these patients for as long as possible. Only 2-4 rows of cone photoreceptor nuclei are required for functional vision, demonstrated experimentally in Chapters 3 & 5, and posthumously in patients with RP (Sieving, 1999) This level of preservation should be our initial aim in a clinical trial using Schwann cells.

7.4 Relevance of the animal models

7.4.1 Royal College of Surgeons (RCS) rat

The cause and progression of the retinal dystropy in the Royal College of Surgeons rat is described in 1.3.1. It has been used for AMD research because it has a primary

retinal pigment epithelium defect with secondary neural retinal damage. While it is not an ideal model of AMD it is a useful bio-assay for potential treatments of retinal pigment epithelial disease. That is the context in which the RCS rat has been used in this series of studies. Experimental strategies can be analysed knowing that without an intervention the photoreceptors will degenerate within 12 weeks (Dowling and Sidman, 1962). The availability of behavioural and functional tests makes the link between photoreceptor preservation and function accessible. In addition to photoreceptor degeneration, the RCS rat is characterised by secondary vascular changes that result in the loss of ganglion cells (Villegas-Peréz *et al.*, 1996, 1998). Similar changes are observed in patients with retinitis pigmentosa (Milam *et al.*, 1998) and a group of patients has been identified with a mutation in the *Mertk* gene with a retinitis pigmentosa phenotype (Gal *et al.*, 2000). This validates using this model to test therapies for the RP group.

The rat model has been used in numerous transplant studies. Its disdvantages are 1) the small size of the rodent eye and the correspondingly large size of the lens; 2) the lack of a macula; 3) vascular changes relate to the remodelling of the inner retinal circulation, not to changes in the choroidal circulation and 4) the absence of drusen. Another rodent model for AMD is the APO (*) E3-Leiden mouse which develops basal laminar deposit (BLD) similar ultrastructurally to human BLD (Kliffen *et al.*, 2000) but secondary changes such as atrophy and/or CNV have not been demonstrated.

7.4.2. The rhodopsin knockout mouse

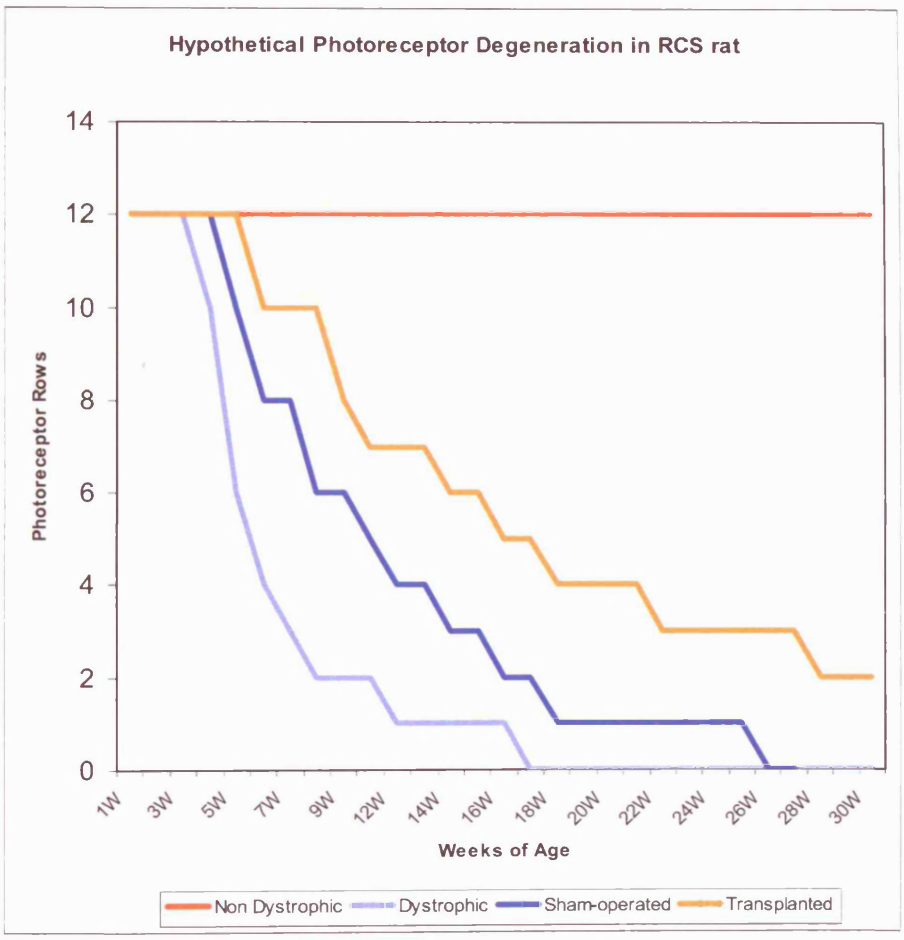
The rhodopsin knockout mouse retinal anatomy and progression of photoreceptor loss has been described in section 1.3.2.

Figure 7.2: Hypothetical Photoreceptor Degeneration in the RCS Rat and the Effect of Transplantation or Sham-surgery

This figure illustrates the rate of photoreceptor degeneration seen in four groups of animals: congenic, dystrophic, sham-operated dystrophic and transplanted dystrophic retinae. The dystrophic RCS rat retina loses photoreceptors rapidly (see section 1.3.1). Sham-operated animals have delayed photoreceptor degeneration but ultimately all photoreceptors are lost (Fig 3.5 and Fig 5.6) by 5 months of age. Transplanted animals display the best preservation of photoreceptors yet there is still a fall off of numbers over time.

Figure 7.2

Hypothetical Photoreceptor Degeneration in the RCS Rat and the Effect of Transplantation or Sham Surgery



Since many of the human forms of retinitis pigmentosa are due to mutations in the rhodopsin gene (e.g. P23H; Daiger RetNet 2004), studies on this rodent model are important and relevant. Although there is no direct genotypic correlation, in rod or rod-cone human RP, rods are also lost first followed by secondary degeneration of the cones. Additionally, this model provides a system whereby interventions designed to protect cones can be examined. The genetic heterogeneity of human RP (in addition to its existence in different races) argues for the development of unifying treatments that tackle the common final pathways in these diseases, such as growth factors (LaVail *et al.*, 1998), Schwann cells (Lawrence *et al.*, 2000 and Chapter 6), retinal prostheses (Rizzo *et al.*, 2001;Margalit *et al* 2002; Humayun *et al.*, 2003) or neural retinal transplants (Kwan *et al.*, 1999).

Apart from the beneficial effects of Schwann cell grafting demonstrated in the rho-/- mouse (Chapter 6), Liang *et al.*, 2001 (using another rho-/- mouse strain, Lem *et al.*, 1999) have demonstrated the potential protective benefit of a gene therapy approach delivering a single growth factor (CNTF) to the retina. Although excellent preservation of photoreceptors was achieved, they were unable to demonstrate ERG recordings in these retinae, in agreement with the findings reported in Chapter 6. Therefore, to preserve retinal function at the electrophysiological level in the rhodopsin knockout mouse, rhodopsin production may have to be introduced by gene therapy such that outer segments are formed, as with peripherin in the rds mouse (Ali *et al.*, 2000).

7.4.3 Other Animal Models

Large animal models for AMD have so far been used to create an RPE deficient environment by debriding the host RPE, leaving Bruch's membrane bare. This has been carried out in rabbits (Lopez *et al.,* 1995; Majji and de Juan, 2000), pigs (Del

Priore *et al.*, 1995b, 1996), cats (Leonard *et al.*, 1997) and monkeys (Valentino *et al.*, 1995). These models are appropriate for studying the efficacy of RPE transplants in geographic atrophy. The studies lasted for about 8 weeks which was probably of too short a duration to draw significant conclusions. What they did demonstrate was the ability of various cell types to attach to host Bruch's membrane and to support overlying photoreceptors, preventing or diminishing underlying choriocapillaris atrophy (Majji & de Juan, 2000). Other strategies for generating large animal models of AMD involve the creation of a choroidal neovascular membrane, usually with argon laser, in monkeys (Obana *et al.*, 2000) and rats (Frank *et al.*, 1989; Kvanta *et al.*, 2000). While these techniques did generate a CNV membrane as occurs in AMD, the nature of the membrane was quite distinct from many seen in true AMD. Laser-induced CNV are typically type II membranes (i.e. subretinal) unlike the majority of CNV in AMD which are type I classic or occult (diffuse) and are sub-RPE (see Fig 1.9). The importance of this distinction is seen when the membranes are removed during submacular surgery. Removal of subretinal membranes tends to be beneficial since an intact RPE is left behind, explaining the success of CNV removal in some of these patients (12%: Thomas *et al.*, 1994). However, with sub-RPE membranes the RPE is removed and Bruch's membrane is damaged, which compromises the ability of RPE to attach (Tezel and Del Priore, 1999 or Tezel *et al.*, 1999). A true model of AMD would couple a compromised RPE layer with an impermeable Bruch's, CNV (Type I and II) and/or geographic atrophy.

Aged monkeys have been identified with naturally occurring drusen (Hope *et al.*, 1992; Olin *et al.*, 1995) and other retinal changes. These monkeys demonstrate many of the clinical, angiographic, functional and histological features consistent with human age-related macular degeneration (Dawson *et al.*, 1989; Hope *et al.*, 1992;

Monaco *et al.*, 1990) though the cohort studied did not demonstrate CNV. These monkeys are the closest animal model that exists to the human form of AMD yet they still do not display all the features found in humans. No transplant studies have been performed on these animals and if they were to be studied, a long-term project, up to five years, would be necessary to determine efficacy.

7.4.4. What is a good model for AMD?

An ideal model of AMD would accurately recreate the slow progressive nature of the disease by gradually destroying the RPE. However, for developing experimental therapies the necessary time course is probably not realistic. Furthermore, the classification of AMD lies largely with the terminal pathology. Some genetic linkages have been identified but these probably reflect small subsets, unlike the RP spectrum where the majority are inherited. Analogous models are acceptable for transplantation studies provided the limitations of the models are respected. Developing good models beyond those currently available clearly deserve further attention. There are two situations in which this should be pursued: firstly, a large animal model in which a type 1 neovascular membrane has been induced. This would permit the removal of the membrane as in humans, and the placement of RPE cells on damaged Bruch's membrane. *In vitro* studies by Del Priore's group suggest that fresh RPE will not attach to outer layers of Bruch's membrane and undergo apoptosis within 48 hours (Tezel *et al.*, 1999). Studies on this type of model would help greatly to predict the behaviour of RPE cell grafts *in vivo*. Leonard *et al.*, (1997) assessed the effect of RPE debridement *in vivo* using two systems, hydraulic and abrasive removal of RPE. The former was associated with regrowth of RPE and restoration of normal anatomy, whereas abrasive removal was associated with poorer recovery, ultrastructurally seen to be due to Bruch's membrane damage and incomplete RPE recovery. Use of the

abrasive technique would enable us to test the ability of transplanted RPE cell lines to survive on damaged host Bruch's membrane and to support overlying photoreceptors, preventing underlying choriocapillaris atrophy. While debridement models are attainable in a large animal eye the exact relevance to AMD is unclear since AMD is characterised by many other functional and histopathological changes (Green and Enger 1993) in adjacent structures such as Bruch's membrane and choriocapillaris. Secondly, the development of a small animal model, such as a mouse or rat, would be beneficial for larger scale studies assessing functional as well as morphological outcome. Methods are currently being developed to deliver patches and suspensions of RPE trans-vitreally to the rodent subretinal space instead of trans-sclerally. This different surgical approach (which may be used in humans) could have a bearing on the immunological response. Trans-scleral allografts (Jiang *et al.*, 1994) could be detected by conjunctival dendritic cells and foreign antigen presented to host T–cells, triggering an immune-mediated destruction of the tissue (van Buskirk *et al.*, 1997).

With the absence of valid animal models for AMD, in particular large animal models, one might argue that it is reasonable to graft into humans now. In initial transplant studies, patients could be used who are not suitable for other available treatments, such as PDT, where the potential, however small, exists to preserve some remaining visual function. These pilot studies would have the primary aim of examining graft survival and host responses.

7.5 Models for Hereditary Retinal Degeneration

As the genotypes for RP are widely known, with many more being discovered almost on a monthly basis (Daiger RetNet 2004), the number of genetically specific models for RP is great. The range of species is broad, as are the loci of disruption, and they have been reviewed extensively (Hafezi et al., 2000; Li *et al.*, 2001). These models

(see 1.3.3.2) provide great opportunities to examine a range of therapies for retinal degeneration and basic mechanisms that underlie these processes. Research into various therapies has been discussed in the Introduction (Section 1.5). While it is exciting that numerous specific gene based therapies have been applied (Ali *et al.*, 2000; Vollrath *et al.*, 2001; Acland *et al.*, 2001) it is advisable to research more broad-based strategies to tackle degeneration globally, thus making them clinically viable, such as using anti-apoptotic agents (e.g. Caspase-3 inhibitors, Bode and Wolfrom 2003), growth factors or Schwann cell grafts.

The Abyssinian cat is a potentially powerful model in pre-clinical studies for potential therapies. The normal cat retina has been well characterised as has its response to experimental retinal detachment (Lewis *et al.*, 2003). Unlike the rabbit, the cat retinal vasculature is similar to humans and is a more viable model. Chong *et al.*, (1999) have described the beneficial effects of multiple intra-vitreal injections of recombinant human CNTF (Axokine™). With pressures to develop clinical therapies this model is an elegant platform to test other broad-based therapies such as Schwann cell transplantation and studies are underway in normal cats.

The Briard dog is another exciting model and recently Bennett and colleagues have demonstrated preservation of retinal function in one of these dogs using gene therapy (Acland *et al.*, 2001). Four years since the initial therapy the animal still portrays goods retinal function electrophysiologically (personal communication).

The transgenic pig (Petters *et al.*, 1997) with an autosomal dominant rhodopsin mutation (Pro347Leu) substitution will prove very useful in transplantation studies as theoretically these pigs could be crossed with pigs with specific immunological genotypes thus permitting detailed immunological studies.

The wide range of animal models for hereditary retinal disease will be valuable tools for investigating potential therapies in pre-clinical and parallel studies.

7.6 Further Requirements for Clinical Trials

The weight of evidence is beginning to suggest that large-scale clinical trials using cell transplant approaches will be feasible within the next 3-5 years. The efficacy of RPE cell lines and Schwann cells, demonstrated in small animal models of retinal degeneration, is encouraging. However, before embarking on clinical trials many other questions need to be considered in relation to both RPE cell transplantation and Schwann cell transplantation. Transplantation of human RPE cell lines to the immunosuppressed RCS rat preserves visual function and morphology (Chapter 5). The allogeneic cell line, LD7.4, also preserved visual function in the non-immunosuppressed RCS rat yet the cells did not survive for a protracted period of time. This observation raises some immediate issues: the validity of this animal model in these studies, the ability of grafted allogeneic cells to survive and the type of response (immune and inflammatory) stimulated by placing grafts in the rodent subretinal space.

While the RCS rat is used as a model of photoreceptor degeneration secondary to RPE dysfunction it is not directly analogous to AMD. The ability of donor cells to preserve photoreceptors in this model, even after their apparent disappearance, poses a problem of interpretation. Do the cells act directly by taking over the necessary functions of RPE, such as phagocytosis and phototransduction? Do they act by producing growth factors (e.g. bFGF) thus maintaining the photoreceptors? And if so is that sufficient to maintain long term efficacy?

They may stimulate activation of resident tissue resident microglia, which in turn take on the phagocytic role (Ng and Streilein, 2001), preventing the build up of toxic

debris. Grafting allogeneic RPE cells may just shift the degeneration curve and not prevent the inevitable photoreceptor loss, as hypothesised in Figure 7.2. The RCS rat is subject to secondary degenerative events including early compromise of the blood-retinal barrier which may negate any immune privilege that could exist in the normal subretinal space. This still has to be investigated fully.

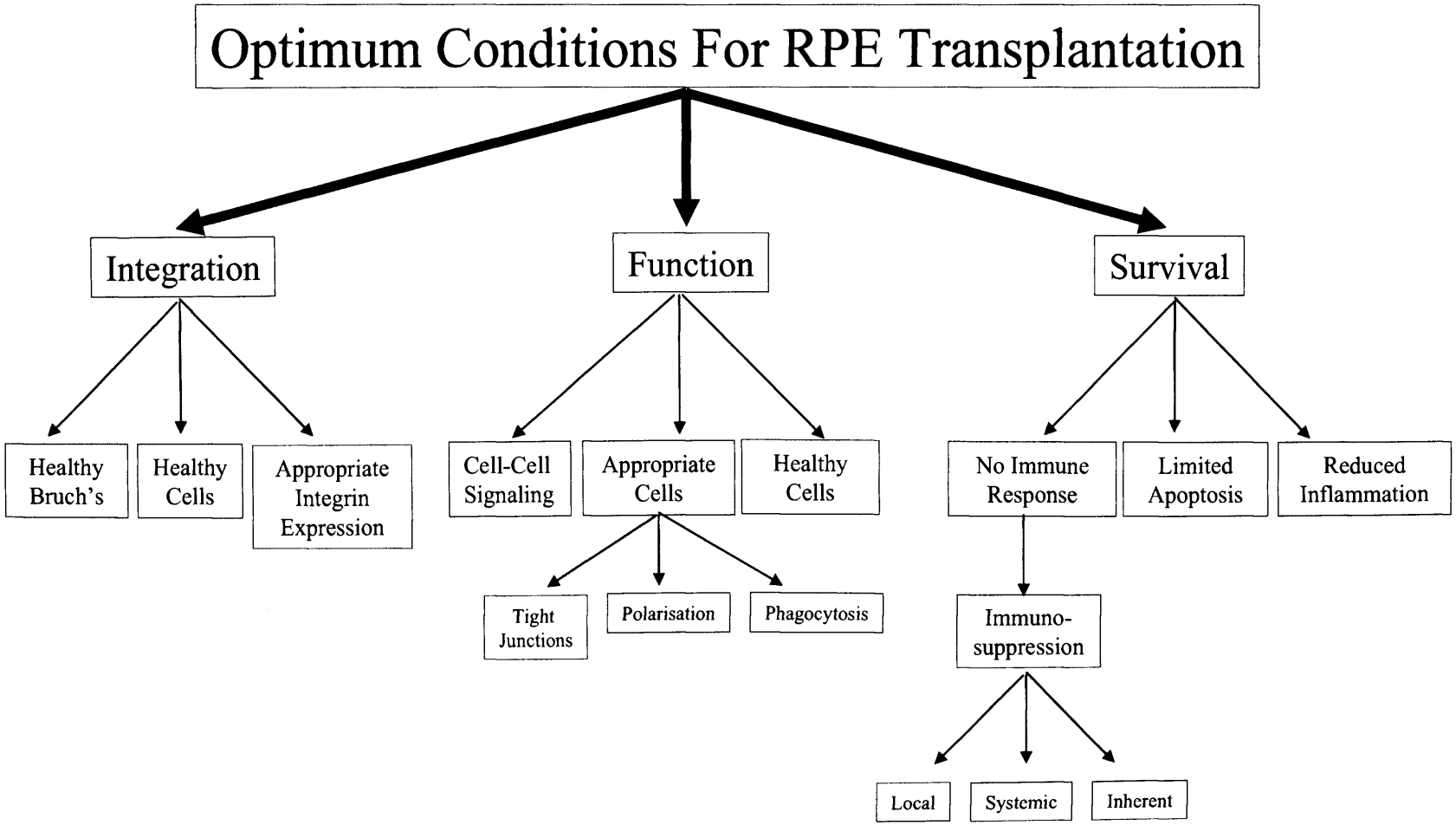
Grafts may fail due to inflammatory and immunological consequences following RPE cell transplantation. In Chapter 4, the influx of ED-1 expressing macrophages and the activation of resident microglia, are significant secondary events accompanying cell transplantation. Modification of these factors as outlined in 7.3.1 and Fig 7.1 will lead to a greater understanding of the mechanisms involved in host responses to RPE grafts. Preliminary studies indicate no benefit in using cyclosporine treatment to manage these alloresponses, unlike the findings with xenografts (Chapter 5) and this is consistent with the precedent literature (Craaford *et al.*, 1999; Craaford *et al.*, 2000). It is clear that the response to subretinal allografting is atypical (Jiang *et al.*, 1994, Chapters 3 and 4) and likely to be macrophage and microglia dependant. The rationale for not using cyclosporine in the allograft studies was to determine the timetable of events and to begin to understand the basic mechanisms involved. Once these have been determined, specific therapies may be utilised or developed (e.g. the use of anti-macrophage agents such as clodronate).

Having identified the loss of cells following transplantation, candidate processes must be investigated. Del Priore's group has published widely on this subject specifically examining apoptosis *in vitro* (Tezel *et al.*, 1999) whilst others studied the apoptotic events *in vivo* (Wang *et al.*, 2001). Failure of grafted RPE cells to attach to Bruch's membrane within 48 hours results in their apoptosis, a process known as anoikis. To

Figure 7.3: Optimum Conditions for RPE Transplantation

There are a number of key problems relating to RPE transplantation that must be addressed. Grafted cells must be able to attach and integrate on Bruch's membrane and for this the membrane must be minimally compromised and appropriate integrins must be expressed (see Chapter 1). Once integrated, the cells must perform all the necessary functions of the RPE cell, such as restoring the integrity of the blood-retinal barrier through cell contact and tight junction formation, phagocytosis and retinal metabolism. Finally, the cells must survive in the host subretinal space and resist both innate and specific immune responses and apoptosis. These processes may be controlled with anti-inflammatory agents, immunosuppressants and anti-apoptotic agents.

Figure 7.3


Optimum Conditions For RPE Transplantation
Integration
Function
Survival
Healthy Bruch's
Healthy Cells
Appropriate Integrin Expression
Cell-Cell Signaling
Appropriate Cells
Healthy Cells
Tight Junctions
Polarisation
Phagocytosis
No Immune Response
Limited Apoptosis
Reduced Inflammation
Immuno-suppression
Local
Systemic
Inherent

ensure maximal graft survival this process must be modified or larger areas of viable substrate must be made available. The process itself may be modified by further genetic manipulation of the cells by inserting anti-apoptotic genes (e.g. bcl-2: Chen *et al.,* 1996). The cells could be grafted already attached to artificial membranes (eg. hydrogel, Singh *et al.*, 2001; poly (DL-lactic-co-glycolic acid) film Lu *et al.*, 1998; Sheridan *et al* 2004), and biological (e.g. amniotic) membrane (Capeans *et al.*, 2003) or anterior lens capsule (Kiilgaard *et al.*, 2002). This may have two benefits: 1) a reduced number of cells would have to be grafted (c.f. suspensions) thus reducing the antigenic load and 2) the cells could be inserted already polarised on a pre-formed substrate.

Considering retinal cell transplantation, specifically RPE transplantation, it is clear that many factors may compromise graft survival. Optimum conditions for graft survival have been outlined schematically in Fig 7.3.

7.7 Limiting Secondary Retinal Effects

Other cellular and vascular changes occur in the degenerating retina (see Introduction). These processes will have to be considered when devising therapies, particularly the timing of transplantation. Grafts need to be inserted prior to the onset of major photoreceptor loss. Once photoreceptors are lost inner retinal remodelling begins and normal retinal connectivity is disrupted. Donor cells are more likely to maintain normal synaptic transmission and useful vision if transplanted at an early stage of the disease. If this is to be the case then the surgeon must be sure that the treatment will be beneficial.

7.8 Demonstrating Real Functional Vision

Data presented here, and in publications arising from this work, uniquely show preservation of a high order of visual function (Lund *et al.*, 2001a, Coffey *et al.*,

2002a). It is of tremendous importance to clinicians, who may consider performing transplants on patients in the future, that these studies demonstrate more than just morphological preservation. Work published from the our group has demonstrated preservation of cortical vision at the single neuron level (Coffey *et al.*, 2002a). In addition, our collaborator, Dr. P.Coffey has recently used cortical optical imaging on RCS rats that had received RPE transplants and shown preservation of global cortical function (Gias *et al.*, 2002). This technique provides more evidence of downstream transplant efficacy in this animal model and could be combined with ERG testing to give a retinal and a cortical image in the same animal. It could also be applied to large animal models such as the cat.

7.9 Previous clinical studies and their shortcomings

The design of a clinical trial for transplantation is very important. Patient selection, study goals and study outcome measures are key to the ultimate success of these therapies. Previous studies on RPE transplants to patients have not been successful. No patients in the published studies have shown improved vision following transplantation, but these were not clinical trails. They were exploratory operations testing safety. The first series of patients received grafts following removal of CNV membranes (Algvere *et al.*, 1994, 1997). Having initially done well, their visual acuity deteriorated accompanied by subretinal fibrosis and retinal oedema. These changes were attributed to graft rejection, but no immunological studies were undertaken in this group. The conclusion was based on earlier monkey studies that examined cellular infiltration in the retinae following grafting and comparing them with fluorescein angiography (Sheng *et al.*, 1995). Rejection was defined as fragmentation, disruption or disappearance of the graft in association with inflammatory cells. The appearance of oedema is confusing, since rejection and loss

of RPE should lead to overlying retinal atrophy, not oedema. Oedema would suggest that a barrier to bulk fluid outflow exists in these patients. The patients were screened as follows: pre- and postoperative colour photos with fluorescein angiography, and retinal function was assessed with a scanning laser ophthalmoscope (SLO) and microperimetry. In another transplant study a patient with geographic atrophy (Weisz *et al.*, 1999) was assessed very thoroughly before and after surgery by visual acuity testing (ETDRS chart), fundus photography, fluorescein angiography, SLO microperimetry and reading rate. The follow-up also included humoral immune response against inter-photoreceptor binding protein (IRBP), S-antigen and whole RPE and cellular immunity was tested against IRBP, S-antigen, phosducin, rhodopsin, mixed retinal antigens and bovine RPE microsomal proteins. Again, there appeared to be no benefit to the patient, the graft site was associated with angiographic leakage (consistent with vasculitis) and was interpreted by the group as evidence of rejection, in spite of the fact that the panel of tests above was either totally normal or near normal.

7.10 Aims of future studies

7.10.1 Introduction

Future clinical trials will have to incorporate the type of pre- and post-surgery assessment used by Weisz *et al.*, 1999, detailed in figure 7.4, and including additional tests such as contrast sensitivity testing, reading vision, static perimetry, multi-focal ERG recording (Hood, 2000), autofluorescence imaging (von Ruckmann *et al.*, 1999), optical coherence tomography imaging and quality of life assessment. Trials will also depend on accurate phenotyping (including natural history) and in cases of retinal dystrophies, genotyping of the disease. Trials that are ongoing at Moorfields Eye Hospital are seeking to develop a database of such AMD patients (Scholl *et al.*,

2003); there is already a database of patients with hereditary dystrophies. These patients would provide a valuable resource in the design of new studies because not only are they needed for the therapeutic group but also for suitable control groups (both interventional and non-interventional). A failing of the previous transplant studies has been the absence of suitable control groups.

7.10.2. Choice of patients for AMD trials and the conduct of the trials

In designing suitable trials a stepwise progression will be the most sensible course. Initial studies should aim to demonstrate the viability of RPE cells in the subretinal space of patients with AMD (for RPE). Once graft viability has been proven in patients with advanced AMD then trials may be designed with the aim of preserving and improving visual function in patients with moderate to severe disease. Finally, a randomised control trial involving established therapies, such as argon laser and PDT in AMD could be incorporated and compared with the experimental procedure (Fig 7.4).

Initial studies should concentrate on restoring RPE to Bruch's membrane following removal of a neovascular membrane (Type 1). The role of autofluorescence imaging and OCT is paramount in post-operative assessment of these patients. Follow-up trials would concentrate on preservation of vision and assessment would be based on acuity and contrast sensitivity testing together with more sophisticated assessments such as microperimetry and/or static macular perimetry. The second tier of studies would involve patients with high-risk second eyes (MPS Group, 1997) since there is a risk of progression to debilitating disease in this group. This particular cohort could be used in a longer-term study (over five years) to examine the longitudinal benefit of preventing progression.

Figure 7.4: Proposed Design for RPE Transplantation Trial for AMD

This figure gives an outline of the trial stages. Trial stage I would recruit a group of patients with advanced age-related macular degeneration. This would primarily assess safety and immunological responses but graft viability and retinal integrity over the graft would be examined using fluorescein angiography (FFA), autofluorescent imaging and optical coherence tomography (OCT) with microperimetry. Later studies (stage II) would involve patients with moderate visual loss and would primarily examine the effect of the graft on visual function. In addition to the tests outlined above, patients would undergo visual acuity, contrast sensitivity, microperimetric and multifocal electroretinographic (mfERG) testing. The stage III trials would hopefully be built on positive results from stage I and II, and would be a comparative randomised, controlled trial against natural history and/or photodynamic therapy (PDT).

Figure 7.4

Proposed Trial Design for RPE Transplantation in AMD

Stage I

Patient Group:-	Cohort of patients with advanced late-stage AMD (Neovascular and Geographic Atrophy) and non-interventional controls
Cohort Size:-	n= 10 in each group
Outcome:-	a) Immunological Response (Cellular & Humoural) b) Viability of graft in each setting Assessed by Fundus Fluorescein Angiogram (FFA), Autofluorescence (AF) and Optical Coherence Tomography (OCT)

Stage II

Patient Group:-	Cohort of patients with moderate-severe late-stage AMD (Neovascular and Geographic Atrophy)
Cohort Size:-	n= 10 in each group
Outcome:-	a) Immunological Response (Cellular & Humoral) b) Viability of graft in each setting (FFA, AF, OCT) c) Visual improvement (Acuity, Contrast Sensitivity (CS), Microperimetry and Multifocal Electro-retinogram (mERG))

Stage III

Patient Group:-	Cohort of patients with mild-moderate late-stage AMD (Neovascular and Geographic Atrophy) in 4 groups (Non-interventional control; PDT, Argon laser, Transplant)
Cohort Size:-	n= 50 in each group
Outcome:-	a) Immunological Response (Cellular & Humoural) b) Viability of graft in each setting Assessed by FFA, AF, OCT c) Visual improvement (Acuity, CS, microperimetry, mERG)

7.10.3 Choice of patients for inherited retinal dystropy trials and conduct of trials

A similar stepwise approach would be adopted for these patients with initial studies to assess the viability of Schwann cells in the SRS. Patient selection is very important since choosing the wrong patient cohort initially could jeopardise any trial, skew results and disguise a specific benefit. As with trials in AMD, investigators must be sure of the study goals. Initial aims should be the preservation of residual cone structure and function, with prolongation of useful vision for 5 years considered a successful outcome. Since studies will be on patients with hereditary dystrophies the fellow eye could serve as the control.

Two approaches would be valid: a) to treat patients whose inherited degenerative disease had an equivalent animal model e.g. a P23H mutation using methods that had been tested on those animals or b) treat patients with a specific disease characterised by a rapid, reliable and symmetrical deterioration in cone function after rod loss such as X-linked RP. A potential first trial would recruit severely affected heterozygotes (X-linked RP) to determine whether central cones could be preserved. If a beneficial effect can be demonstrated these patients' younger relatives with manifest disease may be enrolled in to a second level study to examine the long-term benefit of the same treatment but with earlier intervention.

Post-operative assessment in these patients would concentrate on macular function and should include acuity and contrast sensitivity testing and electrophysiological assessment including pattern ERG (pERG), multifocal ERG and static macular perimetry.

7.11 The Holy Grail

The aim is to build on the studies presented in this thesis, and others presented elsewhere, to provide a treatment or a range of treatment options for patients with

retinal disease, specifically age-related macular degeneration and retinitis pigmentosa. It is important that the momentum is not lost: there are millions of patients world-wide affected by retinal disease desperate for treatments, and the public health and economic benefits for a viable treatment would be great. To be able, in 10 years time, to see a patient in clinic with age-related macular disease or retinitis pigmentosa and list them for a surgical procedure, much as is done now for corneal transplantation, and confidently tell them that they will benefit from the intervention would be a fantastic achievement.

PUBLICATIONS ARISING FROM THIS WORK

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APPENDIX I

BUFFERS

Phosphate buffered saline (PBS)

Stock solution 10x working dilution.

For 1 litre:

900ml distilled water

2g Potassium Chloride

2g Potassium dihydrogen phosphate (KH_2PO_4)

80g Sodium Chloride

11.4g Di-sodium hydrogen phosphate (Na_2HPO_4).

Make up to 1l with distilled water. pH 7.3.

Gomori Phosphate Buffer (0.5M)

For 1 litre:

900 ml distilled water

30g sodium dihydrogen phosphate (NaH_2PO_4)

142g di-sodium hydrogen phosphate (Na_2HPO_4)

Make up to 1l with distilled water. pH 7.4.

Phosphate buffers for electron microscopy

For 1 litre, 0.2M:

Solution A: 31.2g sodium dihydrogen phosphate $2H_20$

Solution B: 28.4g di-sodium hydrogen phosphate

To make 100ml 0.2M phosphate buffer at ph 7.4, mix 23ml solution A with 77ml solution B.

Sodium cacodylate buffer for electron nicroscopy

For 100ml of 0.1M buffer, dissolve 2.14g sodium cacodylate in 100mls. distilled water. pH to 7.4 with 1 or 2 drops of concentrated hydrochloric acid.

FIXATIVES

4% Paraformaldehyde in phosphate buffered saline

For 1 litre:

40g paraformaldehyde (TAAB, Aldermaston, UK)

800ml distilled water

40 drops 1N sodium hydroxide

Heat to 60°C (but no higher, or paraformaldehyde will dissociate).

Add 100 ml stock PBS (10x concentration).

Make up to 1 litre with distilled water.

pH 7.2-7.4. Filter before use.

Paraformaldehyde-lysine-periodate fixative (PLP)

For 1 litre:

Solution A: 13.7g lysine monohydrochloride in 375ml distilled water.

Solution B: 1.4g disodium hydrogen phosphate (anhydrous) in 100ml distilled water.

Solution C: 20g paraformadehyde in 200ml distilled water. Add 2 drops 1N sodium hydroxide and heat to 60°C. When dissolved allow to cool.

Mix A with B and then add C. pH to 6.8.

Make up to 1l with Gomori's buffer which has been diluted to 0.25M.

Add 2.14g sodium periodate solution just before use. Filter before use.

Fixative for electron microscopy

For 1 litre:

25g paraformaldehyde in 300 ml distilled water

25 drops 1N NaOH. Heat to 60°C to dissolve.

When cool add 100mls 25% glutaraldehyde (EM grade).

Make up to 500ml with distilled water.

Add 500ml of 0.2M phosphate buffer (see buffer for electron microscopy).

Check pH (7.4) and filter.

Just before use add 10mls 1% picric acid solution per litre.

HISTOLOGICAL STAINS

Cresyl violet, Nissl counterstain.

0.5% cresyl violet	2mins
Distilled water	2 x 2mins
70% alcohol	2mins
95% alcohol/acetic acid	A few dips
(95mls 95% alcohol and 5ml acetic acid)	
95% alcohol	2mins
100% alcohol	2 x 2mins
Xylene	2 x 5mins

Mount in DPX

Haematoxylin/Eosin

1. Wash sections in tap water.
2. Stain in Mayer's haematoxylin, 2-4mins.
3. Wash in tap water until blue.
4. Differentiate with acid alcohol (95% alcohol, plus 1% hydrochloric acid).
5. Wash in tap water until blue.
6. Counterstain in 0.25% aqueous Eosin yellowish for about 1min. NB. Eosin is water soluble.
7. Dehydrate through 70%, 80%, 95% and absolute alcohol.
8. Clear in xylene.
9. Mount in DPX.

PROTOCOL FOR EMBEDDING TISSUE IN POLYESTER WAX

1. Fix in PLP or 4% paraformaldehyde by immersion or perfusion. Mark dorsal pole with a suture.

2. Wash 2x in PBS over 30 mins.

3. 50% alcohol for 1h.

4. 70% alcohol for 1h.

5. 80% alcohol for 1h.

6. 95% alcohol for 1h. Remove lens.

7. 95% alcohol for 1h.

8. 95% alcohol: polyester wax, 2:1 for 1h at approximately 39°C.

9. 95% alcohol: polyester wax, 1:1 for 1h.

10. Neat wax for 1h.

11. Fresh wax overnight. Make sure tissue sinks.

12. Embed in fresh wax. Cool and store blocks at 4 °C.

PROTOCOL FOR IMMUNOHISTOCHEMISTRY USING DAB AS A CHROMAGEN

Bring cryostat sections to room temperature and dry in a current of cool air for 1h. Dewax polyester sections by dipping briefly in absolute alcohol and 95% alcohol for 10mins. Dry in a current of air for 30mins.

1. For all sections: Block in 5% defatted milk (unless using Jackson secondary antibodies) or 5% serum (appropriate to the animal in which the secondary antibody was raised) in PBS.

2. Drain sections and cover with primary antibody diluted to the required concentration in antibody diluent (1% bovine serum albumen, Sigma, in PBS). NB. Do not use bovine serum albumen if using Jackson secondary antibodies. Place in a humidified chamber overnight at 4°C.

3. Wash 3x 5mins in PBS.

4. Incubate sections in appropriate biotinylated secondary antibody, diluted in antibody diluent or PBS 1:150. Add 1% rat serum. Incubate in humidified chamber for 30mins at room temperature.

5. Wash 3x10mins PBS. Make up Streptavidin/ horseradish peroxidase (HRP) complex by mixing 1drop of solution A with 1drop of solution B in 2.5mls PBS. Allow to stand for 30mins before use.

6. Cover sections with streptavidin complex. Place in humidified chamber for 30mins at room temperature.

7. Wash 3x 10mins in PBS.

8. Place sections in filtered 1% nickel chloride in distilled water for 5mins.

9. Transfer sections directly to filtered DAB solution (50mg/100ml DAB buffer activated with 10µl hydrogen peroxide.

10. Wash 3x 10mins in PBS.

11. Counterstain if necessary, dehydrate through graded alcohols, xylene and mount in DPX.

DAB Buffer:

80g sodium dihydrogen phosphate. $2H_2O$

13.5g imidazole

Make up to 500ml with distilled water. Use at 1+9 for DAB. pH should be 5.8 (optimal for DAB reaction).

BrdU VISUALISATION USING VECTOR BLUE CHROMAGEN

1. If slides have been stored at -70°C, bring to room temperature
2. Dry slides in a current of cool air for 1h.
3. Distilled water (DW) 1 x 5min.
4. Tris Buffered Saline (TBS) pH 7.6, 2 x 5 min.

TBS recipe: 0.05M Tris/HCl (7.88g/l) + 0.15M NaCl (8.34g/l), pH to 7.6 with NaOH.

5. 2NHCL + 0.5% Triton for 30-35min. at 37°C. (2ml. Conc HCl + 10ml DW+ 50µl Triton).
6. Borax (0.1M di-sodium tetraborate, 38.138g/l) for 10min at RT.
7. TBS pH 7.6, 3 x 5min.
8. Block with 5% goat serum in TBS, pH 7.6 (no BSA) + 0.05% Tween for 30min at RT.
9. BrdU antibody, monoclonal (1:1000, Sigma) in TBS, pH 7.6 (no BSA) + 2% rat serum + 0.05% Tween overnight at 4°C.
10. TBS pH 7.6, 3 x 5min.
11. Secondary antibody: Biotinylated preadsorbed goat anti-mouse IgG (Jackson ImmunoResearch). 1:200 in TBS pH 7.6 (no BSA) + 2% rat serum for 30 min at room temperature (RT). Make up Streptavidin/alkaline phosphatase complex using TBS pH 7. 6, no BSA, 30mins before use.
12. TBS pH 7.6, 3 x 5min.

13. Streptavidin/AP for 30 min at RT.

14. TBS pH7.6, 2 x 5min

15. Tris buffer, pH 8.2, no salt. 1 x 5min.

TB pH 8.2, 0.1M recipe: TrisHCL 7.08g/l + Tris Base 6.68g/l.

16. Vector blue (Vector Labs), make up as specified in kit using Tris buffer, pH 8.2 + 5drops levamisole + 0.1% Tween, in the dark at RT, approx. 10mins. Monitor microscopically.

17. Wash in TBS, pH 8.2, 1 x 5 min.

18. Dehydrate through graded alcohols, clear in Neoclear.

19. Mount in Vectamount.

PROCESSING TISSUE FOR ELECTRON MICROSCOPY

1. Fix tissue in 2.5% paraformaldehyde and 2.5% gluraraldehyde in 0.1M phosphate buffer, pH7.2-7.4.

2. Wash in 0.1M sodium cacodylate buffer with agitation for 20 mins.

3. Postfix in 1% aqueous osmium tetroxide for 2h.

4. Rinse in distilled water with agitation for 20 mins.

5. Dehydrate through ascending alcohols, 50%, 70% and 90%, 2x10min each.

100% ethanol, 3x10min with agitation.

6. Epoxypropane, 3x10min each.

7. Equal parts epoxypropane and Araldite epoxy resin (Taab Laboratories) with agitation for at least 2h or overnight.

8. Araldite resin with agitation for at least 2h or overnight.

9. Embed in fresh Araldite and polymerise at 60°C for at least 12h.

10. Allow to cool before cutting.

Araldite epoxy resin mixture: CY212 25ml

DDSA 20ml

DMP 30 0.8-0.9ml. Stir mixture thoroughly.

Stains for Electron Microscopy:

Alcoholic Uranyl Acetate: Dissolve 0.3g uranyl acetate in 10mls methanol.

Reynold's Lead Citrate: Dissolve 1.33g lead nitrate in 30ml distilled water. Add 1.76g sodium citrate $2H_2O$. Shake at intervals over 30mins. Add 8ml 1N NaOH. Add 12ml distilled water. Store in sealed syringes (away from air/CO_2) at 4°C. Sections stained for 5mins in each stain.

NEUROTACS™ II: *IN SITU* APOPTOSIS DETECTION KIT

(R&D Systems Europe, Abingdon, UK).

SOLUTIONS REQUIRED

10x Phosphate buffered saline (PBS):

Sodium dihydrogen phosphate	3.45g
Di-sodium hydrogen phosphate	10.65g
Sodium chloride	86.19g

Dissolve in 800ml DNase-free water and make up to 1litre.

1x PBS:

10ml 10xPBS in 450ml DNase-free water. Check pH 7.4.

NeuroPore solution provided in kit.

Quenching solution:

30% hydrogen peroxide	5ml
Methanol	45ml

Prepare just before use. Ensure that hydrogen peroxide solution is no more than 2 months old.

1x TdT labelling buffer:

10x labelling buffer	5ml
DNase-free water	45ml

Keep on ice.

Labelling reaction mix:

TdT dNTP mix	1µl
TdT enzyme	1µl
50x Mn^2	1µl

1x TdT labelling buffer	50µl

Prepare by thawing TdT dNTP and the 50x Mn^2 at room temperature, and then place on ice. Remove TdT from freezer only long enough to remove required volume (to maintain enzyme reactivity). Prepare labelling reaction mix just before use and keep on ice.

1xTdT stop buffer:

10x TdT stop buffer	5ml
DNase-free water	45ml

Streptavidin-HRP working solution:

Streptavidin-HRP	1µl
1xPBS	50µl

Store on ice until used.

DAB working solution:

30% hydrogen peroxide	50µl
DAB	250µl
1x PBS	50ml.

Prepare no more than 30mins before use. Thaw DAB at 37°C and keep at room temperature.

TACS-Nuclease working solution:

TACS-nuclease	1µl
TACS-nuclease buffer	50µl

Prepare just before use and keep on ice.

Blue counterstain:

Provided in kit. Use ammonium water to blue the counterstain.

Ammonium water:

30% ammonium hydroxide solution	0.25ml
Tap water	500ml.

Prepare just before use.

METHOD FOR LABELLING FIXED FROZEN SECTIONS

1. Remove sections from freezer and bring to room temperature. Dry under a current of cool air for at least one hour or overnight.

2. Immerse slides in 1x PBS for 10 mins at room temperature (RT). Dry glass around sample.

3. Pipette 50µl NeuroPore onto each slide and cover with a coverslip. Incubate in an humidity chamber for 15-30mins at RT.

4. Wash 2x 2mins in DNase-free water.

5. Prepare a nuclease-generated positive control: pipette 50µl TACS-nuclease working solution onto one of the prepared slides. Cover with a coverslip and incubate for 10-60mins in an humidity chamber. Wash 2x 2mins in DNase-free water. Keep remaining slides in DNase-free water.

6. Immerse slides in quenching solution for 5mins at RT.

7. Wash with1x PBS for 1min at RT.

8. Immerse in 1x TdT buffer for 5mins at RT. Dry around sections but it is essential that sections themselves do not dry otherwise there is a risk of staining artifacts.

9. Add 50µl Labelling Reaction Mix to each sample. Cover with a coverslip. For an unlabelled control, cover one sample with labelling mix that lacks TdT. (ie. 1µl TdT dNTP mix in 50µl labelling buffer). Incubate for 1h at 37°C in an humidity chamber.

10. Stop the reaction with 1x TdT stop buffer for 5mins at RT.

11. Wash 2x 2mins in 1x PBS.

12. Dry around each sample and add 50µl streptavidin-HRP solution to each sample. Do not allow to dry out, cover with a coverslip if necessary. Incubate for 10mins at RT.

13. Was 2x 2mins in 1x PBS.

14. Immerse in DAB solution for 2-10mins.

15. Wash 2x 2mins with distilled water at RT.

16. Immerse in Blue Counterstain for 5-30secs.

17. Wash and dehydrate sections by dipping in the following solutions:

tap water (10x)

ammonium water until the stains turns from purple to blue.

70% ethanol (2 changes, 10x each)

95% ethanol (2 changes, 10x each)

100% ethanol (2 changes, 10x each)

Xylene (2 changes, 10x each).

18. Mount in DPX.