# The Role of TGF-ß Receptor and its Ligand in Leukocyte Development, Immune response and Disease:

## Generation of a Mouse Model

## by Conditional Mutagenesis of TGFß-RII.

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# ABSTRACT

The TGF-ß family represents cytokines with profound regulatory effects on many biological processes. TGF-ß1 deficient mice die of a multifocal inflammatory disease, involving most leukocyte subsets. To determine the role of TGF-ß in leukocytes we have used the Cre/loxP system to create mouse models of cell type-specific TGF-ß Receptor Type II (TGF-ßRII) deficiency. The genomic locus of the mouse TGF-ßRII was cloned and characterised. The information obtained was used to construct a targeting vector to flank, ("flox"), exon 3 of the TGF-ßRII locus with loxP sites, through homologous recombination in mouse embryonic stem cells. Clones carrying a floxed copy of the TGF-ßRII exon 3 (TGF-ßRII$^{fl/+}$) were used to generate chimeric mice by morula aggregation. Chimeras transmitting the TGF-ßRII$^{fl/+}$ mutation were crossed with mice expressing the Cre recombinase driven by the *CD19* promoter to generate animals with a B cell-specific (TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$) TGF-ßRII deficiency. B cell-specific Cre-mediated deletion of exon 3 was confirmed by PCR analysis of peripheral blood leukocytes and subsequently analysed by Southern blotting of genomic DNA from purified B and T lymphocytes. TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ animals exhibited 97% deletion of exon 3 in a B cell specific manner. When analysing B cell development in conditional mutant and control littermates, no significant differences were found in the percentages of B cell populations from bone marrow, peripheral blood, spleen and lymph nodes. In contrast, FACS analysis of cells from the peritoneal cavity of TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice, revealed a noticeable increase in total numbers of B-1 cells, in relation to the wild type littermates. To examine if TGF-ß plays a role in B cell proliferation *in vivo* we next carried out a BrdU incorporation assay on TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ and wild type control animals. This showed no difference in the turnover of bone marrow and peritoneal cavity B cells and a two-fold increase in the turnover of TGF-ßRII deficient splenic B lymphocytes. To assess if TGF-ß is essential in directing switch recombination in the IgA immunoglobulin isotype, we carried out an intracellular staining of bone marrow and plasma cells from wild type and B cell specific TGF-ßRII deficient mice. Our results indicate that this immunoregulator is necessary for regular IgA class switch *in vivo*. This study proves the validity of our TGF-ßRII$^{fl}$ model in systematically addressing the roles of TGF-ß in leukocyte commitment *in vivo*.

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# ABBREVIATIONS USED

| | |
|---|---|
| TGF-ß | Transforming Growth Factor-ß |
| TGF-ßRI and II | TGF-ß Receptor Type I and Type II |
| SMAD | Similar to Mothers against DPP |
| ECM | Extracellular Matrix |
| TNF-α | Tumor Necrosis Factor-α |
| IFN-γ | Interferon-γ |
| MIP-1α | Macrophage Inflammatory Protein-1α |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| IL- | Interleukin- |
| Ab | Antibody |
| Ag | Antigen |
| Ig | Immunoglobulin |
| LPS | Lipopolysaccharide |
| CDK | Cyclin Dependent Kinase |
| GC | Germinal Centrè |
| VDJ | Variable Recombined Region |

| | |
|---|---|
| $C_H$ | Constant Region |
| S | Switch Region |
| I | Intervening Region |
| TßRE | TGF-ß Responsive Element |
| Th-1 and -2 | T helper -1 and -2 Cell |
| LC | Langerhans Cell |
| PMN | Polymorphonuclear Cell |
| ES Cell | Embryonic Stem Cell |
| Fc | Fragment of crystallisation |
| SCID | Severe Combined Immuno-Deficiency |
| Lox P | Locus of Crossover in P1 |
| Flox | Flank by lox P |
| kDa | kilo Dalton |
| b.p. | base pair |
| kb | kilobase |
| BrdU | Bromo-2'-Deoxyuridine |

# INTRODUCTION

## Chapter 1 TGF-ß and its Type II Receptor

### 1.1 TGF-ß and its Superfamily

Transforming Growth Factor-ß (TGF-ß) was originally defined in 1983 by its ability to reversibly cause a phenotypic transformation of normal rat fibroblasts (Anzano et al., 1983; Roberts et al., 1983). Since then, it has been distinguished as a multifunctional cytokine with effects on nearly every tissue and cell type. It has become the prototype of a superfamily of over 40 structurally related regulatory proteins, presumably derived from a common ancestral gene (Kingsley, 1994). As a set, these proteins, including the TGF-ßs, the activin/inhibins and the bone morphogenetic proteins (BMPs) subfamilies, are thought to influence cell growth, differentiation, motility, organisation and death, generally serving to limit cell expansion.

In mammals, the TGF-ß subfamily is represented by at least three genes: TGF-ß1, -ß2, and –ß3 sharing approximately 80% homology, with each gene situated on a different chromosome (Roberts AB, Sporn MB. 1990). In certain milieus the –ß1, -ß2 and -ß3 isoforms have similar functions, though with different timings and degrees of influence. Key to all of their effects are not only complex transcriptional regulatory mechanisms governing the expression of ligands and receptors, but also intricate post-transcriptional mechanisms, controlling both the trafficking and activity of the mature peptides. Thus, all three isoforms are secreted by most cell types, generally in a latent form, requiring activation before they can exert biological activity (for a review on the regulation of TGF-ß activity see Bonewald, 1999).

While the three TGF-ß isoforms share a common set of receptors and have cognate biological activities that often overlap *in vitro*, independent gene ablation experiments have

brought to light distinct phenotypes *in vivo* (Kaartinen et al., 1995; Shull et al., 1992; Letterio and Roberts, 1996). Such studies have reflected clear isoform specificity in the developmental functions of TGF-ß1, -ß2 and –ß3 and have highlighted the first isoform as an important immunomodulator. This overview will focus mainly on the TGF-ß1 isoform and its Type II receptor (TGF-ßRII), which are the most relevant to our immunological research interests. The names TGF-ß1 and TGF-ß will be used indistinguishably throughout this work.

### 1.1.1 Biological Effects of TGF-ß1

The TGF-ß1 isoform has regulatory effects on a broad spectrum of cell types, eliciting diverse cellular responses, depending on cell type, state of differentiation and on the total milieu of cytokines present. It acts via autocrine, paracrine and endocrine mechanisms to achieve three major roles:

- Inhibition of cell proliferation, by blocking the cell cycle at the G1 phase. For a review see Ravitz and Wenner, (1997).

- Control of extracellular matrix (ECM), by acting as a mediator of ECM synthesis and degradation. TGF-ß1 has also been shown to have an important role in modulating the response of cells to the ECM, actively participating in the mechanism of dynamic reciprocity between cells and their immediate environment. What is more, this special relationship is intensified by the fact that specific components of the ECM are able to both deliver and regulate TGF-ß1 activity. For a review see Roberts A.B. *et al.*, (1992).

- Immunoregulation, acting as one of the most important modulators in immune and inflammatory responses and modulating hematopoiesis.

As our interest in TGF-ß lies mainly in its influence on leukocytes, this review will concentrate on the third of TGF-ß's roles, i.e. its immunoregulatory properties. More comprehensive reviews on the TGF-ß subfamily and its roles outside the immune system can be seen in Clark and Coker, 1998 and McCartney-Francis et al., 1998.

## 1.2 TGF-ß as an Immunoregulator

All leukocytes express TGF-ß1 and its type -I and -II receptors (Letterio and Roberts, 1998). Expression of this cytokine and its receptors serve both in autocrine and paracrine fashion to control the proliferation and differentiation of both B and T lymphocytes and to alter the differentiated functions of all classes of mature leukocytes. TGF-ß1 can both modulate the production and antagonise the effects of specific inflammatory cytokines, including interferon-γ (IFN-γ), interleukin-1 (IL-1), IL-2 and IL-6. In macrophages, TGF-ß1 acts as a potent suppressor, influencing maturation, activation and blocking superoxide and nitric oxide production. It influences leukocyte migration by altering patterns of expression of adhesion molecules such as integrins and selectins, thus interfering with adhesion of neutrophils, monocytes and lymphocytes to the vascular endothelium. It also acts on migration of inflammatory cell types to the site of inflammation by providing a chemotactic response and inhibiting these cells once they have become activated.

Dysregulated TGF-ß signaling (and/or activation of the cytokine) amongst leukocyte cell populations has been linked to immune malignancy, autoimmune disorders, susceptibility to opportunistic infection and chronic inflammatory conditions.

Ever since TGF-ß1 was first discovered, there have been innumerable *in vitro* studies on its functions and effects on all leukocytes. Unfortunately, a large proportion of these appears to have failed in appreciating the contextual nature of TGF-ß's biological actions. For this reason in the next section we will focus on biological data resulting from studies on the TGF-ß1 knockout model.

## 1.3 The TGF-ß1 Knockout Model

TGF-ß1 knockout mice have greatly aided our understanding of the role that TGF-ß1 plays in immunity and inflammation, with a main phenotype suggesting the loss of a critical regulator of immune function. Numerous studies carried out on these mutant mice have brought to light three principal phenotypes along the different stages of development: an embryonic lethal phenotype at days E9.5-10.5, dysregulated hematopoiesis and severe

multiorgan inflammation starting at days 7-10 post partum and leading to death within the first month of life (Kulkarni et al., 1993; Shull et al., 1992).

### 1.3.1 Embryonic Lethality

The dominant role of the TGF-ß1 isoform in development and early haematopoiesis is highlighted by the high rate of embryonic lethality at days E9.5-10.5 (≥50% for TGF-ß1$^{-/-}$ embryos, 25%for TGF-ß $^{+/-}$ embryos), and is confirmed by the similarity in phenotype shown by the TGF-ß RII$^{-/-}$ embryos (Oshima et al., 1996). It is thought to arise due to defective vasculogenesis and haematopoiesis of the embryonic yolk sac (Dickson et al., 1995).

### 1.3.2 Multifocal Inflammation

Live-born TGF-ß1$^{-/-}$ mice have no apparent developmental defects upon birth. The development and maturation of the lymphoid system are remarkably intact. This is most probably as a result of placental/foetal and lactation maternal TGF-ß1 transfer. This is supported by studies showing a normal pattern of immunohistochemical staining of TGF-ß1 in tissues of young TGF-ß1$^{-/-}$ mice in absence of gene expression (Letterio et al., 1994). Surprisingly however examination of the lymphoid organs of TGF-ß1$^{-/-}$ pups from homozygous TGF-ß1$^{-/-}$ mothers shows very little difference to pups born to TGF-ß1$^{+/-}$ mothers, implying perhaps redundancy between isoform in the early developmental stages.

Within three weeks of life, TGF-ß1$^{-/-}$ mice develop a severe multifocal inflammatory syndrome, characterised by a massive infiltrate of mononuclear cells, (principally lymphocytes and macrophages, with few neutrophils), into the vital organs (heart, lungs, stomach, liver, pancreas, intestine, salivary glands). The symptoms develop rapidly, resulting in wasting and death by the fourth week of age. The earliest lesions develop large tissue infiltrates, characterised by enhanced expression of MHC Class I and II and increased adhesion of leukocytes to vascular endothelium, with ultimate perivascular accumulation (Geiser et al., 1993; Hines et al., 1994). It is not clear whether either of these two processes is directly responsible for the inflammation: both the increased leukocyte adhesiveness and the enhanced expression of MHC are initiated prior to the first histopathological evidence of inflammation. Follow-up studies on the knockout mice have shown that inhibition of

binding to integrin $\alpha_4$ and proteoglycans, by repeated administration of synthetic fibronectin peptides, reduces tissue infiltration and damage, prolonging survival of the mice (Hines et al., 1994; McCartney-Francis et al., 1996). These experiments support previous studies which demonstrate that TGF-ß1 can modulate integrin expression on vascular endothelium and strengthen evidence for increased leukocyte-endothelial cell interactions in the genesis of the TGF-ß1 knockout phenotype.

Although research has shown that TGF-ß1 can act as a potent chemoattractant for all classes of leukocytes during the earliest stages of an inflammatory response, TGF-ß1 knockout mice suggest that it is the cytokine's ability to control and promote the resolution of such processes which may be its most important function. Thus while in the initial recruitment phase of inflammation TGF-ß1 induces production of specific inflammatory cytokines, it suppresses both the production of and the response to these cytokines in the resolution phase. This is supported by PCR analysis of inflammatory cytokine mRNA levels from mutant animals, showing a significant increase in levels of important mediators of inflammation, including TNF-$\alpha$, IFN-$\gamma$ and Macrophage Inflammatory Protein-1$\alpha$ (MIP-1$\alpha$) (Shull et al., 1992). In a further dissection of this complex inflammatory phenotype, breeding the TGF-ß1 knockout mice in a severe combined immunodeficiency (SCID) background dramatically decreased the severity of inflammation, suggesting that the principal cell lineages responsible for its initiation are B and/or T cells (Diebold et al., 1995).

### 1.3.3 Autoimmunity

From the first reports on TGF-ß1$^{-/-}$ mice, this cytokine's role in maintenance of normal immune function was highlighted by increased activation of splenic and lymph node lymphocytes, thymocyte differentiation and hyperactivation of T-cell populations. Because no detectable pathogen was identified in inflamed tissues of TGF-ß1 null mice, studies were carried out to examine whether an autoimmune mechanism may play a role in the initiation and/or maintenance of the phenotype. Several reports demonstrated that shortly after the onset of inflammation, TGF-ß1$^{-/-}$ mice exhibit manifestations typical of autoimmune disease. Serum from the mutant mice was found to contain elevated levels of circulating antibodies to nuclear antigens, immune complex deposition and Sjögren's syndrome-like lymphoproliferation (Dang et al., 1995; McCartney-Francis et al., 1996; Yaswen et al., 1996).

In an elegant study, Letterio *et al*, demonstrated that the abnormal autoimmune response may be partly dependent on the presence of MHC Class II antigen and autoreactive T cells: TGF-ß1$^{-/-}$ mice were crossed with MHC-II$^{-/-}$ ($CD4^+$ T cell-deficient) mice. The resulting strain exhibited a complete lack of autoimmune inflammatory manifestations. Similar results were also recently obtained by Kobayashi et al., (1999), when crossing the TGF-ß$^{-/-}$ model into an MHC class I/$ß_2$-microglobulin background, this time highlighting a possible involvement of MHC class I antigens and $CD8^+$ T cells in the autoimmune manifestations resulting from lack of TGF-ß1.

### 1.3.4 Dysregulated Hematopoiesis

Importantly, the mice resulting from the TGF-ß1$^{-/-}$, MHC-II$^{-/-}$ cross showed a severe myeloid metaplasia and extensive extramedullary hematopoiesis, similar to that seen in the original mutants, revealing that the hemopoietic abnormalities present in TGF-ß1$^{-/-}$ mice are not solely a consequence of systemic inflammation.

In the same report, Letterio *et al*, depleted TGF-ß1$^{-/-}$ mice of $CD4^+$ T cells using anti-CD4 antibodies (Letterio et al., 1996). The treated mice survived twice as long as the undepleted controls, but still displayed a high level of extramedullary myelopoiesis, as indicated by percentages of $GR1^+$ cells in various hematopoietic organs. This is somewhat in contrast with the absence of myeloid hyperplasia found in SCID TGF-ß1$^{-/-}$ mice (Diebold et al., 1995).

### 1.3.5 Other Leukocyte Phenotypes

Alongside dysregulated antibody (Ab) production, TGF-ß1 null mice exhibit other defects in B cell function, such as enhanced B cell proliferation within lymphoid follicles, as assessed by the abundance of CDK and proliferating cell nuclear antigen (PCNA) expression (Christ et al., 1994) and decreased IgA levels (van Ginkel et al., 1999). The role of TGF-ß in B cell biology will be discussed in detail in chapter 2. Other studies have shown that in the absence of TGF-ß1, monocytes are also markedly hyperactivated, with increased expression of inducible nitric oxide synthase (Vodovotz et al., 1996). Interestingly, the TGF-ß1$^{-/-}$ model has also brought to light a requirement for this cytokine in Langerhans cell (LC) biology. The epidermis of TGF-ß1 null mice was found to be devoid of LCs. Abrogation of the

inflammatory phenotype via rapamycin administration did not reverse the LC deficiency (Borkowski et al., 1996), suggesting that it is not secondary to inflammation. In a more recent report, dendritic cells were expanded from the bone marrow of TGF-ß1$^{-/-}$ mice transferred into lethally irradiated recipients, indicating that the LC deficit was not due to an absolute deficiency in bone marrow precursors and that paracrine production of TGF-ß1 is sufficient for their development. In addition TGF-ß1$^{-/-}$ skin was fully repopulated by bone marrow derived LCs when engrafted onto BALB/c nu/nu mice, demonstrating that the impaired development of LCs is not due to epidermal abnormality and implying a direct requirement of these cells for TGF-ß1 (Borkowski et al., 1997).

Just like all other white blood cells, neutrophils express high levels of type I and II TGF-ß receptors, (Brandes et al., 1991; Parekh et al., 1994). *In vitro* research has shown that human peripheral blood neutrophils produce high levels of TGF-ß1 and that its secretion is stimulated by activation with LPS (secretion after stimulation of $10^6$ml$^{-1}$cells with 1 gml$^{-1}$ LPS $\approx$ 4.5 ng ml$^{-1}$per 24 hours), (Grotendorst et al., 1989). Data from various investigations suggests that the inflammatory effects of TGF-ß1 may also be mediated through its effects on neutrophil survival and function. Most researchers have reported large increases in human and murine neutrophil migration in response to TGF-ß1 (Brandes et al., 1991; Fava et al., 1991; Lagraoui and Gagnon, 1997). TGF-ß appears to be specific in attracting neutrophils in suspension without activating them. Fentomolar concentrations of this cytokine are sufficient to stimulate chemotaxis *in vitro* (Brandes et al., 1991; Parekh et al., 1994; Reibman et al., 1991), suggesting that TGF-ß may play an important role in the recruitment of PMNs during the initial phase of the inflammatory response. Parekh et al., (1994) have shown that PMN migration towards TGF-ß is inhibited by monoclonal antibodies specific to the cell-binding domain of fibronectin on the neutrophil. Interestingly, injection of fibronectin repeats in TGF-ß1 null animals reduces the inflammatory cell infiltration, thus decreasing the severity of the phenotype (see section 1.3.3; Hines et al., 1994; McCartney-Francis et al., 1996). This reported effect on neutrophil chemotaxis is consistent with *in vivo* studies in SMAD3 knockout mice, which show a decreased neutrophil migration to the site of inflammation (Yang et al., 1999). This and a decreased monocyte chemotaxis may be in part responsible for the accelerated wound healing in these animals, due to loss of TGF-ß responsiveness. There is no consensus as to this cytokine's role in

stimulation of the neutrophil respiratory burst (Brandes et al., 1991; Lagraoui and Gagnon, 1997). More *in vitro* studies on human peripheral neutrophils have shown TGF-ß1 to enhance their phagocytotic potential and increase neutrophil survival by preventing apoptosis (Lagraoui and Gagnon, 1997). In a more recent investigation, murine IgG-bound TGF-ß1 has been found to markedly suppress PMN function and host defence against *S.aureus in vivo* (Caver et al., 1996).

Very little data on the direct role of TGF-ß1 on granulocyte function are seen in reports on the TGF-ß1$^{-/-}$ model. One interesting aspect of this research, is the apparent unmasking of the role that TGF-ß1 may have in myeloid hematopoiesis (Letterio et al., 1996). The mechanisms by which this may be modulated are not entirely clear. Early studies showed TGF-ß1 to have negative effects on haematopoietic stem cell proliferation (Waegell et al., 1994). This may result from this cytokine's role in modulation of receptor expression for colony-stimulating factors, as was suggested by the increased sensitivity of TGF-ß1$^{-/-}$, MHC-II$^{-/-}$ marrow cultures to GM-CSF and IL-3 (Letterio et al., 1996).

## 1.4 Modes of Action of TGF-ß Isoforms

When analysing this complex phenotype it is important to bear in mind the multiple modes of action of the three TGF-ß isoforms. Data showing the occurrence of maternal transfer of TGF-ß1 protein and circulating levels of TGF-ß1 in adult plasma, clearly demonstrate that this isoform can function in an endocrine–like mode (Ivanovic et al., 1995). Sources of endocrine TGF-ß1 may also include bone, which contains high levels of the protein. It is not known how TGF-ß1 may be transported and delivered in an endocrine manner. An interesting example of this process may be the transfer of TGF-ß1 bound to IgG, that is taken up by macrophages via Fc receptors and delivered in an active form to TGF-ß receptors on interacting lymphocytes (Rowley et al., 1995). Endocrine functions are seemingly specific to the Type I isoform as there are no reports for circulating levels of the TGF-ß2 or –ß3 isoforms.

Data from the TGF-ß1-null mice suggest that although some paracrine functions of the TGF-ß proteins may exhibit redundancy between isoforms, bone marrow derived cells may rely solely on autocrine TGF-ß1. Indeed, the profound dysregulation exhibited by such cells in TGF-ß1$^{-/-}$ mice is not reversed by systemically injected TGF-ß1 (J. Letterio

unpublished, abstract). Furthermore the wild type functions of leukocytes from irradiated mice with transplanted marrow from TGF-ß1$^{-/-}$ donors, were not restored despite the presence of TGF-ß1 produced by non-marrow-derived cells (Yaswen et al., 1996).

In the next chapter we will review in detail the roles of TGF-ß and its receptors in in B cells, as these are the cell type most actively studied by us in this context. Firstly however, the next section will explore what is known of TGF-ß signal transduction to the nucleus.

## 1.5 TGF-ß Signaling to the Nucleus

All three of the major receptors for TGF-ß have been cloned. The type-III receptor, TGF-ßIIIR or betaglycan, is a 250kDa transmembrane proteoglycan, thought to function by presenting TGF-ß to the TGF-ßIIR via a physical interaction which induces a higher affinity for TGF-ß in TGF-ßIIR (Lopez-Casillas et al., 1994; Lopez-Casillas et al., 1993). Recently it was shown to have a non-redundant role in endocardial cell transformation in the heart (Brown et al., 1999).

TGF-ßIR and TGF-ßIIR share a common overall structure with sequence similarity in the extracellular cysteine-rich domain and the intracellular serine/threonine-specific protein kinase domains (Hill, 1996). The type I receptor is a 50-60 kDa protein whereas the type II receptor is approximately 75-80 kDa, the increase in size being due to larger N-and C- termini in the mature TGF-ßIIR molecule (see figure 8.3 for structure of TGF-ßRII). Biochemical data strongly suggests that the TGF-ßIR requires TGF-ßIIR in order to bind ligand, and that signalling is mediated via a hetero-oligomeric complex (Inagaki et al., 1993; Wrana et al., 1992). Researchers have shown that hetero-oligomeric receptor complexes can occur in the absence of ligand, and in certain mammalian cell types, type II receptors can form stable homodimers.

Thus although the functional specificities of TGF-ßIR-TGF-ßIIR and TGF-ßIIR-TGF-ßIIR complexes remain to be characterised, it appears that TGF-ßIIR plays a pivotal role in mediating TGF-ß binding and in orchestrating TGF-ß responses (Hill, 1996).

Figure 1 shows a hypothetical TGF-ß signal transduction pathway. On binding to ligand (figure 3.1.a) the oligomeric TGF-ßIIR recruits the TGF-ßIR to the complex (3.1.b)

and activates it by phosphorylation of the GS Domain (3.1.c). The activated TGF-ß-IR is now able to recruit and phosphorylate SMAD2 or SMAD3 (vertebrate homologues of drosophila Sma and Mad) which are normally anchored in the cytoplasm in homotrimeric form (3.1.c). Activated SMADs 2 or 3 bind SMAD4, forming a heteromeric complex (3.1.d) that is translocated to the nucleus (3.1.d). Here the hetero-oligomeric complex may associate to other DNA-binding proteins and bind to DNA, thus modulating transcription of specific genes (3.1.e). This signal transduction pathway may be inhibited by binding of SMAD6 and -7 to the receptor complex (not shown in figure, Derynck et al., 1998; Heldin et al., 1997).

Although the TGF-ßIIR has highest affinity to the TGF-ß1 isoform, biochemical studies have shown that it may bind and mediate signalling for the –ß3 isoform as well. Further biochemical evidence also suggests very low affinity to the –ß2 isoform *in vitro* (Heldin et al., 1998). It is not clear whether all three isoforms signal through this same receptor *in vivo*.

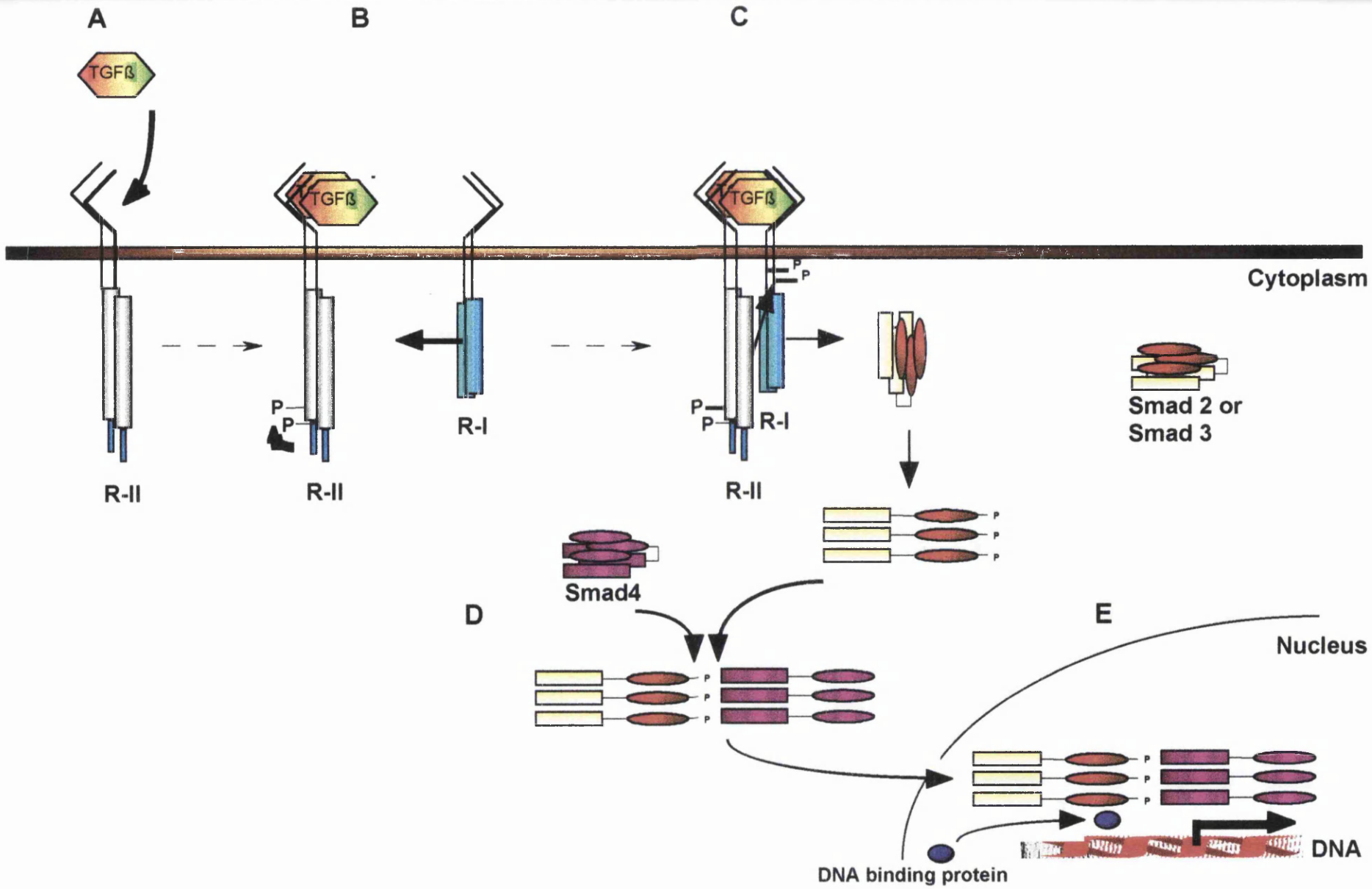



**Figure 1.1 TGF-ß Signaling to the Nucleus**
See text for legend.

# Chapter 2 TGF-ß and the B Cell

Previous *in vitro* studies have shown TGF-ß to control several aspects of B lymphocyte maturation and function, including proliferation, Ig production and secretion, expression of cell surface molecules and activation. As in most leukocyte populations, it appears that the synthesis, secretion and response to TGF-ß in B cells are a consequence of the cell's state of differentiation, activation and even of the activation signals involved. Generally activation of B cells will lead to up-regulation of TGF-ß and TGF-ß receptor mRNA (Kehrl et al., 1986; Matthes et al., 1993; Warner et al., 1992) and to secretion of bio-active TGF-ß1 (Kehrl et al., 1986; Snapper et al., 1993). As will be discussed in the following sections, most B cell *in vitro* models suggest that TGF-ß may act as an autocrine to inhibit B cell proliferation, often leading to apoptosis. Thus it is likely that B cell induction of TGF-ß and its receptors serves as an important regulatory feedback loop to limit the expansion of activated B cell populations. This autocrine inhibitory loop appears to be dysregulated in several murine and human B lymphoid malignancies (Berg and Lynch, 1991; DeCoteau et al., 1997; Kadin et al., 1994).

## 2.1 TGF-ß in B Cell Development

Although TGF-ß and its receptors are found on most nucleated cells in the bone marrow (Lee et al., 1987) it is unclear whether this cytokine has a role in B cell development.

Early studies on bone marrow-derived and transformed cloned pre-B cells found TGF-ß to significantly inhibit kappa light chain and class II molecule expression *in vitro* (Lee et al., 1987) suggesting it as an important negative regulator of B lymphopoiesis. Since then researchers have shown TGF-ß either to inhibit or enhance the growth of hemopoietic progenitor cells depending on their differentiation stage and on the cytokine milieu (Keller et al., 1994; Keller et al., 1992; Ottmann and Pelus, 1988). Most frequently however TGF-ß has been reported to inhibit the proliferation of mouse hemopoietic progenitors (Eaves et al., 1991; Kishi et al., 1989; Lu et al., 1993) and of committed mouse B cell precursors (Lee et al., 1989).

*In vivo*, the picture is more complicated: the early stages of B cell development are dependent on a series of interactions between B cell precursors and a supportive bone marrow microenvironment (McGinnes et al., 1991; Moreau et al., 1993; Whitlock and Witte, 1982). One important role of bone marrow stromal cells is to provide cytokines required for the early phases of pre-B cell proliferation and maturation, such as IL-7 (Lee et al., 1988). A recent investigation showed TGF-ß may have an indirect effect on B cell development in humans, by down-regulating stromal IL-7 mRNA expression and protein secretion (Tang et al., 1997). In mice this effect is at least partly mediated by other factors, as B cell numbers in new-born TGF-ß1$^{-/-}$ mice appear normal if moderately reduced (Christ et al., 1994).

## 2.2 TGF-ß as an Inhibitor of B Cell Proliferation

In most cell types tested, TGF-ß will inhibit proliferation, by arresting the cell cycle in late G1 phase. Progression of cells through the G1 phase is dependent on the sequential formation, activation and subsequent inactivation of cyclin/cyclin dependent kinase (Cdk) complexes. TGF-ß1 cell cycle arrest at the G1 phase is thought to be mediated partly via its effects on levels of expression and activation of different cyclins and Cdks (Ewen et al., 1995; Ewen et al., 1993; Koff et al., 1993; Polyak et al., 1994; Ravitz and Wenner, 1997; Schwarz et al., 1995; Warner et al., 1992) but also, indirectly via induction of different Cdk inhibitors (cdis) which suppress Cdk activity via stoichiometric mechanisms. Inhibition of the cyclin/Cdk complexes results in the maintenance of Rb in the hypophosphorylated growth suppressive state, resulting in the suppression of several genes such as *c-myc*, necessary for transition into S phase. Thus, in a recent investigation Kamesaki et al. (1998) showed that TGF-ß1 induces Cdk inhibitor p27$^{kip1}$ expression in CH31 and WEHI232 murine B cells. Another study (Bouchard et al., 1997) analysed the blockade imposed by TGF-ß1 in murine quiescent mitogen stimulated B lymphocytes. In these cells, the authors found TGF-ß1 to block entry into the cell cycle by inactivation of Cdk2 (again by induction of p27$^{kip1}$ expression) but also by direct inhibition of cyclin A expression. Consistent with these findings, when staining TGF-ß$^{-/-}$ lymphoid organs with anti-PCNA antibodies, which identify cells in the S phase of the cell cycle, Christ et al., (1994) found enhanced numbers of PCNA$^{+}$ cells in TGF-ß$^{-/-}$ spleens and lymph-nodes and a concurrent fivefold increase in the expression levels of the Cdk p34$^{cdc2}$ kinase.

Interestingly TGF-ß may also stop transition to the S phase by repressing *c-myc* transcription by an Rb-independent mechanism, and by repressing synthesis of c-Myb and Bcl-2 (for a review see Stavnezer, J. 1995).

## 2.3 TGF-ß and B cell Apoptosis

Apoptosis plays a crucial role at several stages of B cell development and maturation. Thus in the bone marrow, elimination of self-reactive immature $IgM^{+}IgD^{-}$ B lymphocytes occurs via apoptosis and is essential for the induction and maintenance of tolerance (Hartley et al., 1993). The same fate is thought to be reserved for the vast majority of pro-B cells during Ig gene rearrangement (Cohen, 1991). Mature B lymphocyte homeostasis is also controlled by apoptosis: a large proportion of the B cells in the germinal centre (GC) reaction undergo apoptosis in the selection of B cells during generation of the secondary Ab response (for a review see Liu et al., 1992).

Several *in vitro* studies have shown TGF-ß to induce apoptosis in murine and human lymphocytes (Brown et al., 1999; Chen and Chang, 1997; Choi et al., 1998; Oberhammer et al., 1992; Rotello et al., 1991; Sanchez et al., 1996; Selvakumaran et al., 1994; Yanagihara and Tsumuraya, 1992) suggesting it may be one of the many physiological stimuli to control B cell homeostasis *in vivo*. In fact, the ability of this signal to induce apoptosis in immature B cells is likely to be a major mechanism controlling lymphocyte numbers and subsequently regulating the immune response, as shown by the phenotype of TGF-ß1$^{-/-}$ mice. As discussed in section 1.3.3, although at birth the B cell numbers in these mice are marginally lower than those of their wild-type littermates (Christ et al., 1994), with onset of disease the TGF-ß1$^{-/-}$ mice exhibit extensive lymphocytic hyperproliferation and systemic lupus erytrhomatosus (SLE)-like autoantibodies (Dang et al., 1995; McCartney-Francis et al., 1996). The mechanisms through which apoptosis is regulated by TGF-ß are not well understood and appear to be cell specific, as is often the case with this cytokine. Thus, TGF-ß appears to mediate apoptosis in B cells via mechanisms that may be *bcl-2* dependent (Stavnezer, J. 1995) or independent (Lomo et al., 1995) and which may involve the cleavage of the actin binding cytoskeletal protein αII-spectrin (Brown et al., 1999).

## 2.4 TGF-ß in the Regulation of Ig Synthesis and Secretion

In several experimental systems, TGF-ß has been shown to inhibit immunoglobulin secretion. The addition of TGF-ß during activation of mouse or human B cells inhibits the induction of mRNA for the light chain and secreted form of heavy chain (Briskin et al., 1988; Kehrl et al., 1991; Miller et al., 1991). Although the mechanisms by which TGF-ß regulates light chain and secreted heavy chain mRNA production are unclear, it is likely that they are indirect, as inhibition requires several days of treatment. Interestingly, here there can be a dichotomy in the responses elicited by TGF-ß, as it appears that in small doses, TGF-ß may increase the secretion of most classes of Ig. This was shown by addition of antibodies to TGF-ß to cultures of LPS activated B cells. The result was a ten-fold reduction in IgG2a, a nine-fold reduction in IgG3 and threefold reductions in IgG1 and IgE (Snapper et al., 1993), with no effect on the secretion of IgM. This suggests that small levels of autocrine TGF-ß may serve to increase the secretion of immunoglobulins IgG2a, IgG3 and IgG1 in certain circumstances.

## 2.5 IgA Class Switch and the Role of TGF-ß

Upon stimulation with antigen or mitogen, B cells proliferate and differentiate towards Ab secretion. As these cells proliferate, a proportion of their progeny will switch from expressing IgM to expression of other Ig classes. The process of Ig class switching allows the recombined variable (VDJ) region of the heavy chain to be expressed with a new downstream constant region ($C_H$) gene. At the molecular level, class switching takes place via deletional recombination between switch regions (S) located upstream of each $C_H$ gene. This process is directed to particular $C_H$ genes by different cytokines that induce transcription of germline transcripts from rearranged constant region genes before switch recombination. It is not clear what the function of these germline transcripts is, although they appear necessary for switch recombination. This was shown by gene targeting experiments where deletion of the promoter and first exon (I, intervening region exon) of the germline transcript interrupts switching to that $C_H$ gene (Bottaro et al., 1994; Jung et al., 1993; Zhang et al., 1993) . Ultimately this process results in alteration of the Ab effector function.

Class switching to IgA represents an important component of the immune response, particularly at the host/environment interface. $IgM^+$ $IgD^+$ precursors switch to $IgA^+$ B cells in germinal centres of the spleen, lymph nodes and respiratory- and intestinal tract-associated lymphoid tissues. This gives rise to dimeric IgA antibodies, with effector properties that enable ready transport across respiratory and intestinal mucosae. Specific IgA are critical in hindering the entrance of extramucosal intraluminal pathogens through the respiratory and intestinal surfaces throughout life, starting at the neonate level, when IgA secreted into the mothers' breast milk acts in passive immunisation of the new-born. IgA deficiency is the most common primary immunodeficiency in humans. Patients affected by this condition usually suffer from repeated gastrointestinal and/or upper respiratory tract infections (Grumach et al., 1998). Mice with a targeted deletion of the IgA constant region have normal lymphocyte development, proliferative responses and cytokine production, although they exhibit altered expression of other Ig (Harriman et al., 1999).

The ability of TGF-ß to direct switch recombination in Ig isotypes IgA and IgG2b has been reported by many researchers. In the initial *in vitro* investigation on the effects of TGF-ß on the pattern of isotypes secreted by LPS-stimulated $sIgM^+$ B cells, Coffman et al. (1989) demonstrated that addition of this cytokine (at 0.4 to 2 ng/ml) induced a tenfold increase in the level of IgA secretion concomitantly with a decrease of the same magnitude in the other isotypes. In the same study the authors showed that although addition of IL-2 alone to similarly stimulated B cells caused at best a twofold increase in IgA isotype secretion, addition of both cytokines together caused a 50-fold increase in IgA secretion. A similar synergistic effect was seen after addition of TGF-ß with IL-5 (Coffman et al., 1989; Sonoda et al., 1989). At the cellular level, experimental evidence, including prior depletion of $sIgA^+$ cells, suggested that TGF-ß alone was able to direct switching from $sIgA^-$ to $sIgA^+$, and that IL-2 and IL-5 were only able to stimulate maturation of $sIgA^+$ cells to IgA secreting cells (Coffman et al., 1989; Kim and Kagnoff, 1990; Sonoda et al., 1989) These effects were seen both on splenic and Peyers Patches B lymphocytes.

At the molecular level Lebman et al. (1990) showed that TGF-ß was unique in inducing germline $\alpha$ mRNAs in LPS-stimulated B cells, although it is now clear that the induction of S $\rightarrow$ S$\alpha$ DNA recombination requires additional signals, such as those

provided by bacterial polysaccharides (e.g. LPS) or T cell derived cytokines (e.g. IL-2) (Iwasato et al., 1992; Lebman et al., 1994). (McIntyre et al., 1995) et al. achieved high rate IgA secretion by IgD cross-linking of resting murine B cells *in vitro*, with addition of LPS or CD40 ligand in concert with TGF-ß, IL-4 and IL-5. This suggests that *in vivo* IgA class switch may require multiple inducing stimuli. More recently Zan et al., (1998) have shown that CD40 engagement may be required in this mechanism to induce endogenous TGF-ß production. Work by Shockett and Stavnezer (1991) on the $sIgM^+$ I.29 B lymphoma cell line showed that the induction of germline α transcripts by TGF-ß1 was due to regulation at the transcriptional level. Soon afterwards, a segment of the germline α transcript promoter containing nucleotides –128 to +46 was shown to be sufficient for expression and TGF-ß1 inducibility of a reporter gene in two B cell lines (Lin and Stavnezer, 1992). In particular a novel element (TßRE) within the –128/+46 segment, was shown to be required for TGF-ß1 inducibility of the reporter gene. In a recent investigation Shi and Stavnezer (1998) found that the core-binding factor CBF (AML)-proteins bind the TßRE present within the promoter for germline α transcripts and that CBFα3 (AML2) and TßRE binding activities are regulated by TGF-ß1.

Although the molecular mechanisms by which TGF-ß regulates IgA class switching are slowly being elucidated, it remains unclear whether TGF-ß is essential to this mechanism *in vivo*. Recently, Ginkel et al., (1999) found greatly reduced IgA levels in $TGF\text{-}ß^{-/-}$ mice. They suggested that the little IgA found was due to maternal transfer of TGF-ß. As IgA class switching is highly dependent on Th-2 Type cytokines, and as these are increased in $TGF\text{-}ß^{-/-}$ mice (Christ et al., 1994; van Ginkel et al., 1999), this remains a poor model to study the role of TGF-ß in IgA and other Ig class switching mechanisms.

# Chapter 3 How to Study the Cell-Type Specific Effects of TGF-ß in a Biological Context. Aims of the Investigation. Cre/Lox P Mutagenesis

In a thoughtful review, Sporn (1997) proposed that cytokines such as TGF-ß be regarded as components of the dialogue between cells and their environment. It is becoming increasingly clear that multifunctional cytokines can no longer be looked upon as "little messengers" that act on cells to carry out specific functions. A cell must constantly adjust its behaviour to changes in its surroundings. Cytokines such as TGF-ß can be seen as components of the mechanism which provides this adaptation: they supply negative or positive, autocrine or paracrine signals that stabilise (or sometimes alter) the local microenvironment in which a cell exist.

## 3.1 Problems with Previous Studies and Existing Models

Though previous *in vitro* and *in vivo* studies have done much to elucidate the many facets of TGF-ß, they tend to underestimate the contextual nature of natural regulation. Thus, the murine knockout models have established that TGF-ß isoforms are not as redundant as one might fear, leaving TGF-ß1 as the predominant immunoregulator. The experiments cited above have shown that this cytokine co-ordinates the pathogenesis, progression and resolution of autoimmune-mediated inflammation. The process involves activation and differentiation of leukocytes, recruitment of inflammatory cells and expression of adhesion molecules by lymphocytes, myeloid and endothelial cells. Yet, just as the functions of TGF-ß are highly dependent on the total milieu of cytokines present, so also the balance of this cytokine array is itself dependent on TGF-ß. This has made it difficult to study the cytokine's normal mode of action within the body. These models have not determined whether the dysregulation of bone-marrow-derived cells arising in the TGF-ß1$^{-/-}$ animals is due to a lack of autocrine, paracrine or intracrine function and which different leukocyte sub-populations are affected directly. True, immunosuppresive therapy of the TGF-ß1$^{-/-}$ model may provide hints as to its site of action amongst the different leukocyte subsets. However, can they be expected to provide an adequate physiological model?

## 3.2 Aims of the Study

To investigate the pleiotropic effects of TGF-ß on different leukocyte subsets *in vivo*, we chose to create a model in which TGF-ß signalling deficiencies could be studied in a cell-specific manner, using the Cre/loxP conditional mutagenesis technique (outlined below, in section 3.3). Because we were mainly interested in the cytokine's effect on the responding leukocyte, we set about to conditionally target its type II receptor (TGF-ßRII). As seen in section 1.6, the TGF-ßRII is essential for ligand binding and receptor complex formation. We postulated that inactivation of its gene would interrupt TGF-ß signal transduction to the nucleus.

Once we had attained our principal objective, of generating a murine TGF-ßRII$^{flox}$ model (see next section for Cre/loxP mutagenesis), we aimed to use this conditional receptor knockout to generate B cell specific and neutrophil specific TGF-ßRII deficient strains. We then focused on the characterisation of the B cell specific conditional mutants, to test the efficiency of our model and clarify questions discussed in chapter 2 as to its role in the control of B cell development, differentiation and function.

In the following section, we will briefly review Cre/loxP conditional mutagenesis, the technique used for the generation of the TGF-ßIIR$^{flox}$ cell type specific knockout mice. Although the advantages of this technique over the use of conventional gene targeting will be discussed, the principles of gene targeting will not be described in this introduction. Classical knockout technology techniques are outlined in detail in Gene Targeting, A Practical Approach, edited by A.L. Joyner (IRL, Oxford University Press, 1993).

## 3.3 Cre/loxP Mediated Conditional Mutagenesis

### 3.3.1 The Cre/loxP Recombination System

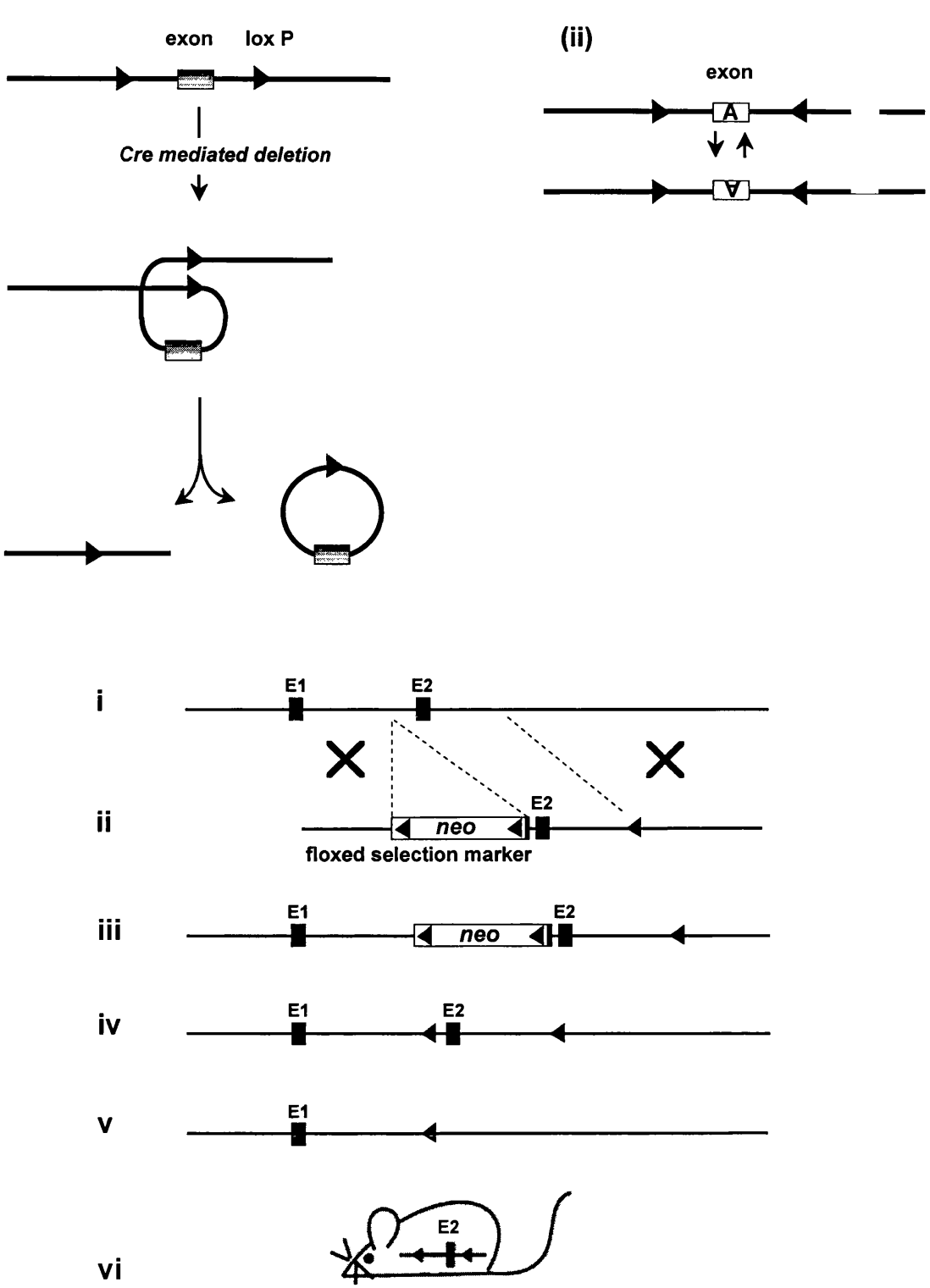
The P1 bacteriophage Cre Recombinase is a 38 kda protein belonging to the λ integrase superfamily of site specific recombinases. Without requiring cofactors or accessory proteins, it is able to recognise 34 b.p. sequences known as loxP (locus of crossover(x) in P1). LoxP sites consist of two palindromic 13 b.p. sequences and an 8 b.p. non-palindromic spacer sequence, dictating the orientation of the overall structure. Cre recombinase will bind

to each of a pair of loxP sites and recombine the sequence between the two sites (Hamilton and Abremski, 1984; Mack et al., 1992). Depending on the orientation of the two sites different types of recombination may occur. If the two sites are placed in the same orientation on a linear piece of DNA, the Cre-mediated intramolecular recombination will result in excision of the lox-P flanked ("floxed") sequence (figure 3.1.A.i). If Cre recognises two sequences in opposite orientation, an intermolecular recombination will occur, resulting in an inversion of the floxed sequence (figure 3.1.A.ii).

### 3.3.2 Use of Cre/loxP in Conditional Gene Targeting

In a typical Cre/loxP mediated gene targeting experiment (figure 3.1.B), the initial step is to incorporate lox P sites into the genome of ES cells (figure 3.1.B.i) via a corresponding targeting vector (figure 3.1.B.ii), in such a way as to allow both the selection marker and a crucial exon of the targeted gene to be "floxed", without interfering with the normal functioning of the gene. Homologous recombinant clones (figure 3.1.B.iii) are transiently transfected with a Cre–expressing plasmid. This gives rise to three different recombination events. In type I recombination (figure 3.1.B.iv), Cre has removed the "floxed" selection marker giving rise to the floxed allele; in type II recombination (figure 3.1.B.v) Cre has fully deleted the floxed locus, giving rise to a knockout clone. A less useful third type of recombination can also occur (not shown in figure 3.1) where Cre removes the floxed allele, leaving only the selection marker. Drug sensitive clones (i.e. those that have undergone either type I or type II Cre-mediated recombination) are selected, characterised and used to generate chimeric mice (figure 3.1.B.vi). The final genetic modification takes place in a conditional manner in mice: the mouse with the floxed allele is crossed with a second mouse in which the expression of Cre is driven by a tissue specific or inducible promoter, giving rise to a Cre-mediated recombination event in which the targeted gene is conditionally inactivated (see figure 3.2.A). Depending on the type of conditional inactivation desired, the floxed strain may be crossed to a variety of different Cre-expressing strains (figure 3.2.B and table 3.1).

Aside from enabling a tissue specific inactivation of the investigated protein, this technique has several advantages over conventional gene targeting. The first becomes apparent when wishing to study a protein such as the TGF-ßIIR. Standard gene inactivation




**igure 3.1 A, Cre Mediated Recombination.** **(i)** Two lox P sites in the same orientation llow for Cre-mediated deletion of intervening DNA. **(ii)** Two lox P sites in opposite rientation catalyse Cre mediated inversion of intervening DNA. **B, Generation of a Floxed llele using *Cre*-lox P Conditional mutagenesis.** The targeting vector **(ii)** is introduced to the genome of wild type ES cells **(i)** by homologous recombination to yield the targeted llele in **(iii)**.Correctly targeted clones are transiently transfected with a Cre plasmid to reate floxed **(iv)** and fully deleted **(v)** clones. Once characterised these clones may be sed for the production of mice **(f)** carrying the above mutations.

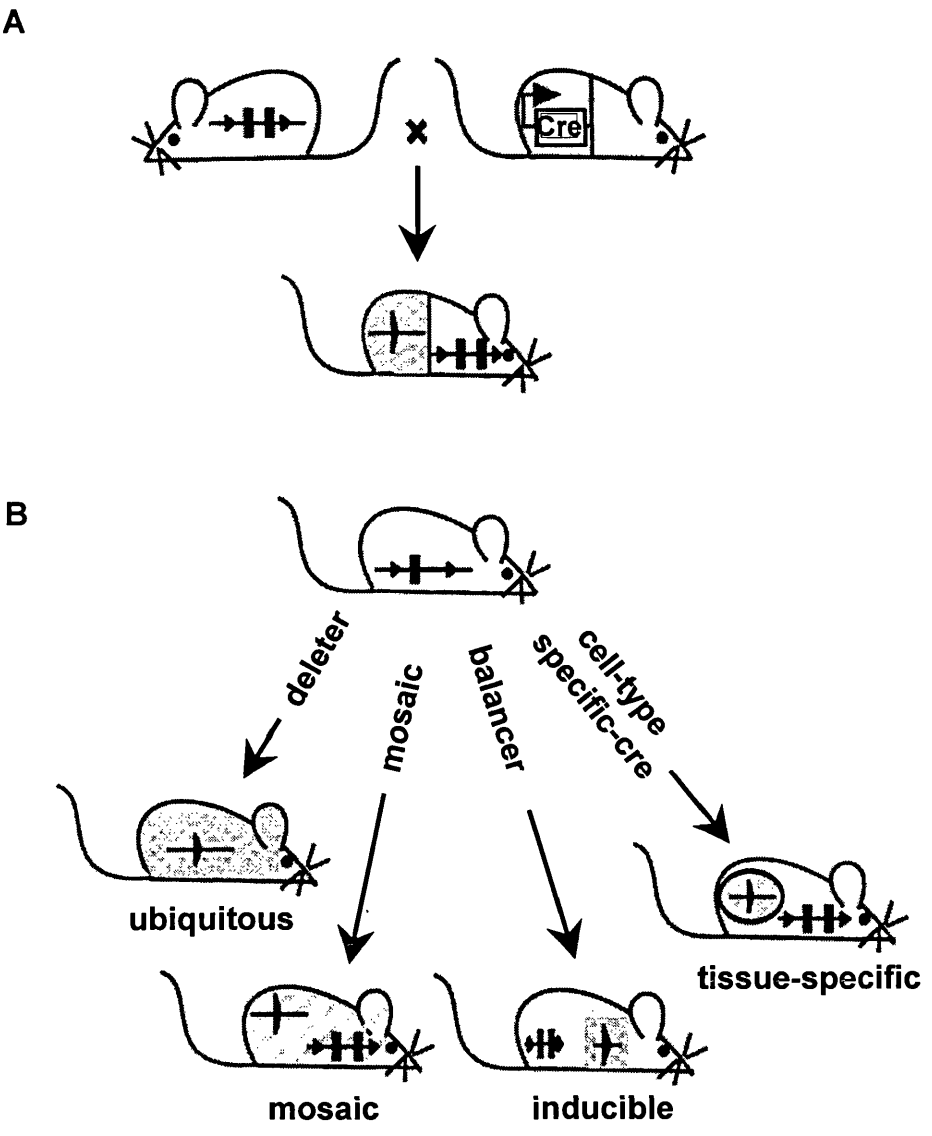



Figure 3.3 Conditional Gene Targeting with the Cre Zoo

of this widely expressed receptor leads to a lethal phenotype during embryonic development (Oshima et al., 1996). A more selective deletion of the receptor gene enables mice to develop to adulthood (see Results section, of this report), and allows for the generation of an altogether "cleaner" experimental model. Another important advantage of this technique is that it allows for removal of the selection marker, which may interfere with the expression of the targeted gene and its neighbours.

Clearly one of the most important components of a Cre/loxP mediated gene targeting experiment is the production of the cell type specific Cre-expressing transgenic mice. Several problems can arise due to inadequate levels of Cre expression in the studied tissue and/or due to uncontrolled expression of the molecule in non-specific tissues. The considerations which must be made when choosing a promoter for the expression of Cre and the principles behind the generation of a Cre expressing strain are described in detail in *Laboratory protocols for Conditional Gene Targeting* (by Torres M. and Kuhn R. Oxford University Press, 1998), and will not be discussed further in this review.

To date several Cre expressing mouse strains have been used, more or less successfully, for the conditional inactivation of genes in different cell types (for reviews see Rajewsky et al., 1996 and Sauer, 1998). Some of the Cre strains used in conditional gene targeting are shown in table 3.1 and in figure 3.2.B.

In addition to its use in conditional gene targeting the Cre/LoxP system has also been used in other experimental systems. A few examples of these are the creation of chromosomal rearrangements such as deletions and translocations (Qin et al., 1994; Smith et al., 1995); the creation of targeted alleles containing subtle mutations (Tores *et al.*, 1996); the insertion of transgenes in defined locations in the genome (Fukushige and Sauer, 1992) and the activation of transgenes in a tissue specific manner (Lakso et al., 1992; Orban et al., 1992).

| Promoter used to express Cre | Pattern of deletion | Efficiency of deletion | Reference |
|---|---|---|---|
| Zp3 | Oocytes | 100% | Lewandoski *et al.*, (1997) |
| αCamkII | Forebrain | Up to 98% | Tsien *et al.*, (1996) |
| Nestin | all organs | Variable but low | Betz *et al.*, (1996) |
| keratin | Dermis | High, close to 100% | (Tarutani et al., 1997) |
| lck | T cells | In $CD4^+$ cells,60-80%;100%in other T cells. | (Hennet et al., 1995) |
| CD19 | B cells | 90 to 95% in splenic B cells | (Rickert et al., 1997) |
| aP2 | Adipose tissue | Not estimated but incomplete | Barlow *et al.*, (1997) |
| G. elastase | Granulocytes | 75% in activated Granulocytes | J.Roes, personal comm. |

**Table 3.1 Examples of Cre Expressing Strains**

# EXPERIMENTAL PROCEDURES

## Chapter 4 Methods for Molecular Biology

### 4.1 DNA Agarose Gel Electrophoresis

Depending on the size of the DNA fragment to be visualised, agarose gels were prepared by dissolving 0.5-2.5% agarose (Gibco) in TBE buffer. Once the agarose had dissolved, ethidium bromide (Sigma) was added (0.5 g/ml). The solution was poured into a gel tray with a comb and allowed to set. The comb was removed and the gel placed in a gel electrophoresis tank (Gibco) containing TBE buffer. DNA samples were diluted in ficoll loading dye and loaded into the wells. The gel was electrophoresed at 100-150 volts and the DNA visualised on a UV transilluminator. Photos were taken using a Polaroid camera or Alpha Imager (Alpha Innotech Com.).

### 4.2 PCR Methods

#### 4.2.1 Standard PCR

For standard PCRs, (those where the product to be amplified was shorter than 2.5kb and/or no polymerase proof reading activity was required), Gibco Taq polymerase and PCR reagents were used. Oligonucleotide primer sequences were from Gibco, Genosys and MWG-Biotech. A Biometra thermal cycler was used. A typical PCR reaction contained template DNA, 20 pico-moles of each of the forward and reverse primers, PCR buffer, magnesium chloride (1.5-2mM), dNTPs (0.2mM) and Taq polymerase (2-3 units). The PCR program varied according to primers used, length of product to be amplified and template DNA. Generally a "hot start was used to increase the specificity of amplification. This would be followed by denaturation (at $94^0$C for 30 seconds), annealing (at a temperature approximately $3^0$C below the $T_m^*$ of the primers, for 30 seconds) and extension (at $74^0$ for 1 minute/kb of product amplified). The number of cycles used varied according to the application and the amount of template used.

*A rough estimate of the $T_m$ for a given primer was obtained by using the following equation:

$T_m = 4 \Sigma (G+C) + 2\Sigma (T+A)$

### 4.2.2 Long Template PCR

In cases where the length of the product to be amplified was longer than 2.5-3.0 kb, or where a proof-reading polymerase was required, PCRs were performed using Boehringer Manheim Expand-Long Template kits. These reactions were set up and run according to the manufacturer's specifications.

## 4.3 Gel Purification

DNA fragments to be purified, such as PCR products or subcloning products, were run on gel electrophoresis gels. The bands containing the required DNA fragments were excised using a short wave u.v. transilluminator and gel purified using QIAquick gel extraction kits by Qiagen.

## 4.4 Restriction Digestion

Restriction enzymes and buffers were from Boehringer, New England Biolabs, Gibco and Promega. For plasmid DNA, P1-clone DNA and PCR products the number of units used was generally four times the amount of DNA to be digested in g. For genomic DNA, the units of restriction enzymes used were ten times the amount of DNA in g.

## 4.5 Southern Blotting

The analysed DNA was digested with the appropriate enzyme (50 ng for P1-clone DNA, 7.5 g for genomic DNA Southerns). Digested DNA was gel-electrophoresed to achieve the appropriate fragment separation. The gel was then soaked in 0.25 M HCl for approximately 30 minutes (or for ten minutes after the gel loading dyes had changed colour) for DNA depurination. After a brief rinse in $ddH_20$ the gel was soaked for 30 minutes in 1.5 M NaCl/0.5 M NaOH, (for DNA denaturation). The gel was newly rinsed in $ddH_20$ and mounted on a capillary transfer set-up using a Hybond $N^+$ membrane (Amersham), as described by the membrane manufacturer. The transfer buffer used was 0.4M NaOH, for alkali transfer and fixation. Following overnight DNA transfer the membrane was rinsed in 2

x SSC to remove residual agarose. The membrane was stored between pieces of 3MM Whatman chromatography paper until required.

The membrane was prehybridised in a tube for 1 hour at 65$^0$C in 10ml of Church hybridisation buffer (1% BSA fraction V, 1 mM EDTA, 0.5 M $NaHPO_4$ pH 7.2) and 200 l of denatured salmon sperm DNA. DNA probes were labelled using a random priming labelling kit (Boehringer Manheim) and $\alpha^{32}$P dCTP (Amersham) according to the kit's manufacturer. Probes were passed through sephadex columns (for removal of unincorporated nucleotides), denatured at 100$^0$C for ten minutes, quenched on an ice-NaCl mix and added to the hybridisation tube. The probe was allowed to hybridise to the membrane overnight at 65$^0$C. The membrane was then washed as recommended by the manufacturer in progressively more stringent washing solutions: wash 1, 0.1% - 2 x SSC for ten minutes at room temperature; wash 2, 0.1% SDS – 1 x SSC for 15 minutes at 65$^0$C; wash 3 0.1% SDS – 0.5% SSC for 10 minutes at 65$^0$C. The washes were carried out until the membrane read 5-10 cpm with a Geiger counter.

The membrane was then rinsed in 2 x SSC, covered with Saran wrap and exposed to X-ray film (Kodak) overnight at -70$^0$C. Films were developed using a Velopex developer. When the membranes were to be re-probed, signal-stripping was carried out by placing them in a tray with a boiling solution of 0.5% SDS and allowing the solution to cool.

## 4.6 DNA Sequencing and Analysis

Automated DNA sequencing was performed using a PRISM sequenase terminator double stranded DNA sequencing kit. Samples were run on ABI 377 and 373 automated sequencers. Sequence analysis was carried out using GCG computer programs (University of Wisconsin).

## 4.7 DNA Cloning and Subcloning

Enzymes used for cloning and subcloning procedures are listed in table 4.1. All enzymes were used as recommended by the manufacturer.

| Cloning Procedure | Enzyme Used | Manufacturer |
|---|---|---|
| Ligation | T4 DNA Ligase | Boehringer Manheim |
| Blunt-Ending:<br>• Removal of 3' restriction fragment overhang.<br>• Filling in of 5' overhang | T4 DNA polymerase<br><br>Klenow Polymerase | Boehringer Manheim<br><br>Boehringer Manheim |
| Dephosphorylation | Shrimp alkaline phosphatase | UBI |

**Table 4.1 Enzymes Used for Subcloning**

### 4.7.1 Transformation of Competent Bacteria

Competent bacteria (*E.coli*, strain DH5α) were prepared by M.Chetty and A. Mcdermott in the laboratory. Aliquots of competent DH5α cells were defrosted on ice, gently mixed with the prepared DNA ligation mix and incubated on ice for 15 minutes. The cells were heat–shocked for two minutes at $42^0$ C, left on ice for ten minutes and added to 1 ml of 2YT medium (1 litre of 2YT medium were made by adding 16 g of bacto-tryptone, 10 g of bacto-yeast extract and 5 g of NaCl to 900 ml of $ddH_20$; the pH was adjusted to 7.0 and the solution made up to 1 litre and autoclaved). The mix was then shaken at $37^0$ C for 30 minutes. The cells were recovered by centrifugation at 3000 rpm for three minutes (Heraeus Megafuge). The pellet was resuspended in 200 l of 2YT and spread onto an agar plate containing the appropriate antibiotic. The plate was incubated inverted at $37^0$ C overnight.

## 4.8 Plasmid DNA Preps

Depending on the amount and purity of DNA required, plasmid mini-preps (approximately 5-10 g of DNA) or midi-preps (100-300 g highly pure DNA) were performed. The composition of the reagents used for plasmid preps is shown on table 4.2.

| Reagents | | Composition |
|---|---|---|
| A | High salt buffer: | 150ml 5M Kac (98.15)<br>28.75ml Acetic Acid<br>ad 250ml water |
| B | Glucose buffer: | 1.25ml Glucose stock (2M)<br>1.25ml 1M Tris (pH 8)<br>1.00 ml 0.5M EDTA (pH8)<br>ad 50ml water |
| C | Lysis buffer: | 5ml 2M NaOH (or equivalent amount)<br>5ml 10% SDS<br>ad 50ml water |
| D | RNAse A: | Sigma |

**Table 4.2 Reagents for Plasmid Preps**

## 4.9 Plasmid Mini-preps

Single colonies were picked from bacterial plates and used to inoculate 2ml of 2YT medium containing the appropriate antibiotic. Cultures were shaken overnight at $37^0$ C (225 rpm). Bacteria were harvested by centrifugation at 13.000 rpm for ten minutes (Heraeus Minifuge). Cell pellets were resuspended in 100 l of glucose buffer. 200 l of lysis buffer was added and the tubes were gently inverted three times. This was followed by addition of 150 l of high salt buffer. After mixing by inversion 400 l of phenol-chloroform were added. The tubes were then vortexed for ten seconds and centrifuged at 13.000 rpm for five minutes. The aqueous phase was transferred to a fresh tube containing 1 ml of ethanol (100%) and the DNA pelleted by centrifugation at 13.000 for ten minutes. The pellets were washed with 70% ethanol, air dried and dissolved in 30 l of ddH $_2$0, containing RNAse (20 g/ml, Sigma). After RNAse treatment (37 $^0$C for 30 minutes), the mini-preps were tested by restriction digestion.

## 4.10 Plasmid Midi-preps

100 ml of 2YT medium containing the appropriate antibiotic were inoculated with 2 l of mini-prep culture or with single colonies from bacterial plates. The culture was shaken

vigorously overnight at 37$^0$C and centrifuged at 3.200 rpm for 15 minutes (Heraeus Megafuge) in 50ml Falcon tubes. The bacterial pellet was resuspended in 4ml of glucose buffer. 10 ml of lysis buffer were added and the tube inverted three times. After incubation for 10 minutes on ice, 7.5 ml of high salt buffer were added and the tube was inverted and centrifuged as previously. The supernatant was filtered through a nylon stocking layer, mixed with 10 ml of propan-2-ol and again centrifuged. The pelleted DNA was resuspended in 2 ml of dd$H_20$ and 2 ml of lithium chloride solution. 30 minutes incubation on ice followed. The tube was then centrifuged and the supernatant phenol-chloroform extracted. The DNA was then ethanol precipitated, washed with 70% ethanol, dried for ten minutes and dissolved in 100 l of TE containing RNAse A (20 g/ml). After RNAse treatment the DNA was newly phenol-chloroform extracted, ethanol precipitated and resuspended in 100 l of TE buffer.

## 4.10 Generation of the TGF-ßRII$^n$ Targeting Vector

Details of the strategy used for the construction of the TGF-ßRII$^n$ Targeting Vector are outlined in section 8.2 of the Results and shown on figure 8.4. The oligoprimers used for the amplification of the targeting vector genomic components are listed in table 4.3.

| Name | Sequence | |
|---|---|---|
| | 5' | 3' |
| i2F.xho | ATA CTC GAG CAG AGA CTG GAG CAG CTG GA | |
| i2R.sal | TCA GTC GAC TCT GTG CAT TCC TTA GGA AGT G | |
| For amplification of Short Arm (SA) and insertion of *Xho1* and *Sal1* sites | | |
| i2F3'.xho | AGA CTC GAG TGC TTC TAT CCA GAG ACT GGT C | |
| FC3'r.sal | CAC GTC GAC ACC GTA TGC ATG AAG CAC CG | |
| For amplification of Long Arm (LA) and insertion of *Xho1* and *Sal1* sites | | |

**Table 4.3 Primers Used for Amplification of Targeting Vector Genomic Components, See Figure 8.4**

# Chapter 5 Methods for Tissue Culture

All tissue culture procedures were carried out in sterilised tissue culture hoods. Dishes and pipettes used were of tissue culture grade and were purchased from Falcon, Triple Red, Costar and Sterilin. Reagents used are listed on table 5.1.

| Reagents | Company/Cat. Number |
|---|---|
| Dulbecco's MEM | (Gibco/BRL; #41965-039) |
| Non-essential aminoacids,100x | (Gibco/BRL; #11140-035) |
| sodium pyruvate (100mM) | (Gibco/BRL; #11360-039) |
| 2-Mercaptoethanol | (Gibco/BRL; #31350-010) |
| L-Glutamin (200mM) | (Gibco/BRL; #25030-024) |
| Penicillin/Streptomycin | (Gibco/BRL; #15140-114) |
| Trypsin/EDTA,10x | (Gibco/BRL; #35400-027) |
| FCS ES Cell Tested | (Sigma; #F-7524) |
| Mitomycin C | (Sigma; #M-0503) |
| Methotrexate | (Sigma; #M-8407) |
| G418 Sulphate | (Roche-Boehringer; #1464981) |
| Ganciclovir | (Syntex; Cymeven) |
| Gelatin | (Sigma; #G1890) |
| Hepes-Buffer (1M, pH7.3) | (Gibco/BRL; 20 ml #15630-056) |
| DNAse I | (Sigma; #D5025) |

**Table 5.1 Reagents for Tissue Culture**

## 5.1 Cell Types Used

Embryonic stem (ES) cells used in this project were of the E14-1 strain (Kühn *et al*, 1991). Unless otherwise stated these were grown on feeder layers (layers of Mitomicin-C treated embryonic fibroblasts) and in the presence of LIF (leukaemia inhibitory factor) to maintain their undifferentiated state and their totipotency. Primary embryonic fibroblast (EF) cultures were prepared by J. Tkalchevic and D. Power in this laboratory. STO cells were kindly provided by S. Moss (UCL) and are from an immortalised embryonic fibroblast cell line. Both fibroblast cell lines are derived from transgenic 129/Sv mouse strains, which

express neomycin resistance genes. Solutions, media used and their compositions are listed in table 5.2. All cells were grown at $37^0$C, 5% $C0_2$ in humidified incubators and were passaged just before reaching confluency. When cells were passaged, the medium was aspirated and the culture was washed with 1 x PBS. The cells were then covered with trypsin and incubated at $37^0$C until they were seen to detach from the dish. The trypsin was quenched with an equal volume of medium and the cells pelleted by centrifugation at 1000 rpm (Heraeus Megafuge). The pellet was gently resuspended and the cells were plated at an appropriate density on the required dish.

| Solution / Medium | Composition |
|---|---|
| Mouse Tonicity PBS (mtPBS) | 16mM $Na_2HPO_4$ MW 141.96 2.27g/l<br>150mM NaCl MW 58.44 8.77g/l<br>made up in 1 L $ddH_20$ and autoclaved. |
| 10x Gelatin stock: | 1% in water, dissolved by autoclaving, stored at 4°C<br>-for use: warm up stock to RT or 37°C and dilute 1/10<br>with sterile water (autoclaved if not sterile). |
| Embryonic Fibroblast (EF) Medium: | Dulbecco's MEM<br>supplemented with 10%FCS<br>1x non-essential aminoacids<br>1mM sodium pyruvate<br>$10^{-4}$M 2-Mercaptoethanol<br>100i.E./ml Penicillin<br>100ug/ml Streptomycin<br>2mM Glutamin |
| ES cell medium: | Made up as EF medium but with 15% FCS, and<br>1 x LIF (leukaemia inhibitory factor) |
| Freezing Medium: | Supplement ES medium (or EF medium,<br>depending on cells to be frozen)<br>with 10 mM Hepes, 10% DMSO) |

**Table 5.2 Solutions and Media Used in Tissue Culture**

## 5.2 Preparation of Feeder Layers for ES cell Culture

Embryonic fibroblasts (EFs and STO cells) were grown on 15 cm dishes in EF medium. When needed for the preparation of feeder plates for ES cells, EF cells were mitomycin-C (MMC) treated. MMC was added to a final concentration of 10 g/ml of medium and the cells incubated for 3-4 hours at $37^0$C, 5% $C0_2$. The treated cells were washed three times in mPBS and plated at 1.5 x $10^4$ cells / $cm^2$ onto the desired gelatin coated dish (0.1 % gelatin solution).

## 5.3 ES cell Transfection

ES cells were grown as described on 6 cm gelatin coated feeder plates. Two days before transfection cells were plated at 2 x $10^6$ per 6 cm dish (twice the normal density) so that they would reach log phase on the day of transfection. Cells were transfected by electroporation using a BioRad Gene Pulser. After trypsinisation, cells were resuspended at $10^7$ / ml of transfection medium (ES medium supplemented with 10mM Hepes pH7) and mixed with the appropriate DNA. 0.8 ml of the cell solution was transferred to an electroporation cuvette (0.4mm, BioRad) and electroporated at 0.625V /cm, with a 500 F capacitance. The cuvette was left at room temperature for 10 minutes. The cells were then diluted and plated at various densities (no more than 4 x $10^6$ / 10 cm feeder plate). Two days after transfection selection was started by complementing the medium with G-418 (175 g/ml of medium). On the fifth day after transfection double selection was applied by adding gancyclovir to the transfection plates (2 x $10^{-6}$ / ml of medium). The medium was changed every three days.

## 5.4 Colony Screening

Resistant colonies were picked 10 days after transfection. The transfection plates were washed once in mPBS and covered with a fresh layer of mPBS. A sterile gilson was used to gently remove and take up single colonies from the feeder layer in 30 ml of mPBS. These were added to individual drops of trypsin (50 l) in a 96 well plate and trypsinized for a maximum of 10 minutes. Each colony was then quenched by addition of two drops of ES medium and resuspended. Half the volume was plated into a 48 well feeder plate and the remainder placed into a PCR tube. Pools of 4 colonies were collected in each PCR tube.

Once collection was over, the PCR tubes were spun down (110g for five minutes), washed once in mPBS, resuspended in 5 l of mPBS and frozen.

The PCR screening was performed by adding 20 l of 1 x PCR buffer including 200 g/ml Proteinase K to the cells. The primers that were used are listed in table 5.3 (also see PCR 1, figures 8.5 and 9.1). The mix was overlaid with mineral oil (sigma) and incubated at $55^0$ C for 30 minutes. The proteinase K was inactivated by heating at $90^0$ C for 10 minutes and the reaction held at $80^0$ C for a "hot start" PCR. The PCR mix was added as previously established with the positive control vector. Positive pools were passaged and rescreened individually. Homologous recombinant single colonies were passaged, frozen and analysed by Southern blotting.

| Name | Sequence | Function |
|---|---|---|
| | 5' 3' | |
| e2f | CAT GAA GTC TGC GTG GCC GTG TG | |
| neo-r | GCG TGC AAT CCA TCT TGT TCA ATG G | PCR-1 |

**Table 5.3 Primers Used for Screening of Homologous Recombinants, PCR-1, See Figure 9.1 for Primer Localisation**

## 5.5 Isolation of G418 Sensitive Clones by Duplicate Selection

After transient transfection with the *cre* expression vector, clones were screened to test if they had lost the neomycin resistance gene. Colonies were isolated as above except that the cells were plated in duplicate on 48 well feeder plates. Selection with G418 (200 g/ml active) was started on one of the plates once cells had settled. Clones that were sensitive to G418 were passaged from the duplicate plate and the deletion event was characterized by PCR and Southern blotting (see figure 9.1).

| Name | Sequence | Function |
|---|---|---|
| | 5' 3' | |
| e2f | CAT GAA GTC TGC GTG GCC GTG TG | |
| pBSlox-r | AGC TTG GCT GGA CGA AAC TCC TCT T | PCR-2 |
| e2f | CAT GAA GTC TGC GTG GCC GTG TG | |
| e3r | TGT AAT CGT TGC ACT CTT CCA TGT | PCR-3 |
| i23'-f | CTG CTT CTA TCC AGA GAC TGG TCA | |
| pBSlox-r | AGC TTG GCT GGA CGA AAC TCC TCT T | PCR-4 |

**Table 5.4 Primers Used for Characterisation of Cre-recombined Clones, See Figure 9.1 for Primer Localisation**

## 5.6 Extraction of Genomic DNA from ES Cells

Genomic DNA was extracted from ES cells using the Miller *et al* method (1988). ES cells were grown on gelatin plates to avoid feeder cell DNA contamination. Cells were trypsinised as usual and resuspended in 3 ml of nuclei lysis buffer (10mM Tris-HCl, 400mM NaCl, 2 nM $Na_2EDTA$, pH 8.2) supplemented with 200 l of proteinase K (10 mg/ml) and 60 l of a 10% solution of SDS. Samples were shaken for three hours at 55 $^0$ C. One ml of saturated NaCl solution was added and the tube shaken for 15 seconds and centrifuged at 3200 RPM for 10 minutes. 2 volumes of ethanol were added to the supernatant in a new tube. The fresh tube was inverted until DNA had precipitated. The DNA was then taken up around a yellow pipette tip, passed through 70% ethanol and dissolved in 200 l of TE buffer (pH 8). The DNA was finally phenol-chloroform extracted, ethanol precipitated and redissolved in TE buffer.

# Chapter 6 Production of Mutant Mice

Correctly targeted and recombined ES cell clones were used in the production of chimeric mice using the morula aggregation technique. All animal procedures outlined in this project were carried out under Home Office Licence. Except for those used for morulae harvest, all animals were kept under SPF conditions.

## 6.1 Morula Aggregation

### 6.1.1 Preparation of ES cells for Aggregation

ES cells were cultured under standard conditions as previously described. The cells were passaged twice before use, on gelatin plates (with no feeder cells). Just before aggregation the cells were trypsinized and resuspended only once to keep lumps of 5-10 cells in suspension.

### 6.1.2 Superovulation

CD1 females at 4-8 weeks of age were injected i.p. with 5 units of gonadotropin (intervet UK) at noon and 2 days later with 5 units of chorionic gonadotropin (intervet UK) at 10 a.m. On the same day mice were mated with CD1 males (6-8 weeks of age) at 5 p.m. The following morning (day 0.5 after plug) females were plug-checked. On day 2.5 females were sacrificed and their uteri isolated for morulae harvest.

The optimum age group for morulae production in female CD1 mice was tested. Three different age groups were superovulated and the average amount of morulae recovered was counted. The results are summarised in table 6.1. 8-week-old females were found to yield the highest number of morulae, with an average of approximately 15 morulae per mouse.

| Age | 4wks | 6wks | 8wks |
|---|---|---|---|
| Morulae/mouse | 8.5 | 8.5 | 15 |

**Table 6.1 Superovulation of female CD1 mice, recovery of morulae**

### 6.1.3 Preparation of Morulae for Aggregation

The uteri were flushed with a 25 gage syringe and M2 medium (Sigma). Morulae were collected in M2 medium. Aggregation plates and zona pellucida removal plates were then prepared. For aggregation plates, a 10cm tissue culture sterile dish was used. 25 aggregation wells were bored along five rows (five wells per row), using a blunt needle. These wells were covered with individual drops of equilibrated M16 medium and the dish filled with sterile embryo-tested mineral oil (Sigma). To remove the zonae pellucidae, morulae were passed from a drop of M2 medium, through 3 drops of tyrode's acid. Immediately after their zonae pellucidas had been dissolved, the morulae were passed through 2 drops of M2 and 1 drop of equilibrated modified M16 medium. The stripped morulae were rapidly transferred into aggregation wells.

### 6.1.4 Aggregation with ES Cells

Using a mouth pipette clumps of approximately 5-10 ES cells were placed on top of each individual morula. The aggregation plates were incubated overnight at 37°,10% $CO^2$.

## 6.2 Collection of Blastocysts and Transfer into Foster Mother

The next morning blastocysts were collected from the aggregation wells. Approximately 20 blastocysts were transferred into the uterus of a 2.5 day pseudopregnant

foster mother in sterile hoods. Fosters were C57/Bl6 x balb/c (at 6-8 weeks) and were mated with sterile males under SPF conditions.

## 6.3 Mouse Breeding

Chimeras were bred with Balb/C mice to test for germline transmission of the TGF-ßRII$^{fl}$ mutation. Offspring were tail-snipped to obtain DNA which was used as template for a screening PCR. Mice heterozygous for the targeted allele (TGF-ßRII$^{fl/+}$) were interbred to give rise to homozygous progeny. Again, homozygous mice were identified by PCR. Mice bearing the *TGF-ßRII$^{fl}$* allele in a homozygous manner were back-crossed into *CD19$^{cre}$* and *GE$^{cre}$* backgrounds in order to obtain B cell (TGF-ßRII$^{fl\fl}$-CD19$^{cre/+}$) and granulocyte (TGF-ßRII$^{fl\fl}$-GE$^{cre/+}$) specific murine models of TGF-ßRII deficiency. The genotyping PCRs for both models are outlined in figure 10.1. The oligo-primers used for genotyping of the TGF-ßRII$^{fl\fl}$-GE$^{cre/+}$ have previously been described, whereas those used for the TGF-ßRII$^{fl\fl}$-CD19$^{cre/+}$ locus are listed in table 6.2.

| Name | Sequence | Function |
|---|---|---|
| | 5' 3' | |
| e2f | CAT GAA GTC TGC GTG GCC GTG TG | TGF-ßRII$^{fl}$ |
| e3r | TGT AAT CGT TGC ACT CTT CCA TGT | locus PCR |
| | | |
| CD19e1f | CCA GAA GTC CTT ACT GGT GGA GGT | CD19$^{cre}$ |
| Cre1x | TTT GGT GCA CGG TCA GCA GAT TGG | locus PCR |
| | | |
| CD19 e1f | CCA GAA GTC CTT ACT GGT GGA GGT | CD19 wt |
| CD19.15 | GTC CTT GAA AGG GGG CCT CTT CTG GC | locus PCR |

**Table 6.2 Primers Used for Genotyping in the TGF-ßRII$^{fl\fl}$-CD19$^{cre/+}$ model**

# Chapter 7 Immunological Analysis

## 7.1 Cytometry

### 7.1.1 Antibodies Used for Analysis of Mutant Mice

The antibodies which were used in immunophenotyping assays in this project are outlined in table 7.1.

| Antigen | Antibody | Company |
|---|---|---|
| Mouse Ig | Polyclonal | Pharmingen |
| Rabbit Ig | Goat | Southern Biotech. |
| IgM | R33-24-12 | |
| IgD | Rabbit-α-mouse IgD | Nordic, NL |
| IgA | Goat α-mouse | |
| IgG1 | G1-6.5 | Pharmingen |
| CD45R/B220 | RA3-6B2 | Pharmingen |
| IA | M5/114 | |
| Thy1.2 | 53-2.1 | Pharmingen |
| CD4 | GK1.5 | |
| CD8 | 53-6.7 | |
| GR1 | RB6-8C5/1 | |
| CD11B | M1-70 | Pharmingen |
| BrdU | | Becton Dickson |
| TGF-ßRII | Rabb. Polyclonal | Upstate Biotech. |
| TGF-ßRII | H-567 | Santacruz |

**Table 7.1 Antibodies Used for Flow Cytometry**

### 7.1.2 Preparation of Leukocyte Cell Suspensions

Bone marrow cell suspension were obtained by flushing femurs with PBS-0.5%BSA-0.01% Sodium Azide. The suspensions were filtered through 60 m nylon mesh. Bone marrow cells were then washed once and incubated for ten minutes at $4^0$ C with blocking agents (e.g. 10% rat, rabbit or goat serum) before antibody staining.

Splenocyte single cell suspensions were prepared by gently rupturing spleens between two microscope slides, in PBS-0.5% BSA-0.01% sodium azide. Red blood cells were then eliminated by addition of erythrocyte quick lysis buffer (155 mM $NH_4Cl$, 0.1 mM EDTA, 10mM $KHCO_3$) and the supernatant filtered through a 60 m nylon mesh. The spleen cells were then washed once and incubated with blocking agents as was done with bone marrow cells, prior to staining.

Peritoneal-cavity leukocyte cell-suspensions were obtained by flushing mouse peritoneums with 8 ml of PBS-0.5% BSA-0.01% sodium azide. The exudate was spun down and the cell pellet washed once, before resuspension in blocking agents, as described above.

### 7.1.3 Cell-surface Antibody Staining for Flow Cytometry

Single cell suspension were aliquoted into multiwell plates (1 x $10^6$ cells x staining). The leukocytes were spun down (350g, for 5 minutes at $4^0$ C) and resuspended in 20 l of a 1x antibody staining mix. After a 30 minute incubation on ice in the dark cells were washed twice and, if required, resuspended in 20 l of the secondary antibody staining mix. Prior to flow cytometric analysis (see section 7.1.5) cells were again washed twice and resuspended in 200 l of PBS-0.5% BSA-0.01% sodium azide.

### 7.1.4 Intracellular Staining

For intracellular staining, leukocyte single-cell suspensions were prepared and depleted of leukocytes (as described above), washed once in mtPBS and fixed in freshly prepared cold 4% paraformaldehyde fix solution (4g paraformaldehyde in 100 ml mtPBS pH 7.4) for 10 minutes at $4^0$ C with occasional agitation (0.5ml of fix solution for 1 x $10^6$ cells). Cells were then washed in PBS, 0.5% BSA, 0.01% sodium azide. After permeabilization in PBS, 0.5% BSA, 0.01% sodium azide, 0.5% saponin (Sigma, S 2149), cells were aliquoted

into multiwell plates (1 x $10^6$ per staining), stained with the appropriate antibodies made up in the same saponin buffer and washed three times with PBS, 0.5% BSA, 0.01% sodium azide. Flow cytometric analysis was carried out as described below.

### 7.1.5 Flow cytometric Analysis

For flow cytometry, all stained leukocytes were resuspended in 200 l of PBS-0.5% BSA-0.01% sodium azide. Cells were analysed with a FACScan (Beckton Dickson). The acquired data were analysed using Cell Quest software (Beckton Dickson).

## 7.2 Analysis of Leukocyte Proliferation Dynamics

B cell proliferation dynamics were assessed by a method adapted from Förster and Rajewsky, (1990). B cell specific conditional mutant (TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$), flox control (TGF-ßRII$^{fl\backslash fl}$-CD19$^{+/+}$), cre control (TGF-ßRII$^{+\backslash +}$-CD19$^{cre/+}$) and wild type mice were given drinking water containing bromo-2'-deoxyuridine (BrdU, Sigma, B5002) at 1mg/ml for 15 days. The mice were then sacrificed and single-cell suspensions prepared and depleted of erythrocytes using standard procedures (see above section 7.1.2). 3 x $10^6$ cells were first stained on the surface with biotin-RA36B2 (anti-B220, spleen and bone marrow) or biotin-R33-24 (anti- , peritoneal cavity cells) antibodies. After fixation in 70% (vol/vol) ice-cold ethanol, cells were washed and resuspended in PBS and an equal volume of PBS, 2% formaldehyde, 0.02 % Tween 20 was added. Cells were left at $4^0$ C overnight, washed in PBS and resuspended in 1ml of 150mM NaCl, 5mM $MgCl_2$ , 10 M HCl supplemented with 300 g/ml DNAse 1 (Sigma D 5025) for ten minutes at room temperature. Cells were then washed with PBS, 0.5% BSA, 0.01% sodium azide and stained with FITC conjugated anti-BrdU antibody (Becton Dickinson). After a final wash in PBS, 0.5% BSA, 0.01% cells were resuspended and analysed by flow cytometry as described in section 7.1.5.

## 7.3 Isolation of B cells

Selection of B lymphocytes was accomplished by magnetic activated cell sorting (MACS; Miltenyi Corp., UK). B cells (B220$^+$,IgM$^+$) were enriched by positive selection using MACS midi-columns according to the manufacturers instructions.

## 7.4 Preparation of Tissues for Pathology

Pathological examination of organs from the B cell specific TGF-ßRII deficient model was carried out by Marco Novelli (UCL). Control and TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ spleens, hearts, livers, lungs, kidneys, brains, skin and intestines placed in formalin (30 mM $NaH_2PO_4$, 45 mM $Na_2HPO_4$, and 4% formaldehyde) immediately after isolation. Intestines were cut open laterally, washed in mtPBS, rolled onto 3MM paper and immersed in formalin. Kidneys and liver were cut in half longitudinally, whereas lungs were gently inflated with formalin, before preservation.

# RESULTS

## Chapter 8 Isolation of the TGF-ßRII Gene and Construction of the Targeting Vector

For cell-type specific abrogation of TGF-ß signalling, we decided to carry out conditional gene targeting of the TGF-ßRII, which is essential for transduction of this cytokine's signals to the nucleus. We set about to construct a Cre-LoxP "flox and delete" style-targeting vector (see section 3.3 of this report and *Laboratory Protocols for Conditional Gene Targeting.* R. M. Torres and R. Kühn, Oxford, 1997). As this style of vector requires an extensive knowledge of the genomic structure of the locus of interest, we first cloned and characterised the murine TGF-ßRII gene.

### 8.1 Cloning and Mapping the TGF-ßRII Locus

The only available information on the murine *TGF-ßRII* locus was the cDNA sequence (Lawler et al., 1994). As the murine cDNA sequence is highly homologous to its human counterpart (cytoplasmic domain: 96%; extracellular domain: 86%), the intron–exon boundaries were deduced from the human sequence (Takenoshita et al., 1996) and later confirmed by sequencing.

Oligoprimers within the cDNA sequence encoding the TGF-ßRII transmembrane domain were used to amplify a 150 b.p. product, "probeA" (shown in figure 8.3). This probe was radiolabelled and used to screen a Bacteriophage P1-129/Sv genomic library (Genome Systems, St. Louis, MO, U.S.A.). One P1 clone, (clone P1-TRII) resulted positive for hybridisation to probe A.

To test if the P1-TRII clone contained the entire sequence for the *TGF-ßRII* gene, PCR primers chosen within exons at both ends of the cDNA sequence, were used to amplify up the 5' and 3' ends of the *TGF-ßRII* locus, from genomic and P1 clone DNA templates

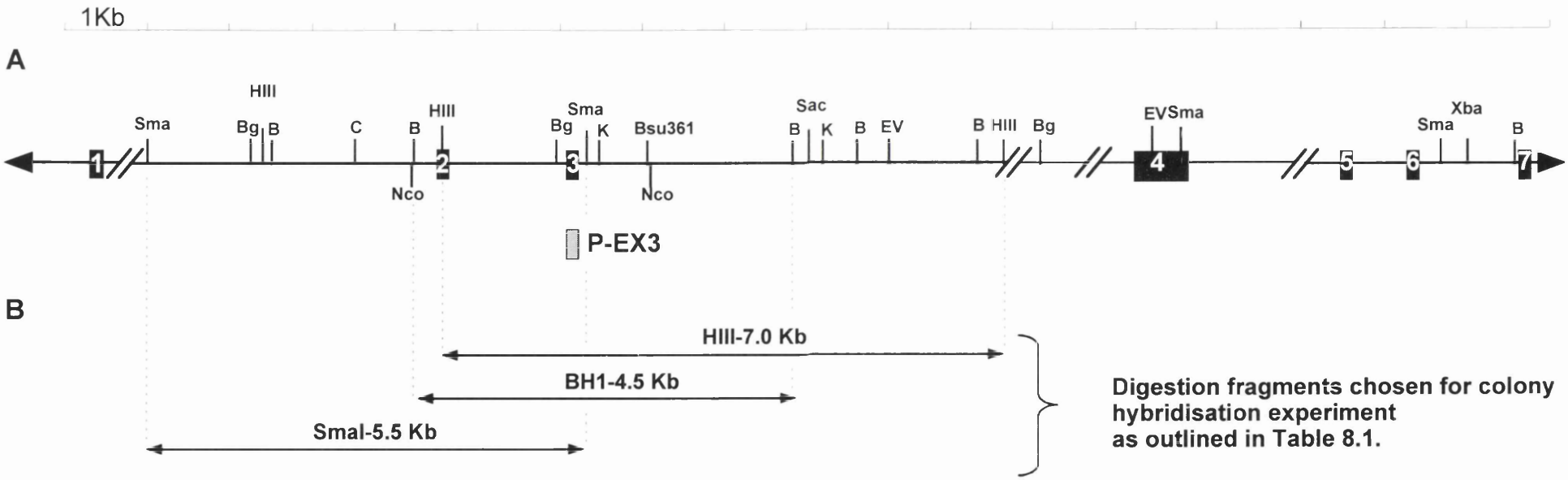
(data not shown). To map the position of important restriction sites on the *TGF-ßRII* locus, a series of restriction digests of genomic 129/Sv and P1-TRII DNA were carried out. The digests were run on an electrophoresis gel and Southern blotting was carried out using TGF-ßRII cDNA probe (see figure 8.1). To subclone and further characterise the *TGF-ßRII* locus, a colony hybridisation experiment was performed (figure 8.1 and table 8.1). The colony screening resulted in several clones positive for hybridisation to TGF-ßRII cDNA probes. These colonies were picked and plasmid DNA was prepared for further analysis. Two overlapping clones, Clone-FC, containing a 5.5 kb *SmaI* fragment and Clone-FD, containing a 7.0 kb *HindIII* fragment, (figures 8.1 and 8.2) were isolated. Once confirmed as true positives by restriction digestion and sequencing analysis, Clones -FC and -FD were mapped by double-digestion experiments. Figure 8.2 shows a representative mapping experiment. The information obtained from Southern analysis, restriction digestion and sequencing was used to create the map of the *TGF-ßRII* locus outlined in figure 8.3.

As predicted, the murine *TGF-ßRII* gene has a genomic structure similar to that of its human homolog, with 7 exons spanning a region of more than 20 kb on mouse chromosome 9. Its high similarity to the human gene and high degree of evolutionary conservation reflect its importance in mammalian development and function.

## 8.2 Generation of the TGF-ßRII$^{fl}$ Targeting Vector

The sequence information obtained was used to construct a targeting vector in which the 3$^{rd}$ exon of the *TGF-ßRII* gene was flanked by lox P sites (*TGF-ßRII$^{fl}$*, the reasons why exon 3 was chosen for floxing are outlined in figure 9.3 and in the Discussion, section 12.4). The targeting vector construction strategy is outlined in figures 8.4 and is described below:

**A**, The short arm of homology (SA) was amplified with primers I2.5'F$^{xho}$ and I2.3'R$^{xal}$. The 1.2 kb product spans the region of intron 2, from 180 bp downstream of exon 2 to 90 bp upstream of exon 3. Primers I2.3'F$^{xho}$ (75 bp upstream of exon 3) and I3.3'R$^{xal}$ (5.4 kb downstream of exon 3) were used to amplify the long arm of homology (LA, 5.5k b).



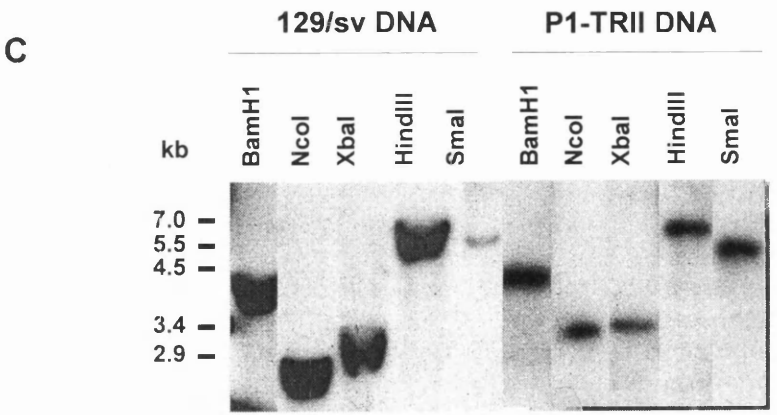
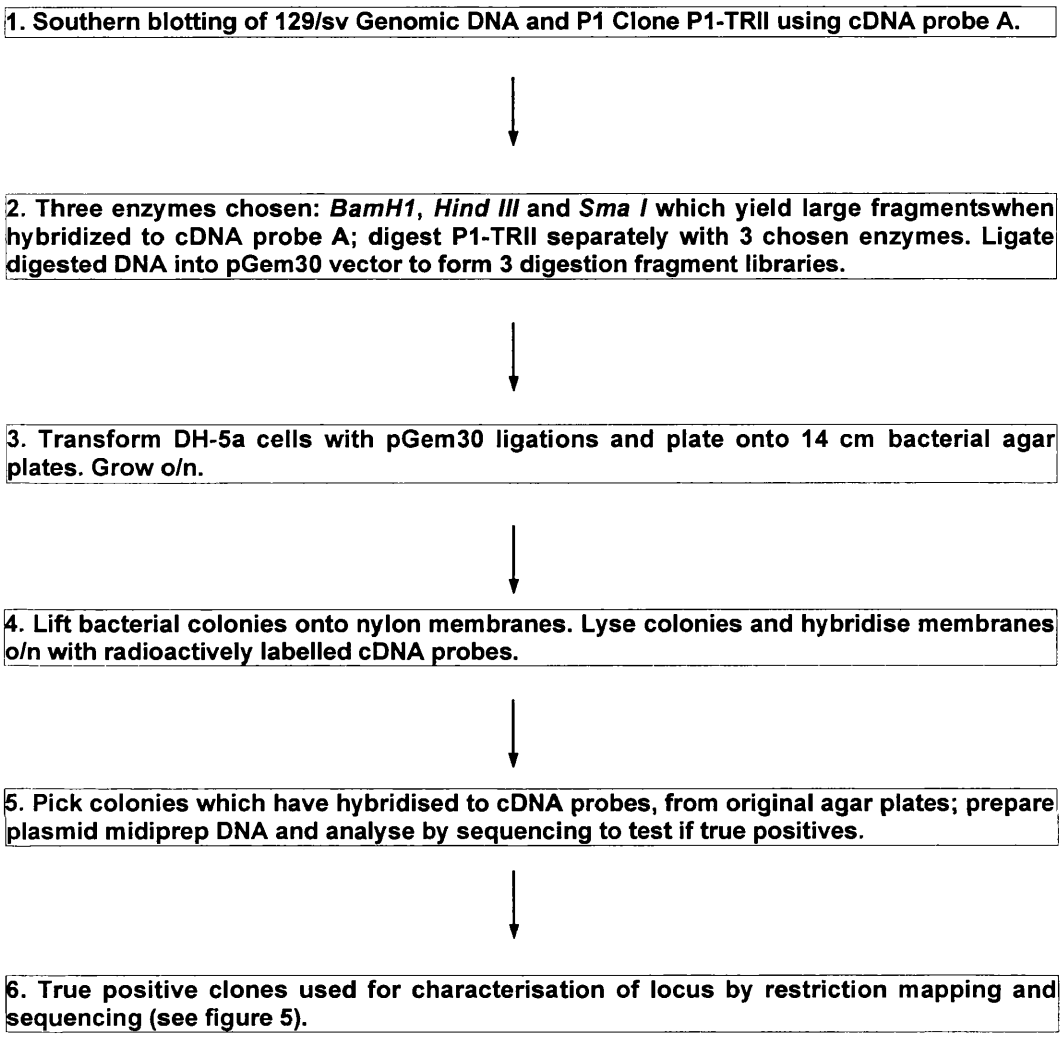




**Figure 8.1 Southern Blot Analysis of P1-TRII DNA** DNA from genomic 129/sv ES cells and from the P1 clone containing the TGF-ßRII locus (P1-TRII, as determined from PCR analysis using cDNA oligonucleotides ), was digested with various rare cutting restriction enzymes. **C**, Southern blot of 129/sv and P1-TRII DNA digested with commonly used restriction endonucleases and probed with cDNA probe P-EX3 (see A). To clone and further characterise the TGF ßRII locus, fragments generated by HindIII, SmaI and BamH1 and identified by Southern blotting (**B**), were chosen for cloning into pGEM-30, and subsequent isolation, by colony hybridisation experiments (see Table 8.1). Their position relative to the TGF-ßRII genomic locus (**A**) is shown.




## Table 8.1 Outline of Colony Hybridisation Experiment

Several true positives were cloned using this technique. Two of these, Clone-F.C and Clone-F.D were used in the construction of a more detailed map of the locus, as shown in figure 5.

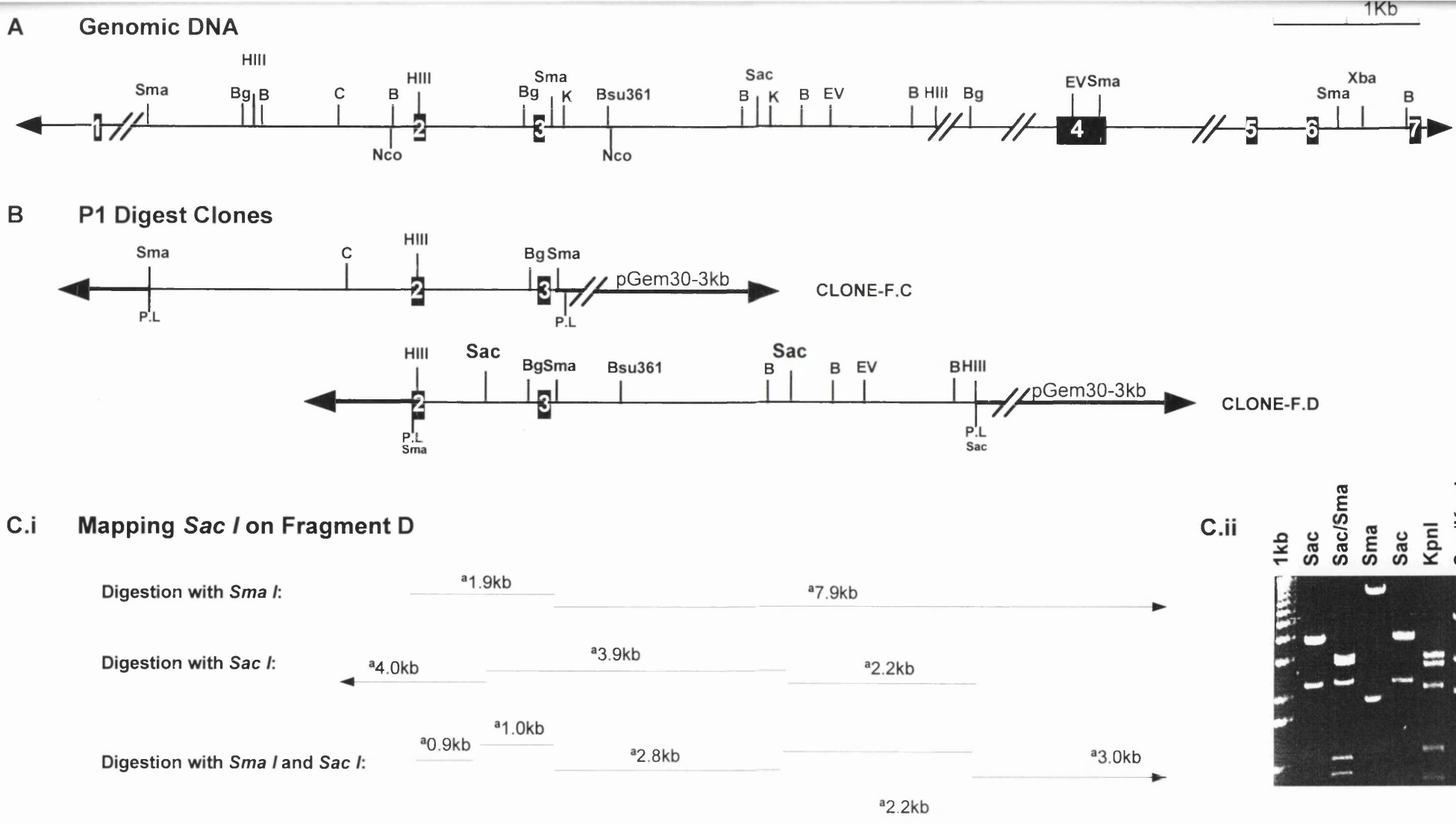



**Figure 8.2 Mapping the TGF-ßRII Locus** **A**,Genomic Structure of theTGF-ßRII Locus as determined by Southern blotting, restriction digestion and sequencing experiments The position of some important restriction sites are shown. **B**, Overlapping fragments isolated as Clone-F.C and Clone-F.D in pGEM30, from P1-TRII colony hybridizatior experiment (see fig.4) **C**, Representative restriction enzyme mapping experiment: the position of *SmaI* and *KpnI* was obtained by Southern digestion of P1-TRII, (see fig 4) the position of *SacI* was deduced by standard double digestion of Clone-F.D with *SmaI* (**C(i)**) and confirmed by double digestion with *KpnI*. **C(i)**, sizes of restriction fragments generated by digestion of Clone-F.D with *SmaI* only, *SacI* only and *SmaI* and *SacI*. **3(ii)**gel electrophoresis of Clone-F.D digested DNA. **P.L.**, pGem30 Polylinker; C,*ClaI* B,*BamH1*; EV, *EcoRV*; Bg, *BglII*; K, *KpnI*; ■ ,Exons; —— 129/Sv genomic DNA; —— ,pGem30 plasmid DNA; 1kb, Gibco 1kb DNA ladder.

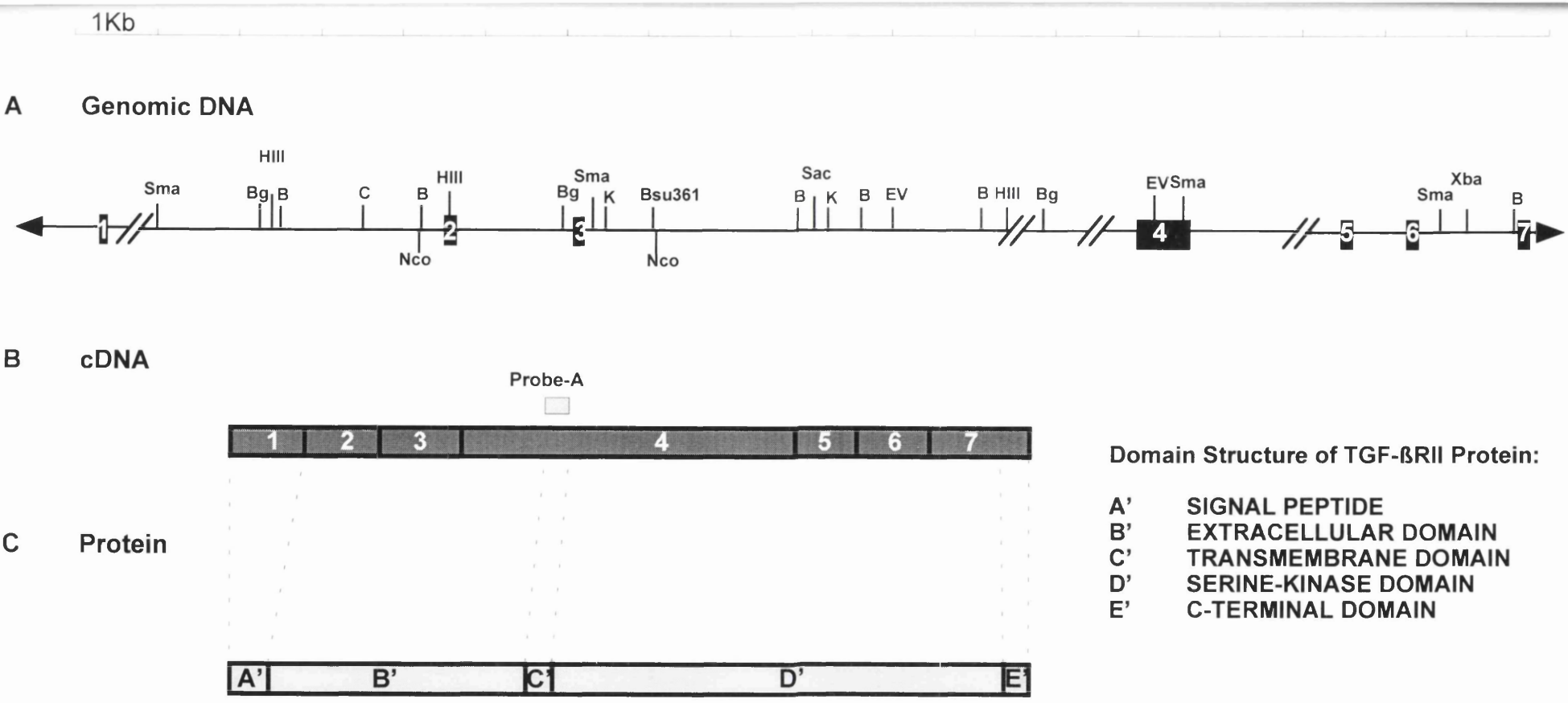



**Figure 8.3 Structure of the Murine TGF-ßRII Genomic DNA, cDNA and Protein** **A.** The murineTGFßRII gene is divided into 7 exons spanning a region of more than 20kb. It is situated on mouse chrom.9. **B.** The cDNA is 1704 bp long. Probe-A, within the transmembrane domain was used to screen a P1-129/Sv library (see methods). **C.** The open reading frame encodes a 567 amino acid (aa) polypeptide. The N-terminal signal peptide (**A'**) is cleaved following residue 23. The mature extreacellular ligand-binding domain (**B'**) contains 12 cysteines and 2 potential N-glycosylation sites, and is encoded by exons 2, 3 and part of exon 4. The extracellular domain (**C'**, aa 160-189) is highly hydrophobic and divides the protein into a 160 aa extracellular compartment and a 378 aa cytoplasmic domain (**D'** and **E'**). The serine-kinase domain (**D'**) has kinase specificity for serine and threonine.

**B**, The LA was blunt-ended, concatamerised and cut with *Bsu361* to generate components LA-1 and LA-2.

**C**, The *Bsu361* sites were blunt-ended and the *XhoI* and *SalI* sites reconstituted. At this point, the section of the *TGF-ßRII* locus to be used in the construction of the targeting vector was ready for subcloning in three DNA fragments: SA, LA-1 and LA-2.

**D**, The tagged *XhoI* (at the 5' end) and *SalI* (at the 3' end) sites in SA allowed for direct insertion into *XhoI* cut pL2Xneo to generate pl2Xneo-SA.

**E**, LA-1 was cloned as a *XhoI-Blunt* fragment into *SalI-SmaI* cut pl2Xneo-SA (pl2Xneo-SA-LA1).

**F**, LA-2 was cloned as a *Blunt-SalI* fragment into *EcoRV-SalI* cut pBSloxP1JD generating pBSloxP-LA2.

**G**, The *XhoI-SalI* fragment from pBSloxP-LA2, incorporating the LA-2 with a loxP sequence at the 5' end, was inserted into *XhoI* cut pIC19RTK. The resulting plasmid, pIC19RTK-LoxP-LA2 was cut with *XhoI*, blunt ended and religated, to remove the *XhoI* site.

**H**, pIC19RTK-LoxP-LA2 was digested with *ClaI* and *HindIII* and cloned into *ClaI –HindIII* cut pl2Xneo-SA-LA1, giving rise to the full length targeting vector.

The targeting vector is approximately 10 kb long. Its short and long arms of homology (6.7 kb) allow for homologous recombination to the endogenous *TGF-ßRII* locus. The construct incorporates a neomycin resistance gene (*neor*) flanked by *loxP* sites, allowing for selection of ES cell transfectants and subsequent deletion of the resistance marker in homologous recombinants, using the Cre/loxP system. The herpes symplex virus-thymidine kinase gene (*HSV-tk*) positioned at the 5' end of the long arm of homology allows for negative selection against random integration (see *Gene Targeting, A Practical Approach*, edited by A.L. Joyner; IRL, Oxford University Press, 1993).

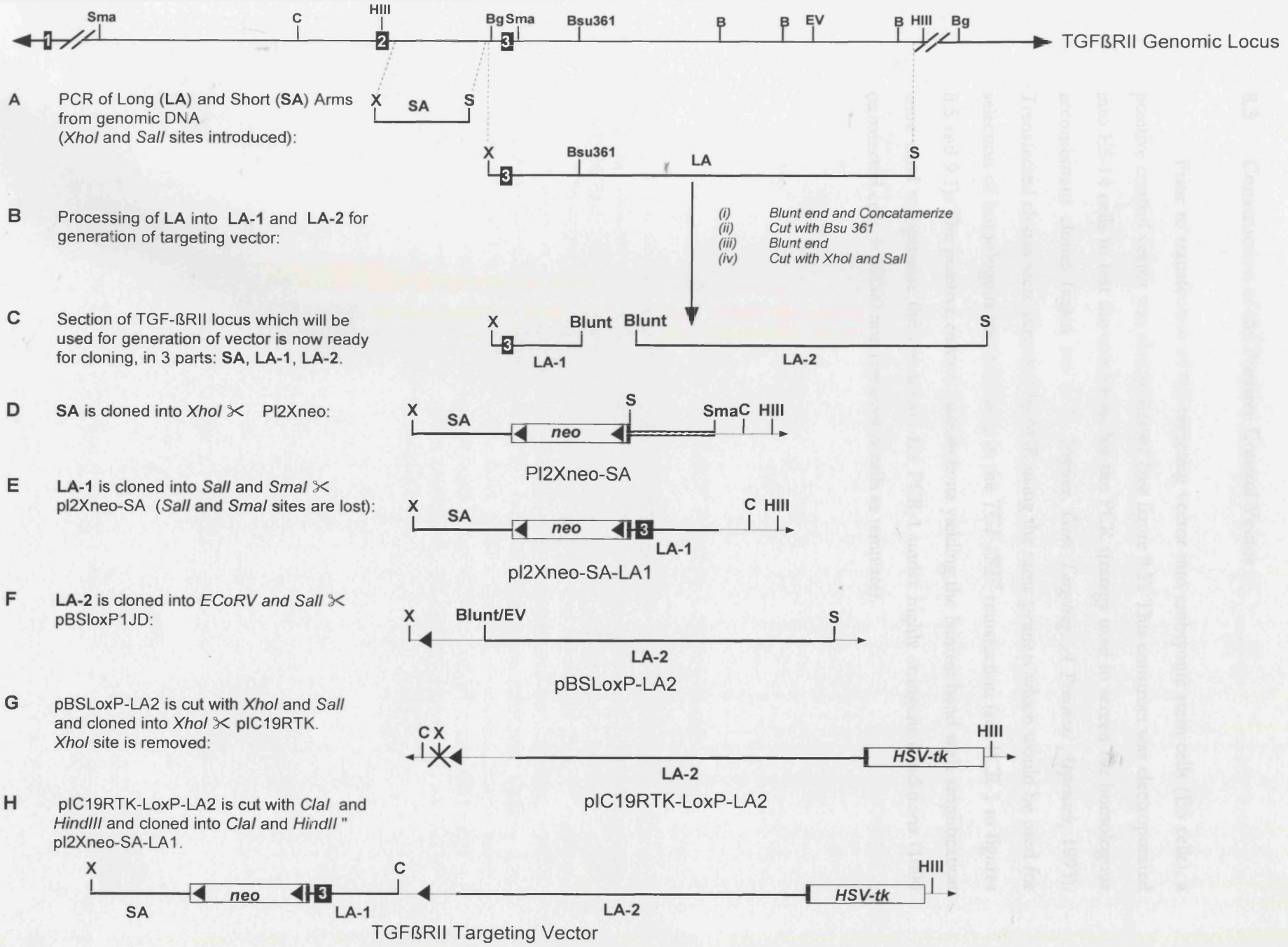



**Figure 8.4 Generation of TGFßRII$^{fl}$ Targeting Vector** (See text for legend)

## 8.3 Construction of the Positive Control Vector

Prior to transfection of the targeting vector into embryonic stem cells (ES cells), a positive control vector was also generated (see figure 8.5). This construct was electroporated into ES-14 cells to test the conditions for the PCR strategy used to screen for homologous recombinant clones (again see A. L. Joyner, *Gene Targeting, A Practical Approach*, 1993). Transfected clones were screened by PCR using the same primers which would be used for selection of homologous recombinants in the *TGF-ßRII*$^{fl}$ transfection (see PCR-1 in figures 8.5 and 9.1). The positive control transfectants yielding the faintest band with amplification were used to optimise the conditions for PCR-1 under highly stringent conditions (1000 transfected cells + 50000 non transfected cells as template).

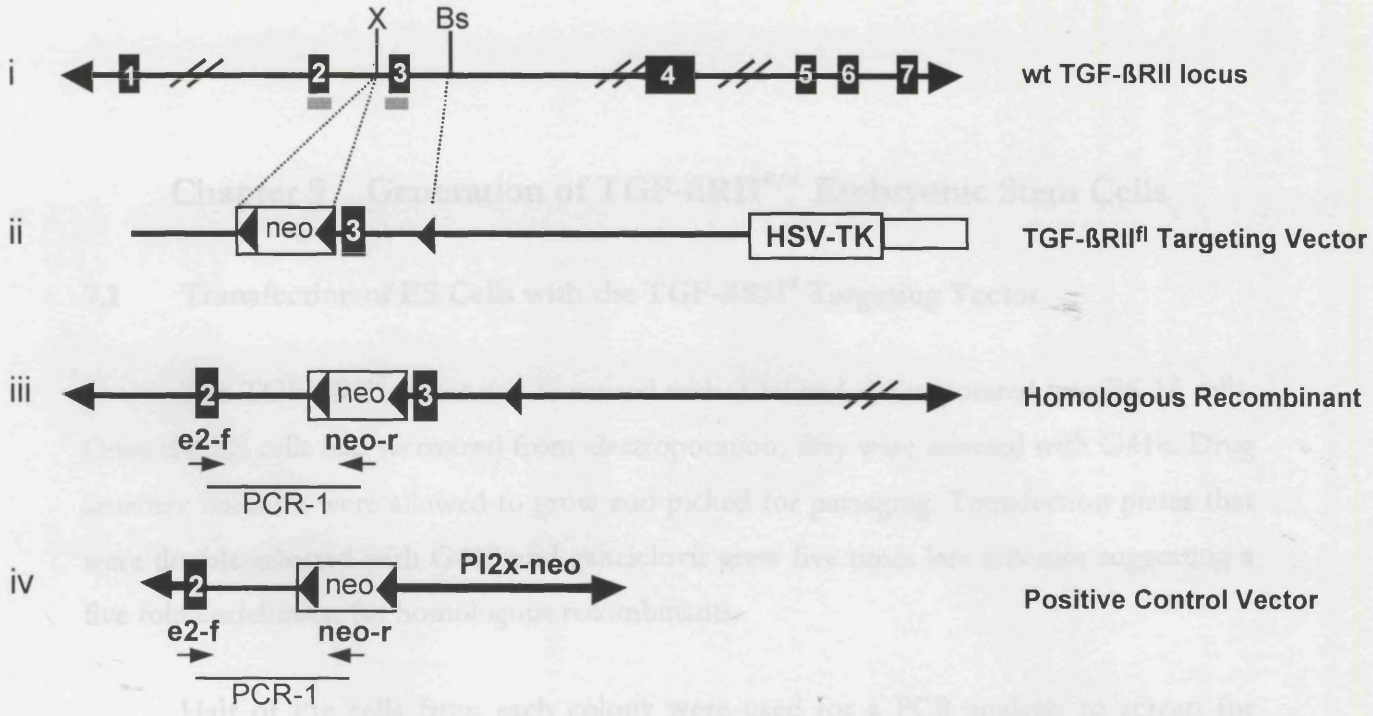



**Figure 8.5 The TGFß-RIIfl Positive Control Vector**

To test the conditions for the PCR to screen for homologous recombinant clones, (shown as PCR-1 in (**iii**)), a positive control vector was generated and transfected into ES cells. This vector, shown in (**iv**), contains an extended short arm of homology including the priming site for the "external" primer used in PCR 1 (E2F in (**iii**)). Clones transfected with the positive control vector were screened by PCR and those giving the faintest band were selected (as they are lilkely to contain less integrated copies of this vector). Selected clones were used to optimise PCR 1 under stringent conditions (1000 transfected cells + 50000 non-transfected cells used as template, 30 cycles), before the real TGF-ßRIIflox targeting vector was electroporated.

# Chapter 9 Generation of TGF-ßRII$^{fl/+}$ Embryonic Stem Cells

## 9.1 Transfection of ES Cells with the TGF-ßRII$^{fl}$ Targeting Vector

The TGF-ßRII$^{fl}$ vector was linearised with *XhoI* and electroporated into ES-14 cells. Once the ES cells had recovered from electroporation, they were selected with G418. Drug sensitive colonies were allowed to grow and picked for passaging. Transfection plates that were double-selected with G418 and ganciclovir grew five times less colonies suggesting a five fold enrichment for homologous recombinants.

Half of the cells from each colony were used for a PCR analysis to screen for homologous recombinants. Details of the PCR strategy used for screening are outlined in figure 9.1 (PCR-1).

Of 78 pools of 4 colonies, 24 pools resulted positive for homologous recombination. Colonies from the positive pools were passaged and re-screened individually. Of 73 single colonies screened, 19 had undergone homologous recombination. The high frequency of homologous recombination events is not likely to be a reflection of the accessibility of the *TGF-ßRII* gene chromosomal structure, as this gene is not expressed in ES cells (Goumans et al., 1998).

Correctly targeted clones were further analysed by PCR to test if they contained the third lox P site (see figure 9.1, PCR-2). Of these clones 9 were selected and passaged for further analysis. Genomic DNA was extracted and used for Southern blotting (see figure 9.2) to show: (i) that the targeting vector had integrated by homologous recombination and that the third lox P was present; (ii) that the 3' and 5' ends of the locus were intact and no mutations had occurred (e.g. no deletions, translocations or incorrect recombinations); (iii) that not more than one copy of the targeting vector had integrated in the ES cell genome.

The characterisation of homologous recombinant clones by Southern blotting is represented on figure 9.2.

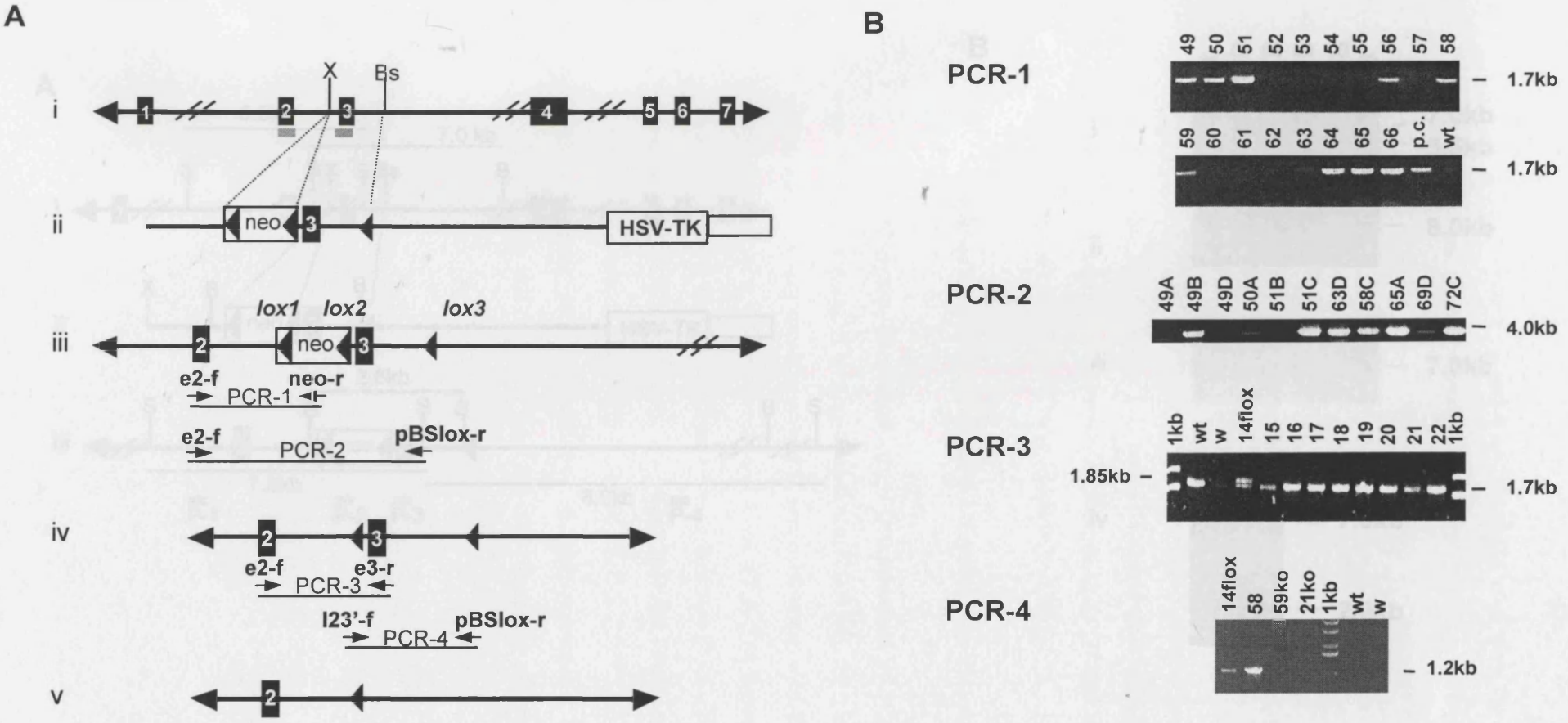



**Figure 9.1 PCR Analysis of Homologous Recombinant, TGFßRII$^{fl/+}$ and TGFßRII$^{+/-}$ ES cell Clones** **A**, To screen for homologous recombina clones (**iii**) ES cells were picked and analysed by PCR using a forward primer (e2-f), external to the targeting vector (**ii**) and a reverse primer within the floxe *neo*$^{r}$ gene (neo-r). Correctly targeted clones yielded a 1.7kb product (**PCR1**). Prior to Southern analysis and *in vitro* treatment with Cre, homologou recombinants were tested to ensure correct integration of the 3rd Lox P site (*lox3*): a PCR was performed on homologous recombinants using forward prim e2-f and reverse primer pBSLox-r (**PCR-2**). Clones carrying a correctly positioned *lox3* yielded a 4.0kb product. To test the type of deletion obtained after *vitro* Cre-mediated recombination, G-418-sensitive clones were analysed by PCR using forward primer e2-f and reverse primer e3-r (**PCR-3**). ES cell clones which one of the alleles had undergone a type I recombination (**iv**) yielded one band at 1.7kb (wt allele, **i**) and a second band at 1.85kb ("floxed" allele, **iv** where a Cre-mediated intramolecular event had deleted the *neo*$^{r}$ gene, leaving behind one loxP site. The same PCR carried out on ES cell clones which ha undergone a type II recombination (full knock-out clones, **v**) gave rise to a 1.7 kb band (wt allele only). To test that type I recombination had occurred witho disrupting the *lox3* sequence in TGF-ßRII$^{fl/+}$ clones 14 and 37, a PCR was performed using primers i2.3'-f and pBSLox-r (**PCR-4**). As expected this PC yielded a 1.2kb product both in the floxed clones (**iv**) and in the initial targeted clone (**iii**) and no product in the full knockout clones (**v**). **B**, Gel electrophores of DNA products from PCRs 1-4. Controls: wt, wild type ES14-1 cells; pc,positive control cells (see fig.8.5); w, water,no DNA. Kb, kilobase.

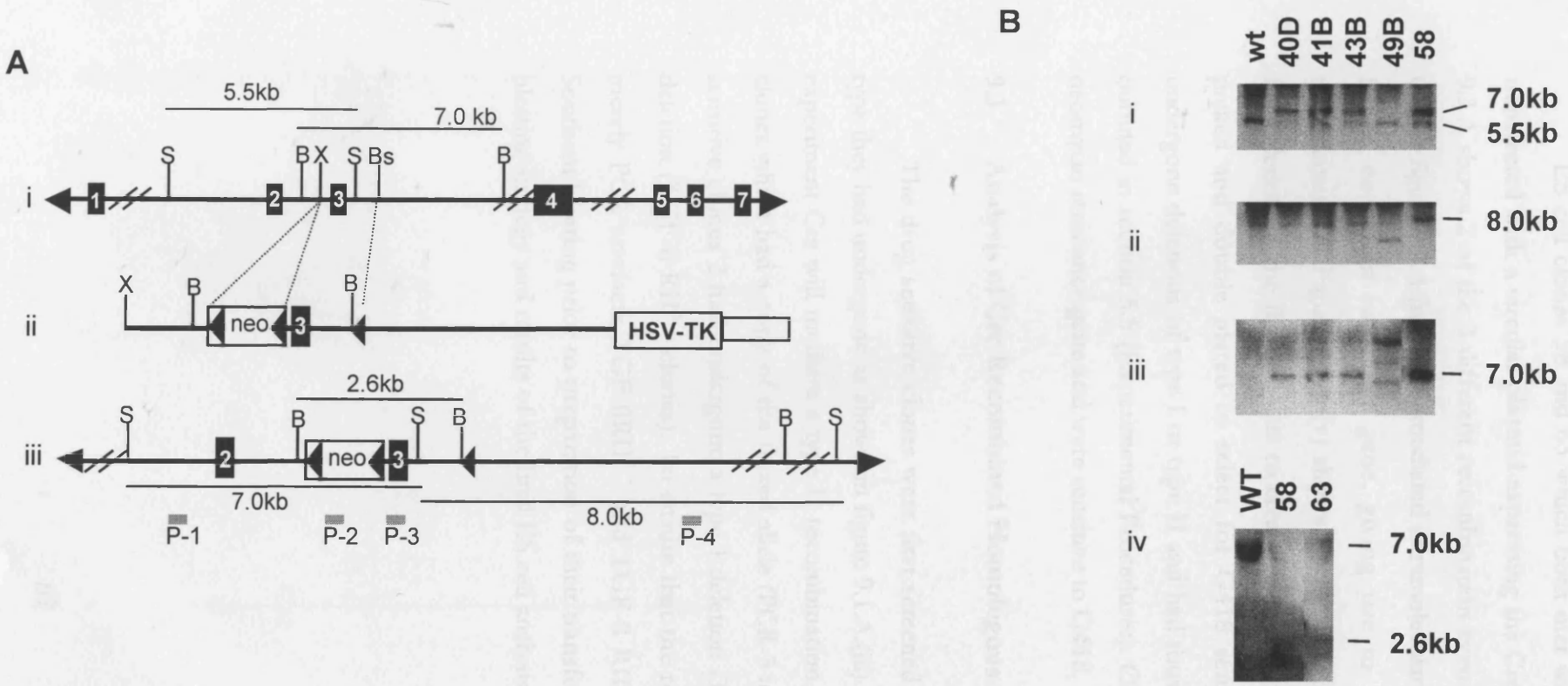



**Figure 9.2 Southern Analysis and Characterisation of Homologous Recombinant ES Cell Clones** Homologous recombinant ES Cell clones identified by PCR (see fig. xx) were analysed by Southern blotting prior to *in vitro* Cre mediated recombination, to ensure: (1) that only one copy of the targeting vector (TGF-ßRIIflox-T.V) had correctly integrated,(2) that the 3' and 5' ends of the locus had not been mutated and (3) that the 3rd LoxP site was present. **A**,Configuration of the locus before (**i**) and after (**iii**) homologous recombination with the TGF-ßRII$^{flox}$-T.V (**ii**). **B**, (**i**) *SmaI* digested wild type 129/Sv DNA (wt) hybridised to Probe-1 (P-1, external to the targeting vector), yields a 5.5kb band, whereas correctly targeted ES cell clones yield a second band at 7.0kb. This southern also suggests that there are no obvious mutations at the 5' targeting vector integration site in clones 40D, 41B, 43B and 58. Clone 49B is shown to be a PCR false positive. In (**ii**) the same membrane probed with P-4 at the 3' end of the locus shows an 8.0kb fragment for wt and homologous recombinant clones but not for incorrectly targeted clone 49B. (**iii**), Hybridisation of *SmaI* digested DNA from homologous recombinant clones with P-2, within the *neo$^r$* gene, shows the expected band at 7.0kb for single integration of the TGF-ßRII$^{flox}$-T.V. Clone 49B appears to have undergone triple random integration of the TGF-ßRII$^{flox}$-T.V. In (**iv**) clones 58 and 63 were tested further by digestion with *BglII* and hybridisation with probe P-3. The targeted clones show a band at 7.0kb (wild type allele) and a second band at 2.6kb (targeted allele) which is not present in the wt control and proves the presence of the 3rd LoxP site.

## 9.2 Transient Transfection with the Cre Recombinase Plasmid

ES cell clones 58 and 63 which both met with the above criteria were transiently transfected with a circular plasmid expressing the Cre recombinase protein (picCre). Figure 9.3.A shows 2 of the 3 different recombination events which may occur in the presence of Cre: in figure 9.3.A.(iv), a Cre mediated intramolecular event has catalysed the removal of the floxed neomycin resistance gene, giving rise to the *TGF-ßRII*$^{fl}$ floxed allele (type I recombination). Figure 9.3.A.(v) shows a type II recombination, where Cre has catalysed a full deletion of the floxed locus to create full knockout alleles (*TGF-ßRII*$^{\Delta}$). Colonies were picked and double plated to select for G418 sensitive clones (i.e. clones which had undergone deletions of type I or type II and had thus lost the neomycin resistance gene) as outlined in section 5.5 (Experimental Procedures). Of 816 colonies picked, 48 had lost the neomycin resistance gene and were sensitive to G418.

## 9.3 Analysis of Cre Recombined Homologous Recombinant ES cell clones

The drug sensitive clones were first screened by PCR to determine which deletion type they had undergone as shown in figure 9.1.A.(iii). As it is most likely that in this type of experiment Cre will mediate a type II recombination, a PCR strategy was devised to select clones which had a copy of the floxed allele (PCR-3 in figures 9.1.A and 9.1.B). Of 48 drug sensitive clones 2 had undergone a type I deletion (TGF-ßRII$^{fl/+}$ clones) and 46 a type II deletion (TGF-ß RII$^{\Delta/+}$ clones). To ensure that the results obtained in figure 9.1 were not merely PCR artefacts TGF-ßRII$^{fl/+}$ and TGF-ß RII$^{\Delta/+}$-ES cell clones were analysed by Southern blotting prior to preparation of their transfer into murine embryos. The Southern blotting strategy and results of the final ES cell analysis are detailed on figure 9.3.

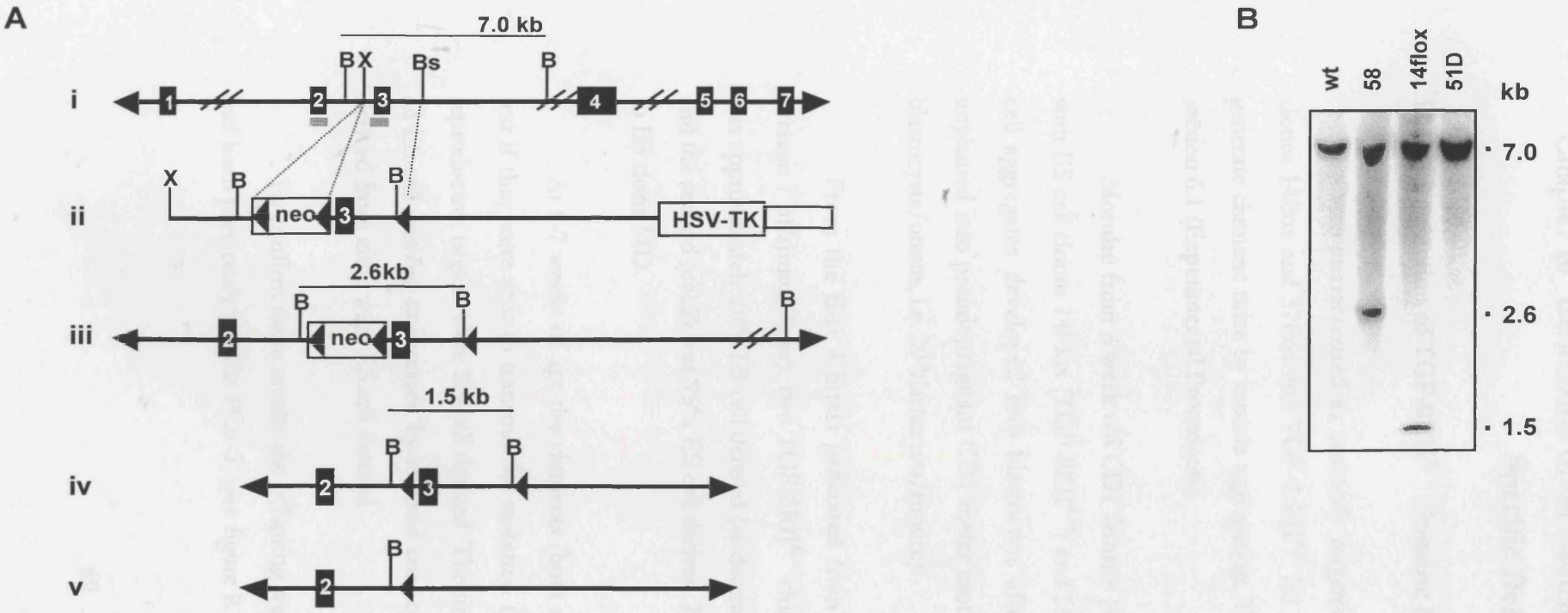



**Figure 9.3 Inactivation and floxing of the TGFß-RII gene in ES cells**

**A**, Configuration of the locus before (TGF-ßRII$^{wt}$, (i)) and after (iii) homologous recombination with the targeting vector (ii). Type I deletion at *in vitro cre*-mediated recombination results in floxed allele (TGF-ßRII$^{fl}$, (iv)); Type II deletion after *in vivo cre*-mediated recombination results deleted allele (TGF-ßRII$^{\Delta}$,(v)). Exon 3 of the TGF-ßRII locus encodes for part of the extracellular ligand binding domain of the receptor. Splic between exons 2 and 4 creates a frameshift mutation which generates a stop codon at the beginning of the exon 4 ORF. Exons (black boxe lengths of diagnostic restriction fragments, and location of probes are shown. The targeting vector incorporates a neomycin resistance ge (*neo*$^r$) flanked by *loxP* sequences (black triangles). The herpes symplex virus-thymidine kinase gene (*HSV-tk*) allows for selection agai random integration. These features allow for selection of ES cell transfectants and subsequent deletion of the resistance marker in homologo recombinants, using the *cre/loxP* system. B, *Bgl* II; X, *Xho*1; N, *Nco*1; Bs; *Bsu* 361; kb,kilobases.

**B,** Southern blot of genomic DNA extracted from ES clones E14-1 (TGF-ßRII$^{+/+}$, wt), 58 (homologous recombinant), 14Flox (TGF-ßRII$^{fl/+}$ ) a 51D (TGF-ßRII$^{+/-}$), digested with BglII and probed with P3, (grey bar, 3). Wild type E14-1 DNA displays the expected band at 7.0 kb (i). Clo 51D shows the 7.0 kb band (wt allele) and a 2.6kb band predicted for a homologous recombination event (targeted allele, iii ). In clone 14F Type I Cre-mediated deletion results in deletion of the floxed *neo r* gene and the 2.6kb band is reduced to 1.5kb (iv). Clone 51D is a prod of *in vitro* Type II Cre-mediated deletion (v): the deleted clone has lost the P-3 probing site. Both clones 14Flox and 51D were derived from C mediated recombination of clone 58.

# Chapter 10 Generation of the Mouse Model of TGF-ßRII Cell Type Specific Deficiency

## 10.1 Production of TGF-ßRII$^{fl/+}$ Chimeric Mice by Morula Aggregation

Once characterised as correctly targeted and recombined, TGF-ßRII$^{fl/+}$ ES cell clones 14flox and 37flox and TGF-ß RII$^{\Delta/+}$ ES cell clones 51D and 63D were selected to generate chimeric mice by morula aggregation. The details of this procedure are outlined in section 6.1 (Experimental Procedures).

Morulae from 8 week old CD1 females (see section 6.1.2) were used for aggregation with ES cell clones 14Flox (TGF-ßRII$^{fl/+}$) and 51D (TGF-ßRII$^{\Delta/+}$). 75 % of the morula-ES cell aggregates developed into blastocysts after overnight incubation. These were re-implanted into pseudopregnant CD1 foster mothers by uterine transfer (approximately 10 blastocysts/uterus, i.e. 20 blastocysts/mouse).

From the first 4 litters generated from aggregation to ES cell clone 14flox (on average 7 offspring/litter), two TGF-ßRII$^{fl/+}$ chimeras were born. The first chimera (chi.1) was approximately 20% ES cell derived (as determined by percentage of sand-coloured coat) and the second (chi.2) was 95% ES cell derived. No chimeras were generated by aggregation to ES clone 51D.

At 6-7 weeks of age the chimeras (both males) were crossed to Balb/C females, to test if they were able to transmit the mutation through to the next generation, i.e. if their reproductive organs were ES-cell derived. The first litter derived from chi.1 was of 12 pups, all ES-cell derived as assessed by eye and coat colour. 7 out of 8 pups from the first litter derived from chi.2 were ES cell derived.

To confirm these results the offspring were genotyped. Primers e2.f and e3.r, which had been previously used in PCR-3, (see figure 9.1) were used to test for the presence of the

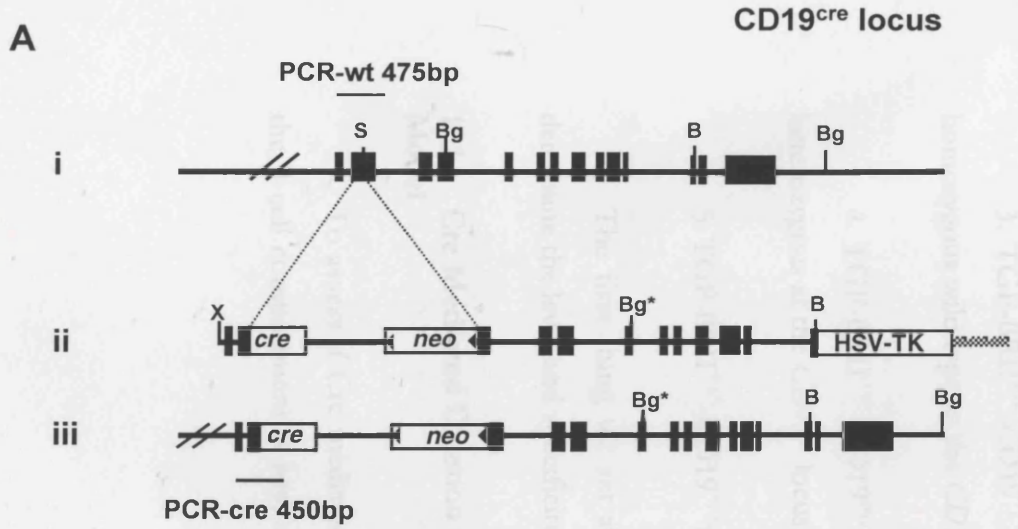
*TGF-ßRII*$^{fl}$ allele. 8 out of 15 mice tested were positive for the *TGF-ßRII*$^{fl}$ band (see figure 10.1.C).

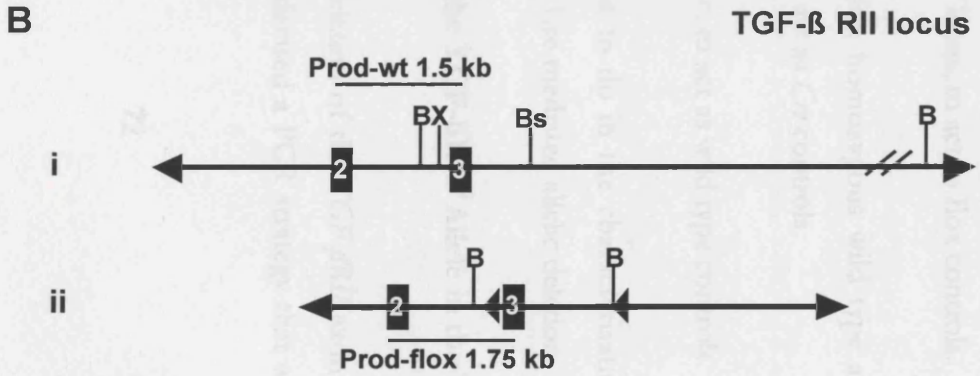
## 10.2 Generation of Cell Type Specific TGF-ßRII Deficient Mouse Models

Transmitting offspring of TGF-ßRII$^{fl/+}$ chimeras chi.1 and chi.2 were mated with mice of the B cell specific Cre expressing strain (CD19$^{cre}$, Rickert et al., 1997). Offspring of these crosses were inbred to give rise to a murine model of B cell specific TGF-ßRII deficiency (TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$). The PCR strategy used to genotype mice of the TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ strain is shown in figure 10.1.

Mice which were genotyped as wild type at the *Cre* allele and homozygous floxed at the *TGF-ßRII* allele (TGF-ßRII$^{fl/fl}$-CD19$^{+/+}$) were born at the expected Mendelian ratio, and were indistinguishable from their wild type littermates, thus demonstrating that the floxed mutation had not affected normal synthesis of the TGF-ßRII protein. If receptor protein levels had been down-regulated, it is likely that these mice would not have survived past day 10.5 of gestation due to defects in yolk sac hematopoiesis and vasculogenesis (Oshima et al., 1996).

In accordance with our decision to concentrate initially on the *in vivo* role of the TGF-ßRII in B cells, the next chapter will describe the work we carried out on TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice. We have also created a granulocyte specific model of TGF-ßRII deficiency, by crossing TGF-ßRII$^{fl/fl}$ mice with mice from the GE$^{cre}$ strain (J. Roes, unpublished). This model will be discussed in chapter 15.



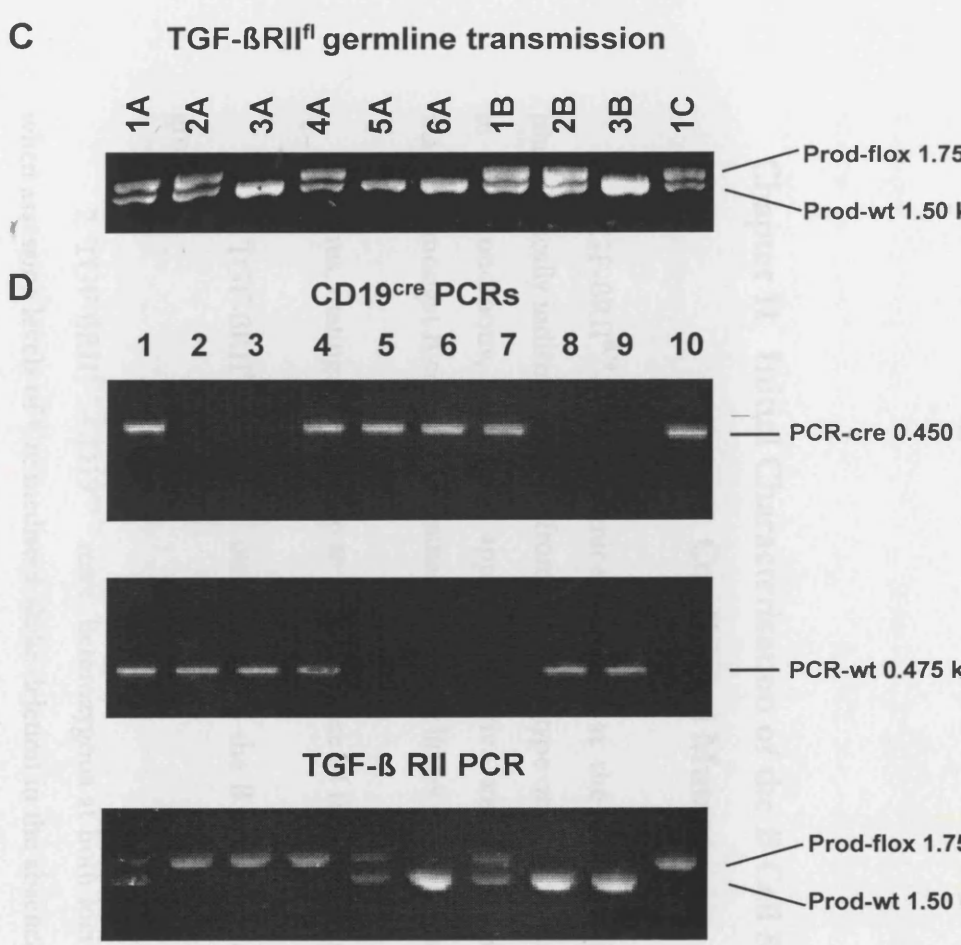




**Figure 10.1 CD19 locus targeting and typing PCR strategy for TGF-ßRII$^{fl/+}$ and for TGF-ßRII$^{fl/fl}$-CD19$^{cre}$ mice.** Offspring derived from crosses between TGF-ßRII$^{fl/+}$ chimeras and Balb/C mice and crosses between CD19$^{cre}$ and TGF-ßRII$^{fl/fl}$ mice were tailsnipped for DNA genoyping by PCR analysis. **A**, CD19 locus targeting. Details of the strategy used have been described in detail elsewhere (Rickert et al. 1995). Briefly, **A** shows the configuration of the CD19 locus before (**i**) and after homologous recombination (**ii**) with the Cre containing vecto (**iii**). Also shown are the PCRs used to test for the presence of the wild type CD19 allele (PCR-wt, (**i**)) and for the CD19$^{cre}$ knock-in allele (PCR-cre (**iii**)). **B**, A PCR between primers e2-f and e3-r in the TGF-ßRII locus was used to test for the presence of wt (prod-wt, (**i**)) and floxed (prod-flox, (**ii**)) TGF-ßRII alleles. **C**, Representative gel electrophoresis of PCR genotyping analysis of offspring of TGF-ßRII$^{fl/+}$ chimeras. **D** Representative gel electrophoresis of TGF-ßRII$^{fl/fl}$-CD19$^{cre}$ typing PCRs.

# Chapter 11 Initial Characterisation of the B Cell Specific TGF-ßRII Conditional Mutant

TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice are born at the expected Mendelian ratio and are phenotypically indistinguishable from their wild type or heterozygous littermates. When kept in s.p.f. conditions, these mice appear to survive well into adulthood and, as expected, matings amongst B cell specific mutants result in litters of normal size.

Thus, matings were set up to generate mice of the following genotypes:

1. TGF-ßRII$^{fl\fl}$-CD19$^{cre/+}$ mice, to act as the B cell specific TGF-ßRII deficient test group.

2. TGF-ßRII$^{fl\+}$-CD19$^{cre/+}$ mice, heterozygous at both loci and required as a control when assessing levels of Cre mediated allelic deletion in the absence of selection.

3. TGF-ßRII$^{fl\fl}$-CD19$^{+/+}$ mice, homozygous floxed at the *TGF-ßRII* allele and homozygous wild type at the *CD19*$^{cre}$ locus, to act as flox controls.

4. TGF-ßRII$^{+\+}$-CD19$^{cre/+}$mice, homozygous wild type at the receptor locus and heterozygous at the *CD19*$^{cre}$ locus, to act as *Cre* controls.

5. TGF-ßRII$^{+\+}$-CD19$^{+/+}$mice, to act as wild type controls.

The first thing we set about to do in the characterisation of this model was to determine the level and specificity of Cre mediated allelic deletion.

## 11.1 Cre Mediated Deletion of the TGF-ßRII$^{fl}$ Allele in the TGF-ßRII$^{fl\fl}$-CD19$^{cre/+}$ Model

To assess if Cre mediated deletion of the *TGF-ßRII* exon 3 locus was occurring in the B cell compartment *in vivo*, we devised a PCR strategy that would enable a distinction

between the wild type (*TGF-ßRII*$^{+}$), floxed (*TGF-ßRII*$^{fl}$) and deleted (*TGF-ßRII*$^{\Delta}$) TGF-ßRII alleles (see figure 11.1.A and 11.1.B). This PCR was carried out on total peripheral blood leukocytes (PBLs) of 4-week-old mice (figure 11.1.B). Encouragingly, this PCR showed a clear 5.5 Kb product corresponding to the *TGF-ßRII*$^{\Delta}$ allele, when amplifying from the conditional mutant template (figure 11.2.B.).

The same PCR was also tested on PBLs from TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$mice, as shown on figure 11.1.C.

To determine the efficiency of Cre mediated deletion of the *TGF-ßRII* allele in B cells of TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ mice, we used a Southern blot strategy, outlined in figure 11.2. Genomic DNA was isolated from populations of thymocytes and splenic MACS (magnetic activated cell sorting) - sorted B lymphocytes (95% B220$^{+}$ and IgM$^{+}$ as assessed by FACS analysis, data not shown), from 12-week old TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ (test group), TGF-ßRII$^{fl\backslash +}$-CD19$^{cre/+}$ (heterozygous control) and TGF-ßRII$^{fl\backslash fl}$-CD19$^{+/+}$ (flox control) mice. The DNA was digested with *NcoI* and hybridised to a radioactively labelled DNA probe spanning exon 2 of the receptor locus in such a way as to distinguish between *TGF-ßRII*$^{+}$, *TGF-ßRII*$^{fl}$ and *TGF-ßRII*$^{\Delta}$ alleles (figure 11.2.A and 11.2.B). Somatic deletion of the *TGF-ßRII* was assessed by quantitation of the loss of *TGF-ßRII*$^{fl}$ and acquisition of the *TGF-ßRII*$^{\Delta}$ allele. The percentages of allelic deletion were calculated as previously described (Kuhn et al., 1995) and are shown in the bottom column of figure 11.2.B. In splenic B cells of TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ mice, we found a level of deletion of 97.6 ± 2 % whereas B cells from heterozygous control mice exhibited a level of deletion of 93.3 ± 5.8 %. Purified T cell DNA from all groups tested showed no deletion at the *TGF-ßRII* locus.

## 11.2 B Cell Development in the TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ Model

To assess the role of TGF-ß signaling in B cell development, bone marrow, spleen, lymph node, peripheral blood and peritoneal cavity leukocytes were analysed (figure 11.3). Single cell suspensions were depleted of erythrocytes using standard procedures and analysed by flow cytometry.

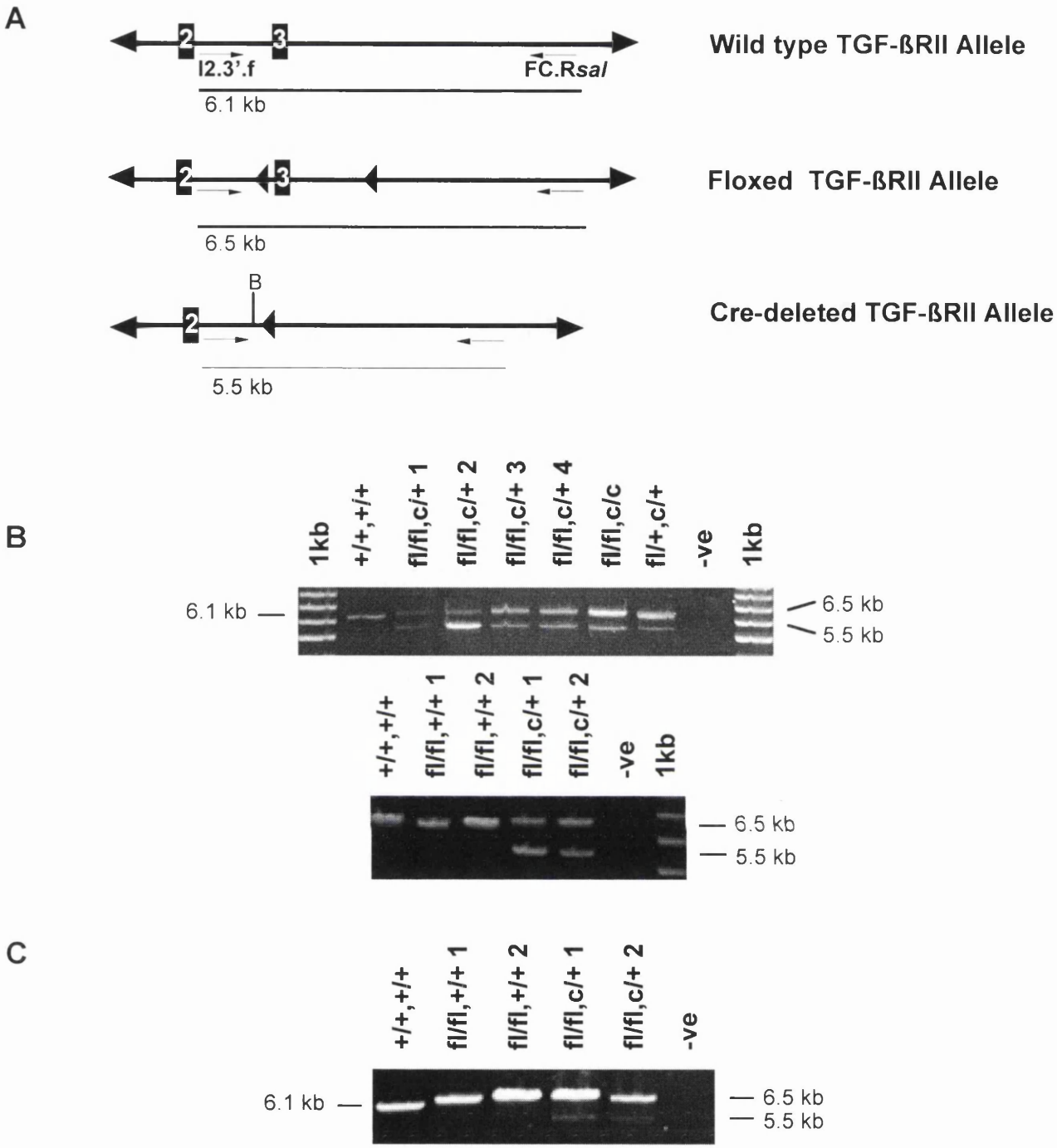



**Figure 11.1 PCR analysis of Cre-mediated deletion at the TGF-ßRII locus in peripheral blood leukocytes from TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ and TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice. A**, PCR strategy devised to qualitatively assess *Cre* mediated deletion at the TGF-ßRII locus in peripheral leukocytes *in vivo.* Amplification of the wild type allele with primers I2.3'.f and FC.R*sal* yields a 6.1kb fragment (*top*). The same primers used to amplify the floxed (*middle*) and deleted (*bottom*) alleles yield a 6.5kb and a 5.5kb band respectively. **B**, Gel electophoresis of products of the deletion PCR carried out on 10000 peripheral leukocytes from 4 wk old TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice and their control littermates. **C**, Deletion PCR on 10000 peripheral leukocytes from 4 wk old TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ mice and their control littermates.
1kb, 1kb DNA ladder (Gibco); +, wild type allele; fl, floxed allele; c, *cre* allele.

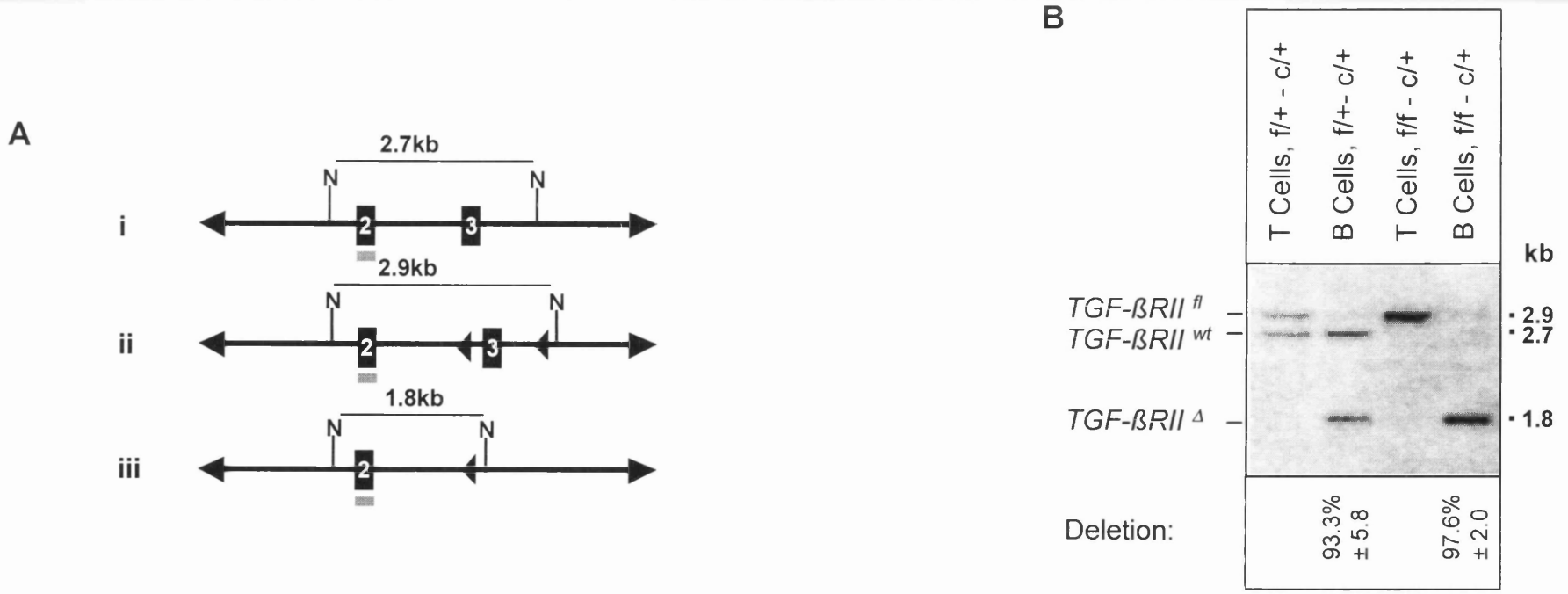



**Figure 11.2 Cre Mediated Deletion in the TGFßRII$^{flox}$ - CD19$^{Cre}$ model**

**A** Southern blot strategy to distinguish TGF-ßRII$^{wt}$ (**i**), TGF-ßRII$^{fl}$ (**ii**) and TGF-ßRII$^{\Delta}$ (**iii**) alleles after *Nco*I digestion and transfer of genomic DNA. The genomic probe (P.Ex-2) spans across exon 2 of the TGF-ßRII locus and is depicted as a grey bar.

**B** Hybridization of *NcoI* digested lymphocyte DNA with $\alpha$ $^{32}$P dCTP labelled P.Ex-2. Splenic B cells (CD45R/B220$^{+}$) were isolated by positive enrichment using magnetic bead separation (MACS, Miltenyi Biotec, Cologne); T cells were isolated from the thymus. B and T populations were from single mice. The numbers in the lower column show quantitation of deletion in B lymphocyte DNA with Image Quant (Storm, Molecular Dynamics). The percentage deletion (mean ± SD) was calculated as described (Kühn et al., 1995) and normalized for the purity of the sorted population which was between 90-98% (as shown by flow cytometry, data not shown).

Despite the early expression of TGF-ß and its type II receptor in lymphoid progenitors (Lee et al., 1987), analysis of bone marrow cells from TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ (figure 11.3, top row) revealed normal development and expansion of B lineage cells, as indicated by the numbers and distribution of pro- and pre-B (IgM$^-$ B220$^{low}$ and IgM$^-$ B220$^+$), immature B (IgM$^+$ B220$^+$), and mature B cells (IgM$^+$ B220$^{high}$). A negligible decrease in the relative numbers of B lineage cells to myeloid progenitors was seen in the conditional mutant animals (data not shown).

As shown in the middle panels of figure 11.3, simultaneous staining for IgM and IgD of splenic lymphocytes revealed normal relative numbers of mature B cells (IgM$^{high}$ IgD$^+$, upper right quadrant), in the absence of TGF-ßRII. A slight increase in the numbers of splenic immature B cells (IgM$^{low}$ IgD$^-$) and of B-1 cells (IgM$^{high}$ IgD$^{low}$) (upper left quadrant), was found in the conditional mutants. The same increase in immature B cells was also seen when comparing lymph nodes from TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ mice, to those from control mice (data not shown).

To evaluate the role of TGF-ß1 in recruitment of B cell progenitors to the CD5 compartment (B-1 cells), which predominate in the peritoneal cavity, we carried out a simultaneous IgM-IgD staining of peritoneal exudate cells (figure 11.3, bottom). We found an increase in relative numbers of B-1 cells (IgM$^{high}$IgD$^{low}$) in TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ mutant animals (74.9 ± 2.0 % mutants, 48.0 ± 8% wild type littermates). The total numbers of nucleated cells recovered from the peritoneum of B cell specific TGF-ßRII deficient mice were (7.56 ± 2.9) x $10^6$, on average threefold higher than their wild type littermates ((2.6 ± 0.3) x $10^6$). Taken together these findings denote a 4.5 fold increase in total numbers of B-1 cells in the peritoneal cavity of B cell specific TGF-ßRII deficient animals, thus highlighting a possible auxiliary role of TGF-ß1 in mediating a regulatory feedback loop to limit the expansion of B-1 cells. To test if other cell types in the peritoneum of TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ mice were affected, we carried out a GR1-CD11B staining (figure 11.4). This showed a marked decrease in the ratio of GR1$^+$ or GR1$^-$-CD11B$^+$ leukocytes to B cells, in the conditional mutants. The total numbers of these peritoneal non B cells (resident macrophages and few neutrophils) were shown to be no different from those of wild type littermates (figure 11.4).

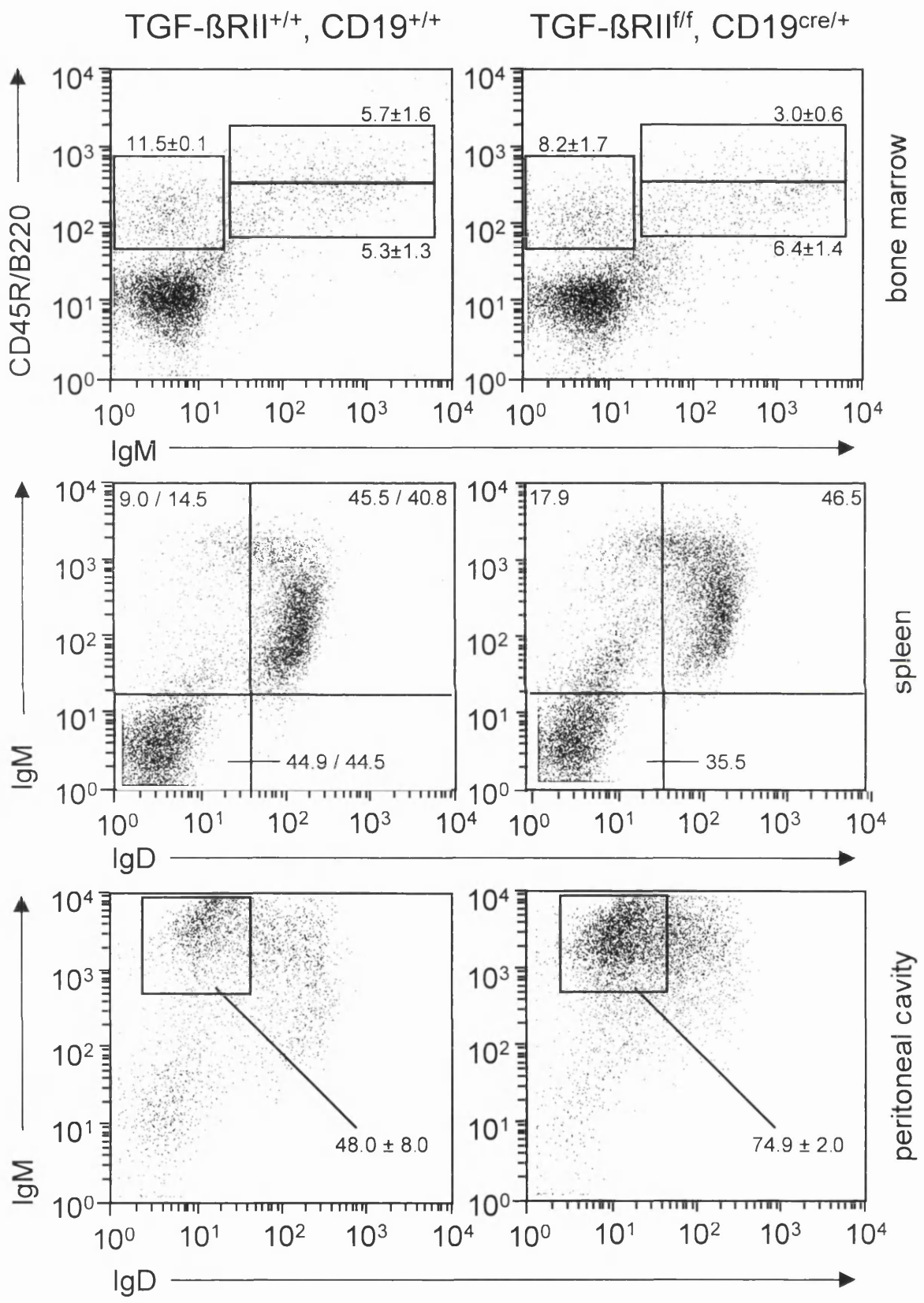



**Figure 11.3 B cell Development in the TGF-ßRII$^{flox}$ - CD19$^{cre}$ model**
(see following page for legend)

**Figure 11.3 B cell Development in the TGF-ßRII$^{flox}$ - CD19$^{cre}$ model**

Representative flow cytometry of bone marrow, spleen and peritoneal exudate cells of wild type (TGF-ßRII$^{+/+}$, CD19$^{+/+}$) and B cell specific TGF-ßRII deficient mice (TGF-ßRII$^{f/f}$, CD19$^{cre/+}$). *Top*, Bone marrow. Populations of pro-B and pre-B (IgM$^{-}$ B220$^{low}$ and IgM$^{-}$ B220$^{+}$), immature B (IgM$^{+}$ B220$^{+}$) and mature B cells (IgM$^{+}$ B220$^{high}$) are framed and percentages noted (mean and SD). The total number of nucleated cells recovered from the bone marrow of TGF-ßRII$^{+/+}$-CD19$^{+/+}$ and TGF-ßRII$^{f/f}$-CD19$^{cre/+}$ were (1.61 ± 0.05) x $10^7$ and (1.39 ± 0.06) x $10^7$ respectively. *Middle,* Spleen. IgM-IgD staining shows normal relative numbers of mature B (IgM$^{high}$ IgD$^{+}$, upper right quadrant), immature B (IgM$^{low}$IgD$^{-}$) and B-1 cells (IgM$^{high}$IgD$^{low}$) (upper left quadrant), in the absence of TGF-ßRII. Leukocyte numbers in lymph nodes and peripheral blood and expression of MHC CII in all B cell populations were also found to be normal in the TGF-ßRII$^{flox}$ - CD19$^{cre}$ model (results not shown). *Bottom,* Peritoneal cavity. IgM-IgD staining shows an increase in relative numbers of B-1 cells in mutants (IgM$^{high}$IgD$^{low}$).The mean percentages and SD are given. The total numbers of nucleated cells recovered from the peritoneum of B cell specific TGF-ßRII deficient mice were (7.56 ± 2.9) x $10^6$, on average threefold higher than their wild type (2.6 ± 0.3) x $10^6$ littermates. Equal numbers of events are plotted for each staining. All stainings were also carried out on flox-control (TGF-ßRII$^{f/f}$, CD19$^{+/+}$) and heterozygous mice (TGF-ßRII$^{f/+}$, CD19$^{cre/+}$) which showed no differences from wild type controls (data not shown).

**METHODS** For each organ FACS profiles are representative of experiments conducted on at least three TGF-ßRII$^{f/f}$, CD19$^{cre/+}$ and TGF-ßRII$^{+/+}$, CD19$^{+/+}$ mice of 10-14 weeks of age. Single-cell suspensions were prepared and depleted of erithrocytes using standard procedures. The antibodies used in the above stainings were: fluorescein (FITC)- and biotin-RA36B2 (anti-B220), PE-R33-24-12 (anti-IgM), and FITC-rabbit anti-mouse IgD. Biotinylated antibodies were detected with streptavidin cy-chrome (Pharmingen). Cells were analysed with a leukocyte gate as defined by light scatter and forward scatter (FACScan, Becton-Dickinson) using Cellquest software (Becton-Dickinson).

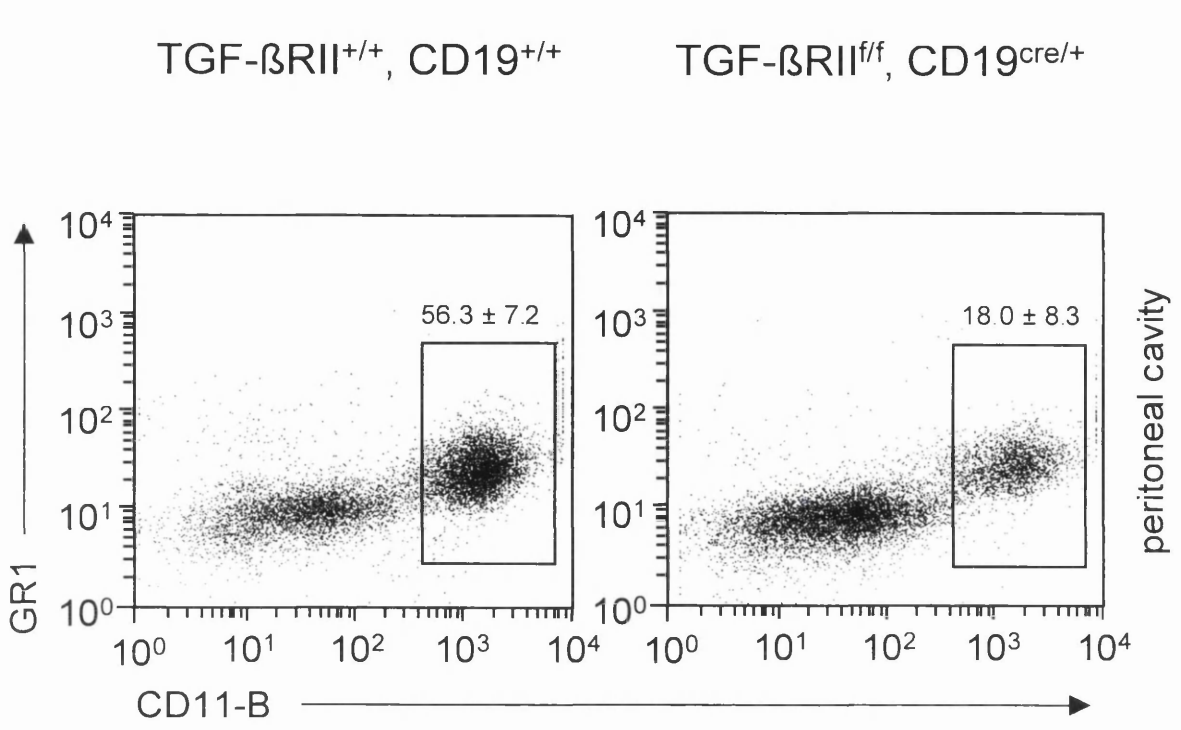



**Figure 11.4 Non B cell populations in the peritoneum of TGF-ßRII$^{flox}$-CD19$^{cre/+}$ mice**

To test the relative numbers of resident non-B cell peritoneal populations, leukocytes from the peritoneum of TGF-ßRII$^{f/f}$, CD19$^{cre/+}$ mice and control littermates, were simultaneously stained with GR1 and CD11B antibodies. CD11B$^{high}$ cells (resident macrophages), are boxed. The numbers above the boxes show mean percentages and s.d. for wild type and conditional mutants. Although the percentage of CD11B$^{high}$ cells was threefold lower in TGF-ßRII$^{f/f}$, CD19$^{cre/+}$ mice, the total numbers of cells recovered from conditional mutant peritoneums ((7.56 ± 2.9) x $10^{6)}$ was found to be threefold higher than that in wildtype littermates ((2.6 ± 0.3) x $10^{6}$), suggesting that numbers of non B-cell leukocytes in the peritoneum are not affected by a B cell specific TGF-ßRII inactivation.

## 11.3 B Cell Proliferation Dynamics in the TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ Model

Generally TGF-ß is thought to inhibit B cell proliferation and to influence B cell apoptosis both in B cells and in fully differentiated plasma cells (Amoroso et al., 1998; Holder et al., 1992; Lomo et al., 1995). To assess the possible influence of TGF-ß signaling on the lifespan and turnover of murine B cells *in vivo*, we measured BrdU incorporation into leukocytes of TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ and control mice fed for 15 days with BrdU in their drinking water (see figure 11.5). By simultaneous flow cytometric detection of cell surface markers and intracellular BrdU incorporation, we found no significant difference in the proliferative behaviour of B cells from the bone marrow and peritoneal compartments of B cell specific TGF-ßRII deficient animals (figure 11.5, top and bottom ). However, the same analysis carried out on splenic lymphocytes showed a two-fold increase in BrdU labelling of B220$^{+}$ spenic B cells (13.1±0.3% of splenocytes in wt, 29.3±1.6% in conditional mutants) indicating an increase in their turnover in the absence of TGF-ßRII.

## 11.4 MHC-Class II Expression in the TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ Model

TGF-ß controls several aspects of the differentiated functions of B cells, including the induction of MHC class II (MHC-II) expression on both pre-B and mature B cells (Stavnezer J., 1996) Furthermore, leukocytes from TGF-ß knock out mice have elevated levels of MHC-II [Shull, 1992 #135; Nakabayashi, 1997 #24]. To test the constitutive expression levels of MHC-II on the surface of B cells lacking the TGF-ßRII, we stained bone marrow, splenic and peritoneal exudate leukocytes from mutant and control animals with antibodies to cell surface markers and to MHC-II. No significant differences were seen in the levels of MHC-II on TGF-ßRII deficient B cells (data not shown).

## 11.5 IgA Switch in the TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ Model

TGF-ß has been shown to induce transcription from the unrearranged murine Iα - Cα and Iγ2b-Cγ2b gene segments, thus directing switch recombination in immunoglobulin isotypes IgA and IgG2b. To examine if TGF-ß is essential for this process *in vivo* we purified lymphocytes from bone marrow and spleen of TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ and control mice, and carried out an intracellular anti-IgA staining (figure 11.6). This revealed a population of

$IgA^{high}$ $B220^{low}$ plasma cells amongst wild type bone marrow and splenic lymphocytes that were virtually absent in the conditional mutants. No difference was seen when we stained bone marrow and splenic B-lymphocytes intracellularly with a polyclonal antibody to total mouse Ig (data not shown).

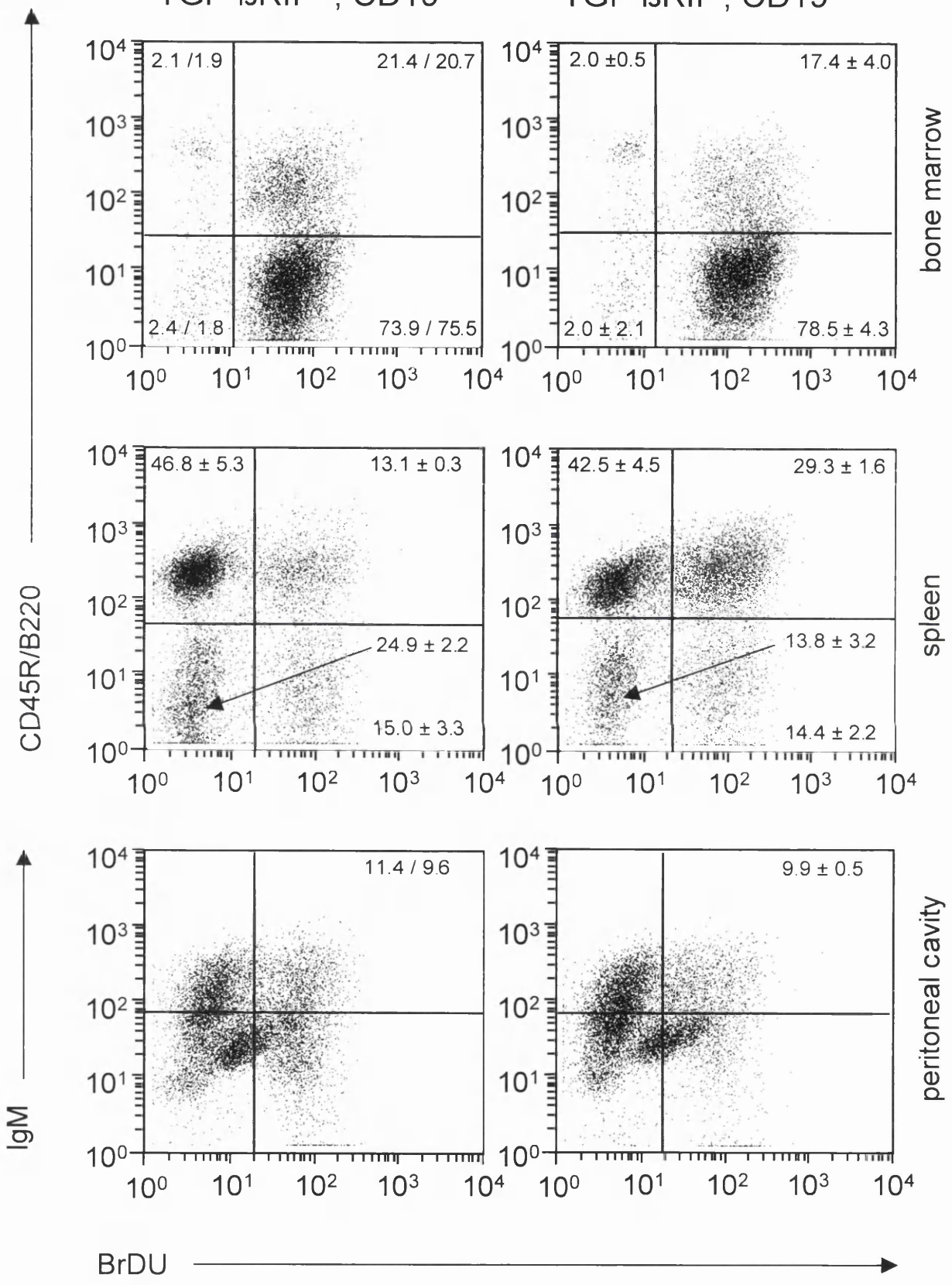



**Figure 11.5 B cell lifespan in the TGF-ßRII$^{flox}$ - CD19$^{cre}$ model**
(see following page for legend)

**Figure 11.5 B cell lifespan in the TGF-ßRII$^{flox}$ - CD19$^{cre}$ model**

Bone Marrow (*top)*, spleen (*middle*) and peritoneal cavity (*bottom*) cells of wild type (TGF-ßRII$^{+/+}$, CD19$^{+/+}$) and B cell specific TGF-ßRII deficient mice (TGF-ßRII$^{f/f}$, CD19$^{cre/+}$) fed for 15 days with 1 mg 5-bromo-2'-deoxyuridine (BrdU) per ml drinking water were analysed by flow cytometry for BrdU incorporation. The numbers shown are percentages of cells in each quadrant (where more than one mouse was analysed mean and sd are noted). No differences were found in the turnover of bone marrow and peritoneal cavity B cells after 15 days of BrdU labelling, whereas a small increase was seen in splenic B lymphocytes. Unlabelled cells from mice that had not been fed BrdU in their drinking water showed no staining with the anti-BrdU antibody (results not shown).

**METHODS** For each organ FACS profiles are representative of experiments conducted on at least three TGF-ßRII$^{f/f}$, CD19$^{cre/+}$ and two TGF-ßRII$^{+/+}$, CD19$^{+/+}$ mice of 10-14 weeks of age. Mice were given drinking water containing BrdU (Sigma, B5002) at 1mg/ml for 15 days. Mice were sacrificed and Single-cell suspensions were prepared and depleted of erithrocytes using standard procedures. 3 x $10^6$ cells were first stained on the surface with biotin-RA36B2 (anti-B220) (spleen and bone marrow) or biotin-R33-24 (anti-µ) (peritoneal cavity cells) antibodies. After fixation in 70% (vol/vol) icecold ethanol, cells were washed and resuspended in PBS and an equal volume of PBS, 2% formaldehyde, 0.02 % Tween 20 was added. Cells were left at $4^0$ C overnight, washed in PBS and resuspended in 1ml of 150mM NaCl, 5mM $MgCl_2$ , 10µM HCl supplemented with 300µg/ml DNAse 1 (Sigma D 5025) for ten minutes at room temperature. Cells were then washed with PBS, 0.5% BSA, 0.01% sodium azide and stained with FITC conjugated anti-BrdU antibody (Becton Dickinson). After a final wash in PBS, 0.5% BSA, 0.01% cells were resuspended and analysed by flow cytometry (FACScan, Becton Dickinson).

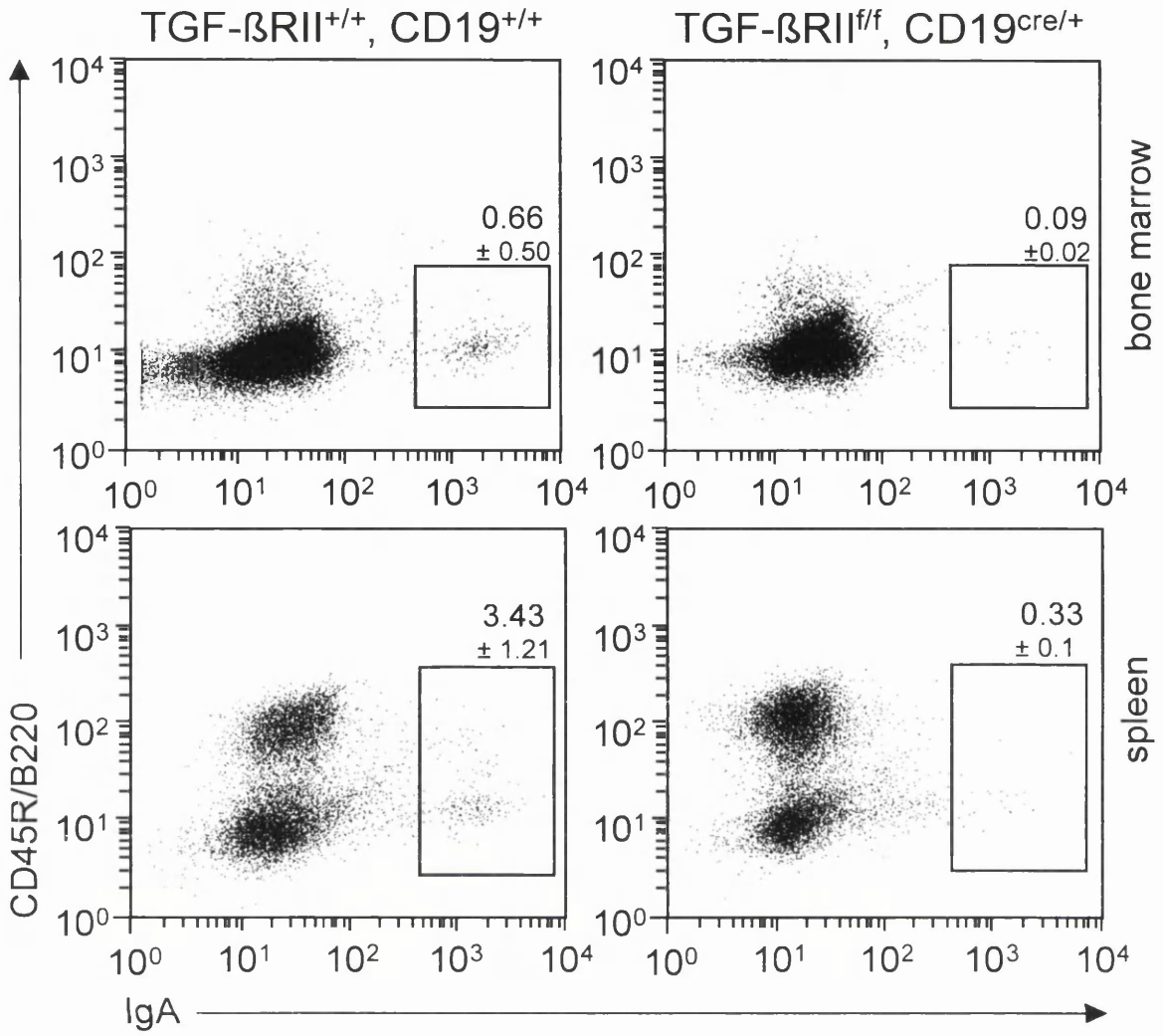



**Figure 11.6 IgA Switch in TGF-ßRII$^{flox}$ - CD19$^{cre/+}$ mice**

Representative intracellular flow cytometric staining of bone marrow (*top*) and spleen (*bottom*) leukocytes of wild type (TGF-ßRII$^{+/+}$, CD19$^{+/+}$) and B cell specific TGF-ßRII deficient mice (TGF-ßRII$^{f/f}$, CD19$^{cre/+}$ ) with anti-IgA antibodies. *Top,* Bone marrow. Intracellular anti-IgA staining revealed a population of IgA$^{high}$ B220$^{low}$ plasma cells in wild type bone marrow cells which is absent in the conditional mutants. The IgA expressing plasma cells are framed and the percentages noted (mean and SD). *Bottom,* Spleen. The same results were seen with intracellular staining of splenic lymphocytes. Intracellular staining of both bone marrow and splenic B cell compartments with policlonal rat anti-mouse Ig antibody showed no differences in total numbers of plasma cells in TGF-ßRII$^{+/+}$, CD19$^{+/+}$ and TGF-ßRII$^{f/f}$, CD19$^{cre/+}$ mice (data not shown).

**METHODS** For each organ FACS profiles are representative of experiments conducted on at least three TGF-ßRII$^{f/f}$, CD19$^{cre/+}$ and TGF-ßRII$^{+/+}$, CD19$^{+/+}$ mice of 10-14 weeks of age. Single-cell suspensions were prepared and depleted of erithrocytes using standard procedures. Cells were then fixed by addition of equal volumes of PBS, 4% paraformaldehyde (10 minutes at 4$^0$ C) and washed in PBS, 0.5% BSA, 0.01% sodium azide. After permeabilization in PBS, 0.5% BSA, 0.01% sodium azide, 0.5% saponin (Sigma, S 2149), cells were aliquoted into multiwell plates ( 1 x 10$^6$ per staining), stained with the appropriate antibodies made up in the same saponin buffer and washed three times with PBS, 0.5% BSA, 0.01% sodium azide. FACS analysis was carried out on a FACScan flow-cytometer (Becton Dickinson). The antibodies used were as follows: fluorescein (FITC)- and biotin-RA36B2 (anti B220); FITC polyclonal antimouse Ig (Pharmingen 12064D) and goat anti mouse IgA (Pharmingen). Biotinylated antibodies were detected with streptavidin cy-chrome (Pharmingen).

# DISCUSSION

## Chapter 12 Characterisation of the TGF-ßRII Locus and Generation of Cell Type Specific Models of TGF-ßRII Deficiency

To assess the importance of the *TGF-ß* gene product to different leukocyte subsets *in vivo*, we decided to create mice in which TGF-ß signal transduction could be abrogated, in a cell type-specific manner. We chose to inactivate the TGF-ß Type II Receptor (TGF-ßRII), as it is essential for signaling by this cytokine (see section 1.5 and references therein). For conditional gene targeting of the *TGF-ßRII* locus we made use of the Cre-*LoxP* system (see section 3.3). As discussed in the introduction, targeting genes with this method requires an extensive knowledge of the genomic structure of the locus of interest. Thus, we first set about to clone the murine TGF-ßRII locus and characterise its genomic structure.

### 12.1 The Structure of the Murine TGF-ßRII Genomic Locus is Highly Homologous to its Human Homologue

By a combination of long template genomic PCR amplification, automated sequencing and Southern blotting experiments we characterised the genomic structure of the murine TGF-ßRII locus (see figure 8.3). As expected from the high degree of cDNA sequence identity with its human homologue (Lawler et al., 1994), the murine TGF-ßRII locus has a genomic structure similar to that of the human. As in the human, the murine TGF-ßRII gene is divided into seven exons spanning a region of > 20 Kb (see figure 8.3). The murine intron-exon boundaries were determined by comparing the genomic sequence with the TGF-ßRII cDNA sequence (Lawler et al., 1994). Aside from few base pair changes, these are highly conserved and all confirm to the "AG/GT" rule (data not shown).

Taking into account the structural similarity of the murine TGF-ßRII genomic locus to that of its human homologue, we predicted that its protein sub-domains would be encoded by the same exons as in humans. This information, coupled with intronic sequence

data obtained from the characterisation of sub-cloned TGF-ßRII genomic fragments, was used in the design and construction of the TGF-ßRII$^{fl}$ targeting vector (see figure 8.4).

## 12.2 Introduction of the TGF-ßRII$^{fl}$ mutation into the germline

The TGF-ßRII$^{fl}$ targeting vector was integrated into ES-14 cells. Through *in vitro* Cre-mediated recombination, homologous recombinant clones were modified to generate TGF-ßRII$^{fl/+}$ and TGF-ßRII$^{\Delta/+}$ ES cell lines, which were screened and selected by a PCR based procedure (figure 9.1). A Southern blotting strategy was devised to ensure that we had attained correct floxing of the TGF-ßRII exon 3 sequence, without undesired modifications of the locus (figures 9.2 and 9.3).

TGF-ßRII$^{fl/+}$ ES cells were used to generate chimeric mice by aggregation to CD1 morulae (see section 10.1, Results) and subsequent uterine blastocyst transfer (see section 10.1, Results). The chimeras were crossed with Balb/C mice to test for transmission and their heterozygous offspring inter-crossed (figure 10.1).

## 12.3 In the absence of Cre Recombinase the TGF-ßRII$^{flox}$ Mutation Does Not Affect Expression of TGF-ßRII protein in vivo

As discussed in the introduction, Dickson et al. (1995) showed that 50% of TGF-ß1$^{-/-}$ mice were dying at day 9.5 – 10 of gestation due to a defect in haematopoiesis and vasculogenesis of the yolk sac, suggesting that there is a pathway which may rescue embryonic development in the absence of TGF-ß1. In a later experiment Oshima et al. (1996) applied classical gene targeting to disrupt the sequence of the murine TGF-ßRII by insertion of neomycin and hygromycin-resistance cassettes, 5' of the transmembrane domain coding-sequence, in exon 4 of the receptor gene. These insertion vectors interrupted the transcription of the TGF-ßRII gene prior to the serine kinase domain sequence, thus disrupting receptor synthesis and blocking signalling by all TGF-ß isoforms. The resultant embryos all died at day 9.5 of gestation, due to a similar defect in yolk sac hematopoiesis and vasculogenesis, thus highlighting an essential role for TGF-ßRII in development.

As anticipated, in contrast to the TGF-ßRII$^{-/-}$ model, mice exhibiting the TGF-ßRII$^{fl}$ mutation in a homozygous manner (TGF-ßRII$^{fl/fl}$) are born at the expected Mendelian ratio,

survive well into adulthood and are indistinguishable from their wild type littermates. This proves that integration of lox P sites 3' and 5' of the TGF-ßRII exon 3 does not disrupt receptor expression *in vivo*, in the absence of Cre recombinase protein.

## 12.4 Mice Bearing the TGF-ßRII$^{fl}$ Mutation Can Be Used to Generate Models of Cell Type Specific TGF-ßRII Deficiency

The TGF-ßRII$^{fl}$ mutation was designed in such a way as to allow for the excision of exon 3 of the TGF-ßRII locus upon *in vivo* tissue specific Cre mediated recombination. Exon 3 encodes for part of the extracellular ligand-binding domain of the receptor. By sequence analysis we predicted that direct splicing of exon 2 with exon 4 would create a frameshift mutation, generating a stop codon at the beginning of the exon 4 ORF. This exon encodes for the transmembrane domain and for a large part of the extracellular and serine kinase receptor domains. Thus we anticipated that even in the event of an alternative splicing reaction (e.g. between exons 2 and 5), Cre mediated excision of the exon 3 sequence would fully disrupt TGF-ßRII translation (see figure 8.3).

Once our floxed model had been crossed to CD19$^{cre}$ mice, we demonstrated, by a PCR strategy on PBLs from the offspring, that excision of the floxed exon 3 gene occurs *in vivo* (see figure 11.1). This result is merely qualitative and cannot be used to extrapolate information as to the level or specificity of Cre mediated deletion in these strains.

In the next chapter we will discuss the results which were obtained from the initial characterisation of the TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ strain and the significance of this model in understanding the role of TGF-ß in B cell differentiation and function.

# Chapter 13 B Cell Specific TGF-ßRII Deficiency in Mice

## 13.1 TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ Mice Can Act as a Powerful Tool to Examine the Role of TGF-ß signaling to the B Cell Compartment

To prove the validity of this conditional targeting model we first tested the level and specificity of Cre mediated deletion of the floxed TGF-ßRII exon 3 allele (see figure 11.2).

CD19 is the hallmark differentiation antigen of the B lineage. It is expressed at the earliest stages and throughout B cell development and differentiation (Krop et al., 1996). The CD19$^{cre}$ strain has previously been tested as a system for B lineage specific targeting by crossing to a strain that bears the DNA polymerase ß gene in a floxed (*poß*$^{flox}$) germline configuration (Rickert et al., 1997). In this investigation, the *poß*$^{flox}$ gene was found deleted in 90-95% of splenic B cells and in 80-85% of bone marrow pre-B cells. As the authors postulated, this indicates that Cre-mediated deletion is an ongoing process during B cell development. This was confirmed by studies of Cre expression in CD19$^{cre}$ mice using Cre-specific antibodies (Schwenk et al., 1997). Other researchers, when using this system for Cre-mediated deletion of different floxed loci, did not achieve the same level of deletion (C. Schmedt, personal communication). Rickert et al. (1997) also tested deletion of the *poß*$^{flox}$ gene in T cells and several non-lymphoid tissues, where no Cre-mediated recombination was found, thus proving that this system is B lineage specific.

We used a Southern blotting strategy similar to that used by Rickert et al. (1997), to distinguish between *TGF-ßRII*$^{+}$, *TGF-ßRII*$^{fl}$ and *TGF-ßRII*$^{\Delta}$ alleles (figure 11.2.A and 11.2.B), in lymphocyte DNA from our model. Splenic B cells of TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ mice exhibited a 97.6±2% level of somatic deletion of the *TGF-ßRII* gene, as assessed by quantitation of the loss of *TGF-ßRII*$^{fl}$ and acquisition of the *TGF-ßRII*$^{\Delta}$ allele. The *TGF-ßRII* locus was maintained in germline configuration in T cells and in all non-lymphoid control tissues tested (figure 11.2.B and data not shown). This correlates with the findings described above (Rickert et al., 1997) and validates the TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ model as an effective tool in understanding the role of TGF-ß signaling to the B cell compartment.

Given that TGF-ß inhibits B cell proliferation and was shown to induce apoptosis in germinal centre (GC) B cells (Holder et al., 1992), in peripheral blood resting B cells (Lomo et al., 1995) and in fully differentiated plasma cells, we thought that deletion at the *TGF-ßRII* locus might give a selective advantage to Cre-recombined B cells, thus affecting the deletion results seen in TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ splenocytes. To test this assumption we carried out the same Southern blotting deletion analysis on B cells from TGF-ßRII$^{fl\backslash +}$-CD19$^{cre/+}$ mice, in which B lymphocytes still retain a functional wild type copy of the receptor gene. Splenic B cells from these mice exhibited a deletion level of 93.3±5.8% (figure 11.2.B) indicating that positive selection of receptor deficient cells is not a consequential factor in the *TGF-ßRII$^{fl}$* deletion levels seen in TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ splenic B lymphocytes. However, given that haploid insufficiency is a common phenomenon in TGF-ß signalling (Shida et al., 1998; Tang et al., 1998) this does not exclude the possibility that positive selection is also acting on TGF-ßRII$^{+/\Delta}$ B cells in heterozygote animals.

## 13.2 TGF-ß Does not Modulate the Development of Conventional B cells, but May Control Numbers of B-1 and B-2 cells in the Adult Mouse

We next assessed the role of TGF-ßRII in B cell development. We immunophenotyped cells from bone marrow, spleen, lymph nodes and peritoneal cavity of conditional mutant and control mice by flow cytometry (figure 11.3 and data not shown).

### 13.2.1 B Cell Development in the Bone Marrow

Despite the early expression of TGF-ß and its type II receptor in lymphoid progenitors (Lee et al., 1987), no significant differences were seen in the relative numbers of pro- and pre-B (IgM$^-$ B220$^{low}$ and IgM$^-$ B220$^+$), immature B (IgM$^+$ B220$^+$), and mature B cells (IgM$^+$ B220$^{high}$) in bone marrow from animals with a B cell specific absence of TGF-ßRII (see figure 11.3, top). Nevertheless, a slight decrease was seen in the total numbers of bone marrow B cells in TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice, with a concurrent increase in the relative numbers of myeloid cells (data not shown). This unexpected result derives from the cumulative decreases in numbers of pro- and pre-B and mature B cells in mutant bone marrow (see figure 11.3). A possible explanation for the slight reduction in B cell progenitors in TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice is that this derives from feedback inhibition, as increased

numbers of B cells were seen in the secondary lymphoid organs of these mice (Mayer et al., 1989).

Loss of TGF-ß responsiveness has been associated with increased production of TGF-ß in a variety of cell lines and *in vivo*. In particular Amoroso et al. (1998) found significant amounts of biologically active TGF-ß in the ascites of plasmacytoma-bearing mice, as well as in media conditioned by pure cultures of these cells, which were shown to have lost TGF-ß responsiveness. Similar results had been found by Berg and Lynch (1991) and Caver et al., (1996). Furthermore transgenic mice in which a dominant-negative TGF-ßRII construct was expressed under the control of a hepatocyte-pancreatic acinar cell-specific promoter, showed a marked increase in TGF-ß1 production by transgenic acinar cells (Bottinger et al., 1997). If bioactive TGF-ß levels are elevated in tissues from TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice it is possible that a down-regulation of stromal IL-7 secretion (Tang et al., 1997) is partly responsible for the decreased numbers of B cell progenitors. Interestingly, no differences were seen in the numbers of myeloid progenitors in this model.

Bone marrow cells from the conditional mutants showed no discernible differences in the levels of BrdU intracellular staining after 15 days of labelling. This is not surprising, as after 15 days of labelling all B cells in the bone marrow will have divided, except for a very small proportion of long lived plasma cells, which in our model were found at the same frequency as in wild type littermates (figure 11.4, top). To assess the B cell proliferation rate it would be interesting to carry out a BrdU pulse chase experiment (Förster and Rajewsky, 1990). In this assay, once most B cells have taken up BrdU, the supply would be removed and the proportion of B cells still labelled with BrdU would be measured by flow cytometry after set time-intervals.

### 13.2.2 Immunophenotyping the Secondary Lymphoid Organs of TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ Mice

Analysis of splenic B cell populations from TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice revealed no significant differences in the numbers of mature B cells (IgM$^+$ IgD$^+$), suggesting that there is no impact from the absence of the TGF-ßRII on the maturation of conventional B lymphocytes. Interestingly however, we found an elevated number of immature B cells (IgM$^{low}$ IgD$^-$) (figure 11.3, middle). This population of immature B cells was also seen in the

lymph nodes of conditional mutant animals (data not shown), implying a possible auxiliary role of TGF-ß in the control of B lymphocyte proliferation in germinal centres of secondary lymphoid organs. This is consistent with our finding of elevated numbers of BrdU labelled cells amongst splenocytes of TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice (see figure 11.4, middle) and with previous *in vitro* data (Smeland et al., 1987).

### 13.2.3 Elevated Numbers of B-1 Cells in the Peritoneal Cavity of B cell Specific TGF-ßRII Deficient Mice.

The B-1 subset of cells is distinguished from the conventional (B-2) B cell in surface phenotype, antigen specificity, signaling, anatomical localization and growth properties (Stall et al., 1996). These cells, predominating in the peritoneal and pleural cavities, are thought to be maintained by self-renewal of mature cells, through a process involving signaling through membrane immunoglobulin and co-stimulatory molecules such as CD19 (Krop et al., 1996). When assessing whether the B cell specific absence of TGF-ßRII would affect B-1 cell development we found a 4.5 fold increase in the numbers of such cells in the peritoneal cavity of conditional mutants (figure 11.3, bottom and figure 11.4). This is consistent with earlier literature suggesting that autocrine TGF-ß signalling may constitute a regulatory feedback loop controlling the numbers of B cells (see sections 2.2 and 2.3), although our model suggest it is particularly important in regulation of B-1 cell expansion. Interestingly, after 15 days of BrdU labelling, analysis of the B-1 cells showed no discernible differences in intracellular anti-BrdU antibody staining, between TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ mice and control littermates (see figure 11.5). This suggests perhaps that loss of TGF-ß responsiveness in these cells is not allowing for a faster proliferation rate, but more probably is rescuing them from apoptosis. It will be interesting to test the apoptotic rates of B-1 cells from these mice.

## 13.3 Does TGF-ß Up-regulate MHC-class II Expression on B lymphocytes?

Previous literature suggests that TGF-ß may control MHC class II expression on both pre-B and mature B cells (for a review see Stavnezer J., 1996). Up-regulation of MHC class II mRNA levels is seen in the TGF-ß1$^{-/-}$ model (Geiser et al., 1993; Nakabayashi et al., 1997; Shull et al., 1992), prior to any evidence of inflammatory infiltrates and prior to up-regulation of any inflammatory cytokine (Geiser et al., 1993). This suggests a causal relationship between increased MHC expression and activation of immune cell populations.

Cell surface expression of MHC molecules in the knockout model, as detected by immunohistochemistry correlates well with mRNA levels. These findings suggest that one natural function of TGF-ß1 is to control the expression of MHC class II. Altered regulation of MHC expression is one of the major causes of autoimmunity in these mice (Letterio et al., 1996).

We found no differences in the levels of MHC class II in B cells from our TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ *ex vivo* (data not shown), suggesting that TGF-ß does not modulate the constitutive expression of MHC class II on non-stimulated B cells by a direct mechanism acting within these cells.

### 13.4 TGF-ß Mediates IgA Switch Recombination In Vivo

As discussed in the Introduction (section 2.5), several reports have shown that TGF-ß directs switch recombination in immunoglobulin isotypes IgA and, to a lesser extent, IgG2b in mouse B cells *in vitro*. This is thought to occur via induction of transcription from the unrearranged Iα-Cα and Iγ2b-Cγ2b gene segments. In a recent report van Ginkel et al., (1999) showed a marked decrease in IgA secretion, especially in the gastrointestinal tract, in TGF-ß$^{-/-}$ mice, accompanied by a concomitant increase in IgM, IgG1, IgE and surprisingly IgG2b isotypes. They postulated that the retention of limited IgA secretion might be due to intake of maternal TGF-ß, via placental/foetal and lactational transfer, as documented by Letterio et al., (1994). Thus it is not yet entirely clear whether TGF-ß is essential for IgA class switch *in vivo*.

We examined IgA class switch in the TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ model, by intracellular staining of splenic and bone marrow plasma cells (figure 11.6). We found a tenfold decrease in the numbers of IgA$^+$ plasma cells in the spleen and bone marrow of conditional mutant mice (see figure 11.6, spleen: 3.43±1.21% IgA$^+$ plasma cells in wild type, 0.33±0.1% in TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$; bone marrow: 0.66±0.5% in wild type, 0.09±0.02% in mutants). To our knowledge this is the first study systematically to assess the direct role of TGF-ß on any instance of lymphocyte lineage commitment *in vivo*. These results also emphasise the importance of this cytokine as a crucial modulator, along with other Th2-type cytokines, of B cell commitment to mucosal immunity.

The result thus validates the technique of cell type-specific receptor inactivation, as a probe for detecting a cytokine's role in the control of activity in a particular cell type of interest.

# Chapter 14 Future Experiments on the TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ Model

## 14.1 Confirmation of the loss of TGF-ßRII expression in deleted cells

As outlined in section 12.4, we anticipated that Cre-mediated deletion of the *TGF-ßRII* exon 3 sequence would create a frameshift mutation due to incorrect splicing between exons 2 and 4, leading to the generation of a transcription stop sequence at the beginning of the exon 4 ORF, and ultimately to ablation of TGF-ßRII protein synthesis. The phenotypes which we have unmasked in these mice, with the initial analysis of TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ B lymphocytes (e.g. the effect on IgA class switching), act as an indirect proof of loss of TGF-ß responsiveness in these cells. Nevertheless they do not directly show absence of the TGF-ß RII protein in B cells of the conditional mutants.

Surface expression of the TGF-ß RII is low in most non-activated mammalian cell types, (approximately 350 receptors/B cell in humans)(Massague et al., 1990). Both the type I and type II receptors are found abundantly in intracellular cytosolic compartments and translocate to the cell surface upon cell activation (Gilboa et al., 1998; Koli and Arteaga, 1997; Wells et al., 1997). The low surface expression levels make direct receptor quantification by flow cytometry very difficult. Thus, to demonstrate the absence of receptor protein, we will employ a TGF-ßRII detection kit (R&D), based on flow cytometric detection of receptor binding, by recombinant biotinylated TGF-ß1. Another method which we may make use of is receptor cross-linking to $^{125}$I-labeled TGF-ß1, followed by immunoprecipitation of receptor-ligand complexes (used by Amoroso et al., (1998) on plasma cells). In this assay TGF-ßRII expressing cells should show strong cross-linking to both the TGF-ßRI and TGF-ßRII, whereas lack of the TGF-ßRII should stop any cross-linking to $^{125}$I-labeled TGF-ß1.

## 14.2 Response of TGF-ßRII$^{\Delta/\Delta}$ Lymphocytes to TGF-ß

The next assay to be carried out on the TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ model will be to test loss of TGF-ß responsiveness in TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ B lymphocytes at the biochemical level. Phosphorylation of SMAD 2 and 4 after stimulation with TGF-ß1, -ß2 and -ß3 in mutant and wild type B cells will be compared. Activin will be used as a positive control for SMAD 4 phosphorylation. SMAD 3 phosphorylation levels will not be examined as this transducer does not appear to be involved in TGF-ß signalling to B lymphocytes (Ashcroft et al., 1999; Yang et al., 1999).

More simply, B cell responsiveness to TGF-ß may be assayed by testing differences in cell viability after treatment with this cytokine. This test was also carried out by Amoroso et al., (1998) on plasma cells. The authors assessed the viability of plasmacytomas that had lost TGF-ßRII surface expression in response to TGF-ß, by flow cytometry. No effect was seen on the viability of these cells after a 48hr treatment with TGF-ß1, at concentrations causing a ≈ 30 % decrease in the viability of control plasma cells expressing normal levels of the receptor.

## 14.3 Serum Immunoglobulin Levels in TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ Mice

In sections 2.4 and 2.5 of the introduction, we discussed the ability of TGF-ß to influence secretion of immunoglobulins. Generally addition of TGF-ß during activation of murine or human B cells *in vitro* will inhibit Ig secretion by an unknown indirect mechanism (Briskin et al., 1988; Kehrl et al., 1991; Miller et al., 1991). We also discussed revealing work by Snapper et al., (1993) which showed paradoxically that small doses of TGF-ß may act to increase the secretion of most classes of Ig, and particularly IgG2a, IgG3, IgG1 and IgE.

*In vivo* results from van Ginkel et al., (1999) showed increased secretion of most classes of Ig in TGF-ß$^{-/-}$ mice (11 fold increase in IgG2b, 1.5 fold increase in IgM, 31 fold increase in IgE levels in serum). In this model it is impossible to understand what derives directly from loss of TGF-ß1 signalling to the B cell compartment and what is due to the marked increase in Th2-type cytokines (van Ginkel et al., 1999). Placental and lactational

transfer of TGF-ß1 to the TGF-ß1$^{-/-}$ offspring of heterozygous mothers (Letterio et al., 1994) does not make analysis of the effects of TGF-ß1 on Ig class switching any easier.

It is our belief that the TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ model will clarify the role of TGF-ß in isotype switching and secretion. We have obtained peripheral blood from tail bleeds of 6 TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ and 6 control mice at 4, 8-10 and 13-15 weeks of age. We will carry out Ig ELISA's to evaluate the serum levels of IgA, IgE, IgG1, IgG2a, IgG2b and IgG3 in the absence of B cell TGF-ß responsiveness *in vivo.*

## 14.4 Further Work on the role of TGF-ß on IgA Class Switch

From our results, we cannot say that loss of TGF-ß responsiveness leads to a total absence of IgA, as a much reduced fraction of IgA$^+$ plasma cells was still visible in conditional mutant samples (figure 11.6). As mutant samples were run on the flow cytometer after wild type samples, it is not clear whether this fraction of IgA$^+$ cells found in organs from conditional mutants (TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$: 0.09±0.02% in bone marrow; 0.33±0.1% in spleen) consisted of true positive IgA$^+$ plasma cells or of flow-cytometric contaminants.

The assay will be repeated, this time on lymphocytes from LPS immunised conditional mutant and control mice, to stimulate an increase in plasma cell production (Groeneveld et al., 1985). If IgA$^+$ cells are again seen in the conditional mutant samples, they will be sorted by fluorescence activated cell sorting and analysed by PCR (see figure 10.2), to test if they have undergone Cre-mediated deletion of the TGF-ßRII exon 3. This, the evaluation of IgA serum levels by ELISA, (as discussed above) as well as anti-IgA staining of sections from gastrointestinal tracts and Peyers patches of TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ and control littermate mice, should clarify if TGF-ß is essential for IgA class switch, or if other secondary mechanisms exist *in vivo.*

## 14.5 Immune response to TI-II and T Cell-dependent Antigen in the TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ model

As outlined in chapter 2 (Introduction), TGF-ß is thought to control several aspects of B cell biology. Thus it will be important to investigate the immune response of our conditional mutant mice to T cell dependent (TD) and independent (TI-II) antigens. For this

purpose we have immunised two groups of 9wk old TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ and control mice.

The first group of mice have been immunised by intra-peritoneal injection of 50 g of alum precipitated (4-hydroxy-3-nitro-phenyl) acetyl (NP) coupled to chicken gamma-globulin (CG) to elicit a T cell dependent B cell response. We are currently taking peripheral blood samples from these mice at different time points after immunisation. These will be used to test the serum concentrations of hapten (NP)- and carrier (CG)-specific antibodies and provide clues as to the importance of B cell TGF-ß responsiveness in mounting primary and secondary T cell dependent immune responses.

The second group have been immunised with the TI-II antigens α(1-3)-dextran (to assess the T cell independent B-1 cell response) and NP-Ficoll (to assess the T cell independent conventional B cell response), (Förster and Rajewsky, 1987).

## 14.6 Modulation of B Cell Gene Expression with Loss of TGF-ß Responsiveness In Vivo

As discussed previously TGF-ß modulates the expression of various genes in B cells and other leukocytes. For example, in the TGF-ß1$^{-/-}$ mice (Shull et al., 1992) mRNA levels for cytokines important in immune and inflammatory responses as well as MHC Class I and Class II, are severely affected (see section 1.3.2). We have also discussed how in many cell types loss of responsiveness to TGF-ß has been associated with a marked increase in the production and secretion of TGF-ß (section 13.2.1). Samples of mRNA will be collected from spleen, bone marrow, lymph node and other tissues from conditional mutant and control mice, which have or have not been stimulated *in vivo* by i.v. injection of LPS. These samples will be examined by gene chip technology to assess the levels of mRNA expression of immunologically relevant proteins, in the absence of normal TGF-ß signaling to the B cell compartment.

## 14.7 Histopathology and Immunohistochemistry

We have prepared formalin sections of all the major lymphoid organs and of gut, lungs, heart, liver and kidneys of conditional mutant and control mice. These are currently

being examined by M. Novelli (UCL), for histophathology. We will carry out immunohistochemical stainings of the gut and Peyers patches with antibodies to IgA, where we expect to see a marked difference in our conditional mutants.

# Chapter 15 Role of TGF-ßRII in Granulocytes, the TGF-ßRII$^{fl/fl}$-GE19$^{cre/+}$ Model

## 15.1 TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ Mice are Morphologically Indistinguishable from their Wild Type Littermates

TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ mice are born at the expected Mendelian ratio and are phenotypically and morphologically indistinguishable from their wild type or heterozygous littermates. In s.p.f. conditions these mice are fertile and survive well into adulthood.

So far hardly anything has been done to assess the effects of this mutation on the granulocyte compartment. As seen on figure 11.1 we have shown by a qualitative PCR assay on peripheral blood leukocytes that deletion is occurring in the periphery, although this result does not tell us about the specificity or level of Cre-mediated excision achieved at the TGF-ßRII locus in granulocytes. We have also shown that there are no discernible differences in white blood cell numbers in the periphery of the TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ mice at 4 weeks of age (results not shown). The mice are currently expanding and will soon be ready for analysis. In the next sections I have outlined a plan for the initial characterisation of this model, which will hopefully help to elucidate the ways in which TGF-ß modulates neutrophil function in health and disease.

## 15.2 A Plan for the Initial Characterisation of the Murine Granulocyte Specific TGF-ßRII Deficient Model

### 15.2.1 Evaluation of the Model, Specificity and Level of Deletion

The level and specificity of allelic deletion will be determined in the TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ strains using the same Southern blotting technique used for the TGF-ßRII$^{fl/fl}$-CD19$^{cre/+}$ model. Previous data suggests that for any given allele, the level of deletion obtained by crossing a mouse harbouring a floxed allele in a $GE^{cre/+}$ background will be highly variable (J. Roes, personal communication). Generally the level of deletion achieved in neutrophils is approximately 70-75%. Genomic DNA will be obtained from neutrophils,

macrophages (resident and inflammatory) and control tissues, and analysed by Southern blotting (as in section 11.1) to quantitate levels Cre-mediated deletion.

## 15.3 Granulopoiesis in TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ mice

Having established the levels of granulocyte specific *TGF-ßRII* allelic deletion, the first process that we wish to investigate in these mice is myelopoiesis. As stated in the introduction, studies by Letterio *et al*, (1996) have reinforced previous data on the importance of TGF-ß in the production and maturation of granulocytes. TGF-ß1$^{-/-}$ and TGF-ß1$^{-/-}$,MHC-II$^{-/-}$ mice exhibit enhanced myelopoiesis and myeloid hyperplasia. As mentioned, no discernible differences were found between the numbers of circulating neutrophils in the peripheral blood of 4 week old TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ and their control littermates. It will be important to immunophenotype the bone marrow and secondary lymphoid organs of older mutants by flow cytometry, using a strategy similar to that used by Liu et al., (1996), to test for possible abnormalities in bone marrow and extramedullary myelopoiesis. This should clarify if the effects seen by Letterio et al, (1996) in TGF-ß1$^{-/-}$, MHC-II$^{-/-}$ and TGF-ß1$^{-/-}$, MHC-II$^{+/+}$ mice are directly due to TGF-ß signaling deficiency to the myeloid cell component, or if other factors are involved.

## 15.4 TGF ß and Neutrophil Proliferation

Neutrophil proliferation in the absence of TGF-ßRII will be assessed by measuring bone marrow neutrophil turnover, using a bromodeoxyuridine (BrdU) "pulse chase" experiment (Förster and Rajewsky, 1990; Förster et al., 1989). In this experiment mice are fed BrdU (in their water supply, as was done for the TGF-ßRII$^{fl\fl}$-CD19$^{cre/+}$ model) until all neutrophils have incorporated BrdU in their DNA (in place of thymidine, during the S-phase of the cell cycle). The BrdU supply is then removed and the proportion of neutrophils which are still BrdU labelled is determined at set intervals by flow cytometry.

## 15.5 PMN Emigration to Sites of Inflammation in TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ Mice

Aside from its effects on leukocyte and endothelial cell-adhesion molecule expression, and aside from its importance in the modulation of processes regulating inflammatory cell infiltration (as reported in TGF-ß1$^{-/-}$ studies, section 1.3 and in section

1.5), previous data have shown that TGF-ß1 may have powerful chemotactic effects on neutrophils. The emigration of PMNs to the site of inflammation in the granulocyte specific TGF-ßRII conditional mutants will be assessed by flow cytometric analysis of cells recovered from the peritoneal exudate, at various time points after thyoglicollate induced peritonitis (Gresham et al., 1991; Liu et al., 1996; J. Roes, personal communication).

### 15.6 TGF-ß and neutrophil adhesion

If differences are seen in the rates of neutrophil migration, it will be important to investigate the mechanisms that are involved. It is clear from previous data that TGF-ß1 influences neutrophil adhesion by modulating on the one hand the expression of integrin molecules on leukocytes, and on the other E-selectin and IL-8 on activated endothelial cells (Kulkarni et al., 1993; Shull et al., 1992; Smith et al., 1996). Thus, it may be interesting to test the ability of TGF-ßRII deficient neutrophils to adhere to endothelial layers. In one such assay, PMNs are made to adhere and migrate across endothelial cell-coated filters. The expression of various adhesion molecules (e.g. Mac-1, Mel-14), on cells that have managed to cross the artificial endothelial layer is determined by flow cytometry (Kuijpers et al., 1992).

### 15.7 TGF-ß and Neutrophil Defence Against Bacterial Infection

The role of TGF-ß1 in granulocyte activation will be investigated by assessment of wild-type and TGF-ßRII-deficient neutrophil defence against bacterial infection. PMN respond to host challenges primarily by releasing toxic metabolites via the respiratory burst and by degranulation of proteolytic enzymes.

#### 15.7.1 Respiratory Burst

The first of these processes, the respiratory burst, is the major mechanism by which neutrophils, at all degrees of biological maturation, kill micro-organisms. Reactive oxygen metabolites (ROS) are generated via the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase system. It is not clear whether ROS act by directly attacking phagocytosed bacteria. They may also assist in their digestion by proteases, by creating the optimal pH in the phagosome. As stated in the introduction, previous data on the effects of TGF-ß on this process are contradictory (Brandes et al., 1991; Lagraoui and Gagnon, 1997).

We will assess respiratory burst rates by an *in vitro* superoxide production assay (Mayo and Curnutte, 1990; Voncken et al., 1995). In this assay, bone marrow neutrophils are purified and activated either with phorbol myristate acetate (PMA, receptor independent) or with N-formyl-Met-Leu-Phe (fMLP, receptor dependent). Superoxide production is followed in a kinetic microplate reader by inhibitable ferricytochrome C reduction. This method initially devised by Mayo and Curnutte (1990) allows for an accurate assessment of the kinetics of the respiratory burst. Furthermore it has the advantage that the microplates can be coated with components of the extracellular matrix, thus mimicking adhesion dependent activation of the NADPH oxidase (Lowell et al., 1996).

### 15.7.2 Neutrophil Priming

TGF-ß is also likely to play an active part is neutrophil priming, a process by which stimulation with very low concentrations of agonist (which by themselves would not be sufficient to activate neutrophils) will cause an upregulation of their function upon subsequent activation (Condliffe et al., 1998). From previous data, it looks as if TGF-ß may act to recruit and prime inflammatory cells in the initial phase of an inflammatory response (Drake and Issekutz, 1993), before playing a part in its resolution and in the general down-regulation of inflammatory activity. It would be useful to find out if TGF-ß receptor-deficient neutrophils are less easily primed. This could be done by following the superoxide production of mutant and wild-type neutrophils which have or have not been previously treated with concentrations of PMA that are too low to cause activation (see Vonken et al., 1995), in the presence or absence of active TGF-ß1. To test if there are differences in the sensitivity to stimuli in mutant circulating neutrophils, another assay optimised by Vonken et al. in the same study, may be used. In this, the superoxide production of peripheral blood neutrophils, which have or have not been primed by injections of low concentrations of LPS, is assessed by a flow-cytometric method. The neutrophils are allowed to take up 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Intracellular oxidation of DCFH to fluorescent dichlorofluorescein (DCF) by reactive oxygen metabolytes is measured.

To test further for possible abnormalities of TGF-ßRII deficient neutrophils in inflammatory processes, we will induce experimental endotoxemia by injecting sublethal doses of lipopolysaccharide (LPS), as optimised in Vonken et al., (1995). Under these

conditions wild-type controls develop mild symptoms that are cleared within 12-14 hours. A lack of the inhibitory effects of TGF-ß in TGF-ßRII deficient neutrophil development and activation, may induce a more severe inflammatory response to the bacterial endotoxin, perhaps leading to septic shock in the TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ mice, as seen in the Bcr$^{-/-}$ mice (Voncken et al., 1995). This would enable us to assess the importance of TGF-ß in the deactivation and clearance of neutrophils from sites of inflammation and in the development of severe inflammatory disorders.

### 15.7.3 Degranulation in the TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ Mice

The other important process by which PMN respond to host challenges is degranulation, i.e. the secretion of cytoplasmic granule contents that contain proteolytic enzymes. It is widely reported that PMN in suspension respond quite differently to cytokines and growth factors than do adherent neutrophils. It is important to understand the effects of different cytokines on PMN in both scenarios, to interpret the regulation of these cells at sites of inflammation. As in most processes, it appears that the role of TGF-ß in neutrophil degranulation is highly context specific. Thus in vitro TGF-ß will not activate and stimulate degranulation of neutrophils in suspension (Reibman et al., 1991). It will however stimulate the degranulation of adherent human neutrophils (Balazovich et al., 1996).

Several assays have been developed to assess degranulation, usually by quantitation of the release of enzymes from neutrophil granules. Granulocytes from wild-type and mutant mice will be isolated, primed with cytochalasin, stimulated in the presence or absence of TGF-ß and left to degranulate for a set period of time. The cells will then be centrifuged, to isolate enzymes released in the supernatant. The quantity of elastase or ß glucoronidase enzymes released can be assessed by measuring the cleavage of colourless precursors into fluorescent or coloured products (Devi et al., 1995; Harter et al., 1994; Smail et al., 1992).

### 15.7.4 The Role of TGF-ßRII in Neutrophil Phagocytosis

The process of phagocytosis is necessary not only for the uptake and intracellular killing of pathogens but also for the clearance of immune complexes, the removal of damaged tissue, and initiation of wound repair. Whereas stimulation of PMN with inflammatory mediators markedly augments phagocytic function, previous data seem to

suggest that TGF-ß1 may act to inhibit phagocytosis (Gresham et al., 1991). Increased phagocytosis may also be in part responsible for the multiple inflammatory phenotype in TGF-ß1 knockout mice. We will assess neutrophil phagocytosis *ex vivo* by testing the ability of neutrophils recovered from the peritoneal cavity, to phagocytose ethidium bromide stained heat-shocked bacteria by a flow cytometric method.

## 15.8 Neutrophil Cytokine Production

Countless reports have shown TGF-ß1 to be a powerful modulator of cytokine production in many cell types. It will be important to assess neutrophil inflammatory cytokine production in TGF-ßRII$^{fl/fl}$-GE$^{cre/+}$ mice. Initially we will test the levels of TNF-α, IL-1B, IL-6 and IL-4 using Quantikine kits (R&D). TGF-ß production by mutant neutrophils will also be assessed: as discussed previously loss of TGF-ß responsiveness has been associated with increased production of bio-active TGF-ß in several cell types (section 13.4). Increased levels of TGF-ß1 production and their effects on neighbouring receptor expressing cells will have to be taken into account in the study of any TGF-ßRII$^{-/-}$ deficient cell type.

## 15.9 TGF-ß and Neutrophil Apoptosis

Apoptosis in neutrophils is by no means a clear-cut process (Homburg and Roos, 1996). Neutrophils are the most short lived form of leukocyte, constitutively programmed to undergo apoptosis, followed by a rapid recognition and internalisation by macrophages. This limits their inflammatory potential and stops them from releasing their toxic contents into the surrounding tissues. It is now recognised that most neutrophil priming agents have the added function of delaying apoptosis, thus increasing the neutrophil's functional longevity at the inflamed site (Condliffe et al., 1998).

*In vitro*, TGF-ß1 has been shown to prolong neutrophil survival by suppressing apoptosis, with inhibitory effects comparable to those of GM-CSF and dexamethasone (Ward et al., 1997). Furthermore it has been shown to augment the recognition of apoptotic neutrophils by macrophages (Ren and Savill, 1995). In other cell types, on the contrary, it promotes apoptosis (e.g. transformed fibroblasts and resting B lymphocytes, Lomo et al., 1995; Picht et al., 1995). New Zealand Black (NZB) mice, which secrete an aberrant amount

of TGF-ß, and express an increased level of extracellular TßRII on B cells, have an increased level of B cell apoptosis (Douglas et al., 1997).

Several assays exist for the assessment of neutrophil apoptosis. One method relies on the detection of apoptotic markers by flow cytometry. Phosphatidylserine (PS) is confined to the inner leaflet of the plasma membrane in viable cells. During apoptosis, it is translocated to the outer leaflet of the plasma membrane, where it becomes available for Annexin V binding. Annexin V is a 35-36kD $Ca^{2+}$ dependent phospholipid binding protein. Under defined salt and calcium concentrations, Annexin V binds specifically to PS residues with high affinity and is a reliable indicator of early apoptosis (Liu et al., 1996). It may at times be difficult to asses the apoptosis rate *in vivo*, as apoptotic cells tend to be readily cleared in vivo by the reticulo-endothelial system. To overcome this problem, mutant and control bone marrow mononuclear cells can be cultured in serum-containing media with or without TGF-ß, and assessed by flow cytometry using anti-GR1 and Annexin. Another useful method for the assessment of apoptosis is terminal dUTP nucleotide end-labelling (TUNEL). As the name suggests, this assay relies on the staining and visualisation of 3' ends of DNA fragments generated by DNAses in apoptotic cells. This enables *in situ* detection and comparisons of apoptotic rates in wild-type and mutant organs.

# CONCLUSIONS

Clearly a cleaner and more systematic approach is needed to study mechanisms by which TGF-ß and its receptors can modulate immunological homeostasis. It was for this reason, and with particular reference to our interest in the way in which TGF-ß regulates B cell and neutrophil function, that we made use of Cre/LoxP conditional mutagenesis to generate the TGF-ßRII$^{flox}$ strain. By crossing this strain with mice bearing the Cre recombinase gene (*Cre*) under transcriptional control of the *CD19* and *GE* promoters we have created respectively B cell (TGF-ßRII$^{fl\fl}$-CD19$^{cre/+}$) and granulocyte (TGF-ßRII$^{fl\fl}$-GE$^{cre/+}$) specific murine models of TGF-ßRII deficiency.

We have shown by a PCR based method that Cre-mediated deletion of the floxed *TGF-ßRII* locus occurs in peripheral blood leukocytes from TGF-ßRII$^{fl\fl}$-GE$^{cre/+}$ mice (figure 11.1.C). As discussed, we hope that this model can be used to bring insight not only into the specific mechanisms by which TGF-ß controls granulocyte biology but also into the importance of neutrophils in the inflammatory response.

The main progress has been made in characterising the TGF-ßRII$^{fl\fl}$-CD19$^{cre/+}$ model, as applied to B cells. By Southern blotting analysis of Cre-mediated recombination at the *TGF-ßRII$^{fl}$* locus, we have found a deletion efficiency of >95% in splenic B-lymphocytes from these mice (figure 11.2). Inactivation of the Cre-recombined floxed allele, leading to loss of TGF-ß responsiveness in these B cells, has been confirmed by our result showing a difference in B-1 and B-2 cell proliferation (figures 11.3, 11.4 and 11.5) and abnormal IgA$^+$ plasma cell production in TGF-ßRII$^{fl\fl}$-CD19$^{cre/+}$ mice (figure 11.6). The latter result makes this the first study to assess systematically the role of TGF-ß on an instance of lymphocyte lineage commitment *in vivo*.

The nature of this model will now allow us to test whether TGF-ß is essential for IgA class switch *in vivo* (14.4) and to assess the role of this potent cytokine, in the mechanisms of Ig class switch and secretion (section 14.3). This, and the role of TGF-ß in

controlling and limiting the expansion of B cells after an immune response, should become clearer with measurement of serum Ig levels.

Last but not least we emphasise that loss of sensitivity to TGF-ß is associated with the malignant transformation of many cell types, including lymphocytes (Amoroso et al., 1998; Kumar et al., 1991; Sing et al., 1990). The possibility that B cell tumours may develop in the TGF-ßRII$^{fl\backslash fl}$-CD19$^{cre/+}$ mice remains open: an exciting prospect.

Aside from the B cell and granulocyte specific models, with expanding availability of different tissue-specific and inducible Cre-expressing strains (see section 3.3), it is hoped that the TGF-ßRII$^{flox}$ mouse will be of use more generally for analysing the impact of TGF-ß on the many other cell types which express the receptor.

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