CONDITIONAL MUTAGENESIS IN THE IMMUNE SYSTEM

Targeting the Expression of the *iCre2* Recombinase to Neutrophils and Macrophages.

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ABSTRACT

Conditional mutagenesis allows the introduction of tissue specific mutations in the mouse and is of crucial importance in converting genome sequence information into functional data for biomedical research. Mice expressing the Cre recombinase in a spatially controlled manner are essential in creating such conditional knock-outs. A wide variety of Cre mice have been generated, but there is a distinct lack of models expressing the recombinase faithfully and at high levels in cells of the innate immune system. To address this need, three target genes, *Itgb2l, Marco* and *Msr1*, were chosen to create novel neutrophil and macrophage specific knock-in models harbouring *iCre2*, a recombinase engineered for increased expression levels. Two strategies were employed. Initially gene specific bacterial artificial chromosomes in which the *iCre2* fragment replaced the endogenous translation start codon were created by Red/ET recombineering. Utilization of these BAC vectors for embryonic stem cell targeting successfully created knock-ins but the identification of homologous recombinants was complicated by the vectors' large size. As the discovery of mutations impeding *iCre2* functionality in the knock-in lines necessitated repeating the vector creation process, novel shorter vectors were designed. These vectors achieved targeting frequencies of around 10% and facilitated the isolation and verification of 9 *Itgb2l* and *Marco* specific *iCre2* knock-in murine embryonic stem cell lines on the 129 genetic background. To determine tissue specific *iCre2* expression before generating mouse models, an *in vitro* haematopoietic differentiation system, utilising three-dimensional embryoid body formation and selective expansion of progenitors in the presence of IL-3 and MCSF, was adapted. Embryonic stem cells were successfully differentiated into macrophages as assessed by CD11b and F4/80 marker expression. Collectively, this work has established the foundations for obtaining viable myeloid specific Cre producer mouse strains and discusses the potential of their future application in elucidating the role of macrophages and neutrophils in innate immune function.

DECLARATION

I, Jennifer Doreen Könitzer, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Jennifer Doreen Könitzer

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RESULTS & DISCUSSION

ABBREVIATIONS

2xYT	Yeast Tryptone broth
AGM	Aorta Gonads Mesonephros
AM	Alveolar Macrophages
ATP	Adenosine Triphosphate
att	Attachment Site
BAC	Bacterial Artificial Chromosome
BFU	Blast Forming Unit
β geo	β Galactosidase Neomycin Phosphotransferase Fusion Construct
BMP	Bone Morphogenetic Protein
bp	Base Pair
BSA	Bovine Serum Albumin
CAG	CMV Early Enhancer/Chicken β Actin Promoter
CD	Cluster of Differentiation
CFC	Colony Forming Cell
CFU	Colony Forming Unit
CGE	Capillary Gel Electrophoresis
CHO	Chinese Hamster Ovary
CMV	Cytomegalovirus
d	day(s)
DC	Dendritic Cell
dCTP	Deoxycytidine Triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ds	Double Strand(ed)
E	Days in Embryonic Development
EB	Embryoid Body
EDTA	Ethylenediaminetetraacetic Acid
ER	Estrogene Receptor
(m/h)ES(C)	(Murine/Human) Embryonic Stem (Cell)
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FISH	Fluorescence in situ Hybridisation
FITC	Fluorescein Isothiocyanate
floxed	flanked by loxP
FRT	Flp Recombinase Target
(E)GFP	Enhanced Green Fluorescent Protein
HIV	Human Immunodeficiency Virus
hm	Homology Arm
HR	Homologous Recombination
HSC	Haematopoietic Stem Cell

Hsp90	Heat Shock Protein 90
ICM	Inner Cell Mass
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco Medium
iNOS	Induced Nitric Oxide Synthase
iPS	Induced Pluripotent Stem
KAc	Potassium Acetate
kb	Kilo Base Pair
kDa	Kilo Dalton
LB	Luria Bertani broth
LBD	Ligand Binding Domain
LCR	Locus Control Region
LDL	Low Density Lipoprotein
LIF	Leukemia Inhibitory Factor
loxP	Locus of Crossing Over
LTRHSC	Long Term Repopulating Haematopoietic Stem Cell
Mb	Megabase
MBP	Maltose Binding Protein
MCM	Methyl Cellulose Medium
MCS	Multiple Cloning Site
MCSF	Macrophage Colony Stimulating Factor
MIP	Macrophage Inflammatory Protein
MMC	Mitomycin C
MMP	Matrix Metalloproteinase
MTG	Monothioglycerol
n/a	not assessed
NaAc	Sodium Acetate
NADPH	Nicotine Adenine Dinucleotide Phosphate
Neo^R	Neomycin Phosphotransferase Gene
NLS	Nuclear Localisation Signal
nt	Nucleotide(s)
OHT	4-Hydroxytamoxifen
PAC	Phage Artificial Chromosome
pA	Polyadenylation
pAp	polyA/pause
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PFGE	Pulse Field Gel Electrophoresis
PGK	Phosphoglycerate Kinase

pH	Potentia Hydgrogenii
pSp	Para-aortic Splanchnopleura
RA	Rheumatoid Arthritis
RFP	Red Fluorescent Protein
RMCE	Recombinase Mediated Cassette Exchange
RNA/mRNA	Ribonucleic Acid/Messenger RNA
RNAi	RNA Interference
ROI	Reactive Oxygen Intermediates
RNI	Reactive Nitrogen Intermediates
RT	Reverse Transcriptase
SCF	Stem Cell Factor
SDS	Sodiumdodecylsulphate
SNP	Single Nucleotide Polymorphism
SSC	Saline Sodium Citrate Buffer
TAE	Tris-Acetate-EDTA
TB	Terrific Broth
TBE	Tris-Borate-EDTA
TβR	TGFβ Receptor Type
TE	Tris-EDTA
Tet	Tetracycline
TGF	Transforming Growth Factor
TK	Thymidine Kinase
TNF	Tumor Necrosis Factor
UV	Ultra Violet
VEGF	Vascular Endothelial Growth Factor
w/o	Without
wt	Wild Type
YAC	Yeast Artificial Chromosome
YFP	Yellow Fluorescent Protein

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OVERVIEW

Among the currently available Cre expressing mouse models for conditional mutagenesis in the haematopoietic systems, those mediating recombination in the lymphoid compartment dominate. The few models available targeting cells of the innate immune compartment largely suffer from low expression levels and/or additional ectopic recombination in non-myeloid tissues. The GE-Cre strain previously created by this lab, for example, designed to express the enzyme from the pro-myelocyte restricted *ela2* locus (Tkalcevic et al., 2000), achieved less than 50% deletion levels in macrophages and granulocytes in a model devised to remove the TGF-β receptor type II (S.Mahbub et al, unpublished data). With such low recombination frequencies resulting in mixed cell populations no phenotype was observed, highlighting the need for new and improved models. The objective of this work is therefore to create such mice utilizing the novel engineered recombinase *iCre2*, which, through the integration of heterologous mouse introns, reaches significantly higher expression levels (Lacy-Hulbert et al., 2001).

As no available myeloid Cre strain can selectively mediate recombination in either neutrophils or macrophages, three novel target loci were chosen as locales for *iCre2* knock-in. *Itgb2l* is expressed in all neutrophils, *Msr1* in all macrophages and *Marco* in subsets of tissue resident macrophages. The knock-in strategy is set up such that *iCre2* replaces the endogenous translation start codon ensuring minimal interference with the host gene's regulatory sequences.

The first three chapters of the introduction focus on the technical evolution of the Cre/loxP system, the essentials in creating a new mouse model and the particulars of conditional mutagenesis in the haematopoietic compartment respectively. The final introductory Chapter 4 briefly covers basics in stem cell biology and blood development from the embryo *in vivo* and *in vitro* as a macrophage differentiation assay was utilized to assess tissue specific recombinase expression prior to the creation of a murine model.

After a brief summary of employed techniques in Chapter 5; Chapters 6 to 9 summarize and discuss the experimental findings of this project, with the key individual objectives and achievements recapitulated here:

Chapter 6: The First Generation of *iCre2* Knock-ins

Objectives	Key Achievements
• Identification of Homologous Recombinants Among Previously Created G418 Resistant ES Cell Colonies Using Gene Specific BAC Knock-ins	• Design of PCR Strategies to Reduce Number of Candidate Clones • Application of Southern Blots to Identify Knock-in Lines for *Itgb2l* • Discovery of Faulty loxP Sites in All Vectors and Targeted Clones, Precluding Removal of the Resistance Marker

Chapter 7: Constructing the Second Generation *iCre2* Targeting Vectors

Objectives	Key Achievements
• Redesigning of Targeting Vectors to Utilize Shorter Homology Arms • Vector Construction in Two Rounds of Red/ET Recombinogenic Engineering	• Successful Construction of BAC knock-ins by First Round of Recombineering • Discovery and Repair of Several Interfering Mutations • Construction and Verification of Plasmid Targeting Vectors Harbouring *iCre2*, a Selection Cassette and 4kb and 7kb Homology Arms for *Itgb2l*, *Marco* and *Msr1*

Chapter 8: Creating 2nd Generation *iCre2* Embryonic Stem Cell Knock-ins

Objectives	Key Achievements
• Establishment of Knock-in 129 ES Cell Lines • Removal of the G418 Selection Marker	• Isolation of 650 Drug Resistant Clones • Application of PCRs & Southern Blots to Identify 9 Knock-ins for *Itgb2l* and *Marco* • Removal of Neo^R • Discovery of Basal *Marco* Expression in Undifferentiated Embryonic Stem Cells

Chapter 9: Generating Embryonic Stem Cell Derived Macrophages

Objectives	Key Achievements
• Implementation of a Haematopoietic Differentiation Protocol to Test *iCre2* Expression *in vitro*	• Testing, Analysis & Optimisation of Conditions to Produce Embryonic Macrophages • Creation of a Reporter Cell Line to Trace *iCre2* Expression • Discovery of Dysregulated *Marco* Expression in Knock-in Cell Lines

Finally, the closing Chapter 10 provides further discussion of the next steps required to produce the novel murine models, including possibilities for resolving technical obstacles encountered in the course of this project and for verifying tissue specific *iCre2* expression *in vivo*. The Chapter also supplies a brief overview of potential applications. In the context of the models' original intent, the cell specific ablation of TGF-β receptor type II, the current knowledge about TGF-β function in neutrophils and macrophages is reviewed and other interesting avenues for conditional mutagenesis in the innate immune system are explored.

INTRODUCTION

CHAPTER 1: CONDITIONAL GENE TARGETING IN THE MOUSE

Mutational modification or inactivation of genes followed by phenotypic analysis remains the perhaps most important method in elucidating gene function in mammals. Prior to the development of directed mutagenesis, this technique was restricted to phenotypes resulting from rare spontaneous mutations, for example those causing hereditary diseases (Capecchi, 1989). The establishment of mouse embryonic stem cell technology (Bradley et al., 1984) allowed the targeted introduction of mutations, a method previously restricted to bacteria, into more complex organisms (Thomas and Capecchi, 1987). The classical experiment (for further details see Chapter 2) uses homologous recombination to deactivate a chosen gene in all cells of the organism. Several disadvantages arise from this approach. About 15% of complete knock-outs prove to be lethal during mouse embryonic development (Copp, 1995), leaving no viable model for studying. Furthermore, genes may play important roles at various stages of ontogeny; but conventional knock-outs show only the target gene's earliest purpose. Gene function can also vary among tissues, which can make organism-wide null phenotypes too complex to analyse. Conditional gene targeting (Rajewsky et al., 1996) utilises the properties of site specific recombinases, in particular the Cre/loxP system, to circumvent these difficulties. Genes of interest can be inactivated in a spatially and/or temporally controlled manner. The technicalities, advantages and drawbacks of this method are described in the following chapter, together with available alternatives for the conditional control of gene expression in the mouse system.

1.1 Site-specific Recombination

Site-specific recombination is a process of breaking and re-connecting DNA segments that commonly results in the integration, excision or inversion of sequences. The exchange of DNA strands is mediated by a protein, the site-specific recombinase, which recognizes a pair of distinct recombination sites (Grindley et al., 2006). Many site-specific recombinases have been described, all of which are responsible for maintaining genetic stability, altering gene expression or inducing genetic diversity in their host organisms. Site-specific recombinases have evolved from topoisomerases; enzymes controlling the winding and unwinding of DNA (Cheng et al., 1998; Van Duyne, 2009). They fall in two distinct families depending on whether the amino acid forming an intermediate bond with the DNA's phosphodiester backbone during recombination is a tyrosine or a serine. Site-specific recombinases need no energy rich co-factors such as ATP, because the energy released during the breakage of the phosphodiester bonds is sufficient to drive recombination.

The tyrosine recombinase family (Argos et al., 1986; Nunes-Düby et al., 1998), also called λ integrase family, contains Cre and Flp, both of which have been used in conditional

mutagenesis in the murine system. A generalized mechanism of action is illustrated in Figure 1.1. ϕ31, a member of the serine recombinase family (Smith and Thorpe, 2002), also known as the resolvase family, has also been utilised, particularly in order to facilitate integrations in mammalian genomes.

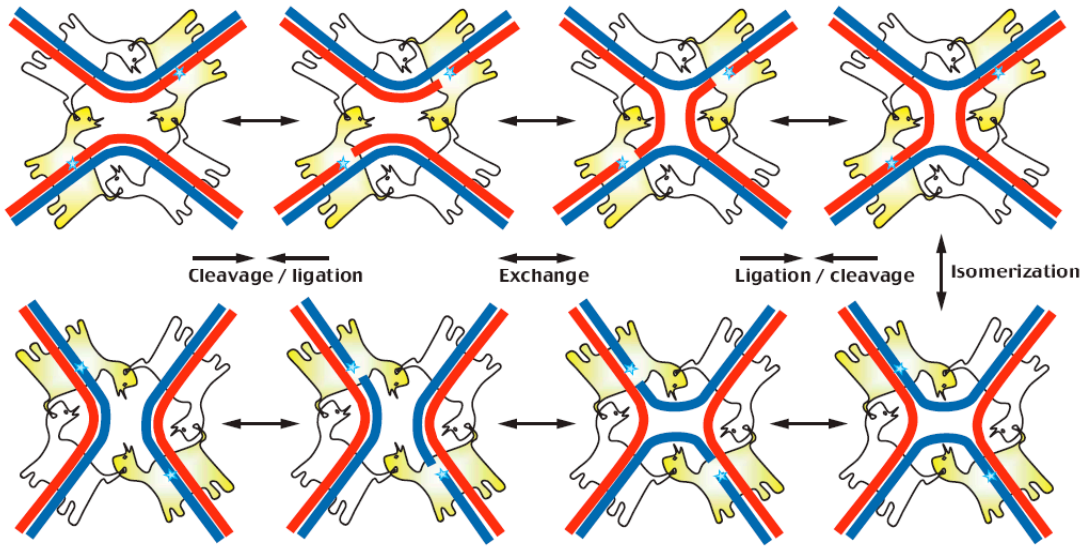


Figure 1.1 Tyrosine Recombinase Mechanism
Four recombinase subunits (white & yellow) and two DNA duplexes (red and blue) form a synaptic complex. Cleavage (blue stars) of one strand each by a nucleophilic tyrosine initiates recombination. The cleaved strands are exchanged and re-ligated forming a Holliday junction. Isomerization of the complex enables the second strand to undergo the same process and resolves the junction. Illustration taken from (Grindley et al., 2006).

1.2 The Cre/loxP System

The Cre recombinase was first described in 1981 as the factor responsible for mediating recombination in the filamentous bacteriophage P1 (Sternberg and Hamilton, 1981). The enzyme catalyses site-specific recombination between sites termed loxP (locus of X over P1) (Hoess et al., 1982). The system plays an important role in maintaining genetic stability throughout the phage's life cycle by controlling the integration into and the excision from bacterial chromosomes as well as recombination aimed at increasing genetic diversity (Austin et al., 1981).

The loxP sites are 34 base pairs (bp) long and are characterised by an 8bp asymmetric core sequence defining orientation (Hoess et al., 1986) flanked by 13bp long palindromic inverted repeats (Figure 1.2A). The recombination process can have three distinct outcomes depending on the spatial position of the loxP sites (Figure 1.2B). Two parallel loxP sites on the same double-stranded DNA molecule result in deletion of the intermediate segment, with one loxP site, made from the two halves of the pre-recombinatory sites, left behind. LoxP sites with an anti-parallel configuration, however, cause the intervening DNA stretch to be excised, inverted

and re-inserted at the same location (Abremski et al., 1983). Sites located on different DNA molecules cause translocation or integration depending on whether both fragments are linear or one is linear and one circular (Sternberg et al., 1981). Excision and reintegration form an equilibrium, favouring excision in presence of the enzyme (Baer and Bode, 2001).

Several groups have attempted to analyze the role of loxP sequence in recombination. Spacer length, but not sequence, is considered essential for efficient recombination (Hoess et al., 1986). Nucleotides 2 to 7 of the 8bp spacer were found to be crucial in recombination. Mutations at these positions abolished Cre mediated strand exchange between wild type and mutant sites. Base pairs 1 and 8 could be mutated without affecting recombination too severely (Lee and Saito, 1998). Several mutant loxP sites with single or double base pair changes in the spacer region are able to efficiently recombine with each other but not with the wild type site. Contrariwise, another study demonstrated that only spacer positions 4 and 5 are essential, whereas all other nucleotides could be mutated with recombination still taking place, if at a lower frequency (Araki et al., 2002). Mutations in the left and right inverted repeats have also been described as functional, particularly those involving the initial and terminal 5bp. The TATA sequence, immediately adjacent to the spacer on the left and right, however, seems indispensable for recombination as mutations of that area have not been reported (Missirlis et al., 2006). Modified loxP sites are useful when multiple genes in the same model are to be targeted or the rapid re-excision of an integrated product is to be avoided (Hoess et al., 1986). For an overview of loxP variants commonly used see Figure 1.2C.

The Cre enzyme is a 38.5kDa protein that binds the inverted repeats of loxP sites with its carboxy-terminal fragment (Hoess et al., 1990). The resolution of the Cre protein structure (Guo et al., 1997) revealed that the enzyme is folded in two distinct domains consisting primarily of α-helices. The enzyme's active site is made up of a catalytic triad with arginines at positions 173 and 292 and histidine at position 289, as well as a conserved nucleophilic tyrosine at position 324 and a tryptophan at 315. Two Cre monomers bind to one loxP site forming a C-shaped clamp around the DNA and subsequently aggregate as a tetramer with the Cre molecules bound to the second loxP site to initiate recombination (Figure 1.2D) (Gopaul et al., 1998).

Recently, it was discovered that Cre recombination occurs primarily during the early S-phase of the mammalian cell cycle (Hashimoto et al., 2008), similar to the endogenous process of double-stranded DNA break repair by homologous recombination (Mao et al., 2008). Since its first successful expression in a mouse cell line (Sauer and Henderson, 1989), the Cre/loxP system has been widely used to modify mouse genomes (Branda and Dymecki, 2004; Orban et al., 1992). Furthermore the system has been applied in yeast (Sauer, 1987), drosophila (Siegal and Hartl, 2000), zebrafish (Pan et al., 2005) and plants (Sieburth et al., 1998).

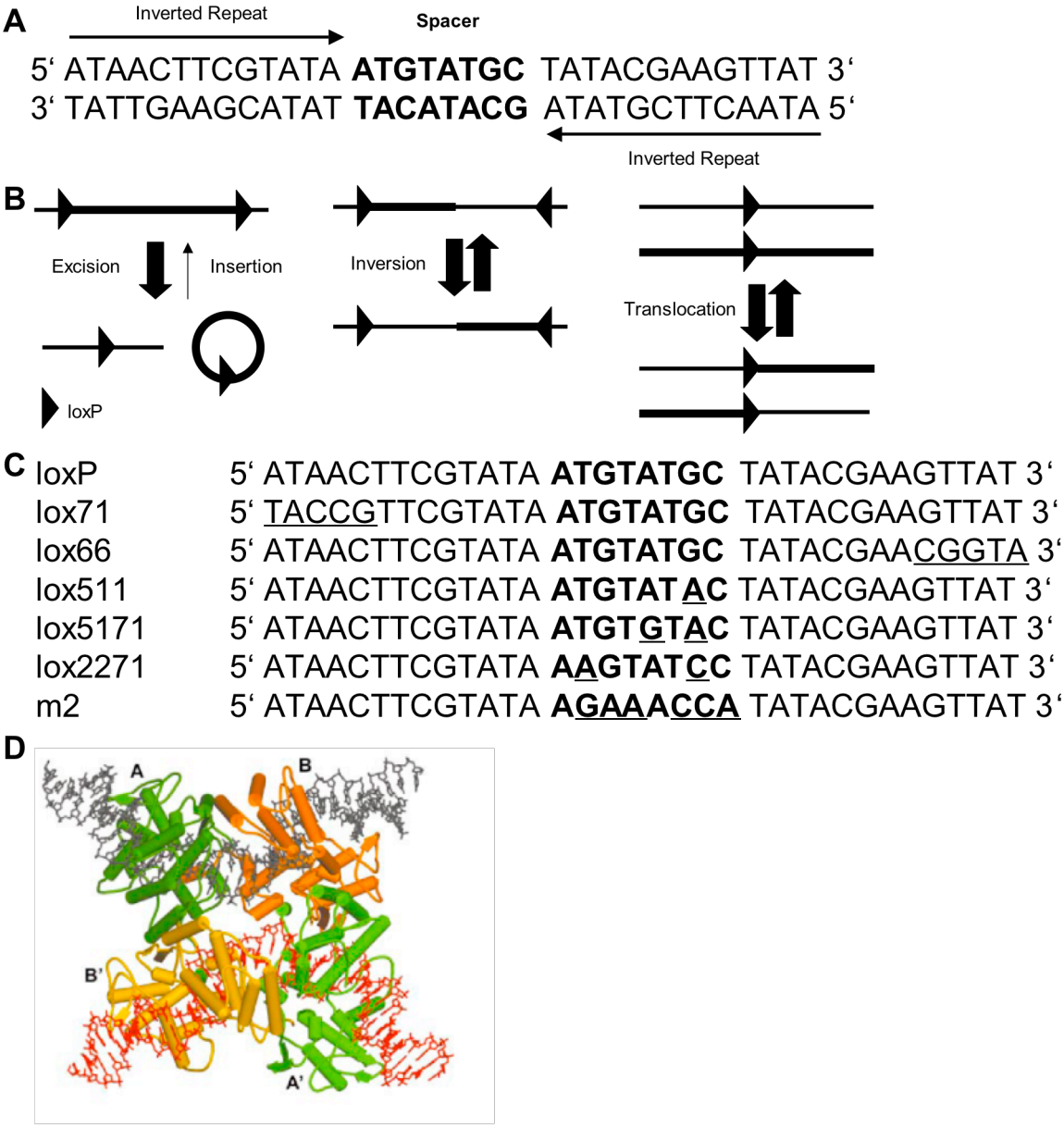


Figure 1.2 The Cre/loxP System
LoxP sites consist of 34bp **(A)** with an orientation-defining 8bp spacer flanked by 13bp-inverted repeats. The spatial orientation of loxP sites **(B)** determines whether excisions, insertions, translocations or inversions occur. Several loxP mutants **(C)**, carrying one or more mutated nucleotides (underlined), are in use. The Cre protein binds to the inverted repeats and forms a tetramer. This juxtaposes the DNA strands and makes recombination possible **(D)**. Illustration D is taken from (Ennifar et al., 2003).

1.2.1 Modifications of the Cre Recombinase

The Cre enzyme has been modified extensively in order to enhance its functionality. Modifications are typically either meant to increase expression levels of the protein in experimental models or to enable inducibility of expression.

1.2.1.1 Modifications of the Cre Coding Sequence

The Cre gene originates from a prokaryotic background. Codon usage differs between prokaryotes and eukaryotes and high CpG contents typically found in prokaryotic genes can lead to epigenetic silencing by methylation in mammals (Cohen-Tannoudji et al., 2000). Silent mutations have been employed to adapt the Cre sequence to mammalian codon usage and to minimize GC pairs (Koresawa et al., 2000; Shimshek et al., 2002). Protein expression levels and recombination events observed increased by about 50% and 80% respectively.

The Cre version used in this work (Figure 1.3) has further been optimized by the insertion of introns. Introns have been shown to enhance gene expression in transgenic mice considerably (Palmiter et al., 1991) by creating a spliceable structure reminiscent of mammalian genes, which improves mRNA stability. Short introns from the inmunoglobulin loci, more specifically from genes encoding the heavy chains of IgM and IgE, were inserted into a partially codon-improved Cre version lacking GC pairs (Lacy-Hulbert et al., 2001). The intronic sequences, 109 and 89bp in size, are compatible with high expression levels and do not increase the size of the Cre gene significantly. mRNA expression levels of this construct termed *iCre2* were 30 fold higher than observed with the codon improved Cre version alone. *iCre2* also contains an E1α nuclear localisation signal (NLS) at its 5' end to enhance transfer into the nucleus, the location of Cre-catalysed recombination. It has recently been shown, however, that Cre is able to cross the nuclear membrane without an extra NLS (Will et al., 2002). This is possibly due to Cre being a basic protein carrying an intrinsic, bipartite NLS-like structure (Andreas et al., 2002).

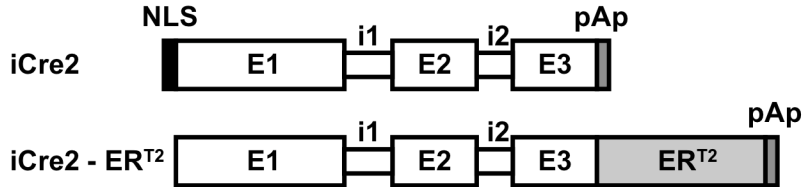


Figure 1.3 Gene Structure of *iCre2*
The Cre version applied in this work has been engineered to remove GC pairs and adapt the codon usage to mammals. Furthermore two heterologous introns (i1:109bp; i2:89bp) have been inserted to create an artificial exon structure (E1:567bp; E2:213bp; E3:264bp). In addition, *iCre2* carries a NLS for better translocation into the nucleus and a polyA/pause (pAp) for efficient termination of transcription. The inducible version is fused to a human estrogen receptor fragment (ER^{T2}) and does not carry the NLS. Figure modified from (Lacy-Hulbert et al., 2001).

1.2.1.2 Inducible Cre Fusion Constructs

Numerous attempts have been made to render Cre temporally inducible. Though the 34bp recognition site is stochastically unlikely to occur randomly in mammalian genomes, similar "pseudo-loxP" sites have been found in both mouse (Schmidt et al., 2000) and human (Thyagarajan et al., 2000). In murine genomes, pseudo-loxP sites occur every 1.2 Megabases and, while having a much lower Cre affinity, can promote illegitimate recombination compromising chromosomal integrity (Semprini et al., 2007). In cell culture, prolonged exposure to high doses of Cre can be cytotoxic due to chromosomal degradation (Loonstra et al., 2001). Some Cre mice, like the pancreas specific RIP-Cre that is glucose intolerant (Lee et al., 2006), show phenotypic abnormalities, though whether this is due to the transgene, insertion locus or a result of Cre toxicity is unknown.

Temporal inducibility can be partly achieved by coupling Cre expression to a promoter only active during a certain period of development or regulable by endogenous molecules. The first inducible Cre model was the Mx1-Cre mouse, in which expression was coupled to the interferon-responsive promoter Mx1 (Kuhn et al., 1995). As this system is potentially leaky, others, using hormone-inducible receptor fragments, have been conceived. Fusion constructs between Cre and steroid receptor ligand binding domains (LBD) are popular. The mouse (Zhang et al., 1996) and human (Metzger et al., 1995) estrogen receptor LBDs have been widely used. Without induction the fusion construct is held in the cytoplasm bound to Hsp90, not able to translocate to the nucleus and is therefore not able to mediate site-specific recombination.

Initial attempts utilized wild-type receptor fragments whose interactions with endogenous estrogen were deleterious. Later constructs employed mutated receptors, particularly the G400V/M543A/L544A (Feil et al., 1997) version of the human estrogen LBD, designated ER^{T2}. Induction only occurs via the estrogen antagonist tamoxifen or its metabolic derivate 4-Hydroxytamoxifen (OHT) (Williams et al., 1994). In cell culture, the system is able to achieve 100% recombination (Zhang et al., 1996). *In vivo*, mouse models are fed or injected with tamoxifen or OHT over a course of several days at differing stages of development. Background Cre function could not be observed, but recombination levels following induction typically do not reach 100% and are highly tissue dependent (Casanova et al., 2002; Hayashi and McMahon, 2002). In the initial stages of this project a fusion construct between *iCre2* and ER^{T2} (Figure 1.3), was used to achieve inducibility.

Other inducible Cre systems include fusion constructs with mutant human progesterone receptor LBD (Wunderlich et al., 2001), mutant human glucocorticoid receptor LBD (Brocard et al., 1998), or doxycycline inducible Cre (DAI-Cre; also see section 1.6.1.) (Holzenberger et al.,

2000b), regulated by the prokaryotic tetracycline repression system (Belteki et al., 2005). A conceptually new approach mediates inducibility by splitting the Cre recombinase in two inactive parts, each fused to proteins that are able to dimerize (DiCre) and thus activate Cre function in the presence of rapamycin (Jullien et al., 2007). Most recent efforts rely on Cre induction by light. Coupling the nucleophilic tyrosine residue in the enzyme's active centre to an o-nitro benzyl residue renders the recombinase inactive. UVA radiation removes the inhibitory group and activates Cre function (Edwards et al., 2009).

1.3 The Flp/FRT System

Flp is a site-specific recombinase originally discovered in the yeast *Saccharomyces cerevisiae* (Broach and Hicks, 1980). The 43kDa enzyme recognizes FRT sites, which have a topology similar to the loxP sites. An 8bp spacer is flanked by two 13bp inverted repeats. However, a further 13bp direct repeat is necessary for complete Flp function. Shorter FRT sites lacking the direct repeat can mediate excision but not integration. Three Flp molecules, one bound per repeat, are required for DNA cleavage (Bode et al., 2000).

Flp is used to a somewhat lesser extent than Cre, possibly due to its lower temperature stability. Cre is fully active at 37˚C and can survive temperatures of up to 46˚C. Flp on the other hand, has a thermo optimum of 30˚C and is labile above 39˚C. Flp also has been reported to need higher expression levels than Cre in order to function (Buchholz et al., 1996). Engineered Flp versions, exhibiting higher temperature stability (Buchholz et al., 1998) and improved expression levels following codon optimization (Kondo et al., 2009), help overcome these drawbacks. In an assay analyzing codon improved Flp recombination activity on a chromosomal target, however, it only reached 10% of the recombination levels observed with Cre (Andreas et al., 2002). Ligand-inducible versions of Flp, similar to those described for Cre, have also become available (Hunter et al., 2005). Flp has been successfully utilized in mammalian cell culture (O'Gorman et al., 1991), drosophila (Theodosiou and Xu, 1998), plants (Luo and Kausch, 2002) and mice.

1.4 The ϕ31/attP/attB System

The ϕ31 integrase is derived from a *streptomyces* phage (Kuhstoss and Rao, 1991). It mediates recombination between dissimilar attachment (attB/attP) recognition sites. AttP is found in the phage genome and attB on the bacterial chromosome. Recombination results in the phage being integrated into its host genome, which generates two chimeric sites; attL and attR. The minimal sizes for recombination are 39bp (attP) and 34bp (attB) in size respectively, characterized by inverted repeats resembling the loxP and FRT sites, and a core consisting of the 3 nucleotides

TTG. Whereas integrations catalysed by Cre and Flp are reversible due to the creation of two identical parallel sites which undergo rapid re-excision, integration carried out by ϕ31 is unidirectional (Groth et al., 2000).

Mammalian genomes have been shown to possess a significant number of pseudo-att sites (about 100-1000 in both mouse and human), which are also recognized by the integrase and could serve as targets for the directed integration of genes without having to introduce attachment sites first (Thyagarajan et al., 2001). ϕ31 functions in both human and mouse ES cells and could be stably integrated and transmitted via the germline (Belteki et al., 2003).

1.5 Creating Conditional Knock-outs

In practice the creation of conditional knock-outs requires a Cre producer mouse, expressing the recombinase, and a Cre responder mouse carrying a floxed (flanked by loxP sites) version of the gene of interest (Branda and Dymecki, 2004). Crossing a female homozygous for the floxed allele to a male heterozygous for Cre results in 50% of the offspring carrying the Cre allele. All F1 offspring are heterozygous for the floxed allele. A F2 generation obtained by intercrossing the Cre positive F1 animals establishes the conditional knock-out (Figure 1.4). When Cre is transmitted maternally, deletions have sometimes been observed without inheritance of the Cre allele due to maternal imprinting, e.g. when Cre mRNA or protein were transferred into the oocyte (Cochrane et al., 2007; Matthaei, 2007).

Cre mice are created by random transgenesis or targeted insertion of Cre into an endogenous locus (knock-in; further details in Chapter 2). Cre expressing strains are classified by their deletion patterns as tissue specific; inducible; deleter and balancer (Kuhn and Schwenk, 1997). Tissue specific and/or inducible strains are supposed to mediate target gene deletion only in defined cells at a given time in development. Deleters possess an ubiquitously expressed Cre, active from the early stages of embryonic development and are consequently able to generate organism wide deletions that resemble conventional knock-outs. Balancer mice create mosaic knock-outs as their Cre recombinase is active in various tissues at levels below maximum.

Numerous Cre expressing mice have been created over the past 15 years, the recently established Cre-X-database (Nagy et al., 2009) lists several hundred entries with specificities spanning virtually all tissues and developmental periods. About half are Cre knock-ins with the other half being randomly inserted transgenes. The number of Cre strains is too broad for a complete detailed discussion. Mice used for studying the immune system, in particular myeloid tissues, like the models proposed by this thesis, are discussed in Chapter 3.

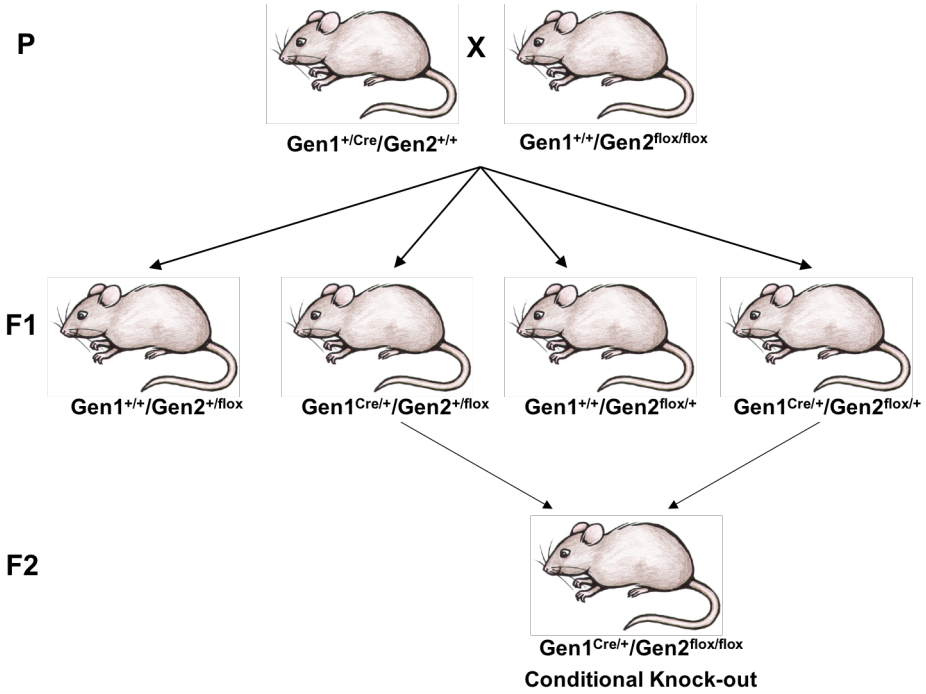


Figure 1.4 Generation of Conditional Knock-outs
F1 Offspring from heterozygous Cre mice ($Gen1^{+/Cre}/Gen2^{+/+}$) and homozygous floxed ($Gen1^{+/+}/Gen2^{flox/flox}$) mice are all heterozygous for the flox allele. 50% also carry the Cre allele heterozygously. Inter-crossing Cre+ F1 individuals is necessary to obtain conditional knock-outs in F2. The chance of obtaining the shown phenotype, heterozygous for Cre and homozygous for the floxed allele is 1 in 8 or 12.5%. Temporal and/or spatial restriction of Cre expression will be reflected in the observed deletion pattern. Gen1 represents the Cre knock-in site, whereas Gen2 is the chosen floxed target gene.

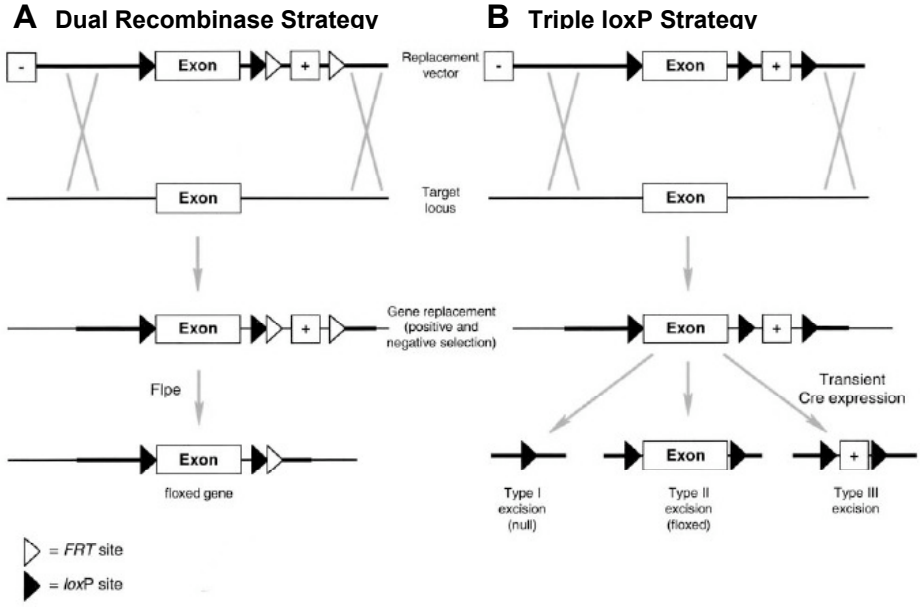


Figure 1.5 Options for the Generation of Floxed Alleles
Exons can be flanked by loxP sites with several strategies based on homologous recombination. Replacement vectors carrying the recombinase sites, positive (+) and negative (-) selection marker recombine with the target locus. After isolating correctly targeted clones, floxed alleles are generated by transient expression of a recombinase. In the Dual Recombinase Strategy the exon is flanked by loxP sites, whereas the positive selection marker is surrounded by FRT sites. Flp action deletes the marker leaves the floxed exon and one FRT site behind **(A)**. The Triple LoxP Strategy relies on three loxP sites encircling exon and marker. Transient Cre action results in three possible alleles. If the whole construct is deleted; a null allele is left behind. Similarly, either the exon or the selection marker can be removed. The latter results in a floxed allele. The graphic is modified from (Branda and Dymecki, 2004).

Likewise, many mouse strains carrying floxed versions of genes have been created, though these have yet to be indexed. Typically, loxP sites are placed in intron sequences by homologous recombination (Figure 1.5); surrounding one or more exons essential for gene function. Cre mediated deletion renders the gene inactive. So far intronic loxP sites have not been reported to influence the phenotype. The accessibility and distance of unidirectional loxP sites in the genome, however, play an important role in whether complete deletion is achievable. Floxed alleles for *Rb*, *Brca2* and *p53*, for example, showed very different and also tissue dependent recombination frequencies (from barely detectable to 100%) when crossed to a tamoxifen-inducible ubiquitous Cre deleter mouse. Therefore the usefulness of a particular Cre strain has to be assessed for each floxed target gene independently (Vooijs et al., 2001).

Apart from using genetically engineered mice expressing Cre endogenously, several virus-based methods to deliver the recombinase have been described, especially for tissues like the brain or uterus were few specific promoters are known. Temporal and spatial control is commonly achieved by choosing the route of virus administration, varying cellular susceptibility to infection, and the promoter driving expression.

Adenoviral Cre, for example, transfused through the tail vein mediated target gene deletion in numerous somatic tissues except the brain (Akagi et al., 1997). Microinjections of similar vectors in the brain resulted in selective localized knock-outs (Sinnayah et al., 2004). Infused adenoviral Cre was used to restrict target gene deletion to the mouse uterus (Wang et al., 2006). Finally, adenoviral Cre served to delete the floxed G418 selection marker during embryonic development at the 16 cell morula stage (Kaartinen and Nagy, 2001). Retroviral Cre mediated recombination on a single cell level in adult mouse neurons after injection in the brain (Tashiro et al., 2006). Similarly, self deleting Cre lentiviruses were successfully directed to the brain (Pfeifer et al., 2001) and trophoblast tissues of the mammalian placenta (Georgiades et al., 2007). Drawbacks of viral delivery are potential unwanted immune side effects caused by infection and targeting not being as precise as when Cre is expressed from an endogenous locus.

Another technique, used primarily *in vitro* in cell culture, utilizes transducible Cre protein. Unmodified Cre recombinase is able to cross the plasma membrane and to facilitate low recombination levels of around 10% depending on protein concentration (Will et al., 2002). Fusing Cre protein to HIV derived basic translocation peptide TAT improved recombination levels to more than 90% when added to the culture medium (Joshi et al., 2002; Nolden et al., 2006; Nolden et al., 2007; Peitz et al., 2002).

1.6 Conditional Gene Expression as Alternative to Conditional Knock-outs

Conditional knock-outs by site specific recombinases switch off gene expression by physically removing the target from the mouse genome. However, alternative methods exist to generate knock-out phenotypes on a transcriptional or post-transcriptional/translational level, preferably in a reversible switch-like manner.

1.6.1 The TetR System

The most widely used system for transcriptional transactivation is based on the tetracycline operon regulatory system originally found in *Escherichia coli*. Similar systems include Gal4/UAS from *Saccharomyces cerevisiae* and lac from *E. coli*. They are less commonly applied in mouse models and are not further described here but are extensively reviewed in (Mallo, 2006).

The TetR system was first shown to be functional in HeLa cells and consists of a mutant Tet repressor (TetR) fused to the Herpes simplex viral VP16 transcriptional transactivation domain. The repressor binds both tetracycline derivatives and the tetO operator sequences inserted in front of the target gene. The system exists in two versions, tet-off and tet-on. In the tet-off system (Figure 1.6A), the tetracycline controlled transactivator (tTA) does not bind the operator in the presence of tetracycline, thereby switching off transcription (Gossen and Bujard, 1992). The tet-on system (Figure 1.6B) works in a reciprocal manner. The transactivator is mutated (rtTA), so that it only binds the operator and induces transcription in presence of the inducer (Gossen and Bujard, 1992; Gossen et al., 1995). The current inducer of choice is doxycycline as it is cheap, widely available, reaches most tissues effectively and is active below cytotoxic levels. The kinetics of the rtTA system are much faster than those of tTA, with induced expression occuring within an hour compared to as much as several days for tTA (Kistner et al., 1996).

Similar to Cre and loxP mice, a large number of tet-on/off mouse models have been generated where tissue specifity is mediated by the selected promoter controlling tTA or rtTA. Problems encountered are remarkably similar to the Cre/loxP system. Regulation in conditional mouse models can be leaky, resulting in incomplete gene activation or inactivation. High expression levels of the transactivator can be cytotoxic due to pleiotropic gene regulation effects (Morimoto and Kopan, 2009; Zhu et al., 2002).

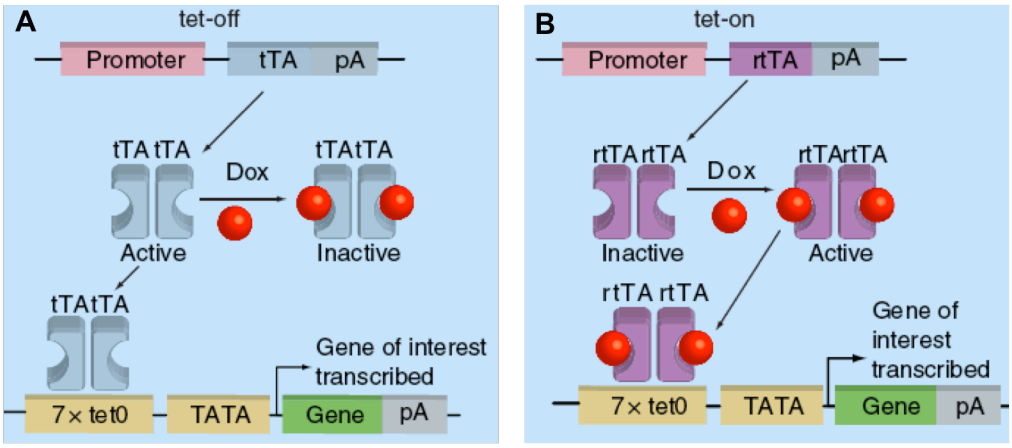


Figure 1.6 Regulation of Gene Expression by the TetR System
In the tet-off system **(A)** the transactivator (tTA) binds to the response element (7xtet0) and switches on gene expression. Doxycycline (red), bound to tTA, silences the gene of interest by inhibiting its binding to the response element. The mutated transactivator (rtTA) activates gene expression, only in the presence of doxycycline **(B)**. Illustration taken from (Bockamp et al., 2008).

The TetR system has also been used to render Cre temporally inducible. The LC-1 transgenic line (Schonig et al., 2002), for example, carries a bi-directional doxycycline inducible construct that expresses Cre and luciferase upon induction. When crossed to a transgenic line expressing rtTA in a liver specific manner, 100% tissue-specific recombination could be observed following induction in one out of five lines. Similar results were obtained for a cross of a TetR inducible transgenic Cre line with a tTA transgenic line expressing the transactivator from the brain-specific CAMKIIα promoter (Lindeberg et al., 2002). Here, Cre mediated recombination could be switched off effectively by continuously feeding the mice with doxycycline.

In an inverse scenario, the expression of transactivators themselves has been engineered to be inducible by Cre, due to placing an excisable STOP cassette in front of rtTA. In a recently described triple transgenic model (Belteki et al., 2005), a knock-in mouse line carrying a STOP-rtTA cassette controlled by the ubiquitously expressed ROSA26 promoter (Soriano, 1999) was crossed to a double transgenic line expressing tissue-specific Cre and tetracycline responsive vascular endothelial growth factor (VEGF-A). Cre-mediated recombination enabled spatial, and doxycycline administration conferred temporal control of transgene expression. Using a Cre activatable rtTA locus abrogates the need to create novel tissue-specific lines in cases where useful Cre lines already exist. In another lately described mouse model, however, where Cre and rtTA were knocked into the ROSA26 locus, tightly controlled generalized expression could only be achieved during early embryonic development. Adult mice showed significant leakage of Cre activity and failure of doxycycline induction (Backman et al., 2009).

1.6.2 RNA Interference

RNA interference (RNAi) is a eukaryotic mechanism in which double-stranded RNA is degraded in a sequence-specific manner. RNAi evolved to protect cells from viral RNAs (Waterhouse et al., 2001) but has since also been implied in the endogenous regulation of gene expression. Double-stranded RNA (dsRNA) is rapidly broken down into short duplexes varying between 21 and 28 nucleotides, which ultimately results in global translational silencing (Meister and Tuschl, 2004). Several types of small RNAs occuring in cells have been discovered: siRNAs (short interfering RNAs), microRNAs (miRNAs) and repeat-associated short interfering RNAs (rasiRNAs). rasiRNAs and siRNAs originate from viral sequences or overlapping transcripts, whereas miRNAs are the degradation products of endogenous transcripts with 20-50bp sequence homology that form dsRNA hairpins.

Since the first report of gene knock-down in mice, where small siRNA expressed from a transgene successfully silenced reporter GFP expression in all assayed tissues (Hasuwa et al., 2002), RNAi has been popular as a means of achieving gene inactivation. *In vivo* mouse models usually rely on transiently infused or injected synthetic short siRNA oligonucelotides matched to an endogenous transcript, stably inserted DNA fragments expressing miRNAs ubiquitously from tRNA or RNA polymerase III promoters or shRNAs (short hairpin RNAs) expressed tissue-specifically from appropriate promoters (Gao and Zhang, 2007). Drawbacks of this method include incomplete and variable knock-downs, non-specific silencing known as an off-target effect (Denovan-Wright et al., 2008) and the induction of interferon responses usually only seen as an *in vivo* defence mechanism against viral infections (Cao et al., 2005).

Several conditional knock-down models combining RNAi with the Cre/loxP or TetR systems have been established. For example, a transgenic mouse line harbouring RNA-PolIII promoter (U6) inactivated by the insertion of a floxed Neo^R gene was coupled to a RNAi construct for *Fgrf2*. Crossing to a deleter Cre mouse (EIIa-Cre) activated RNAi and resulted in a 95% reduction of *Fgrf2* transcripts, creating a lethal phenotype, just like the conventional *Fgrf2* null knock-out. Crossing to a mesenchyme specific Cre line (AP2-Cre) generated a viable model with a distinct phenotype (Coumoul et al., 2005). The combination of two independent RNAi constructs inactivated by stop cassettes and flanked by distinct mutant loxP sites enabled the knock-down of two genes, *Gsk-3*α*/Gsk-3*β and *Erk1/Erk2* simultaneously with a brain specific Cre line (Nestin-Cre) (Steuber-Buchberger et al., 2008). Lentiviral constructs in which the RNAi inserts are antisense to the promoter, but invertible by Cre mediated recombination, have also been utilized successfully in a conditional *p53* knock-down strategy (Stern et al., 2008).

CHAPTER 2: MAKING GENETICALLY ENGINEERED MICE

After the completion of the human and mouse genome projects, the need to transform genetic sequence information into functional data is currently the central issue in biomedical research. Traditionally, spontaneous mutation provided interesting mouse phenotypes for analysis. However, the spontaneous rate of mutagenesis ($5x10^{-6}$ per locus) (Stanford et al., 2001) proved far too low for extensive studies. Genetically engineered mice have consequently grown into a major tool for elucidating gene function.

The first genetically engineered mouse model was created in 1980 by injecting a plasmid vector in the pro-nucleus of a fertilized egg. Mice born after implanting these embryos into pseudo-pregnant females showed stable integration of the extraneous sequences throughout development (Gordon et al., 1980). This pioneering work started the era of transgenesis, the random integration of genetic sequences into organisms. The next major development, gene targeting, occurred in the 1990s. The method allows the directed modification of the murine genome and was honoured with the 2007 Nobel Prize for Medicine. Two major discoveries paved the way for this success. First, murine embryonic stem cells could be isolated successfully and were shown to contribute to the mouse germline when re-introduced into the embryo (Evans and Kaufman, 1981; Martin, 1981; Smithies et al., 1985). Second, homologous recombination was established in those cells, providing a technique to access and engineer any chosen gene (Smithies et al., 1985).

Random transgenesis and targeted modification have been used to create thousands of modified mice. They include both loss-of-function (knock-out) and gain-of function (knock-in, transgenes[1]) models. Both approaches are introduced in this chapter with regards to their advantages, drawbacks and most recent developments.

A further strategy to generate mouse models is random chemical mutagenesis. Rather than establishing defined genotypes, random mutations are introduced by treating mice with N-ethyl-N-nitrosourea (ENU). A number of offspring mice will exhibit an interesting phenotype and reverse genotypic analysis is then used to illuminate gene function. The technique has no direct relevance to the herein presented work and is therefore not discussed further, but has been extensively reviewed elsewhere (Acevedo-Arozena et al., 2008; Cook et al., 2006; Soewarto et al., 2009). All major mouse mutagenesis methods are summarized in section 2.4.

[1] This work uses knock-in as a synonym for a gene inserted into a defined locus as a single copy by homologous recombination. Transgenes represent random, non-homologous recombinations.

2.1 Transgenesis

Classic transgenesis enables the over-expression of endogenous or heterologous genes and relies on relatively short plasmid or viral vectors that are injected into fertilized eggs. Vectors are no more than 10-20kb in length and carry the transgene together with a promoter-enhancer sequence determining expression pattern and level (Rulicke, 2004). The DNA is randomly inserted into the genome in one, or more typically, multiple copy numbers and stably transmitted to offspring. The insertion of multiple copies, often hundreds, commonly results in a head to tail tandem structure.

Such transgenic mouse models can be created relatively quickly. The major drawbacks, however, are unpredictable expression patterns due to variegational position effects that have the potential to disturb the chosen promoter (Williams et al., 2008). For example, when a transgene integrates into a gene with a strong transcriptional control element, the endogenous site-specific regulatory region can interact with the transgenic promoter in a detrimental fashion. Integration into sites of genetic silencing during development, for instance through heterochromatin formation, can abolish expression of the transgene (Wilson et al., 1990). Co-introduced prokaryotic fragments, left behind from assembling the vector in *E. coli*, can have significant non-specific inhibitory effects as they trigger cellular responses such as genetic silencing by methylation or sequence deletion in somatic tissues, originally intended to protect the mammalian genome from foreign bacterial or viral DNA (Chada et al., 1985; Clark et al., 1997; Scrable and Stambrook, 1999). Furthermore transgenes can modulate the expression of unrelated genes in the neighbourhood (Woychik and Alagramam, 1998).

Advances in overcoming positional effects include the use of insulating sequences (Geyer and Clark, 2002) that reduce transgene interactions with neighbouring regulatory elements. Homologous (Brinster et al., 1988) and heterologous (Palmiter et al., 1991) introns have also been utilized in order to increase transgene expression, as have scaffold-matrix attachment regions (Gutierrez-Adan and Pintado, 2000) and locus control regions (Festenstein and Kioussis, 2000; Gutierrez-Adan and Pintado, 2000).

In 1993 the first transgenic mice using large artificial chromosome vectors were reported (Jakobovits et al., 1993; Schedl et al., 1993; Strauss et al., 1993). Artificial chromosomes derived from bacteria (BAC); yeast (YAC) and the P1 phage (PAC) have a vast cloning capacity compared to plasmids and viruses. Thus much larger DNA fragments, including more regulatory sequences, can be used for gene transfer. BAC, YAC and PAC transgenes show fewer positional effects and due to their size only few copies (1-5) are typically inserted. Expression levels from those transgenes are frequently comparable to the wild type (Giraldo

and Montoliu, 2001). Due to these characteristics, BAC transgenic mice have sometimes been referred to a pseudo-knock-in models (Sparwasser et al., 2004). A drawback is, however, that other genes, present in the vector, are co-introduced and may affect the phenotype. As the inserts are large, intra-molecular re-arrangements can occur and affect genomic structure and expression (Kaufman et al., 1999).

One problem with random transgene insertion remains, no matter what type of vector is used; the destruction of endogenous loci by insertional mutagenesis is a distinct possibility. Influences on the phenotype, or, in extreme cases lethality, are likely to be observed.

2.1.1 Gene Trapping

A recent expansion of traditional transgenesis is gene trapping in ES cells. Introduced trap vectors integrate randomly into the genome and disrupt (knock-out) and concomitantly label endogenous genes (Stanford et al., 2001). The method is ideally suited for high-throughput projects, as one vector can be used genome-wide. (Nord et al., 2006).

Trapping vectors are introduced into murine ES cells either by electroporation or as retroviruses (Stanford et al., 2006). Two main classes exist, promoter trap and gene trap vectors (Figure 2.1A&B). Promoter trap vectors contain a promoter-less reporter gene – typically lacZ – that needs to insert into an exon to produce a fusion transcript. Gene trap vectors contain an additional splice acceptor site immediately upstream of the reporter and can thus also create a fusion transcript when inserted into introns by directing the splicing process. Due to low trapping frequencies, resistance markers are commonly used in addition to reporters. Such vectors tag the trapped gene and thus facilitate its identification but can only be used for genes actively transcribed in undifferentiated ES cells. Trap vectors for non-transcribed genes direct a constitutively expressed resistance marker to an endogenous polyA signal or co-transfer enhancer sequences in order to up-regulate expression at the insertion site (Niwa et al., 1993; Schnutgen et al., 2008). The trapped gene is often rendered inactive and thus constitutes a knock-out, though alternative splicing, particularly with trap vectors inserted in introns or towards the 3' region of a gene, can result in maintained function or hypomorphic alleles (Voss et al., 1998).

Of late, gene trap vectors have been engineered to contain strategically placed site-specific recombinase sites (Figure 2.1C). These vectors enable sophisticated genetic modification in conjunction with recombinase activity; like the switching between null-alleles and functionally restored conditional alleles (Floss and Schnutgen, 2008; Schnutgen and Ghyselinck, 2007; Xin et al., 2005). The sites can also be used for recombinase mediated cassette exchange (RMCE) in

order to insert other fragments, for example those encoding Cre itself (Hardouin and Nagy, 2000).

Transposons, DNA fragments with the inherent ability to move around the genome, have also been adapted to serve as vectors for gene trapping in mice. Particularly the Sleeping Beauty and PiggyBac systems, which are transposons of zebrafish and insect origin respectively, were modified to function in mammalian organisms (Ding et al., 2005; Ivics et al., 1997). Transposons insert non-randomly as single copies with many hot and cold spots found across the genome and thus take a place between random mutagenesis and site-directed gene targeting (Ivics et al., 2009).

2.2 Gene Targeting

Gene targeting refers to the directed modification of a defined locus in the mouse genome. It can be used to delete gene function (knock-out), insert novel fragments (knock-in), engineer subtle changes like point mutations or create large chromosomal rearrangements. In case of a knock-in, only one copy of the selected gene is introduced and expression is typically regulated from an endogenous promoter, which solves virtually all problems arising from chromosomal position effects. The technique combines homologous recombination in embryonic stem cells with the ability of those cells to re-populate the mouse germ line. While a gene knock-in model is superior to a transgenic model when looking at overall genetic integrity, creating such a mouse is much more costly and time intensive.

2.2.1 Homologous Recombination

The targeted insertion of sequences relies on the mammalian enzymatic machinery that mediates exact recombination between identical, also called homologous, DNA fragments. *In vivo* this mechanism is one of the central processes for repairing double-strand breaks and interstrand crosslinks during DNA replication in the S phase of the cell cycle prior to cell division. As such homologous recombination (HR) is essential in maintaining chromosomal stability and avoiding tumorigenesis. Mouse knock-out models involving genes needed for HR largely exhibit early embryonic lethality (Thompson and Schild, 2001).

One of the mechanisms of HR is depicted in Figure 2.2. Double strand breaks initiate the process followed by enzymatic degradation of the 5' DNA ends. The new single strands then pair with homologous 3' sequences resulting in Holliday junctions. DNA synthesis fills in the gaps and the junctions are resolved in either gene conversions or crossovers (Waldman, 2008).

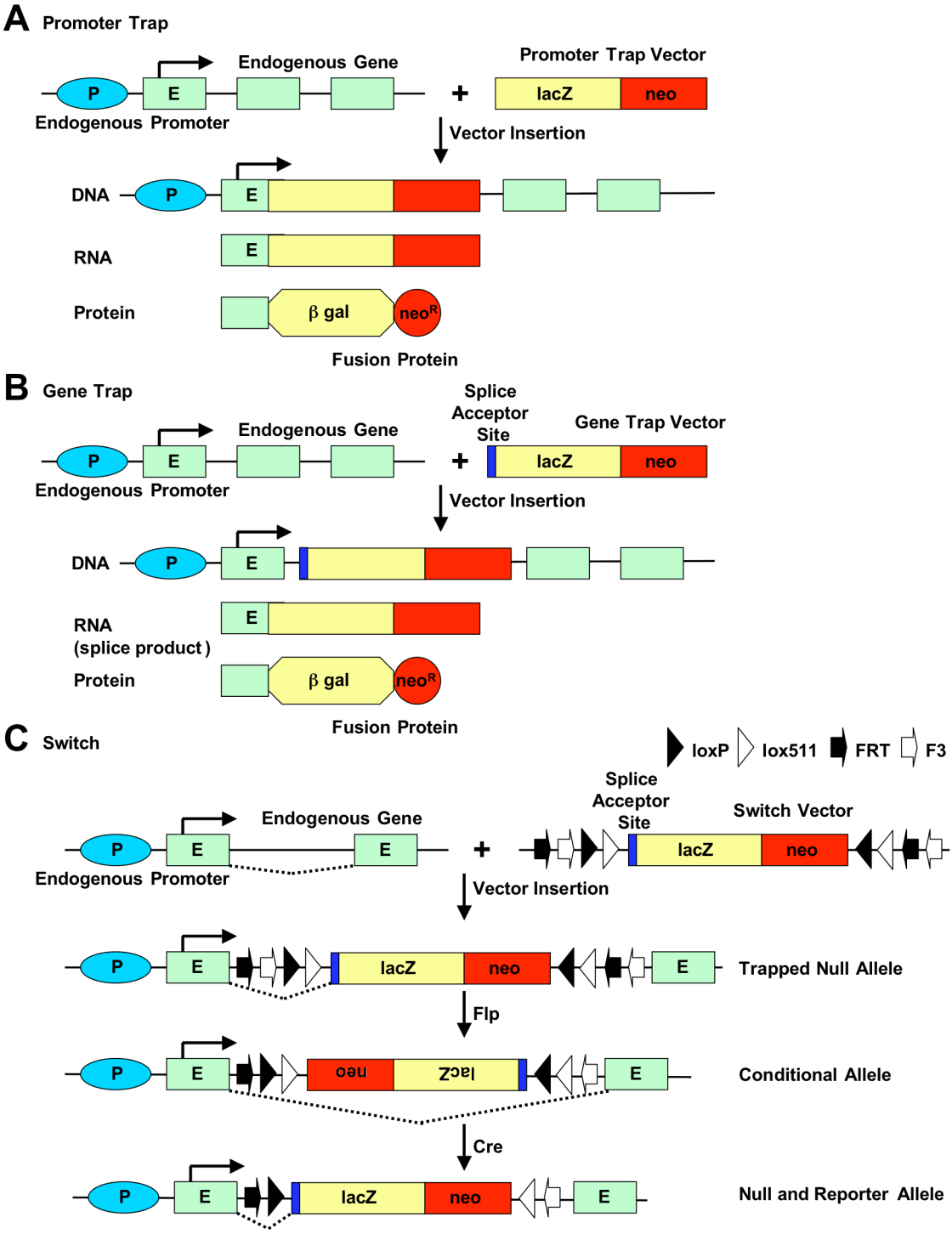


Figure 2.1 Trapping Vectors

A and **B** illustrate the structure of promoter and gene trap vectors respectively. Provided the endogenous promoter (P) is active, the insertion into an exon (E) or, with the help of a vector provided splice acceptor site, into an intron, enables the production of fusion transcripts and proteins. In this example the lacZ gene product β galactosidase (β gal) and neomycin resistance (neo) are fused (β geo). A separate expression, however, is possible by providing neo with its own promoter. **C** shows an example for a switchable construct flanked by pairs of heterotypic lox and FRT sites. As only identical sites can recombine with each other, several complex alleles can be engineered. The trapped null allele can be inverted using the Flp recombinase. This re-establishes the wild type splicing pattern (dashed lines) and yields a conditional allele. A second Cre-recombinase mediated switch, for example during a defined period of development, can re-generate the null allele. Illustrations A and B are adapted from (Stanford et al., 2001), whereas C is modified from (Schnutgen and Ghyselinck, 2007).

The discovery that linear, cloned, modified DNA, introduced into mammalian cells, could also participate in HR resulted in the onset of gene targeting (Capecchi, 1989; Smithies et al., 1985). The DNA, in form of a targeting vector, is transferred into cultured ES cells using electroporation, lipofection or microinjection. If cells are successfully transfected, three outcomes are possible. In the majority of cases the DNA is not inserted into the genome at all. If insertion takes places, it is most likely to be random following non-homologous recombination. Early experiments showed that microinjected linear DNA encoding the Herpes simplex thymidine kinase (TK) was incorporated by 20% of TK deficient mouse cells (Capecchi, 1980). In few cells, however, the targeting vector will pair with the cognate chromosomal segment and undergo successful homologous recombination. Frequencies have been estimated at between 10^{-5} to 10^{-8} correct events per targeted cell (Donoho et al., 1998).

It is accepted that the length of the homology regions flanking the desired insert impacts on the success rate of homologous recombination. Numerous attempts have been made to ascertain how long homology arms need to be to achieve high frequencies. In early experiments, designed to modify the mouse *hprt* gene in ES cells, for example, a five-fold increase of homology, from 2.9kb to 14.3kb, resulted in a 100-fold higher targeting frequency, equalling one correct HR event per 30.000 cells (Capecchi, 1989). Saturation was observed for homologies in excess of 14kb (Deng and Capecchi, 1992). Another similar study noted a 200-fold increase in targeting efficiency, when the total homology was lengthened from 1.3 to 6.8kb (Hasty et al., 1991).

Selection strategies are essential in identifying and enriching correctly recombined cells and can also help to increase targeting frequencies several hundred-fold. Commonly used positive selection markers include the gene for the neomycin phosphotransferase (neo^R), driven by a strong promoter, which renders cells resistant to the antibiotic G418. Using the insertion of neo^R in conjunction with homologous recombination to modify the mouse gene *en-2* resulted in one correctly targeted clone per 300 analyzed resistant cells (Joyner et al., 1989). A negative selection marker, usually the TK gene from Herpes simplex virus (HSV-tk) can be combined with G418 selection to further enrich targeted clones. The negative selection marker is placed adjacent to the homology region and is thus only inserted when the whole vector undergoes random integration. The selective agent, ganciclovir, is phosphorylated by HSV-tk and endogenous kinases resulting in a nucleoside analogue that arrests DNA synthesis causing cells carrying random integrants to die. In one study, homologous recombinant clones could be enriched up to 2000-fold (Mansour et al., 1988), though 10-20 fold enrichments are more commonly reported (Zimmer, 1992). An alternative marker is the diphteria toxin A gene (Yanagawa et al., 1999). Negative selection markers form a large fragment of non-homology flanking one or both homology arms. Nevertheless they appear to not adversely affect recombination efficiency (Deng and Capecchi, 1992).

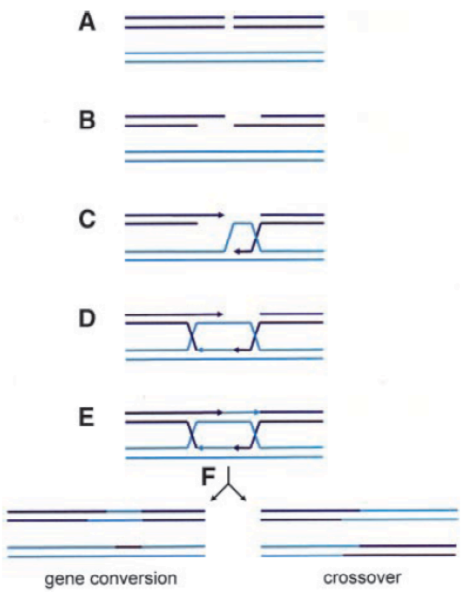


Figure 2.2 Homologous Recombination
A double strand break **(A)** initiates the repair process. 5' ends are resected first **(B)** followed by pairing of the single stranded 3' ends with their homologous counterparts on the cognate strand **(C)**. DNA synthesis leads to the formation of Holliday junctions **(D)** and the single strand gaps are filled in **(E)**. The junctions can be resolved by gene conversion or crossover **(F)**. The illustration is taken from (Waldman, 2008).

A further enrichment possibility is a promoter-less neo^R gene. Vectors are designed such that neo^R inserts in-frame with an exon of the target gene upon successful homologous recombination – akin to the trapping approach. Expression of a fusion protein is driven by the endogenous promoter and confers G418 resistance. Similar strategies rely on targeting the resistance marker to endogenous polyadenylation (pA) signals or using artificial splice sites to direct resistance gene transcription. These approaches can only be employed if the target gene is expressed in the embryonic stem cell line (Ledermann, 2000).

Homologous recombination frequency on the whole is dependent on the intended chromosomal targeting site, making it difficult to compare individual experiments. The local chromatin structure influences the accessibility for the recombination machinery and may affect expression levels of the selection marker (te Riele et al., 1992). Hotspots for recombination impacting on gene-targeting have also been reported (Hasty et al., 1991).

2.2.2 Vectors For Gene Targeting

In general, two different types of vectors, for either insertion or sequence replacement are used (Figure 2.3). Most commonly utilized linear sequence replacement vectors carry the extraneous DNA segment and selection marker flanked by target specific homology arms. Precise HR is the result of two crossing over events towards the ends of the flanking sequences. Contrariwise, insertion vectors are linearized within the homology region and participate in a single cross over, which causes the insertion of the entire vector and thus the duplication of homologous

sequences. They are often used to engineer subtle mutations through a second step of intrachromosomal recombination (Morrow and Kucherlapati, 1993).

The universal recommendation is to use isogenic DNA (matched to the mouse strain from which the chosen ES cell line originates) for vector construction as non-isogenic DNA can lead to lower recombination rates due to localized polymorphisms causing mismatches. Isogenic targeting vectors directed towards exons 3 and 8 of the mouse *hprt* gene, were shown to be 4-5 fold more efficient than non-isogenic vectors (Deng and Capecchi, 1992). Similarly, targeting vectors aimed at *Rb* in 129 embryonic stem cells were 20-fold more efficient when derived from 129 DNA compared to BALB/c DNA. Targeting of the *mu* opioid receptor locus with non-isogenic constructs in 129 and C57BL/6 embryonic stem cells revealed a 15% decrease in targeting frequency. The intronic sequence between those two strains was found to possess a divergence of 2.5% (Zhou et al., 2001). The presence of sufficiently long stretches of homologous DNA, however, seems to be more important in enabling efficient HR than the overall number of mismatches (te Riele et al., 1992). Thus, if non-isogenic DNA constructs are used, longer targeting arms can compensate for the presence of polymorphisms.

Sequence replacement vectors are typically designed to contain a short and a long homology arm. This asymmetry facilitates screening, for example by PCR, across the shorter end. The employed vector type and the subsequent screening strategy dictate the overall length. Nevertheless, the short homology arm should be in excess of 1kb, as shorter fragments are linked to illegitimate recombination (Thomas et al., 1992).

Conventional gene targeting vectors are relatively short and are assembled in standard plasmids by normal cloning techniques or derived from target specific cDNA. BAC vectors, however, have become more popular since several libraries covering the whole mouse genome became available as a result of the genome sequencing projects. Furthermore, methods for modifying large DNA molecules are more firmly established. The vector generation in this work also relied on modifying BACs, therefore these techniques are introduced in the next section.

2.2.2.1 Engineering Large BAC Vectors

The main method for BAC engineering utilizes bacterial and phage recombination systems and has been popularly termed recombinogenic engineering or simply recombineering (Copeland et al., 2001). Large molecules of DNA can be modified without relying on conventional restriction enzymes and DNA ligases.

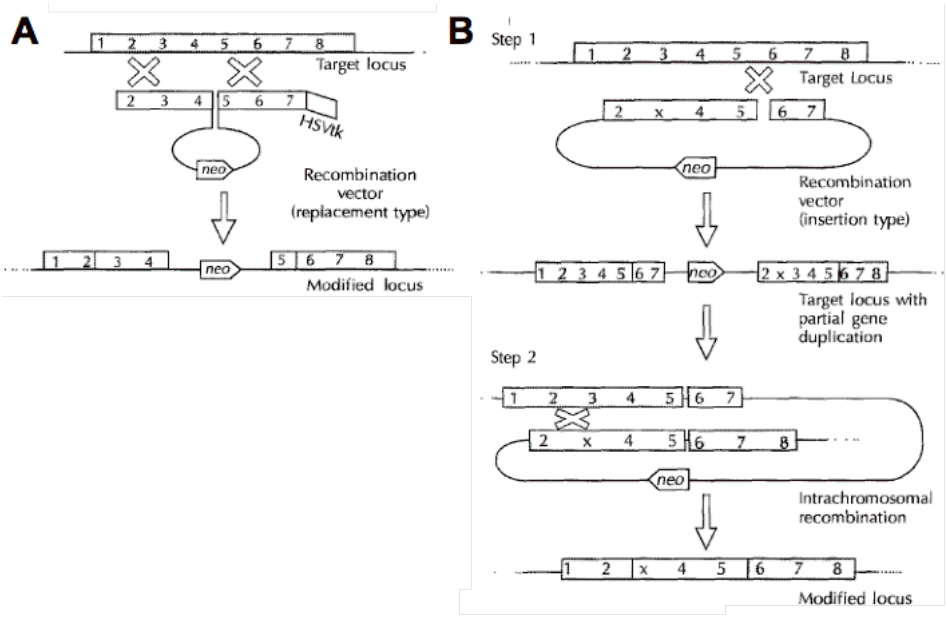


Figure 2.3 Insertion and Sequence Replacement Vectors
A linear sequence replacement vector **(A)** undergoes two crossover events with the homologous sequences (numbered boxes). The positive selection marker neo is introduced whereas the negative selection marker HSV-tk is lost in the modified locus. Insertion type vectors **(B)** contain the double strand break within the homology region and consequently insert as a whole (Step 1). Partial gene duplications can be reversed by another round of recombination (Step 2). The second method is particularly suited to introduce subtle mutations (X), such as base pair exchanges. Common synonyms for this strategy are "hit and run" or "in and out". The scheme is adapted from (Morrow and Kucherlapati, 1993).

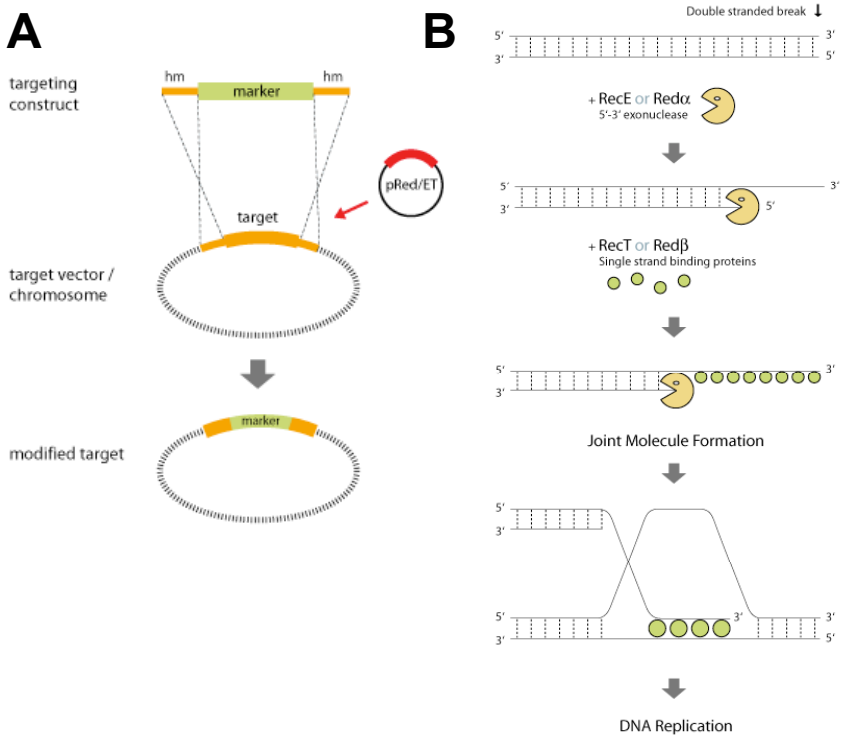


Figure 2.4 Red/ET Recombineering
Using Red/ET recombineering, a linear DNA fragment carrying a positive selection marker flanked by short 50bp homology arms (hm) can be inserted into a target BAC **(A)**. Necessary proteins are expressed from the pRed/ET plasmid vector. Sequential steps carried out by the Red (λ phage) or Rec (Rac prophage) proteins mediate a recombination process that is similar to the one observed in eukaryotes **(B)**. Pictures were retrieved from www.genebridges.com.

The presented study used the λ phage derived Red/ET recombineering system in *E.coli* (Angrand et al., 1999; Muyrers et al., 2000; Muyrers et al., 1999; Testa et al., 2003; Zhang et al., 1998; Zhang et al., 2000). The strategy relies on heterologously expressing the Red proteins encoded by the phage RecA operon in *E.coli* to mediate recombination.

The process is illustrated in Figure 2.4. A DNA fragment containing the desired modification is amplified by high fidelity PCR using primers that introduce 50bp homology arms (Zhang et al., 2003) analogous to the desired BAC modification location. This linear DNA piece is partially digested by the 5' – 3' endonuclease Redα. The 3' single strand is then bound by single stand binding protein Redβ and forms a joint molecule with the target DNA after which recombination occurs. Redγ inhibits RecBCD function in *E.coli*, which stops the linear DNA fragment harbouring the modification being degraded prematurely (Muyrers et al., 2000).

In the Red/ET system, the Red proteins are expressed from a commercially available plasmid (pRed/ET; GeneBridges) in a L-arabinose-inducible and temperature-dependent manner providing temporal control of recombineering activity. More process-related details can be found in the Materials & Methods Chapter. Recombineering systems vary with regards to how the recombination proteins are expressed in *E.coli* (from a transformed plasmid or integrated prophage) and the origin of the recombinogenic proteins (aside from the λ function, the analogous Rac phage system is popular) (Copeland et al., 2001).

2.3 Making Mice From Embryonic Stem Cells

Irrespective of the method used to engineer murine embryonic stem cells, a mouse model often needs to be created. The general procedure is outlined in this section and depicted in Figure 2.5. After the construction of a suitable targeting vector and its transfer into the stem cells, drug resistant colonies are isolated. The screening of those clones aims at establishing successful homologous recombination and the integrity of the targeted locus. Specific PCRs are frequently employed in a first round of analysis and allow the screening of large numbers of clones while being sensitive enough to use only a fraction of an embryonic stem cell colony. Follow-up Southern blot analysis confirms PCR results, excludes the possibility of additional inserts and ensures correct recombination especially at the break-points of crossing over.

The neo^R resistance marker is commonly removed from the targeted embryonic stem cell line. Transient Cre or Flp transfection, depending on whether loxP or FRT sites were used to flank the neo^R cassette in the initial vector construction, can achieve this objective.

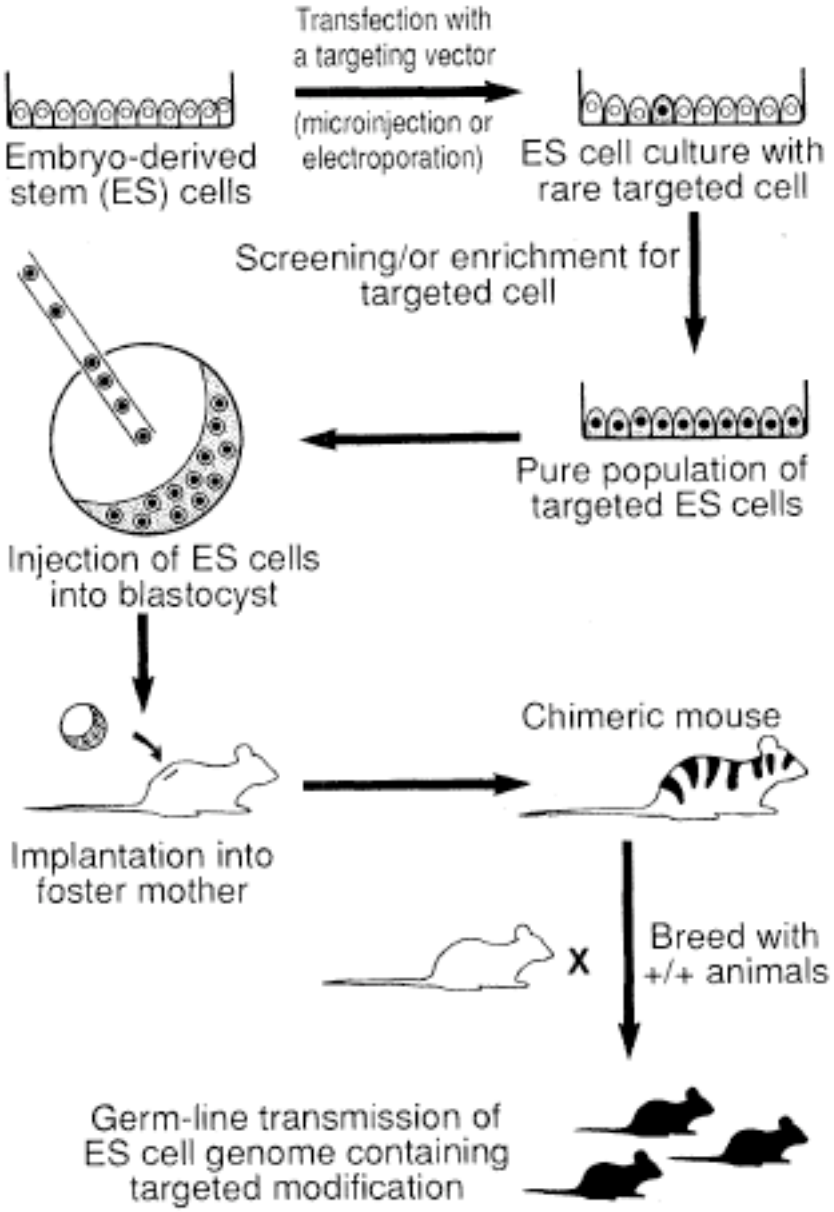


Figure 2.5 Producing Mice from Embryonic Stem Cells
The production of germline chimera and subsequently genetically engineered mice is a multistep process including vector generation, embryonic stem cell transfection and screening and blastocyst injection. Further details can be found in the text. Graphic taken from (Capecchi, 1989).

The step was introduced after *neo*^{R} resistance genes, often coupled to their own strong promoters such as that of the phosphoglycerate kinase (PGK), were shown to influence the gene expression pattern of surrounding genes and thus impact on the phenotype. A PGK-Neo cassette, inserted into the granzyme B and β–like globulin loci, for example, severely reduced the expression of other genes in the respective clusters, even at large distances of more than 100kb. An insertion into cathepsin G residing in the same cluster, however, had no discernible effects (Pham et al., 1996). Similarly, three independent alleles of a *MRF4* knock-out, distinguished by the area targeted and the orientation of the selection cassette, showed gross differences in phenotype (Olson et al., 1996). In one published report, however, the removal of the resistance marker from the CD19Cre mice, resulted in 20% lower Cre-mediated recombination in the majority of B cell lineages. It has been suggested that the presence of the selection cassette improves Cre expression in this particular model, either due to contained enhancer sequences or by conferring increased RNA stability (Schmidt-Supprian et al., 2007).

Once all genomic modifications are complete, homologous recombinant clones are expanded and re-integrated into an early mouse embryo. The embryo is then implanted into a foster mother and chimeric offspring is born. Chimaera whose germline is derived from the engineered embryonic stem cells will transmit the genetic modification to their progeny. The most popular method for embryo re-integration is blastocyst injection, where the cells are injected into the cavity of 3.5d old blastocysts. The process is relatively complicated and time consuming, needing a specialist technical setup. Advantages, however, include that ES cell clones can be carefully selected for morphologic criteria and blastocysts need not be cultured for an extended period of time. Another, technically less demanding technique, is morula aggregation where clumps of embryonic stem cells are aggregated with 2.5d old morula stage embryos. This method, however, requires longer periods of embryo culture and individual cells cannot be selected easily (Nagy et al., 2003).

Embryos for either aggregation or injection are selected so that the coat-colour markers differ from those of the ES cells and that a high probability of germ line transmission is achieved. 129 ES cells (agouti or chinchilla fur colour), for example, are commonly injected into C57BL/6 (black fur colour) embryos. Chimeras can be easily identified by their mixed black and white coat. Chimeras are crossed to each other to obtain germ line transmission, which, again, is judged by fur colour.

2.4 Overview of Mouse Mutagenesis Strategies

Strategy		Success Quota	Possible Mutation Types	Major Disadvantages	Major Advantages
Spontaneous		$5x10^{-6}$ per locus	Point mutations; chromosomal re-arrangements	Very low frequencies; needs large mouse colonies	Selection by distinct phenotype
Chemical (ENU)		$150x10^{-5}$ per locus	Point mutations; small deletions	Numerous mutations per mouse, needs large mouse colonies, Mapping of mutations difficult	Fast; high-throughput possible
Trans-genesis	Micro-injection	10% of all animals	Insertion of heterologous genes; disruption of endogenous genes	Low frequencies; unpredictable phenotypes; positional effects	Medium speed; overexpression of heterologous genes possible
	Gene Trap	up to 100% of all animals*	Insertion of heterologous genes; disruption of endogenous genes	Time and labour intensive; unpredictable phenotypes	Slow; high-throughput possible, disrupted genes are labelled; easy vector design
Gene Targeting	Conven-tional	up to 100% of all animals*	Targeted insertion or deletion of endogenous/he-terologous genes or complex chromosomal re-arrangements	Time and labour intensive; complicated vector design & screening; no temporal or spatial control	Highest level of genome integrity; any gene can be targeted
	Condi-tional			Time and labour intensive; complicated vector design & screening; needs two mice – one floxed one Cre; potential leakiness	Highest level of genome integrity; any gene can be targeted; temporal- and spatial control

Table 2.1. Comparison of Mouse Mutagenesis Methods
*following selection and screening

2.5 Large Scale Mouse Mutagenesis Projects

The creation of an individual mouse model is still time consuming, expensive and laborious. Therefore recent years have seen the emergence of several large scale international projects with the ambitious target of mutating all protein expressing mouse genes by 2011 and thus providing a standardized and indexed resource for biomedical research (Collins et al., 2007; Gondo, 2008). Efforts have been made to streamline the model generation process, moving away from individual researchers producing single to few engineered mice with a wide array of methods in favour of a partially automated; high-throughput; centralized approach.

Several international consortia, for example the International Mouse Knock-out Consortium (IMKC) consisting of European EUCOMM (European Conditional Mouse Mutagenesis Programme), North American KOMP (Knock-Out Mouse Project), Canadian NORCOM (North

American Conditional Mouse Mutagenesis Project) or ITGC (International Gene Trap Consortium) are, together with commercial partners, in the process of establishing libraries for general access. The objective of these efforts is that, following completion of these resources, individual researchers can request a modified version of their gene of interest; as vector; ES cell line or completed mouse model, to either begin with phenotypic analysis directly or at least significantly shorten the time until a new model is available.

Envisaged numbers are staggering, the three members of IMKC, for example aim to produce about 40.000 unique ES cell lines and 920 mouse models by the end of 2010. An evaluation of success will be difficult before the project is concluded and all created resources have been entered into the repositories. By looking at the published results of the EUCOMM partner to date, however, it would appear that the projected numbers were slightly optimistic. As of early 2010 (Table 2.2), less than half of all projected ES cell lines and about 80% of mouse models were completed.

Resource Type	Type of Modification	Projected Cumulative by 2011	Projected Share EUCOMM by 2011	Achieved EUCOMM Early 2010[1]
ES cell	Trapped Conditional	22.000	12.000	5724
ES cell	Targeted Deletion	3500	N/A	N/A
ES cell	Targeted Conditional	15.000	8000	2981
Mouse	Mixed	920	420	380

Table 2.2 Overview of Projected Resource Generation by the IMKC
Divided by totals, share of EUCOMM partner and actually achieved numbers to date. Table adapted from (Collins et al., 2007). [1] Numbers are derived from EUCOMM website www.eucomm.org and are current as of March 2010. N/A not applicable

According to the Sanger Institute High Throughput Gene Targeting resource (www.sanger.ac.uk/htgt); the common problems are unsuccessful vector design, generation or purification; failed ES cell electroporation and homologous recombination; modified ES cell lines not meeting quality control standards for injection; and unsuccessful germ line transmission. Nevertheless, the number of available resources for the production of genetically engineered mice is increasing drastically. Therefore it will become more and more important to search available repositories before embarking on a project aimed at creating a new mouse model. As a significant portion of the high throughput projects constructs conditional alleles, novel Cre expressing mouse strains, such as the ones proposed by this work, will become more important to obtain knock-outs for phenotypic analysis.

CHAPTER 3: CONDITIONAL MUTAGENESIS IN THE IMMUNE SYSTEM: MACROPHAGES AND NEUTROPHILS

As the envisioned *iCre2* mouse strains are meant to enable neutrophil and macrophage specific conditional mutagenesis, this chapter very briefly introduces the basic biology of those cell types. The target genes chosen as knock-in sites are also discussed and contrasted to other Cre strains used in immunological research.

3.1 Macrophages and Neutrophils: Phagocytes of the Immune System

Macrophages and neutrophils are major phagocytic cell types in the immune system. As such they play an important role in innate immunity and provide the first line of defense against pathogens such as bacteria and fungi. In addition, they are involved in tissue homeostasis and link the innate to the adaptive immune system.

Both cell types derive from a common myeloid progenitor in the bone marrow. In case of macrophages, this progenitor turns into a pro-monocyte and later a monocyte circulating within the blood stream. Monocytes migrating into tissues become long-lived macrophages. Granulocytes, one of which is the polymorphonuclear neutrophil, develop when the common progenitor induces promyelocyte differentiation. Neutrophils appear as cells with distinctly shaped nuclei and densely staining granules. In flow cytometry, two markers are predominantly used to identify them, Gr-1 (Fleming et al., 1993) and the polymorphic neutrophil specific antigen (Hirsch and Gordon, 1983). Similarly, monocytes and macrophages are characterized by Mac-1 (Springer et al., 1979) and F4/80 receptor expression (Austyn and Gordon, 1981).

Both respond to a wide variety of pathogens via pattern recognition receptors (e.g. Toll-like receptors, lectin family receptors) or via the Fc mediated recognition of antibody/antigen complexes. Resident tissue macrophages are the first cells to encounter pathogens, triggering phagocytosis and the secretion of pro-inflammatory cytokines and chemokines such as IL-1β, TNF- α, CXCL8 or IL-6 and establish a state of inflammation.

Phagocytosis, a key component in macrophage and neutrophil biology, describes the engulfment and internalization of microorganisms and cellular debris. Phagocytosed microorganisms can subsequently be killed and degraded in an enclosed vesicle, the phagosome. This process is supported by acidification (pH 3.5-4.0), the release of antimicrobial peptides and enzymes (i.e. defensins, lysozyme, proteases) and the generation of reactive oxygen intermediates (ROI; i.e. superoxide, nitric oxide, oxygen radicals) by a respiratory burst. Peptides and enzymes are released when lysosomal storage granules merge with the phagosome,

whereas oxygen intermediates are produced by designated enzymes such as the membrane associated NADPH oxidase complex.

Factors released by macrophages cause further leukocytes to be recruited to the site of infection. The first cells are circulating neutrophils, followed by monocytes, which enter the tissue by crossing the barrier between blood vessels and infected tissue. Extravasation, the migration of leukocytes from the blood vessels via the endothelium, occurs as a result of inflammation. First, endothelial cells lining the vessels begin expressing adhesion molecules (i.e. P-Selectin, E-Selectin) that allow leukocytes to attach. Both selectins are recognized by leukocyte glycoproteins, like L-selectin, which cause them to reversibly adhere to and roll along the vessel wall. Second, leukocyte integrins interact with additional adhesion molecules on the endothelium to confer a tighter binding and to stop further rolling. Third, the leukocyte squeezes between endothelial cells in a process called diapedesis and migrates, in response to chemokine signals, to the location of infection.

Once within the tissue, neutrophils like macrophages are avidly phagocytic, and release a battery of microbicidal molecules both within the phagolysosome and into the extracellular spaces. However, unlike macrophages, neutrophils are short lived, and after phagocytosis rapidly die, and are themselves taken up and degraded by tissue macrophages. In addition, macrophages play essential roles in tissue homeostasis, for example by ingesting dead cells during injury and normal cell turn over or in the extensive remodelling needed during embryogenesis (Murphy et al., 2008).

3.2 Candidate Genes for Tissue-Specific *iCre2* Expression

Three target genes were chosen as loci for knocking-in *iCre2*, based on published reports of stringent tissue specific expression, which should render Cre mediated recombination restricted to either macrophages (*Marco* and *Msr1* genes) or neutrophils (*Itgb2l*). Furthermore, connventional knock-outs of all three genes exhibit no or only very mild phenotypes, indicating that knock-in mice, still carrying one functioning allele, should develop normally. Haploinsufficiency has not been reported for any of the chosen genes. Target gene characteristics are summarized in table 3.1.

3.2.1 Itgb2l

The mouse protein Pactolus, expressed by the *Itgb2l* genomic locus, was first discovered in 1998 in bone marrow cells matured with stem cell factor (SCF) or IL-3. The protein's similarity (~60% identical) to the CD18/β2 and CD18//β7 integrin subunits, apart from lacking the

characteristic metal binding unit (Chen et al., 1998), suggests it as their evolutionary paralogue (Hale et al., 2006). Humans, unlike rats, have no comparable gene.

Itgb2l resides on murine chromosome 16 and consists of 16 exons encompassing 24kb. The translation start codon is located in exon 2. Three transcripts are known due to alternative splicing in exon 13 caused by a single base pair mutation found in certain mouse backgrounds. Transcript A encodes the full-length protein, B lacks the trans-membrane domain, and transcript C encodes a prematurely terminated protein only present during neonatal development (Margraf et al., 1999). C57BL/6 mice only produce the full-length transmembrane form whereas BALB/c, C3H/HeJ and 129/Sv mice transcribe both full-length and truncated versions. The promoter strength does not vary between these alleles as there is no significant difference in the total amount of transcripts between the independent strains (Garrison et al., 2001).

The promoter sequence is somewhat unusual as it lacks a TATA box. Other transcriptional sites, however, are present, for example AP-1, AP-4, ELK-1, C/EBP, E47, GATA-1 and NF-κB sites. The GATA-1 site is suspected to be a central regulator in Pactolus expression. GATA-1 is only expressed in cells of the haematopoietic line (Sposi et al., 1992) – thus the Pactolus expression is believed to be restricted to cells of this descent. Regulation by NF-κB suggests Pactolus can be induced following inflammatory stimuli (Margraf et al., 1999). Two tandem PU.1 transcription factor binding sites are present but appear to be strictly negatively controlled in non-neutrophilic cells. Snail and smad family transcription factors have also been implied in the regulation of *Itgb2l* expression (Hale et al., 2006).

Literature data to date shows Pactolus expression as being restricted to mature neutrophils in the periphery and maturing neutrophils in the bone marrow. Its expression pattern is thus far more limited than that of the CD18 integrin subunits which are expressed in T- and B-cells as well as macrophages and granulocytes (Walzog et al., 1999). It has actually been suggested that the expression of Pactolus in cells other than neutrophils is lethal as the stable transfection of selectable Pactolus expression vectors into B- , T- and macrophage cell lines failed to yield drug resistant cells (Hale et al., 2006). The Pactolus protein consists of a single chain and varies in size depending on whether it is membrane bound (98kDa) or cytoplasmic (130kDa) (Garrison et al., 2001). Unlike the integrins, it does not associate with an α subunit to form a functional heterodimer as it contains a 24-29 amino acid deletion rendering it unable to dimerize (Sen and Legge, 2007; Takagi et al., 2002).

Pactolus function has not been completely elucidated. The majority of the protein is held in dense granules and is heavily N- and O-glycosylated and sialylated. Similar to β2 integrins, it is released after inflammatory activation, when constitutively expressed Pactolus on the plasma

membrane is cross-linked. Pactolus is stored in granules differing from those of the β2 integrins and does not respond to the same stimuli. It does not act as an activating phagocytic receptor and its membrane translocation does not increase total calcium flux or protein phosphorylation. Pactolus expression levels were up-regulated in mouse model of pneumonia (Rosseau et al., 2007). It has been proposed that Pactolus serves as a ligand to mark dead and dying neutrophils as it translocates to the cell surface during necrotic or apoptotic neutrophil death without being shed (Garrison et al., 2003). Its extensive coverage with sialic acid residues suggests Pactolus could serve as a ligand for macrophage lectins, further implicating it as marker for phagocytic macrophages.

Itgb2l knock-out mice show no aberrant phenotype suggesting redundant functional equivalents. Their neutrophils are present in normal numbers and perform appropriate migration and combat of infections. They do, however, promote the loss of resident cells producing chemokine CXCL13 from the peritoneal cavity following inflammatory response by an unknown mechanism (Hojgaard et al., 2006).

3.2.2 Msr1

Msr1 (murine scavenger receptor 1) is a mouse gene encoding one of the various class A scavenger receptors first identified in the murine macrophage cell line P388D1. The gene contains 11 exons spanning 60kb on chromosome 8. Two transcripts are known, one consists of exons 1-9, the other links exons 1-8 to 10 and 11, which results in two receptor variants I and II. The translation initiation codon is located in exon 2 (Aftring and Freeman, 1995). The gene's promoter sequence contains no typical TATA box but is characterized by AP-1 and GATA-1 sites, known to be cytokine response elements (Wu et al., 1994).

Msr1 expression patterns identify the protein as a pan-macrophage marker. Expression levels depend on the genetic background due to polymorphisms that cause varying glycosylation patterns and amino acid substitutions (Daugherty et al., 2000; Fortin et al., 2000). C57BL/6J mice, for example, exhibit 1.6-fold higher protein levels than A/J mice (Fulton et al., 2006). Expression has been found in the red pulp and marginal zone of normal adult mouse spleens, the thymus medulla, the subcapsular region of the lymphnodes, Kupffer cells in the liver, alveolar macrophages and lamina propria macrophages in the gut. The only non-macrophage cell types where low level expression of *Msr1* could be observed was the sinusoidal endothelium of the liver (Hughes et al., 1995). The rabbit orthologue of *Msr1* is also expressed in smooth muscle cells (Li et al., 1995). Expression in endothelial and smooth muscle cells appear to be dependent on oxidative stress and endogenous growth factors such as TNF-α and IFN-γ (Mietus-Snyder et al., 1997). Exposure to lipoproteins increased expression levels of

Msr1 in macrophages 12-17 fold (Han and Nicholson, 1998). IL-1, IL-4 and MCSF also appear to upregulate Msr1 levels in macrophages (Cornicelli et al., 2000). Studies of the human orthologue revealed that expression of receptor variant I is low in circulating monocytes, but significantly upregulated at both mRNA and protein level when maturation into adherent macrophages occurred. Variant II is constitutively expressed at low levels in both monocytes and macrophages. In this study Msr1 could not be detected in peripheral human neutrophils and lymphocytes (Geng et al., 1994).

Scavenger receptors are trimeric cell surface glycoproteins whose major function is the recognition of molecules with polyanionic structure, such as LDL (low density lipoprotein), polyribonucleotides, polysaccharides, phospholipids, asbestos or silica (Freeman et al., 1990; Krieger and Herz, 1994; Platt and Gordon, 2001). Scavenger receptors, belonging to the pattern recognition receptors, are found in many mammalian species such as cattle, rabbits and humans, but have low sequence conservation (Ashkenas et al., 1993). The Msr1 protein contains 6 domains and carries a highly conserved cysteine-rich carboxyterminal fragment with a collagen-like structure that mediates ligand binding (Kodama et al., 1990).

Msr1 is involved in the regulation of anti-inflammatory processes as it induces IL-10 upon stimulation with LPS, which inhibits septic shock (Fulton et al., 2006; Manning-Tobin et al., 2009; Tsujita et al., 2007). Scavenger receptors have also been implied in the pathological deposition of cholesterol during atherogenesis by receptor-mediated uptake of modified low-density lipoproteins by vessel-wall macrophages and smooth muscle cells (Linton and Fazio, 2001). Furthermore *Msr1* has been connected to clearing bacteria and apoptotic cells as well as to cell adhesion (Kosswig et al., 2003; Platt et al., 1996).

Msr1 knock-out mice appear to be normal in both morphology and development but their macrophages exhibit impaired uptake and degradation of lipoproteins and apoptotic cells and adhere slower than wild type cells to tissue culture plastic. *Msr1*$^{-/-}$ animals are also more susceptible to *Listeria monocytogenes* and herpes simplex virus type-1 infections (Kunjathoor et al., 2002; Ling et al., 1997; Suzuki et al., 1997).

3.2.3 Marco

The gene expressing Marco (Macrophage receptor with collagenous structure) is located on chromosome 1. It consists of 17 exons encoding a 210kDa disulfide-trimeric protein (Kangas et al., 1999). The translation start codon lies at the end of exon 1 and is preceded by a Kozak sequence (Kraal et al., 2000). Marco belongs, like Msr1, to the class A scavenger receptor proteins and shares its binding specifities (Elomaa et al., 1995). The protein differs from Msr1

in that it lacks an α helical coiled-coil domain and possesses a longer extracellular subunit of collagenous structure (Dahl et al., 2007). Successful protein folding and delivery to the cell membrane depend on chaperones in the endoplasmic reticulum (Kvell et al., 2006).

Where *Msr1* is expressed in all macrophages, *Marco* expression is restricted to certain subsets in distinct tissue compartments. It has been found on macrophages in the medullary cord of lymph nodes, the marginal zone of the spleen, peritoneum and particularly in alveolar macrophages. No further expression has been observed (Dahl et al., 2007; Hamilton et al., 2006; Kanno et al., 2007). Induced ectopic expression of Marco in other cell lines brought on changes of morphology, such as the formation of long dendritic processes, implying a role in adhesion and migration (Pikkarainen et al., 1999). LPS binding upregulates Marco expression independent of proinflammatory cytokines such as IFN-γ, IL-6, TNF- α and IL-1 (Kangas et al., 1999). Following stimulation, Marco also appears transiently on macrophages in the liver, the red pulp of the spleen and Kupffer cells, where it is not normally found in healthy individuals. (Elomaa et al., 1995). LPS or bacterial activation has also been shown to switch on expression of Marco in splenic dendritic cells and microglial cells in the brain, which are otherwise negative for the protein. In these cells Marco appears to play a role in rearranging the actin cytoskeleton and thereby changing cellular adhesive properties (Granucci et al., 2003; Grolleau et al., 2003; Milne et al., 2005). Marco also performs a critical part in the protection against inhaled antigens by taking up and clearing of inhaled oxidized surfactant lipids and bacteria, thus forming a part of the lung innate immune system (Dahl et al., 2007).

Marco knock-out mice display an impaired ability in clearing bacteria from the lungs, increased pulmonary inflammation and diminished survival in a murine model of pneumococcal pneumonia, but otherwise appear to be normal (Arredouani et al., 2004). In general, their macrophages possess substantially diminished capabilities in the uptake of bacteria like *Escherichia coli* or *Neisseria meningitidis* as well as acetylated LDL (low density lipoproteins) (Mukhopadhyay et al., 2006) and exhibit a 2.7 fold lower production of IL-12 (Jozefowski et al., 2005). Reduced levels of Marco and resulting impaired clearance of apoptotic cells have recently been implicated as a major contributor in a murine model of Systemic Lupus Erythematosus (Rogers et al., 2009).

	Itgb2l	*Msr1*	*Marco*
Protein	Pactolus	Murine Scavenger Receptor 1	Macrophage Receptor with Collagenous Structure
Chromosomal Location	16C4	8A4	1E4-5
Gene Structure	16 exons, start codon in exon 2	11 exons, start codon in exon 2	17 exons, start codon in exon 1
Expression Pattern	Maturing and mature **neutrophils**	**Macrophages** in spleen, thymus, lymphnodes, liver, alveoli, gut Ectopic: liver endothelium	**Macrophages** in spleen, lymphnodes, alveoli
Function	Marker for dead and dying neutrophils	Macrophage adhesion, uptake of bacteria and LDL	Clearance of inhaled pathogens
Knock-out phenotype	Normal	Mild, impaired uptake and degradation of lipoproteins and phagocytosed cells; more susceptible to *Listeria monocytogenes* and herpes simplex	Mild, increased susceptibility to pneumonia, impaired uptake of lipoproteins and bacteria

Table 3.1 Summary of Target Gene Characteristics

3.3 Cre Lines Used in Immunology

A variety of immune-specific Cre lines have been generated in recent years. A selection, sorted by tissue specificity, is listed in Table 3.2. The majority of available strains direct Cre expression to the lymphoid compartment. The novel Cre strains proposed in this work aim at providing myeloid specific recombination, in particular in neutrophils and macrophages. The thus far available Cre donor mice targeting myeloid cells; LysMCre (Clausen et al., 1999), CD11b-Cre (Ferron and Vacher, 2005) and GE-Cre (Thomas et al., 2004), all suffer from ectopic expression and varying expression levels. In addition, none is able to distinguish between neutrophils and macrophages.

3.3.1 LysM-Cre

The LysM-Cre mouse line is arguably the most widely used strain for conditional mutagenesis in granulocytes and macrophages. The model, based on a 129 background, was first developed in 1999 and carries the Cre cDNA knocked into the endogenous *Lysozyme M* locus (Clausen et al., 1999). In mice *Lysozyme M* (*LysM*) is specifically expressed in cells belonging to the myeloid lineage and is specifically up-regulated when tissue macrophages become activated (Keshav et al., 1991).

In an approach similar to the one presented herein, the Cre cDNA carrying a NLS sequence was recombined into the ATG start codon in exon1 of *LysM* to avoid interference with the gene's regulatory elements. The original paper showed high deletion levels using two floxed target

genes. A floxed version of the β DNA polymerase was deleted in 95% and 99% of peritoneal macrophages and neutrophils respectively, while deletion levels in the bone marrow were slightly lower with 75% and 79%. Deletion was also observed in 16% of splenic and 31% of bone marrow dendritic cells (DCs). This ectopic recombination is attributed to early expression in the myeloid DC progenitor. Low levels of deletion also occurred in lung and spleen, the authors, however, attribute this effect to resident tissue macrophages. A second floxed target, *RFX5*, a transcription factor involved in MHC II expression, showed lower deletion factors of 83% in peritoneal macrophages, once more emphasizing the importance of the target locus in recombination efficiency (Clausen et al., 1999). Another group observed additional ectopic expression when a floxed SOCS1 allele was not only deleted in the myeloid lineage, but also in B and T lymphocytes (Chong et al., 2005).

Due to its comparatively good expression levels, the LysM-Cre model has been broadly employed to scrutinize innate immune function. Many studies have been aimed at dissecting the signalling events occuring during inflammation processes. In a LysM-Cre conditional knock-out model of SOCS3 (suppressor of cytokine signalling), it became clear that SOCS3 plays a major role in negatively regulating G-CSF signalling in mature neutrophils, with deleted mice having more neutrophils than wild-type ones due to lower apoptosis levels (Kimura et al., 2004). SOCS1 deficient macrophages were found to have a higher tumor killing ability (Hashimoto et al., 2009). Specific disruption of STAT3, confirmed its suspected role as a repressor in resident macrophages during acute inflammation in a mouse peritonitis model in response to IL-10. STAT3 absence resulted in increased leukocyte infiltration and heightened production of pro-inflammatory cytokines and chemokines such as TNFα and MIP-2 (Matsukawa et al., 2005). This finding was also confirmed in a mouse colitis model, where it was shown that the interaction of STAT3 deficient macrophages with lymphocytes leads to a more severe inflammatory response (Reindl et al., 2007). The conditional deletion of IL4-receptor α led to a higher arthritis incidence accompanied by an up-regulation of pro-inflammatory cytokines IL-1β and IL-6 as well as chemokines MIP-1 and MIP-2, indicating IL-4 as another suppressor of chronic inflammation (Cao et al., 2007). The same deletion caused an increased mortality in a mouse model of schistosomiasis due to an impaired alternative macrophage activation process and increased T_H1 response (Herbert et al., 2004).

LysM-Cre has also been successfully used in a macrophage ablation study designed to analyse the role of macrophages during skin repair. Cre mediated recombination led to the myeloid specific activation of the diptheria toxin receptor, which renders cells susceptible to diptheria toxin. After toxin administration, skin wound macrophages were efficiently depleted, wounds were infiltrated by large numbers of neutrophils resulting in high persisting levels of pro-

inflammatory cytokines. Due to impaired neo-vascularisation and wound contraction, healing was seriously disturbed in non-sterile environments (Goren et al., 2009).

3.3.2 CD11b-Cre

The C57BL/6 derived CD11b-Cre mouse carries a plasmid transgene in which Cre expression is regulated by a 1.7kb long fragment of the human *CD11b* promoter (Ferron and Vacher, 2005). *CD11b* is expressed in myeloid cells, osteoclasts and subsets of T and B cells. Expression levels were shown to be highly dependent on the chromosomal insertion position as a transgenic line carrying only 4 copies achieved much higher frequencies than one harbouring 40. Transgene specificity was assessed using the Z/EG reporter mouse (Novak et al., 2000) in which the deletion of lacZ activates EGFP expression in targeted tissues. Flow cytometry analysis showed GFP activation in 38% of peritoneal macrophages and 35% of bone marrow granulocytes and macrophages. 50% of B220+ B cells in the spleen and 33% of CD4+ T cells in the thymus were also fluorescent. Interestingly, DNA quantitation by Southern Blot estimated the total recombination frequency in peritoneal macrophages to be 100% whereas very low levels in testis, kidney, liver, brain heart and lung were attributed to peripheral blood contamination. Microglial cells and osteoclasts, derived from the common macrophage progenitor, exhibited significant recombination as well (Ferron and Vacher, 2005).

Unlike the LysM-Cre mouse, the CD11b-Cre model has not been extensively used in generating myeloid specific conditional knock-outs, very likely due to its lower levels of recombination and significant ectopic activity. In one study, however, the strain was used to generate an ostecoclast specific knock-out of the microRNA processing enzyme Dicer, which resulted in mild osteopetrosis caused by reduced osteoclast numbers and impaired bone resorption (Sugatani and Hruska, 2009).

3.3.3 GE-Cre

The 129 based GE-Cre mouse strain, previously designed in this lab, harbours Cre as a knock-in into the granulocyte elastase locus *ela2* (Tkalcevic et al., 2000), which is expressed at the pro-myelocyte stage. Crossing of GE-Cre to a mouse harbouring a floxed version of *csk*, the C terminal Src kinase, resulted in 55% deletion in bone marrow and peritoneal granulocytes. Deletion could not be observed in resident peritoneal macrophages or lymphocytes, however, once macrophages became activated, they also displayed significant levels of recombination (Thomas et al., 2004). In another mouse model, carrying a floxed version of TβRII, a receptor subtype for TGFβ, recombination levels in bone marrow derived granulocytes only reached 30%, climbing to 52% after stimulation (Mahbub et al, unpublished).

The GE-Cre mouse served to ascertain the role of csk in regulating phagocyte recruitment. Conditional *csk* deficiency causes enhanced acute inflammation and sensitivity to LPS by rendering granulocytes hyperresponsive and -adhesive. Both phenotypes result from a dropped neutrophil activation threshold caused by spontaneous de-granulation and the localisation of integrins to the cell surface. Mice show acute inflammation of the skin and lung (Thomas et al., 2004). In a model where the target was the conditional deletion of TβRII however, the low deletion levels precluded the study of a specific phenotype regarding the migratory capabilities of neutrophils in response to TGFβ (Mahbub et al, unpublished).

In conclusion, the available blood specific Cre mouse models provide varied tools for studying the immune system. If one is interested in the myeloid cell lineage, the LysMCre mouse appears to be the most popular choice at present. That model, however, does not allow to selectively delete floxed target genes in myeloid subpopulations, as Cre is expressed in all macrophages and granulocytes. In addition, ectopic expression in non-myeloid compartments has been reported. The herein proposed models are an attempt to close this gap by providing Cre mice that enable selective and strong expression in neutrophils (*Itgb2l*) and macrophages (*Marco* and *Msr1*). We hope that *Msr1* controlled Cre expression results in a superior model allowing pan-macrophage conditional knock-outs. The *Marco* model, on the other hand, will hopefully provide a superior tool to engineer deletions in macrophage subsets.

Strain Name	**Target Gene/ Promoter**	**Type**	**Main Expression in**	**Ectopic Expression in**	**Expression Levels**	**Reference**
Lymphoid specific Cre lines						
Lck-Cre	*lck* promoter	Plasmid transgene	Immature T cells	Splenocytes	90%	(Orban et al., 1992)
CD19-Cre	*CD19*	Knock-in	B lymphocytes	None observed	75-80% pre-B cells, 90-95% splenic B cells	(Rickert et al., 1997)
hCD2-iCre	*hCD2* LCR	Plasmid transgene	T and B cells	Testis and ovaries	≈100% in all B and T Cells	(de Boer et al., 2003)
CD21-Cre	*CD21*	BAC transgene	Mature B cells	Liver, pancreas, kidney, gut, ovary	90% in mature B cells, 4-40% immature B cells, 55-70% bone marrow B cells	(Kraus et al., 2004)
Rag1-Cre	*Rag1*	Knock-in	T and B cells	n/a	100% spleen partial in bone marrow	(McCormack et al., 2003)
Foxp3^{YFP}-Cre	*Foxp3*	Knock-in	Regulatory T cells	T and B cells, myeloid cells, bone marrow precursors	100% in regulatory T cells 2-10% in other tissues	(Rubtsov et al., 2008)
CD4-Cre	*CD4* promoter/ enhancer	Plasmid transgene	Maturing T cells	Macrophages, B cells, Granulocytes, Microglia	90% in T cells	(Lee et al., 2001)
Strain Name	**Target Gene/ Promoter**	**Type**	**Main Expression in**	**Ectopic Expression in**	**Expression Levels**	**Reference**
Mb1-	*Mb1*	Knock-in	B Cells	Low levels in	99% splenic B cells,	(Hobeika et

Cre				kidney, liver, T cells	97% bone marrow B cells, 100% pre B	al., 2006)
GZMB-Cre	*Granzyme B* promoter	Plamsid transgene	Activated CD4+ T cells	n/a	n/a	(Jacob and Baltimore, 1999)
Cγ1-Cre	*γ1 constant region* promoter	Knock-in	Germinal B cells	IgM+ B cells	>85%	(Casola et al., 2006)
Ox40-Cre	*Ox40* promoter	Knock-in	Active T cells, regulatory T cells	Testes, naive T cells	90% in regulatory T cells, 8-70% in T cells	(Klinger et al., 2009)
Aicda-Cre	*AID* promoter	BAC Transgene	Germinal B cells	n/a	n/a	(Kwon et al., 2008)
E8I-Cre	*CD8a* Promoter *E8I* enhancer	Plasmid transgene	CD8+ T cells	n/a	n/a	(Maekawa et al., 2008)
Myeloid Specific Cre Lines						
LysM-Cre	*M Lysozyme*	Knock-in	Monocytes, macrophages, neutrophils	Dendritic cells	95% peritoneal macrophages, 99% peritoneal neutrophils, 75% bone marrow, macrophages, 78% bone marrow granulocytes	(Clausen et al., 1999)
GE-Cre	*Ela2*	Knock-in	Granulocytes	Macrophages	55% granulocytes	(Thomas et al., 2004)
CD11b-Cre	*CD11b* regulatory elements	Plasmid transgene	Macrophages, granulocytes, mature osteoclasts	T and B cells,	100% peritoneal macrophages, 35% bone marrow, 35% thymic T cells	(Ferron and Vacher, 2005)
Mcpt5-Cre	*Mcpt5* regulatory elements	BAC transgene	Mast cells	None observed	99.1% peritoneal mast cells	(Scholten et al., 2008)
Haematopoietic Stem Cell Specific Cre Lines						
vav-iCre	*Vav* promoter	Plasmid transgene	All hemato-poietic tissues	Testis and ovaries, gut, brain, muscle	n/a	(de Boer et al., 2003)
Fes-Cre	Human *c-fes* promoter	Plasmid Transgene	Definitive hematopoietic stem cells	Heart, muscle, lung, kidney, brain	52% erythrocytes, 69% granulocytes, 56% B cells, 77% T cells	(Keller et al., 2001)
Erythrocytes/Megakaryocyte Specific Cre Lines						
pEV-Cre	*Erythroid specific β globin* promoter and LCR	Plasmid transgene	Erythrocytes	Fetal mast cells, macrophages	100% in fetal liver	(Gutierrez et al., 2004)
Pf4-Cre	*CXCL4* regulatory elements	BAC transgene	Megakaryo-cytes	None oberserved	100% in megakayocytes and platelets	(Tiedt et al., 2007)
Gata1-Cre	*Gata1* promoter	Plasmid transgene	Erythrocytes	Megakaryo-cytes, eosino-phils mast cells	100% in peripheral erythrocytes	(Jasinski et al., 2001)
Dendritic Cell Specific Cre Lines						
CD11c-Cre	*CD11c* regulatory elements	BAC transgene	Dendritic cells	Lymphocytes, myeloid cells	>95% splenic dendritic cells	(Caton et al., 2007)

Table 3.2 Cre Lines Used in Immunology

CHAPTER 4: HAEMATOPOIETIC DIFFERENTIATION OF MURINE EMBRYONIC STEM CELLS

Embryonic stem cells are able to give rise to almost any cell type. Recent years have seen major advances regarding *in vitro* culture protocols aimed at producing a multitude of tissues. It has become clear that ES cells follow some of the key regulatory processes governing *in vivo* embryonic development. Differentiation can consequently serve as a model for mammalian development and also provide cells for regenerative medicine (Murry and Keller, 2008). Simultaneously, the knowledge about the cellular processes distinguishing embryonic stem cells has evolved considerably. Haematopoietic differentiation was used as a method in the herein presented work to assess expression of the knocked-in Cre recombinase *in vitro* prior to investing the time and resources in creating mouse models. The basics of murine stem cell biology and differentiation, as well as key concepts surrounding *in vivo* and *in vitro* haematopoiesis are summarized in this final introductory chapter.

4.1 Basic Stem Cell Biology: Pluripotency and Self-Renewal

Murine embryonic stem cells are derived from the inner cell mass (ICM) of the 3.5d pre-implantation blastocyst. They are able to differentiate into all three germ layers and can thus complement embryos that give rise to adult mice. The key characteristics of embryonic "stemness" are pluripotency[2] and self-renewal (Boheler, 2009). By definition, pluripotency portrays a cell's ability to form all cell types of the adult body without possessing the self-organising capability needed to build a whole organism. Self-renewal describes the capacity to undergo repeated cell divisions while maintaining the undifferentiated state and not entering senescence (Niwa, 2007). So far "stemness" has been traced to three distinct cellular properties – a defined set of transcription factors, a unique epigenetic state and a shorter cell cycle – that differentiate embryonic stem cells from somatic cells.

Of the transcription factors implicated in maintaining pluripotency, Oct3/4, Nanog and Sox2 are most important. Oct3/4 is essential for the initial development of pluripoteniality in the ICM. Oct3/4 knock-out mice die soon after implantation (Nichols et al., 1998). Suppression of Oct3/4 in ES cells leads to trophoblast-like differentiation (Niwa et al., 2000). The mechanism of action seems to be dose-dependent, as overexpression results in mesodermal and endodermal differentiation and Oct3/4 has also been linked to the regulation of cardiac differentiation (Stefanovic and Puceat, 2007; Zeineddine et al., 2006). Likewise, Nanog is expressed in pre-implantation embryos but not in differentiated cells (Chambers et al., 2003; Mitsui et al., 2003).

[2]Pluripotent cells can create all embryonic and thus adult tissues, but not extraembryonic structures. Only the zygote is able to generate both and is therefore totipotent.

The factor acts as a dimer that suppresses endodermal differentiation (Wang et al., 2008a). Overexpression of Nanog enables *in vitro* embryonic stem cell cultures to remain undifferentiated in the absence of leukemia inhibitory factor (LIF) and sera (Liu et al., 2009; Ying et al., 2003). The third factor, Sox2 (Ivanova et al., 2006; Kopp et al., 2008), curbs throphoectoderm formation in a dose-dependent manner (Kopp et al., 2008) and is further able to dimerize with Oct3/4 in order to regulate gene expression (Chew et al., 2005). Sox2 deletion can be rescued by Oct3/4 administration, suggesting that its major role is governing Oct3/4 (Masui et al., 2007). Oct3/4, NANOG and Sox2 interact in complex feedback loops and regulate each other's expression as well as their target genes.

Plenty of other transcription factors have been connected to pluripotency and self-renewal, though they are not restricted to ES cells. These include, but are not limited to, c-Myc (Kidder et al., 2008), B-Myb (Kidder et al., 2008; Liu and Labosky, 2008), Foxd3 (Liu and Labosky, 2008), Klf4 (Chan et al., 2009), Sall4 (Yuri et al., 2009), Zfp42 (Scotland et al., 2009) and Gbx2 (Chapman et al., 1997). Overall, the maintenance of "stemness" in ES cells appears to be controlled by an intricate transcriptional network.

The transcriptional network is complemented by elaborate and, in the context of stem cell biology, poorly understood signalling pathways. Cultured murine ES cells, for example, are maintained in an undifferentiated state through growth on a fibroblast feeder layer in the presence of extrinsic LIF (Williams et al., 1988). LIF has recently been shown to hierarchically affect Jak/STAT3; Grb2/MAPK and PI(3)K/Akt (Niwa et al., 2009) pathways. It has been long known that stimulation of the Jak/STAT3 pathway is sufficient to maintain pluripotency in culture (Matsuda et al., 1999). Signalling controls Klf4 and Tbx3 transcription factors, which in turn influence the major pluripotency markers Sox2, Oct3/4 and Nanog (Figure 4.1). Supporting evidence comes from the discovery that enhanced expression of Nanog (Chambers et al., 2003), renders embryonic stem cells independent of LIF. Parallel auxiliary conduits encompass signalling through BMP4 (bone morphogenetic protein 4) and Smad to activate the Id (inhibitor of differentiation) proteins that repress lineage specific commitment (Ying et al., 2003) as well as activation of the Wnt/β-catenin system (Ogawa et al., 2006; Sato et al., 2004).

A recent study supporting the hierarchical structure of the signalling network postulates that embryonic stem cells represent a basal cellular state, which self-maintains if shielded from differentiation stimuli (Ying et al., 2008), as blocking cellular differentiation pathways (i.e. autocrine stimulation of the mitogen-activated protein kinase ERK1/2 by fibroblast growth factor FGF4) with small molecule inhibitors maintains ES cells in the absence of LIF (Kunath et al., 2007; Stavridis et al., 2007).

ES cells have a distinct pattern of histone acetylation and methylation as well as DNA methylation, comprising a unique epigenetic state. Embryonic stem cell chromatin is in an active shape, poised for transcription, which is evidenced by a looser structure with fewer tightly wound heterochromatin foci that can be easily accessed by pluripotency-maintaining transcription factors; a higher proportion of loosely bound architectural proteins (Meshorer et al., 2006) and increased levels of acetylated histones implicated in rendering chromatin transcriptionally permissive (Lee et al., 2004). Simultaneously, discrete patterns of histone lysine methylation and the co-operative binding of polycomb protein complexes efficiently repress lineage specific transcription factor expression (Boyer et al., 2006; Pietersen and van Lohuizen, 2008). Once differentiation commences; pluripotency markers such as Nanog and Oct3/4 are rapidly silenced by DNA methylation (Farthing et al., 2008; Li et al., 2007). Furthermore, embryonic stem cells express a unique set of microRNAs[3] which are down regulated with the onset of differentiation (Houbaviy et al., 2003). Their mode of action is unclear, they might serve to silence pluripotency factors as embryonic stem cells leave the pluripotent state (Chivukula and Mendell, 2009). Transcriptional and epigenetic regulators interact extensively to maintain both pluripotency and the induction of differentiation (Figure 4.2).

A final defining feature of embryonic stem cells is their rapid division and thus comparatively short cell cycle with a particularly short G1 phase controlled by constitutively active Cdk2 (Koledova et al., 2009). ES cells exhibit a non-functional mitotic-spindle checkpoint in G2 that normally induces apoptosis if chromosomal integrity is compromised. Consequently, embryonic stem cells are more likely to develop karyotypic abnormalities than adult cell lines, a trait that has been repeatedly observed in long term *in vitro* culture (Mantel et al., 2007).

A direct result of understanding what governs stem cell biology is the recent development of the induced pluripotent stem (iPS) cell technology through which somatic cells are reprogrammed into an induced pluripotent state by overexpressing certain transcription factors, modifying the cell's epigenetic state and/or altering defined signalling pathways (Muller et al., 2009; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007).

[3] microRNAs are a 22nt long subset of mammalian transcriptomes, They have been found to regulate gene expression by degradation of transcripts or inhibition of translation.

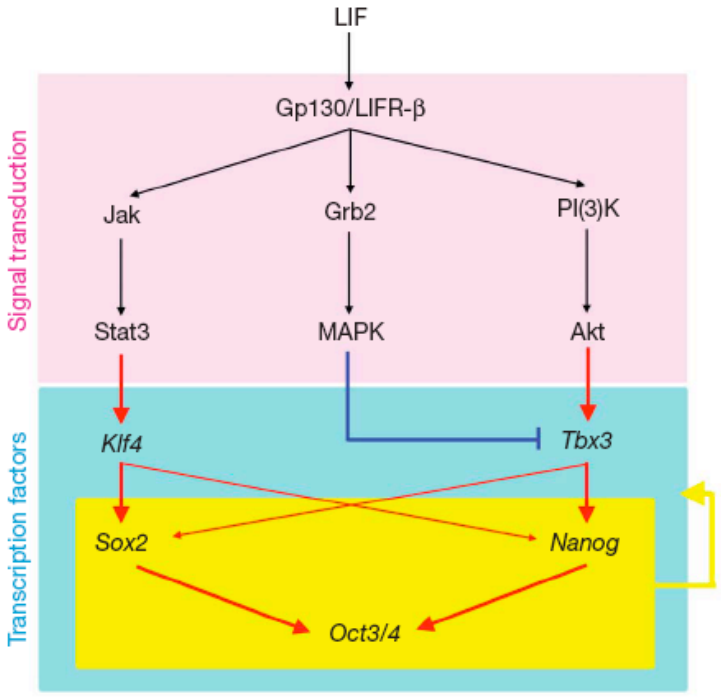


Figure 4.1 LIF-controlled Parallel Signalling in Cultured Embryonic Stem Cells
LIF interacts with its receptor gp130/LIFRβ and controls three pathways. The Jak/STAT3 pathway mainly activates Klf4, whereas the PI(3)K/Akt pathway regulates Tbx3. The Grb2/MAPK pathway is proposed to have a balancing effect on Tbx3. Klf4 and Tbx3 interact in regulating Sox2 and Nanog, which then direct Oct3/4. Illustration taken from (Niwa et al., 2009).

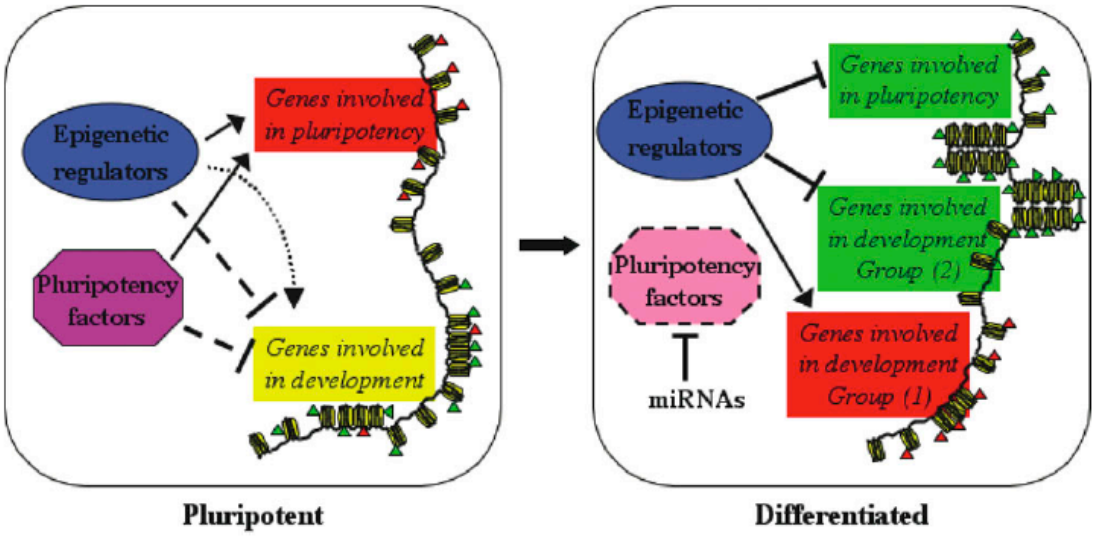


Figure 4.2 Regulatory Networks in Pluripotency and Differentiation
Epigenetic (blue) and pluripotency regulators (dark pink) interact to activate pluripotency-inducing genes (red) and suppress lineage specific genes (yellow) when the cell is in a pluripotent state. During differentiation, however, the epigenetic regulators activate transcription of defined developmental genes relevant for lineage commitment (red) and inhibit pluripotency and non-related developmental genes. MicroRNAs (miRNA) serve to silence pluripotency transcripts (light pink). Cartoon taken from (Chen and Daley, 2008).

4.2 Embryonic Haematopoietic Differentiation *in vivo* and *in vitro*

4.2.1 Basic Murine Embryonic Development

Murine pre-natal development has been studied for decades as a model for vertebrate and especially human embryogenesis. A brief summary, focussing on the early stages, is presented here, for more extensive reviews, the reader is referred to (Duranthon et al., 2008; Gridley, 2006; Nagy et al., 2003; O'Shea, 2004; Rossant and Tam, 2009; Tam, 2004; Tam et al., 2006).

Embryo development begins with the fertilization of an ovum resulting in the formation of a zygote covered by the zona pellucida. Progressing cell divisions commence the morula stage (4-16 cells), with a compaction observed at the 8-cell phase around E2.5. The morula enters the uterine cavity via the fallopian tubes and forms a liquid-filled cavity, the blastocoel, at 32 cells or around E3.5. This period (40-150 cells) is called the blastocyst stage. The zona pellucida degenerates and is replaced by the trophoblast built from the outer layer of the blastocyst. The trophoblast serves to generate the trophoectoderm, an epithelial-like structure essential for implantation and later placenta development (Watson and Barcroft, 2001). The blastocyst's inner layer forms the embryoblast and is the embryo's source pluripotency (i.e. inner cell mass; ICM) (Rossant, 2004; Yamanaka et al., 2006). It differentiates into an epiblast, adjacent to the trophoblast, and a hypoblast, next to the blastocyst cavity. The epiblast is the starting point for all three embryonic germ layers. The hypoblast gives rise to a primitive endoderm.

The embryo implants into the uterine lining around E5 and forms a central pro-amniotic cavity. Shortly thereafter, around E5.5, epiblast cells loose their pluripotency. The primitive endoderm develops into the visceral endoderm at gastrulation and subsequently into the visceral yolk sac. Gastrulation, the invagination of the blastocyst, begins with the formation of the primitive streak at E6.5 and establishes the spatial organisation of the embryo. The process ultimately results in the extraembryonic mesoderm and the three definitive germ layers mesoderm, endoderm and ectoderm (Rivera-Perez, 2007). The germ layers then begin with the complex course of organogenesis, with each layer, giving rise to a specific set of tissues until birth at E19.5 (Figure 4.3).

4.2.2 *In vivo* Haematopoeitic Development

A functioning cardiovascular system is essential for mammalian embryos to develop beyond implantation (Copp, 1995). Haematopoietic tissues are consequently, together with cardiac and vascular structures, the first to arise in the embryo. Early embryonic haematopoiesis begins with

the onset of gastrulation. Future mesodermal cells move along the primitive streak[4] and exit at either the posterior or anterior end. The former contribute to several extraembryonic tissues including the yolk sac (Lawson et al., 1991). The latter serve to form an intraembryonic region called the para-aortic splanchnopleura (P-Sp), which later develops the aorta, gonads and mesonephros (AGM) (de Bruijn et al., 2000; Medvinsky et al., 1993). Both of these sites play a vital role in embryonic blood production. Several other locales of haematopoietic potential have been suggested. These include the allantois, a transient sac-like extraembryonic structure involved in excretion (Lawson et al., 1991); aortic clusters in the vitelline and umbilical arteries which supply blood to the yolk sac and placenta (de Bruijn et al., 2000) and, later in development, the placenta (Gekas et al., 2005). The respective tissues and time frames are summarized in Figure 4.4.

The yolk sac produces haematopoietic tisssues in several staggered waves. The first generates primitive[5] erythroid cells from so-called blood islands, formed by blood cells and endothelium, starting from E7.25 (Palis et al., 1999) and plateauing by E12.5. These cells derive from a unique progenitor (EryP-CFC), enter circulation, mature and can be detected until several days after birth (Kingsley et al., 2004). The yolk sac also produces macrophages and megakaryocytes during this phase (Xie et al., 2003; Xu et al., 2001a). The second wave of yolk sac haematopoiesis begins at E8.25 and generates definitive erythroid progenitors (BFU-E and CFU-E[6]) which enter the blood stream and begin populating the fetal liver at E10 (Palis et al., 1999). The liver becomes the main organ for the maturation of definitive erythrocytes which go into circulation around E11.5 (Kingsley et al., 2004). Mast cell and granulocyte-macrophage progenitors appear around the same time whereas the megakaryocyte and macrophage progenitors found during primitive yolk-sac haematopoiesis persist throughout the definitive stage (Keller et al., 1999). The yolk-sac thus provides myeloid and erythroid precursors to the fetus and helps to populate the liver with haematopoietic potential (McGrath and Palis, 2005). Whether or not yolk sac derived cells play a role in long-term haematopoiesis is still debated, though it sometimes has been found to be capable of forming haematopoietic stem cells (Kumaravelu et al., 2002).

After E10 (Lux et al., 2008) the AGM region begins to play a distinct part in embryonic haematopoiesis. It is thought to provide haematopoietic stem cells, including those of lympho-myeloid potential, that populate the liver, thymus, spleen and eventually initiate the switch to

[4]The primitive streak is the first sign of mammalian gastrulation. It forms as a transient furrow across the embryo and plays a role in defining the anterior and posterior orientation.

[5] Primitive erythrocytes are nucleated and resemble the red blood cells found in non-vertebrate species. In addition, they are much larger than the definitive enucleated erythrocytes produced later during embryonic development or in the adult mammal.

[6] BFU-E (burst forming unit - erythroid) and CFU-E (colony forming unit – erythroid) are both progenitor cells for erythroblast and erythrocyte development. BFU-E differentiates into CFU-E when stimulated by Erythropoietin.

long-term blood generation in the bone marrow. The involved mechanisms and cell types are not yet clearly elucidated (Cumano et al., 1996; Cumano et al., 2001).

The early steps of embryonic haematopoiesis, irrespective of the locale, are still a matter of debate. It is well known that blood cells differentiate from the mesoderm, but exactly how is still under discussion. An established paradigm postulates the existence of the haemangioblast, a cell type capable of producing both endothelial and blood cell lineages as the common mesodermal precursor. This assumption is supported by the finding that embryonic stem cells can differentiate into cells expressing the mesodermal marker Flk-1 which then mature into blast colony forming cells (BL-CFC) that in turn can develop into both haematopoietic and endothelial cells (Huber et al., 2004). Flk-1 is also expressed during embryonic yolk-sac haematopoiesis. Yet, a newer theory suggests a haemogenic endothelium as the intermediate state. It is based on the discovery that Flk-1+ cells progress through a transient stage expressing exclusively endothelial markers before moving on to develop haematopoietic lineages (Lancrin et al., 2009). Furthermore it has been found that endothelial cells from the AGM region give rise to cells populating the fetal liver and bone marrow (Zovein et al., 2008) in a Runx-1[7] controlled process (Chen et al., 2009) and that individual cells could be tracked *in vitro* from the mesodermal through the endothelial to the haematopoietic level (Eilken et al., 2009).

4.2.3 *In vitro* Haematopoietic Differentiation from Embryonic Stem Cells

Haematopoietic differentiation of ES cells is one of the best-studied differentiation processes and its investigation began as soon as embryonic stem cells had been isolated. It was shown early on that ES cells can differentiate in culture to generate several haematopoietic lineages (Doetschman et al., 1985). One of the central concepts of *in vitro* haematopoietic differentiation is to model the *in-vivo* haematopoietic niche. Several techniques have been employed in cell culture to mimic those surroundings: three-dimensional embryoid body culture in suspension (4.2.3.1), growth on feeder layers and matrices (4.2.3.2) and the application of soluble growth factors (4.2.3.3) (Orlovskaya et al., 2008).

Experiments have shown that early haematopoietic cells, primitive and definitive erythroid; myeloid and endothelial lineages, reminiscent of *in vivo* yolk-sac haematopoiesis, can be generated with comparative ease. In accordance with the dogma that all blood originates from mesoderm, differentiation cultures begin to express mesodermal markers such as Flk-1 and CD34, before haematopoietic markers appear (Keller et al., 1993). The gene expression patterns observed during *in vitro* differentiation also mimic those of the early embryo with the

[7] The transcription factor Runx-1 is needed for haematopoietic stem cell formation in the mouse embryo.

transcription factors of the GATA family (Shimizu and Yamamoto, 2005; Tsai et al., 1994; Weiss et al., 1994) and SCL/tal-1 (Porcher et al., 1996) playing essential roles.

Lymphoid, haematopoietic stem cell (HSC) populations and mature blood cell types such as neutrophils are much more difficult to derive *in vitro*. This is in part due to their later appearance *in vivo*. As such they are believed to require a more complex microenvironment, which is difficult to model in culture. One of the crucial factors appears to be the oxygen pressure, as one study found that lymphoid progenitors could only be derived in a low-oxygen environment (Potocnik et al., 1994). Recent years, however, have seen an emergence of complex; multi-step; long-term culture protocols, able to produce most blood cell lineages, if with low efficiencies (Wassarman and Keller, 2003). Figure 4.5 shows an overview of the order of differentiation seen in murine cultures. Early blood development *in vitro* was found to be independent of growth factors with the exception of those provided by batch tested serum (Keller et al., 1993; Wiles and Keller, 1991) or, more recently, serum replacements (Ma et al., 2008). The reproducible creation of mature lineages, however, heavily depends on soluble growth factors, in particular cytokines and extracellular matrix proteins.

4.2.3.1 Suspension Culture and Embryoid Body Formation

ES cells cultured in suspension in the absence of pluripotency maintaining LIF form small clumps surrounded by an visceral-endoderm like structure (Keller, 1995). These are referred to as embryoid bodies (EB). EBs are reminiscent of early *in vivo* post-implantation development when considering size, gene expression patterns and differentiation abilities. They contain precursors of all germ layers, including haemangioblast-like cells expressing Flk-1, which are commonly viewed as blood progenitors (Doetschman et al., 1985; Kabrun et al., 1997; Keller, 1995). EBs cultured for more than 10d commonly develop a cystic structure, characterized by a liquid filled cavity and a polar morphology, much like the late blastocyst *in vivo*.

Techniques for EB generation include liquid suspension culture in ultra-low attachment plates, culture in semisolid media (i.e. methylcellulose, MCM), hanging drops or porous scaffolds. Formation efficiency depends on the cellular density, with dilute suspensions working better than dense ones (Dang et al., 2002). Murine embryonic stem cells are, unlike their human equivalent (Ng et al., 2005), capable of forming EBs with haematopoietic potential from single cells. Irrespective of the starting cell numbers, EBs grow to a threshold size in suspension, which is very likely due to them reaching a developmental block or not being able to extract enough nutrients and oxygen from the medium (Dang et al., 2002).

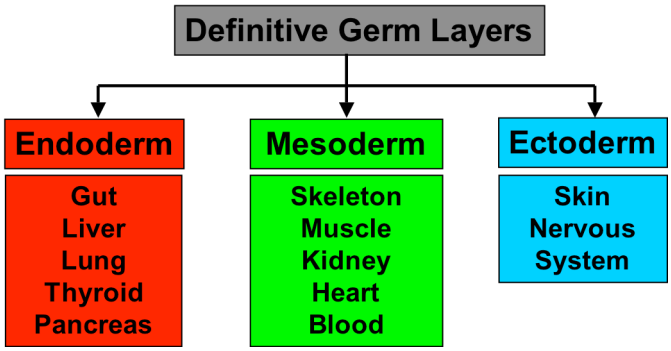


Figure 4.3 Murine Germ Layers and Tissue Origination
The three different germ layers endoderm (red), mesoderm (green) and ectoderm (blue) give rise to distinct tissues in the adult organism. Adult blood is ultimately derived from the mesoderm.

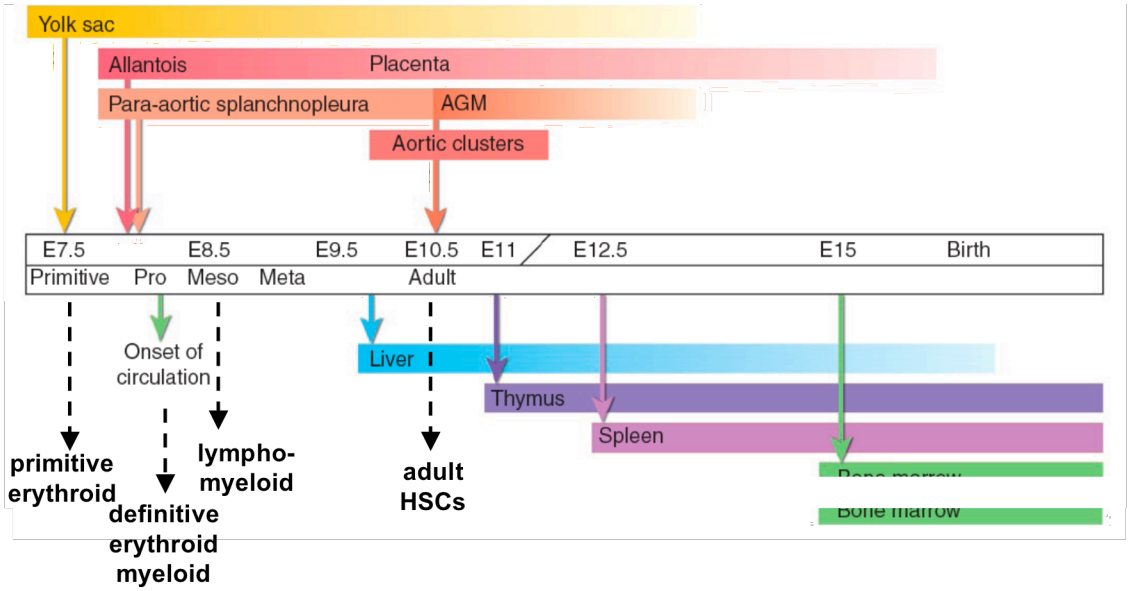


Figure 4.4 Stages of Murine Embryonic Haematopoiesis
The upper panel illustrates active haematopoietic tissues during the embryonic development timeline (middle panel). The lower panel indicates the earliest time points (dashed arrows) at which certain blood cell types appear and when definitive haematopoietic organs are first colonized. AGM: aorta-gonads-mesonephros; HSCs: hematopoietic stem cells. Figure modified from (Dzierzak and Speck, 2008).

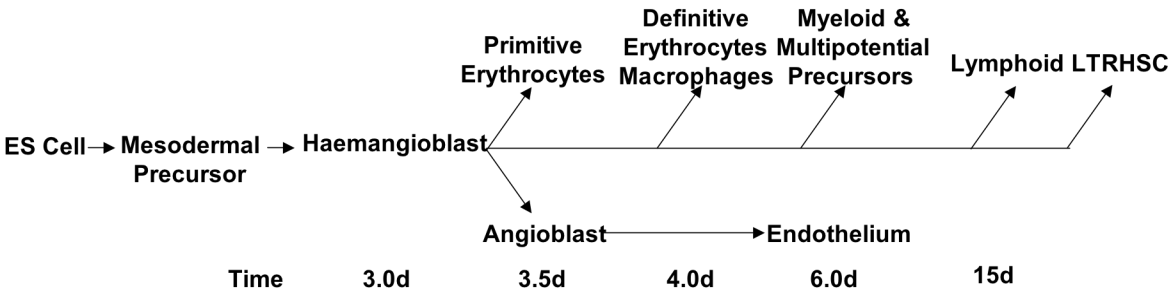


Figure 4.5 Order of Haematopoietic Differentiation in Murine ES Culture
The time-line indicates days from which onwards the first population of lineage progenitors can be observed. Timings will be heavily dependent on individual culture protocols. Obtaining mature cells often requires further periods of maturation in the presence of specific growth factors. LTRHSC: long term repopulation haematopoietic stem cell, describes a transplantable population that can replace or supplement *in-vivo* haematopoiesis in the recipient. The graph is modified from (Keller et al., 1999).

Keller et al (Keller, 1995; Wiles and Keller, 1991) pioneered the EB technology for haematopoietic growth. In their cultures, based on semisolid methylcellulose supplemented with growth factors (Table 4.1), early erythrocytes were observed around day 7, as indicated by EBs turning red from produced haemoglobin. Early macrophages became apparent after 12-18d. At later stages mast cells and very few neutrophils appeared, around days 12-20. The initial experiments proved that EBs can initiate both primitive and definitive haematopoiesis. The former, however, is quantitatively dominant, possibly due to the lack of anatomic complexity needed to expand the latter (Lengerke and Daley, 2005). In practical terms, this means that if mature blood cells are to be differentiated, they require multifarious and lengthy culture procedures beyond the formation of EBs.

A key factor in the growth of haematopoietic EBs is culture in suspension. Attachment to cell culture plastic and subsequent spreading abolishes haematopoietic potential, possibly by inhibiting the formation of early mesodermal precursors (Dang et al., 2002). This theory is supported by the observation that attached EBs exhibit a drastic down-regulation of the mesodermal markers CD34 and Flk-1. Interestingly, this problem occurs only in early cultures. If EBs attach after 4 days or later, haematopoietic development is not compromised significantly and attachment culture of late EBs is indeed widely used for longer term haematopoiesis *in vitro* (Bautch et al., 1996; Dang et al., 2002).

4.2.3.2 Feeder Cells and Matrices

Feeders and matrices model the stromal environment of the adult bone marrow and have been used particularly in differentiation assays aimed at creating haematopoietic populations that appear later in development, such as neutrophils, lymphocytes and re-populating stem cells. They are thought to support differentiation by providing soluble factors and extracellular matrix molecules. The OP9 cell line (see below), for example, has been shown to express IL-7 and SCF, both of which are relevant to granulopoiesis (Cho et al., 1999; Veiby et al., 1996).

Several murine stromal-like fibroblast cells have been utilized. They are commonly isolated from the adult bone marrow, the fetal liver or intraembryonic regions of the early embryo. One of the first lines employed was the murine stromal cell line OP9, derived from adult bone marrow that is deficient in macrophage colony stimulating factor (Nakano et al., 1996). Due to the lack of M-CSF, it is still popular in creating non-macrophage lineages such as megakaryocytes (Eto et al., 2002) and neutrophils (Lieber et al., 2004). Other described cell lines include S17, supporting B-lympho- and myelopoiesis (Collins and Dorshkind, 1987); MS-5 (Issaad et al., 1993), AFT024 (Hackney et al., 2002) and AGM lines (Krassowska et al., 2006; Weisel et al., 2006) used in HSC maintenance.

Collagen IV is an effective matrix protein that enables the production of Flk-1+ blood progenitors without the step of three-dimensional EB formation (Nishikawa et al., 1998). Similarly, the portrayed cell lines are able to support certain haematopoietic processes independently of EB formation. The majority of differentiation protocols, however, still rely on the assembly of EBs and their further expansion, either as whole bodies or as trypsin or collagenase dissociated single cells, followed by maturation with the help of co-cultured cells and/or growth factors.

4.2.3.3 Soluble Growth Factors and Differentiation

Whereas the initial stages of differentiation, up to the formation of early mesoderm, appear to be factor independent, soluble growth factors, in particular cytokines and chemokines, are broadly used to maintain longer term haematopoietic differentiation *in vitro*. Table 4.1 summarizes combinations employed for a panel of murine differentiation experiments sorted by culture method and time frame. Murine haematopoietic differentiation is still heavily reliant on batch tested bovine serum. While this has been blamed for the high rate of variability between experiments (Keller, 2005), it is likely set to continue due to its cost-effectiveness. Also xeno-free culture protocols are a much lower priority in murine experiments compared to the differentiation of human embryonic stem cells for potential transplantation. Curiously, almost all protocols recommend the use of reducing agents, such as monothioglycerol (MTG), ascorbic acid or vitamin E due to their beneficial effects on haematopoietic development. The mechanism through which these substances exert their influence are currently unknown. Additionally, insulin is often added as it improves cell plating efficiency.

4.2.4 *In vitro* Production of Murine Macrophages and Neutrophils

4.2.4.1 Macrophages

As described previously, macrophages are the first murine myeloid lineage to appear in differentiation cultures. Very low rates have even been seen without any added growth factors beyond those provided by the serum (Loureiro et al., 2008). The general protocol, however, relies on EB formation and expansion of macrophage progenitors by IL-1, IL-3 and M-CSF. Embryoid bodies cultured in liquid or semisolid suspension experience primitive and definitive erythropoiesis during the first 10 days illustrated by the EB core going red due to the production of haemoglobin. Macrophages appear over the following 10 days. They present as highly refractive cells forming a halo around the EB and are eventually shed into the cell culture medium. Disrupted EBs can also be expanded as attached single cell cultures in which macrophages appear after similar time frames. Attached EBs were shown to be able to

continuously produce macrophages for several weeks (Wiles, 1993). The ES derived macrophages express markers CD11b and F4/80 as well as RNA for lysozyme and for the M-CSF receptor and are able to phagocytose (Inamdar et al., 1997; Wiles and Keller, 1991).

4.2.4.2 Neutrophils

Neutrophils appear late in embryonic development. Their derivation from murine embryonic stem cells is consequently much more difficult than for macrophages. Nevertheless, a complex three-step culture protocol, able to generate mature neutrophils with a yield of around 75%, has been described recently (Lieber et al., 2004). First, EBs were formed in liquid suspension culture for several days. Day 8 EBs were trypsinized and plated onto semi-confluent OP9 cells with serum based medium supplemented by growth factors OSM, bFGF, LIF, KL, IL-6 and IL-11. After two replating steps over the next 3 days, adherent cells were transferred into medium containing G-CSF, GM-CSF and IL-6 for maturation. Mature neutrophils could be harvested after additional 4-20 days, bringing the total culture period to 15-35 days. The phenotype of those differentiated cells agrees well with that of bone marrow derived neutrophils. They express neutrophil markers Gr-1 and the neutrophil specific antigen and a large fraction stains positive for gelatinase, a late stage neutrophil maturation marker. In addition, they are able to produce super oxide when stimulated and react chemotactically to MIP-2 (Lieber et al., 2004).

Soluble Growth Factor	Differentiation Protocol	Predominant Cell Type	Time Frame	Reference
Erythropoietin (Epo)	Embryoid body/MCM	Erythrocytes	5-7 days	(Wiles and Keller, 1991)
IL-3	Embryoid body/MCM	Macrophages, mast cells	10-12 days	(Wiles and Keller, 1991)
IL-3 and/or IL-1; M-CSF or GM-CSF	Embryoid body/MCM	Macrophages	8-14 days	(Wiles and Keller, 1991)
IL-3; IL-1; GM-CSF; Epo	Embryoid body/MCM	Broad range of colony forming cells (haematopoietic progenitors)	8-14 days	(Keller et al., 1993)
SCF and/or IL-11	Embryoid body/MCM	M-CFC; E-CFC; GM-CFC; mix CFC	8-14 days	(Keller et al., 1993)
IL-6; IL-11; Thrombopoietin (Tpo)	OP9 co-culture	Megakaryocytes	8-12 days	(Eto et al., 2002)
Epo	OP9 co-culture of Flk1+ cells, no serum	Erythrocytes	7 days	(Otani et al., 2004)
IL-3; IL-7; Epo	Embryoid body/MCM; ST2 co-culture	Lymphoid precursors	18 days	(Nisitani et al., 1994)
Tpo; FGF2; SCF; IL-3; IL-6; IL-11; G-CSF; Epo	Embryoid body; MS-5 co-culture	Myeloid progenitors, megakaryocytes	>12 days	(Berthier et al., 1997; Uzan et al., 1996)
OSM; bFGF; IL-6; IL-11; LIF; SCF; G-CSF; GM-CSF;	Embryoid body; OP9 co-culture	Neutrophils	15-35 days	(Lieber et al., 2004)
Flt-3L	OP9 co-culture	B-lymphocytes	19-28 days	(Cho et al., 1999)
IL-3; GM-CSF	Embryoid body	Dendritic cells	>14 days	(Fairchild et al., 2003)

Table 4.1 Cytokines Used in the Haematopoietic Differentiation of Murine ES Cells

MATERIALS AND METHODS

CHAPTER 5: EXPERIMENTAL PROCEDURES

5.1 Molecular Biology Methods

5.1.1 Electrophoresis Related Methods

5.1.1.1 Agarose Gel Electrophoresis

Gel concentrations varied between 0.7-2% agarose (Sigma) depending on the nucleic acid fragment size to be visualized. Gels, containing 50µg/ml ethidium bromide, were poured and run in 1xTAE buffer. Samples were diluted in 6x loading buffer (20% Ficoll, 30mM EDTA, 0.01% Bromphenolblue). Size standards (Promega) were run with every gel to estimate nucleic acid concentration and size. Gels were run at varying field strengths for a duration of 45min to 1h. Samples were visualized using a UV transilluminator; pictures were taken using the Alpha Imager (Alpha Innotech).

5.1.1.2 Pulse Field Gel Electrophoresis (PFGE)

Pulse field gels contained 0.8% agarose and were run in 0.5xTBE using the BioRad Chefmapper according to manufacturers protocols. All conditions were set to "AUTO". Gels were dyed post-run in 0.5xTBE containing 50µg/ml ethidium bromide for 15min whilst shaking. To enhance contrast, gels were de-stained in water for further 10min.

5.1.1.3 Gel Purification

Samples to be isolated were run in low melting point agarose (Sigma) gels, excised, weighed and then purified with the Qiagen Mini Elute kit according to the manufacturer's protocols. The protocol was modified to use doubled elution buffer volumes and eluting at 65˚C.

5.1.2 PCR Related Methods

Sequences of all primers used in this work can be found in Appendix A. Primers were designed using Primer3 (Rozen and Skaletsky, 2000). Primers used were de-salted with the exception of targeting primers, which were gel and HPLC purified.

5.1.2.1 Standard PCR

All PCRs designed to yield product sizes of up to 3kb and not requiring proofreading activity were carried out as follows. Reactions were performed in 10-50µl using the GoTaq Polymerase (Promega) according to the manufactures protocols. Primer (Sigma, MWG) concentrations were 10 or 20pmol per reaction. Nucleotides (Invitrogen) were added at 200µM. Magnesium concentration was 1.5mM. The template amount varied from 100pg in case of plasmids to 200ng for genomic DNA per reaction. The annealing temperature was typically set about 5˚C below the melting temperature of the primer. 25-35 cycles were carried out.

5.1.2.2 Long Range PCR

PCRs for products over 10kb were performed using the Takara LA Taq (Takara Bio Inc) according to the manufacturer's protocols. PCRs were performed in 10µl with 2.5mM Magnesium, 0.5U enzyme, 10pmol per primer and 20-50ng template genomic DNA per reaction. A special auto-segment extension programme (Ohler and Rose, 1992) was employed to increase product yield. All PCRs were performed as Hot Start. Primer annealing and extension were combined in one step at 68˚C.

5.1.2.3 Intermediate Length PCR

PCRs intended to generate fragments between 3kb and 10kb, those for sequencing or subcloning were performed mixing the previously mentioned GoTaq polymerase with proofreading polymerase Pfu (Promega) to reduce errors at a ratio of 16:1. Reactions were carried out in Pfu reaction buffer as described before.

5.1.2.4 Colony PCR

PCRs using mES colonies were carried out as follows. Cells were harvested and aliquoted into PCR tubes at 10^4, $2*10^4$ or $5*10^4$ cells/tube; alternatively one half of an ES cell colony provided the PCR template with the other half enabling further expansion. After centrifugation the medium was removed and the pellet was washed once with PBS. Following a second centrifugation and removal of the supernatant, the cells were dislodged from the bottom of the tube and frozen overnight at -20˚C before being used as PCR template. Tubes intended for amplification were thawed and the cell pellet re-suspended in 20µl 1xPCR buffer supplemented with 100µg/ml ProteinaseK (Sigma). Cells were lysed for 30min at 55˚C in a thermocycler (MWG). 10min at 90˚C served to inactivate the proteinase. Tubes were held at 80˚C for a Hot Start PCR to add 30µl of PCR master-mix containing all other necessary components.

5.1.2.5 RT PCR

1µg of total RNA was treated with RQ1 DNAseI (Promega) according to the manufacturer's protocols before being subjected to reverse transcription with either oligodT or random primers (both Promega) employing the Superscript III enzyme (Invitrogen) according to the manufacturer's protocols. 1/20 of the reaction volume was used in PCR amplifications.

5.1.2.6 DNA Sequencing

The Wolfson Institute of Biomedical Sciences performed all sequencing analyses using the dye terminator sequencing method.

5.2 Cloning Related Methods

5.2.1 Bacterial Strains

The following *E.coli* strains were used: DH10T-B_1 (Invitrogen) (Narayanan et al., 1999) for BAC propagation and XL1BMR (Stratagene) for all other plasmid related work.

DH10B-T_1: *F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 lacX74 recAI endAI araD139 Δ (araleu) 7697 galU galK rpsL nupGλ- tonA*

XL1BMR: *recAI endAI gyrA96 thi-1 hsdR17 supE44 relAI lac[F-proABlacI^{q}ZΔM15 Tn10 amy Cam^r]*

5.2.2 Bacterial Culture

Bacteria were cultured in LB, 2xYT or TB broth containing the appropriate antibiotics overnight at 37˚C whilst shaking. Initial cultures for plasmids usually were 1.5ml, miniprep cultures for BAC preparation 6ml and cultures for large scale production of plasmids/BACs about 150ml.

Antibiotic	Supplier	Stock Solution	Working Concentration
Ampicillin	Sigma	20mg/ml	100µg/ml
Kanamycin	Sigma	30mg/ml	30µg/ml
Tetracycline	Sigma	15mg/ml	3-12.5µg/ml
Chloramphenicol	Sigma	10mg/ml	15µg/ml

Table 5.1 Antibiotics for Prokaryotic Selection

5.2.3 Bacteria Transformation

5.2.3.1 Heat Shock

$CaCl_2$ competent XL1BMR *E. coli* were thawed on ice. Per transformation 80µl were mixed with DNA and kept on ice for 10min. Following incubation at 42˚C for 2min, cells were returned to the ice for further 10min. 1ml of LB medium was added to each transformation. Samples were incubated at 37˚C whilst shaking for 1h. Subsequently bacteria were plated in dilutions on agar plates containing the appropriate antibiotic and grown overnight at 37˚C.

5.2.3.2 Electroporation

Electrocompetent DH10T-B_1 *E. coli* were thawed on ice. Desalted DNA was mixed with 70µl of the bacteria and transferred into a 0.1cm electrocuvette (Sigma). Cells were shocked at 1.88kV, 25µF and 200Ω using an electroporator (BioRad) and immediately topped up with 1ml of LB medium. Bacteria were kept on ice for 10min before growing 1h at 37˚C whilst shaking and being plated in dilutions on agar plates containing the appropriate antibiotics to be grown overnight at 37˚C.

5.2.4 Plasmid Mini Preparation

Plasmids were isolated by alkaline lysis. 1ml of a TB or LB culture was spun down and re-suspended in 100µl glucose buffer (50mM glucose, 25mM Tris, 10mM EDTA), mixed with 200µl lysis buffer (0.2N NaOH, 1% SDS) and precipitated by adding 150µl neutralization buffer. (3M KAc pH 5.2) After adding 400µl Phenol/Chloroform the sample was vortexed and centrifuged. The aqueous phase was separated, re-extracted with Chloroform and precipitated. Precipitates were dissolved in water containing 10µg/ml RNAse A (Sigma).

5.2.5 Plasmid Midi & Maxi Preparation

Large-scale plasmid preparation was carried out using Qiagen Midi and Maxi kits. For BAC preparation all lysis buffer volumes were doubled and elution was carried out with aliquots of buffer preheated to 65˚C to increase yield.

5.2.6 Nucleic Acid Precipitation

Samples were mixed with 1/10 3M NaAc pH 5.2 and 1 volume isopropanol or 2.5 volumes ethanol before centrifuging them 30min at 4˚C. Precipitates were washed twice with 70%

ethanol, dried at room temperature and re-suspended in an appropriate amount of 10mM Tris-Cl pH 8, TE or water.

5.2.7 Nucleic Acid Quantitation

DNA concentrations were either estimated by running samples on a gel and comparing band intensities to the λ *HindIII* size standard (Promega), or by measuring absorption at 260nm using the NanoDrop spectrophotometer (Thermo Fisher Scientific).

5.2.8 Restriction Digest

Enzymes were purchased from Promega, NEB or Sigma. DNA, at no more than 300ng/µl, was digested in the appropriate buffer at 37˚C for 1h to 16h. Enzymes were added at 5U per µg plasmid DNA/PCR product or 10U per µg genomic DNA. 100µg/ml acetylated BSA was present in all reactions.

5.2.9 Dephosphorylation & Ligation

DNA was dephosporylated by the Shrimp Alkaline Phosphatase (Promega). Ligations were performed using the T4 DNA Ligase (Promega) according to the manufacturer's protocols. Ratios of vector to insert ranged from 1:1 to 1:6.

5.2.10 Southern Blot

DNA for Southern Blots was isolated from confluent ES cell cultures that had been transferred to gelatinized plates during the previous passage to minimize contamination with feeder cell DNA. Following harvest, cells were re-suspended at $5*10^6$ cells/ml in lysis buffer (0.2%SDS, 0.1M Tris pH 8.5, 5mM EDTA, 200mM NaCl, 200µg/ml ProteinaseK) and incubated in a shaker at 55˚C for 4h or overnight. Subsequently, one volume of Phenol/Chloroform was added, and samples were vortexed before being centrifuged to separate the phases. Following re-extraction with Chloroform, the aqueous phase was precipitated with 1 volume of isopropanol or 2.5 volumes of ethanol. The genomic DNA was removed with a yellow pipette tip, washed twice in 70% Ethanol and dried at room temperature. DNA was re-suspended in 1xTE buffer at no more than 300ng/µl. Following restriction digest and gel electrophoresis, Southern gels were blotted on a GeneScreen membrane (Perkin Elmer) using the salt transfer protocol (Sambrook et al., 1989). Membranes were pre-hybridized for 2h in Church Buffer (1% BSA fraction V, 1mM EDTA, 0.2M $NaHPO_4$ pH 7.2, 7% SDS) containing 1µg/ml denatured salmon sperm DNA 65˚C. Southern probes were labelled with α^{32}P dCTP (Amersham) using the NEBlot kit (NEB)

according to the manufacturer's protocol. Probes were purified with microspin columns (Amersham) to remove excess radioactivity and denatured at 95˚C for 10min. The Church buffer was replaced, probes were added and the membranes were hybridized overnight at 65˚C. Membranes were washed using progressively more stringent conditions (1st 2xSSC/0.1% SDS, 2nd 1xSSC/0.1% SDS, 3rd 0.5xSSC/0.1%SDS, 4th 0.2xSSC/0.1% SDS, 5th 0.1xSSS/0.1% SDS, at 50˚C or 65˚C) until the background radioactivity reached 10-20 cpm measured by a Geiger counter. After the last wash, membranes were wrapped in plastic foil and exposed to a PhosphorImager Storage Screen (Kodak) for 12h to 5 days. Screens were scanned with the Storm PhosphorImager (Molecular Dynamics). Band intensities were quantified using ImageJ (Abramoff et al., 2004).

5.2.11 Red/ET Recombineering

The construction of the targeting vectors was accomplished in two steps by Red/ET recombineering (Genebridges) according to the manufacturer's protocol. First, a gene-specific BAC *iCre2* knock-in was created followed by transfer of the modified insert into plasmid vectors. Further details can be found in Chapters 2, 6 and 7. Briefly, the *iCre2-loxP-Neo-loxP* cassette was amplified by PCR with primers introducing 50nt homology regions corresponding to the chosen BAC insertion point. The selection cassette (Genebridges) carries procaryotic (P_{gb2}) and eucaryotic (P_{PGK}) promoters and can thus be used in bacteria and mammalian cells. The PCR product was purified and electroporated into *E.coli*, together with the wild-type BAC and pRED/ET, which expresses all proteins necessary for recombination. pRed/ET is temperature sensitive and can be maintained at 30˚C but is lost at 37˚C. Expression is controlled by a sugar-inducible promoter. Recombineering is thus induced by a temperature shift and adding L-arabinose. Successfully recombined BACs are selected for their newly acquired kanamycin resistance and verified by PCR. To transfer the insert with appropriate homology arms into a plasmid vector, pUC19 was PCR amplified with primers adding 50nt homology regions corresponding to the gene-specific insert. The linear PCR product is transferred into the cells harbouring the knock-in BACs and a second recombination reaction is induced (illustrated in Figure 5.1). Correctly recombined clones are selected for acquiring ampicillin resistance (encoded from the pUC19 backbone) in addition to kanamycin resistance (from the insert). Plasmid vectors are isolated by miniprep and verified by restriction digest and sequencing.

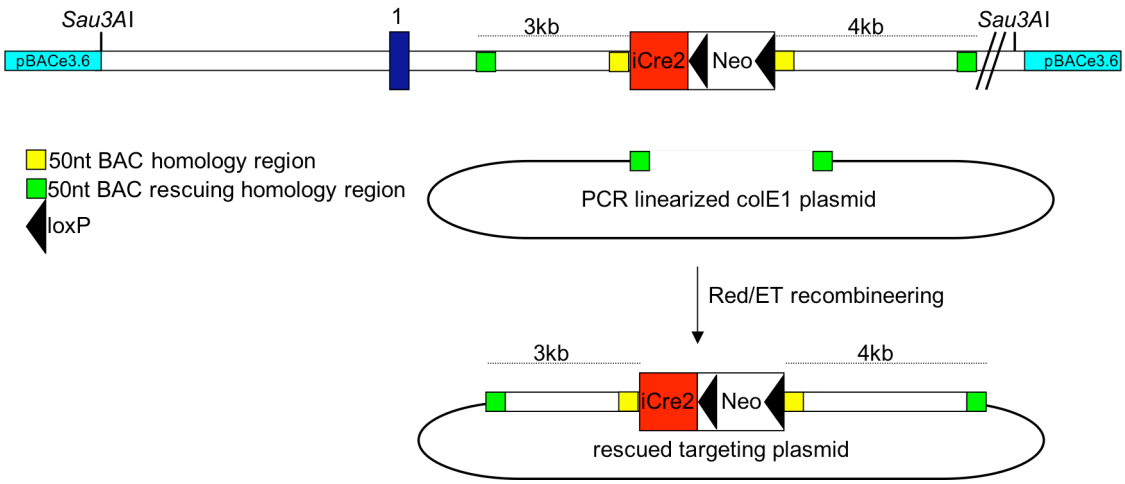


Figure 5.1 Illustration of the Red/ET Recombineering Process
The map at the top shows an *iCre2* knock-in BAC. The homology regions used to create this knock-in are illustrated in yellow. The regions utilized for transferring the insert and adjacent homology arms are in green. The middle panel illustrates the linearized plasmid with attached homology regions. At the bottom, the rescued targeting plasmid is shown after successful recombination.

5.3 Tissue Culture Methods

All procedures were carried out in sterile tissue culture hoods. Tissue culture grade dishes and pipettes were from Nunc, Sterilin, or Costar. Reagents and media compositions are listed below.

5.3.1 Reagents

Dulbecco's MEM	GibcoBRL, 41965-039
Non-essential aminoacids 100x	GibcoBRL, 10829-018
Sodium Pyruvate 100mM	GibcoRL, 11140-035
ß-Mercaptoethanol	GibcoBRL, 31350-010
L-Glutamin 200mM	GibcoBRL, 25030-024
Penicillin/Streptomycin	GibcoBRL, 15140-114
Trypsin/EDTA 10x	GibcoBRL, 35400-027
FCS ES cell tested	Sigma, F-7524
Mitomycin C	Sigma, M-0503
G418 Sulphate	Roche-Boehringer, 1464981
Gelatin	Sigma, G-1890
HEPES 10mM	GibcoBRL 15630-056
PBS	GibcoBRL, 14190-086
LIF	Supernatant of LIF producing 293 cell line, titrated
IMDM	GibcoBRL, 12440-079
FBS for Differentiation	Kind gift from Alexandre Protocnik
Monothioglycerol (MTG)	Sigma, M6145

Ascorbic acid	Sigma, A4403
Insulin	Sigma, I1882
rIL-3	R&D Systems, 403-ML-010/CF
rMCSF	R&D Systems, 416-ML-010/CF
Collagenase IV	Sigma, C2674
Methyl cellulose	Sigma, M7027

5.3.2 Media Composition

STO-NEO Medium	Dulbecco's MEM supplemented with 15% FCS, 1x nonessential amino acids, 1mM sodium pyruvate, 0.1mM ß-Mercaptoethanol, 100i.E./ml penicillin, 100µg/ml Streptomycin, 2mM Glutamin.
ES Medium	STO-NEO Medium, but with 15% FCS and 1xLIF (leukemia inhibitory factor).
Freezing Medium	STO-Neo or ES Medium supplemented with 10mM HEPES and 10% DMSO.
ES Transition Medium	Iscove's MDM supplemented with 15% differentiation FCS, 100i.E./ml penicillin, 100µg/ml Streptomycin, 2mM Glutamin. 450µM MTG (added fresh from stock), 50µg/ml Ascorbic Acid, 10µg/ml Insulin, 1x LIF
ES Differentiation Medium	ES Transition Medium without 1xLIF, 1ng/ml IL-3, 5ng/ml MCSF (for semisolid media 1% methyl cellulose was added)

5.3.3 Cell Lines

The mouse ES cell line used in this work is designated IB10. It is a subclone of the 129P2 E14 line and has the genetic background 129Ola/Hsd. Embryonic stem cells were grown on gelatinized plates on top of growth inhibited STO-NEO feeder cells. STO-NEO is an immortalised embryonic fibroblast cell line possessing G418 resistance. All cells were grown at 37˚C, 5% CO_2 in a humidified incubator.

5.3.4 Passaging of Cell Lines

Cells were passaged just before reaching confluency. The medium was aspirated and the cell layer washed once with PBS. Enough Trypsin was added to cover the cell layer and the dishes were returned to the incubator for 10min. STO-NEO cells were split 1/3 to 1/10 whereas ES cells were plated to yield densities of 1-7*10^4 cells/cm^2.

5.3.5 Thawing and Freezing of Cells

Cells for freezing were harvested by trypsinzation, spun down and re-suspended at $1*10^7$ cells/ml freezing medium and transferred to cryovials (Nunc). Samples were placed in a polystyrene box at -20˚C overnight to facilitate slow freezing. After a few days, vials were transferred to liquid nitrogen for long-term storage. Cells to be thawed were incubated at 37˚C and then transferred to a 15ml tube. 5ml of medium were added drop-wise, cells were centrifuged, re-suspended and plated in the appropriate medium.

5.3.6 Preparation of Feeder Layers for ES Cell Culture

10µg/ml MitomycinC (Sigma) was added to the medium of confluent STO-NEO plates and incubated for 3h at 37˚C. After washing the cells thrice with PBS, they were trypsinized and plated at $1.5*10^4$ cells/cm^2 on gelatinized dishes to serve as feeder layers. MitomycinC treated cells were aliquoted, frozen and thawed as needed during embryonic stem cell culture.

5.3.7 Eukaryotic Cell Transfection & Selection

mES cells were plated at $2*10^6$ cells/6cm dish two days prior to transfection. On the day of transfection cells were harvested, washed once in medium and re-suspended at $0.5*10^6 – 1*10^7$ cells/ml transfection medium. The transfection medium consists of ES medium supplemented with 10mM HEPES. 2-10µg of the appropriate vector was added and cells were transferred into a 0.4cm electrocuvette (BioRad). Cells were electroporated at a voltage of 625V/cm and a capacitance of 500µF. The cuvette was immediately topped up with 1ml of ES cell medium and left at room temperature for 10min. Cells were subsequently plated in varying dilutions. Selection with the appropriate antibiotic was started after two days and selection medium was changed every three days until resistant colonies could be observed. Mass cell death was observed the next day with Puromycin or after 5 days with G418. ES cell colonies were typically isolated between days 10-12.

Antibiotic	Supplier	Stock Solution	Concentration
G418	Sigma	100mg/ml	175-250 µg/ml
Puromycin	Sigma	30mg/ml	2µg/ml

Table 5.2 Antibiotics for Eukaryotic Selection

5.3.8 Embryonic Stem Cell Differentiation

A method for macrophage differentiation of embryonic stem cells was adapted and established, details can be found in Chapter 9. Briefly, embryonic stem cells were transitioned into IMDM

based medium by plating them at low densities (2.5*10E5/6cm dish) for 1-2 passages. ES cells were then harvested and washed thrice to remove LIF and re-suspended in differentiation medium. Embryoid body (EB) aggregation was carried out for 2 days in hanging drop culture. Then EBs were transferred into bacterial grade dishes for suspension culture for further growth with added growth factors Interleukin-3 (IL-3) and Macrophage Colony Stimulating Factor (MCSF). After 7-10 days EBs were attached to tissue culture plastic either as whole structures or single cell suspension until macrophage formation was observed. Single cell suspensions were obtained by protease digestion of embryoid bodies.

5.3.9 RNA Isolation

Total RNA from embryonic stem cells was isolated with the Qiagen RNAeasy mini kit following the manufacturer's protocol. Cellular samples were harvested and re-suspended in 350µl lysis buffer and stored at -80˚C before extraction.

5.4 Flow Cytometry

5.4.1 Antibodies

Antibody	Fluorophor	Isotype	Clone	Manufacturer
F4/80 (rat anti mouse)	FITC	IgG2b	CI:A3-1	Serotec
CD11b (rat anti mouse)	PE	IgG2b	M1/70	Pharmingen
Marco (rat anti mouse)	none	IgG1	ED31	Serotec
Star69 (goat anti rat)	FITC	IgG1	N/A	Serotec
Gr-1 (rat anti mouse)	PE or FITC	IgG2b	B6-8C5	Pharmingen
CD45R/B220 (rat anti mouse)	PE or FITC	IgG2a	RA3-6B2	Pharmingen
CD31 (rat anti mouse)	PE	IgG2a	MEC13.3	Pharmingen
CD117 (rat anti mouse)	PE	IgG2b	2B8	Pharmingen
CD16/CD32 (rat anti mouse)	none	IgG2b	2.4G2	Pharmigen

Table 5.3 Antibodies for Cell Stainings
All antibodies were used at pre-tested titrated quantities or diluted as recommended by the manufacturer. FITC: fluoresceine isothiocyanate; PE: phycoerythrine.

5.4.2 Cell Surface Staining and Data Analysis

Cells were harvested by protease digest and washed once in PBS. Single cells suspensions were aliquoted into 96well round bottom plates, spun down for 10min at 4˚C and 2000rpm and re-suspended in FACS buffer (PBS pH 7.2, 0.5% BSA, 2mM EDTA, 0.01% sodium azide). Cells were blocked for 20min using 20% rat serum (Sigma) and CD16/CD32 antibody (FcBlock, Pharmingen) prior to staining. Cells were stained with the antibodies listed in Table 5.3. Briefly, directly conjugated antibodies were added at the appropriate concentrations and incubated for 30min at 4˚C in the dark. Where a secondary antibody was used, the procedure was the same after a 30min incubation with the primary antibody and two rounds of washing. Finally, cells were washed twice and re-suspended in FACS buffer. Data was acquired on a FACS Calibur flow cytometer (Becton Dickinson). Data was analyzed with FlowJo (Treestar).

RESULTS AND DISCUSSION

CHAPTER 6: THE FIRST GENERATION OF i*CRE2* KNOCK-INS

6.1 Setup of the Project

The objective of presented work is the generation of neutrophil and macrophage specific Cre recombinase knock-in mice as novel tools for conditional mutagenesis. Therefore, prior to the start of this project, a total of 6 different targeting constructs (modified BACs) had been created and verified in which the recombinase is inserted into neutrophil (*Itgb2l*) and macrophage (*Marco* and *Msr1*) specific genes. BAC modification was carried out such that the recombinase replaces the endogenous translational start codon, an example map for *Itgb2l* can be found in Figure 6.2A. Details about the target genes are summarized in Chapter 3.2, whereas the process of generating BAC knock-ins is described in depth in Chapter 2.2.2.1 and Figure 2.4 as well as Chapter 5.2.12. For each of the 3 targets, two vectors were created, one carrying an improved Cre (*iCre2)* (Lacy-Hulbert et al., 2001) and the other one harbouring a tamoxifen-inducible version (*iCre2ER^{T2}*). BACs were used directly to target ES cells, as their long homology regions promised higher targeting frequencies[8].

The vectors were designated as follows:

Itgb2l iCre2
Itgb2l iCre2ER^{T2}
Msr1 iCre2
Msr1 iCre2ER^{T2}
Marco iCre2
Marco iCre2ER^{T2}

IB10 mES cells (a subclone of E14-1) (Hooper et al., 1987; Kuhn et al., 1991) had been transfected with the linearized gene-specific knock-in BACs. 257 drug resistant clones had been isolated; a further breakdown is given in Table 6.1. The herein presented work started with the expansion of G418 resistant embryonic stem cell colonies, extraction of genomic DNA and the screening employed to establish homologous recombinants, all of which is covered in this chapter. Figure 6.1 on the next page gives an overview of the screening regime.

[8] Unless otherwise specified, this work uses the term "targeting frequency" to describe the percentage of verified knock-in clones out of all drug resistant clones assayed.

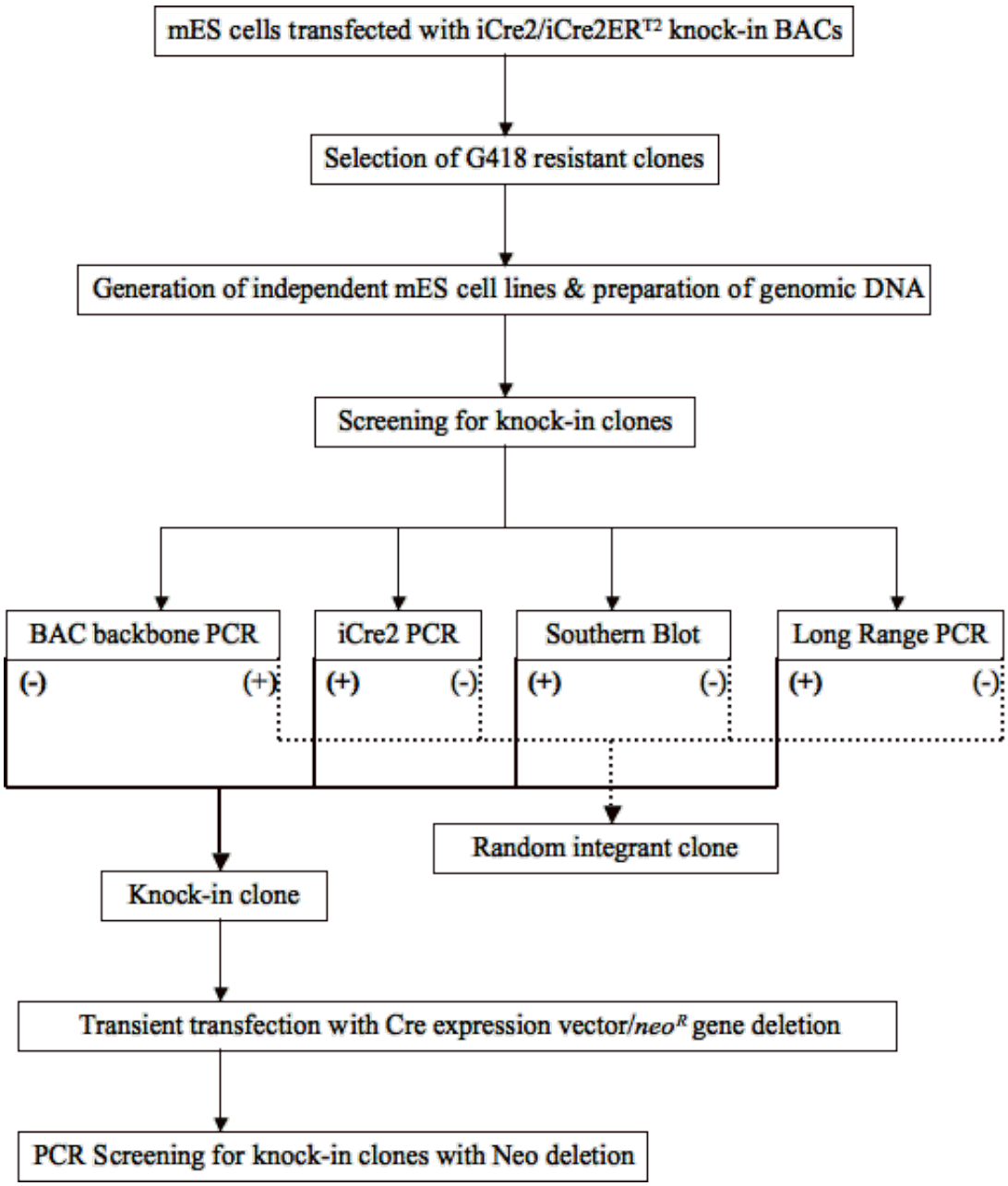


Figure 6.1 Knock-in Screening Process
The flowchart outlines the process used for generating *iCre2* knock-ins in this project. mES cell transfection and selection were followed by PCR and Southern Blot screenings to identify homologous recombinants. The final step of mES cell manipulation is the deletion of the G418 resistance marker. The positive (+) or negative (-) outcome of each screening step helped to classify the resistant clones into knock-ins or random integrants. Only clones with results -/+/+/+ (left to right) were assessed further.

6.2 PCR Screening Reveals Potential Homologous Recombinants

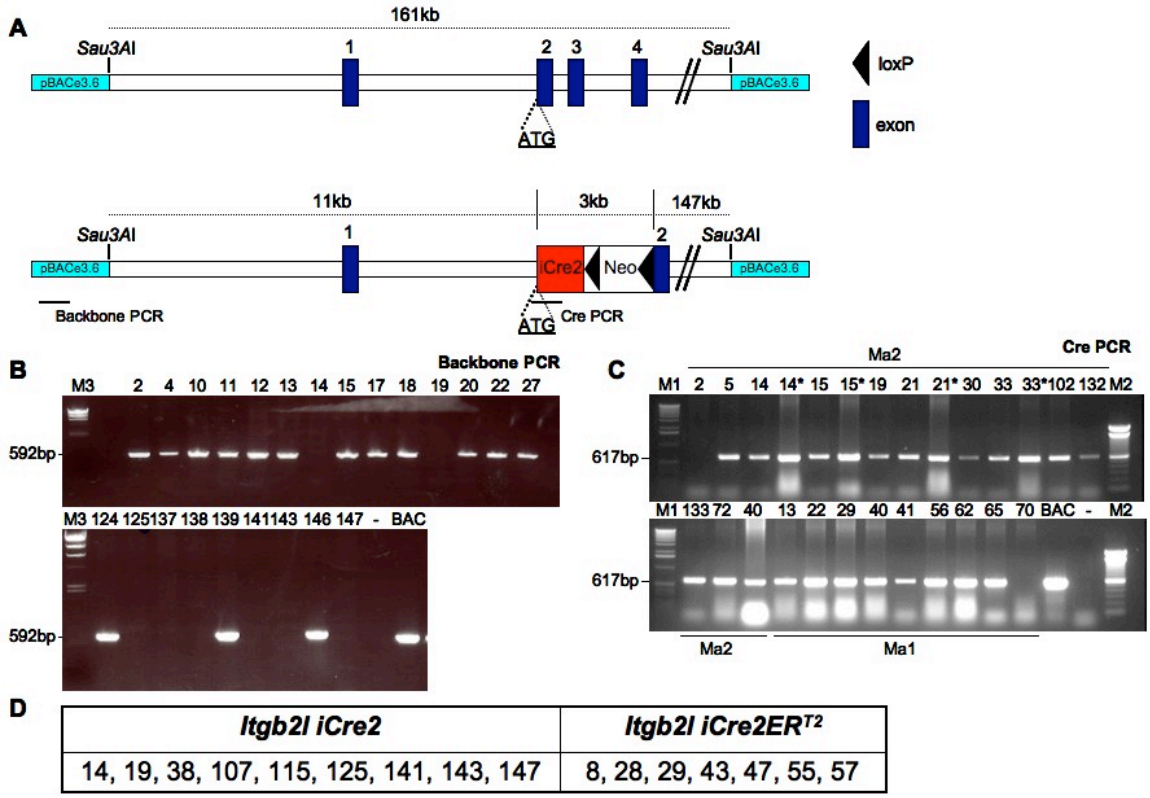
The G418 resistant clones can either be the desired homologous recombinants, or, much more frequently, have inserted the entire BAC vector randomly into the genome. In an attempt to narrow down the 257 isolated clones, a two-tiered PCR strategy was employed (Figure 6.2A).

The first step aimed at ascertaining whether clones carried the BAC backbone. Homologously recombined clones should have lost those sequences. Primers were designed to amplify a 692bp long fragment (backbone PCR) of pBACe3.6, from which all used BACs descend. An absence of a band was counted as a first indicator for successful knock-in. An example for backbone PCR of *Itgb2l iCre2* is given in Figure 6.2B. A further PCR amplified a segment of *iCre2* (Figure 6.2C, results for the *Marco* targeting BAC). The appearance of this band is the second indicator for successful homologous recombination. 6.2D lists the identified candidate clones for the *Itgb2l* locus, as these are the focus in the remainder of this chapter.

Only clones that have yielded the expected results in both PCRs were assayed further. As shown in Table 6.1, the fraction of potentially homologous recombinant clones varied considerably between targeted genes. About one third of the cells targeted with the *Marco* constructs yielded promising results after PCR screening, compared to a quarter of those targeted for *Itgb2l*. The lowest fraction was observed for *Msr1*, only about 10% of mES cell clones were knock-in candidates. Fractions observed for *iCre2ER^{T2}* were only slightly lower for *iCre2* alone, with the exception of *Itgb2l iCre2ER^{T2}*.

Targeting construct	Number of G418 resistant clones isolated	Number of knock-in candidates after PCR	Fraction in %
Itgb2l iCre2	38	9	24
Itgb2l iCre2ER^{T2}	68	7	10
Marco iCre2	30	10	33
Marco iCre2ER^{T2}	31	9	29
Msr1 iCre2	24	2	8
Msr1 iCre2ER^{T2}	66	4	6

Table 6.1 Number of Isolated Clones and Initial PCR Screening Results



Itgb2l iCre2	*Itgb2l iCre2ER^{T2}*
14, 19, 38, 107, 115, 125, 141, 143, 147	8, 28, 29, 43, 47, 55, 57

Figure 6.2 Initial PCR Screening of G418 Resistant Transfectants[9]

A depicts the bMQ203N9 *Itgb2l* BAC before and after modification. Lines underneath the construct illustrate the placement of the backbone and Cre screening PCRs that were employed to determine whether ES cell clones were potential homologous recombinants. Primer pairs used were bacbckgrd3'/backbckgrd5' for the backbone PCR, and MaF;PactF;Msr1F/iCrecheck2 for the Cre PCR. Expected product sizes were equivalent for both the *iCre2* and *iCre2ER^{T2}* constructs. In **B** genomic DNA isolated from clones targeted with *Itgb2l iCre2* was analyzed for presence of the backbone. The Cre PCR is shown for both *Marco iCre2* (Ma2) and *Marco iCre2ER^{T2}* (Ma1) in **C**. Positive controls (BAC) were the respective targeting vectors, negative controls either wild type genomic DNA of 129 mice or no template in the PCR reaction. **D** summarizes the candidate clones for *Itgb2l*.

Numbers indicate individual clones. M1: 1kb marker; M2: 100bp marker; - w/o template, *indicates independent DNA preparations.

[9] Maps in this document may contain elements that are not drawn to scale.

6.3 Two Southern Blots Identify Homologous Recombinants for *Itgb2l*

The previously described PCR exclusion strategy served to reduce the number of candidate clones from 257 to a more manageable 41. However, loss of the BAC backbone and presence of *iCre2* alone are not sufficient to prove homologous recombination. For instance, random integrants could have lost the short stretch of backbone being used for PCR whilst containing *iCre2*. Therefore, the correct chromosomal position of *iCre2* needs to be ascertained independently.

To achieve this, external Southern Blot strategies targeting the 5' homology region were devised for all 6 constructs (Figure 6.3). The approach relies on restriction enzymes cutting outside of the homology arm (between *Sau3AI* and the beginning of *iCre2*) and fragments are identified by hybridization with an external probe. Only homologous recombinant clones possess juxtaposed restriction sites that result in the expected fragment length. The long homology arms of the targeting BACs severely restrict the choice of restriction enzymes and also create large DNA fragments, which are difficult to resolve and visualize.

To test the method, initially only *Itgb2l* knock-in candidate clones were assessed. An *XhoI/SalI* strategy (Figure 6.3A) generates 29kb wild type and 13kb/14kb mutant products. The technique proved to be challenging. In three independent experiments wild type bands could not be clearly distinguished. A re-occuring problem was incomplete digestion of genomic DNA, which may have been due to enzyme inhibition by genomic methylation (data not shown). Eventually, weak bands of the correct size (13kb) indicating homologous recombination (Figure 6.4E) could be observed in some of the *Itgb2l iCre2*, but none of the *Itgb2l iCre2ER*^{T2} samples. Clones exhibiting this faint signal were 19, 107 and 141.

As there were no feasible alternative enzyme choices for the external Southern Blot, an internal Southern blot strategy was devised for *Itgb2l* to generate additional data (Figure 6.3A). The approach relies on *BamHI* restriction sites. The expected wild type band has a size of 3.5kb, compared to 2kb for the *iCre2* knock-in. Bands are visualized by hybridization with an internal probe. Both knock-ins and random integrants will exhibit either band. The analysis of relative band intensities, however, allows distinguishing properly targeted clones. A band intensity ratio - wild type to mutant - of 1:1 indicates successful homologous recombination, as one wild type allele corresponds to one mutant allele. Band intensity ratios deviating from 1 suggest random integration. One random integration, with two intact wild type alleles, would lead to a ratio of 2:1. More than two random integrants would cause a ratio of less than 1.

The results of the *BamHI* Southern Blot are shown in Figures 6.4A and 6.4C. It was performed for all *Itgb2l* clones previously identified as candidates by PCR exclusion (top numbers). Some clones considered to be random integrants (bottom numbers) were also included for control purposes. As expected, the DNA of 129 wild type mice shows only the 3.5kb band, whereas both bands are present in the DNA isolated from most of the candidate ES cell clones. The exceptions were two of the *Itgb2l iCre2* (38, 115 Figure 6.4A) and three of the *Itgb2l iCre2ER*^{T2} clones (8, 28, 29; Figure 6.4C) that had no discernible mutant band and were not further assayed.

Relative band intensities were determined in all lanes exhibiting the wild type and mutant band by measuring integrated pixel densities and ratios are given in Figure 6.4B for *Itgb2l iCre2* and 6.4D for *Itgb2l iCre2ER*^{T2}. In case of *Itgb2l iCre2*, 3 of the candidate clones (BAC negative) were close to a ratio of 1: clones 19, 125 and 141. Most of the control samples (BAC positive) deviated from the ratio of 1 affirming them as likely random integrants. For *Itgb2l iCre2ER*^{T2}, all 4 clones that showed 2 bands in the Southern Blot (43, 47, 55 and 57) exhibited a band intensity ratio of approximately 1. Some of the *Itgb2l iCre2ER*^{T2} control clones also showed this result. This can be partially explained by the low quality of the *Itgb2l iCre2ER*^{T2} Southern Blot, which makes density measurements less reliable. Control samples, i.e. random integrants, reaching a ratio of 1, can occur when 2 random integrations correspond to 2 intact wild type alleles.

Overall, the *XhoI/SalI* Southern Blot suggests *Itgb2l iCre2* clones 19, 107 and 141 as knock-ins. Data for *Itgb2l iCre2ER*^{T2} could not be obtained. The *BamHI* Southern Blot identifies *Itgb2l iCre2* clones 19, 125 and 141 and *Itgb2l iCre2ER*^{T2} clones 43, 47, 55 and 57 as potential homologous recombinants.

6.4 Long Range PCR Confirms Homologous Recombination in *Itgb2l* Clones

Due to the technical difficulties encountered during Southern Blot screening and overall low data quality, a further PCR-based screening was used to corroborate homologous recombinants among the *Itgb2l* clones. It relies on a special polymerase designed to enable the amplification of very long PCR products (Takara LA Taq). Placing PCR primers in a way that allows the amplification of a genomic region from outside the 5' homologous arm to within *iCre2* enables the assessment of correct chromosomal insertion (12.1kb external product, Figure 6.5A). A second PCR, creating a slightly shorter product (internal product) of 10.5kb beginning within the homology region, serves as control.

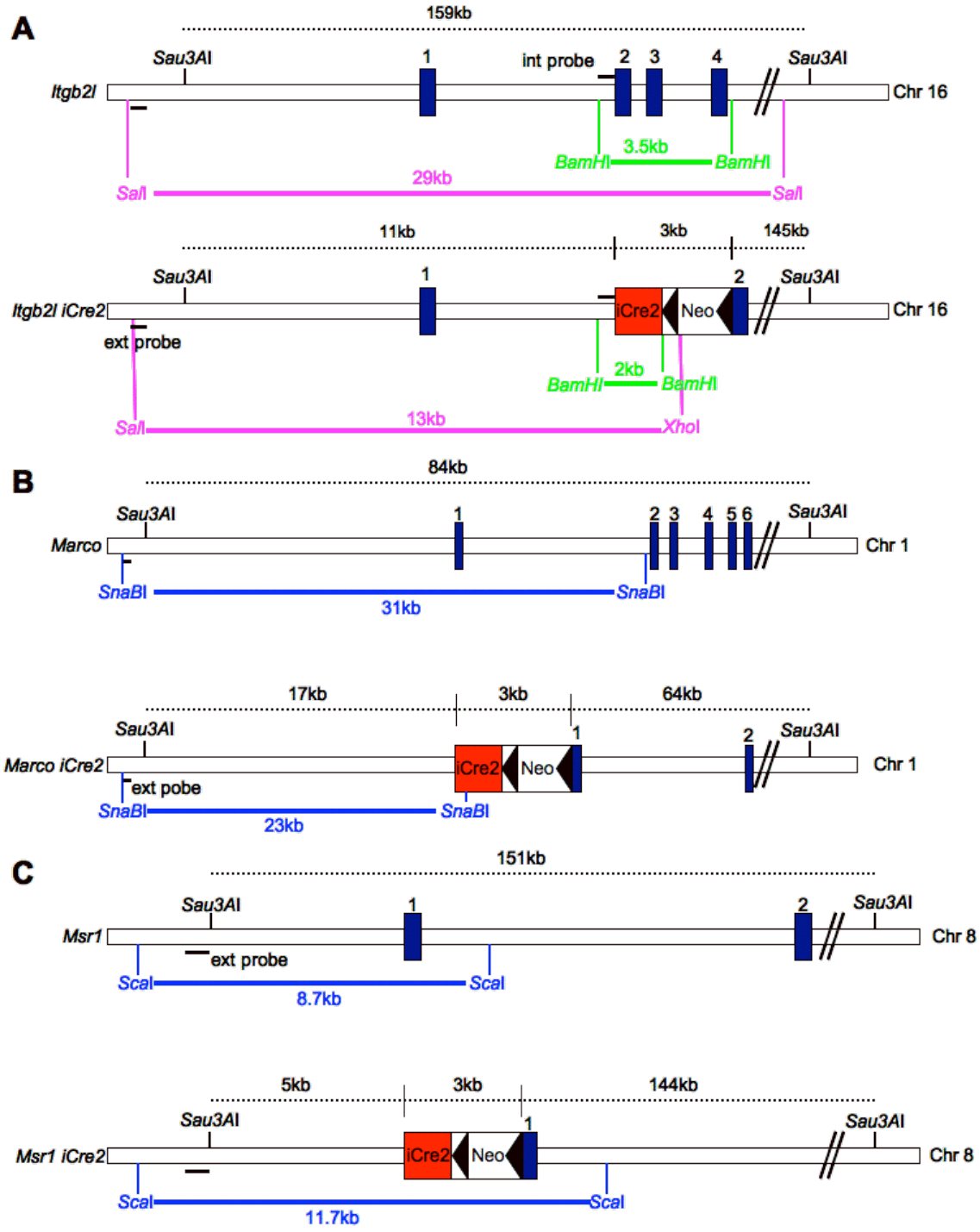


Figure 6.3 Southern Blot Strategies for the Three Targeted Loci

The structure of the genomic loci before and after homologous recombination is illustrated in **A** to **C** for *Itgb2l, Marco* and *Msr1* respectively. Given are the chromosomal locations of the wild type genes or the corresponding Cre knock-ins, suggested restriction enzymes and expected fragment sizes. The *Sau3AI* restriction sites determine the borders of the inserted mouse genomic DNA as provided by the BACs used for targeting. Probe locations are depicted as bold black lines. Only maps for the *iCre2* knock-ins are shown, the respective DNA fragment sizes and restriction enzymes for the *iCre2ER*^{T2} constructs are as follows: *Itgb2l iCre2ER*^{T2}: 3.5kb (*BamHI*); *Itgb2l iCre2ER*^{T2}: 14kb (*XhoI/SalI*); *Marco iCre2ER*^{T2}: 23kb (*SnaBI*); *Msr1 iCre2ER*^{T2}: 12.6kb (*ScaI*).

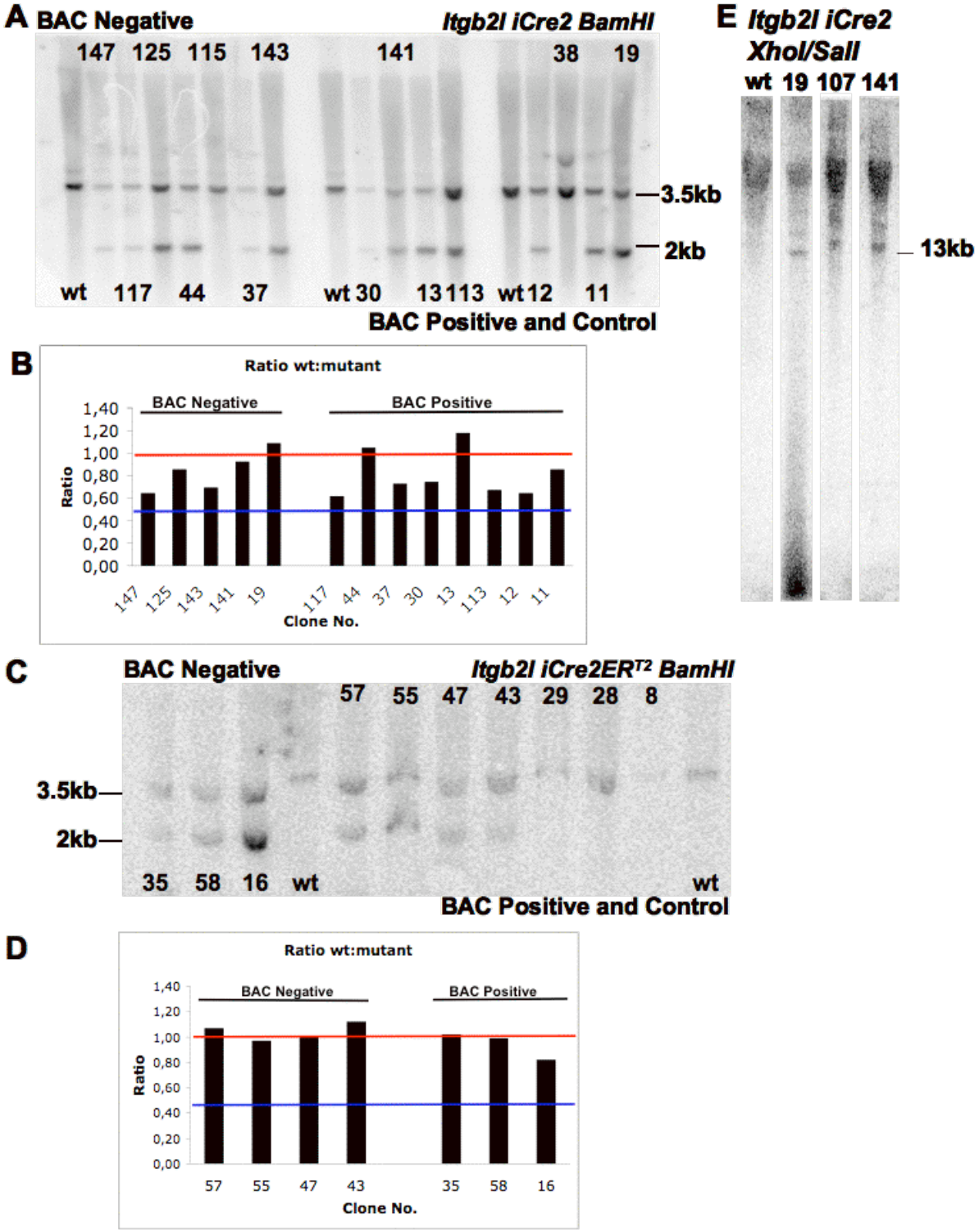


Figure 6.4 Southern Blot Results for *Itgb2l* Clones
BamHI Southern Blots were performed for a selection of suspected *Itgb2l iCre2* clones (**A, B**) *Itgb2l iCre2ER*^{T2} and (**C, D**). Numbers at the top indicate samples that were previously screened as potential recombinants by PCR (BAC negative). Lanes numbered at the bottom indicate samples still harbouring the BAC backbone (BAC positive) and served as an internal control. The ratios of band intensities given in **B** and **D** indicate the copy number of wild type and targeted loci. Clones closest to ratio of 1 are most likely to have undergone homologous recombination. Control DNA was isolated from wild type 129 ES cells. Examples for an *XhoI/SalI* Southern Blot of *Itgb2l iCre2* clones are illustrated in **E.**

All clones containing the target sequence, whether as random integrant or homologous recombinant, should yield the internal PCR product. Only clones that have the insert in the right chromosomal position, however, will additionally generate the external PCR product. An example for this PCR is shown in Figure 6.5B. Clone *Itgb2l iCre2* 141 served as a template for both internal and external PCR products and thus is a homologous recombinant. A *BamHI* restriction digest of the PCR product (Figure 6.5C) generated the expected pattern. Notably, clone 19 initially only displayed the internal PCR product (data not shown), indicating an unsuccessful recombination event. After further optimization of the reaction conditions (in particular reduction of template quantity) and repeated isolation of genomic DNA, however, clone 19 could also be identified as a homologous recombinant by long range PCR. Additional clones yielding this positive result were *Itgb2l iCre2* 107 and *Itgb2l iCre2ER^{T2}* 47 (Figure 6.5D). All clones positive for the long range PCR were also positive by Southern Blot (Figure 6.4).

6.5 Cre-mediated Deletion of the Resistance Marker in *Itgb2l iCre2* Knock-ins

The combination of all obtained screening data allowed the identification of knock-in clones among the *Itgb2l* candidates (see section 6.6 and Table 6.3). The next step was the removal of the G418 selection marker. *Neo^R* is a fully functional expression unit with its own promoters (Figure 6.6A) and can have detrimental effects on *iCre2* expression. To facilitate deletion, *Neo^R* is flanked by parallel loxP sites. Cre mediated recombination excises the intervening DNA and leaves just one loxP site behind.

A PCR strategy was designed to screen for such deletion events. After excision of *Neo^R*, the size of the amplification product is reduced from 2.5kb to 1kb (Figure 6.6A). The deletion was attempted using two different Cre expression vectors for transient transfection of ES cells: pIC-Cre; (Gu et al., 1993) and pCre-Pac (Taniguchi et al., 1998). Cells of two *Itgb2l iCre2* recombinant clones (19, 141) were electroporated with the expression plasmid. After 48h, cells were harvested for PCR to assess Cre activity at the level of bulk cultures before the isolation of individual colonies. In case of pCre-Pac, an additional transient Puromycin selection was applied to enrich transfected cells. However, as shown in Figure 6.6B-D, deletion of the *Neo^R* gene could not be achieved in any of the samples despite carrying out several repeat experiments with varying amounts (1-10µg) of the expression vector.

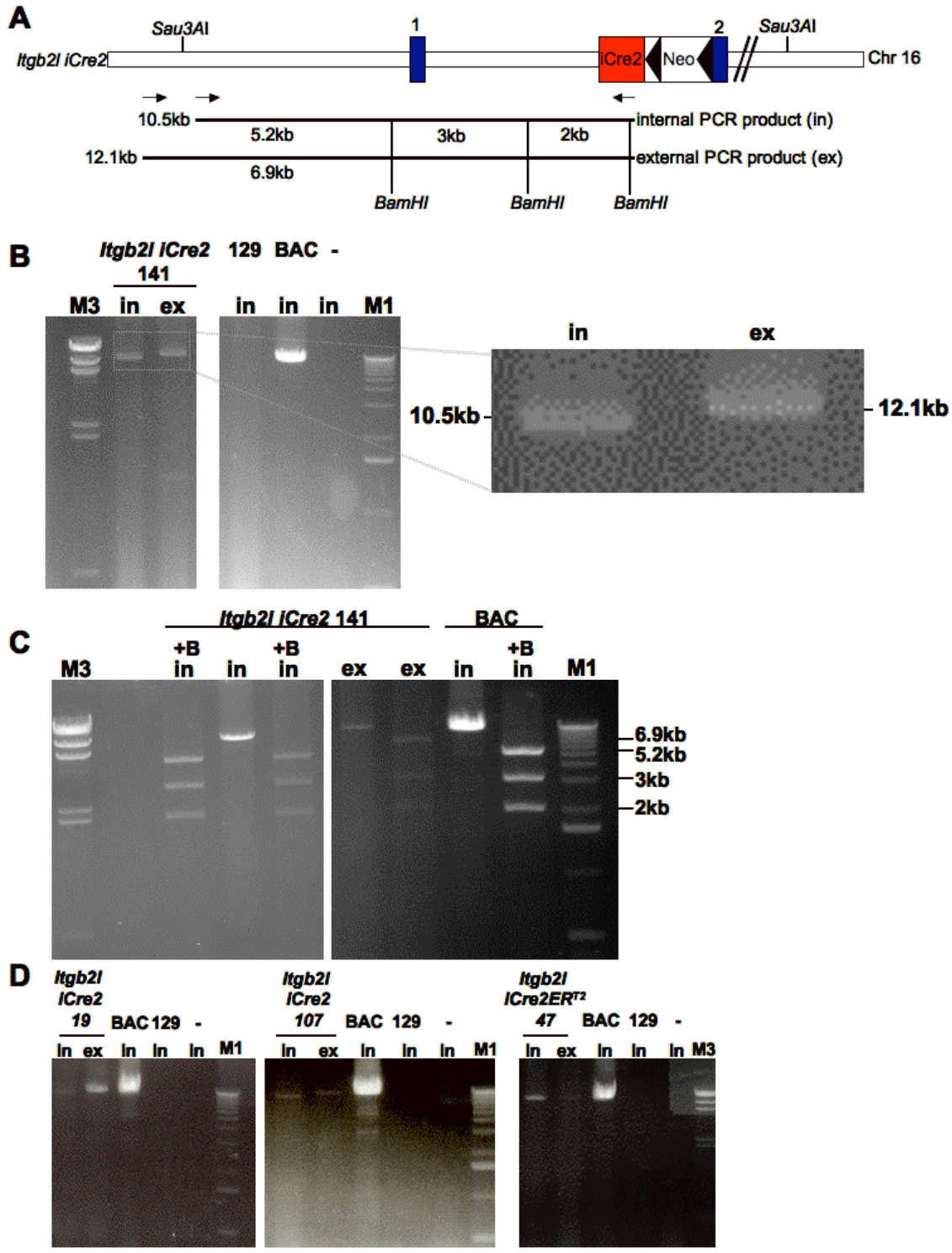


Figure 6.5 Long Range PCR of *Itgb2l* Clones

Map, primer locations (black arrows), product sizes and restriction enzyme sites for the *Itgb2l iCre2* locus are depicted in **A**. PCR products for *Itgb2l iCre2* and *Itgb2l iCre2ER*^{T2} are equivalent. **B** illustrates the PCR results for *Itgb2l iCre2* clone 141. The positive control was the targeting BAC. Wild type 129 genomic DNA or water served as negative controls. In and ex show where internal or external primer pairs have been used. The two-fold zoom illustrates the small size difference between internal and external PCR products. A *BamHI* restriction digest (**C**) resulted in expected fragment sizes. All other clones exhibiting both bands are illustrated in **D**. The external primer pair was Pacttarg/iCrecheck2; the internal pair was Pactin2/iCrecheck2. M1: 1kb marker; M3: λ*HindIII* marker; - w/o template; +B: *BamHI*; BAC: bMQ203N9 *iCre2*; - no template DNA.

Sequencing of the plasmid *pSP72-iCre2-pAp-NeoLox* revealed, that the loxP sites as defined by the central 8bp orientation-defining core sequences, had an inverted orientation relative to each other (Figure 6.6E&F). As this plasmid had initially been used to generate every targeting vector prior to the start of this project, all recombinant ES cell clones contain these antiparallel loxP sites. Neo^R can thus not be excised in any of the ES cell lines as antiparallel loxP sites cause Cre-mediated deletion, inversion and re-integration of the intervening DNA. The error was apparently introduced prior to the start of this project by the primer pair employed to tail the Neo^R fragment with loxP sites, before cloning it into the *iCre2*-containing vector *pSP72-iCre2-pAp-NeoLox*.

6.6 Discussion

6.6.1 The Use of Linearized *iCre2* Knock-in BAC as Targeting Vectors

During the initial planning of the presented project, bacterial artificial chromosomes had been chosen as targeting vectors for several reasons:

1. An indexed 129Sv based genomic library in BAC format spanning the entire mouse genome had just become available, providing a public isogenic DNA resource for targeting vector construction (Adams et al., 2005). Since then an additional library based on the 129/Ola background and thus even more closely matched to the used E14.1 ES cells has been published (Ohtsuka et al., 2006).
2. Methods for the recombinogenic engineering of BACs were becoming more accessible due to the emergence of new kits (Chapter 2.2.2.1).
3. The very long homology arms (Table 6.2) provided by BACs were expected to positively influence homologous recombination and consequently increase gene targeting frequencies.

Gene	BAC	Upstream Homology in kb	Downstream Homology in kb
Itgb2l	bMQ203N9	10.7	145
Marco	bMQ294K23	16.7	64
Msr1	bMQ239G13	5	144

Table 6.2 Length of Homology Arms in BAC Vectors
The area of homology is measured up- and downstream from the *iCre2-loxP-Neo-loxP* insertion point to the closest *Sau3AI* site. *Sau3AI* had been used to partially digest the genomic DNA and clone it into the BAC vector during the library creation process and thus marks the BAC insert end points (also see Chapter 7.2). The listed BACs for each target gene had been chosen for having the shortest upstream homology region of all available candidates.

When this work started with screening the 257 isolated G418 resistant clones obtained after transfecting E14.1 mES cells with the targeting constructs (Table 6.1), however, it became clear that the use of BACs complicates the identification of homologous recombinants.

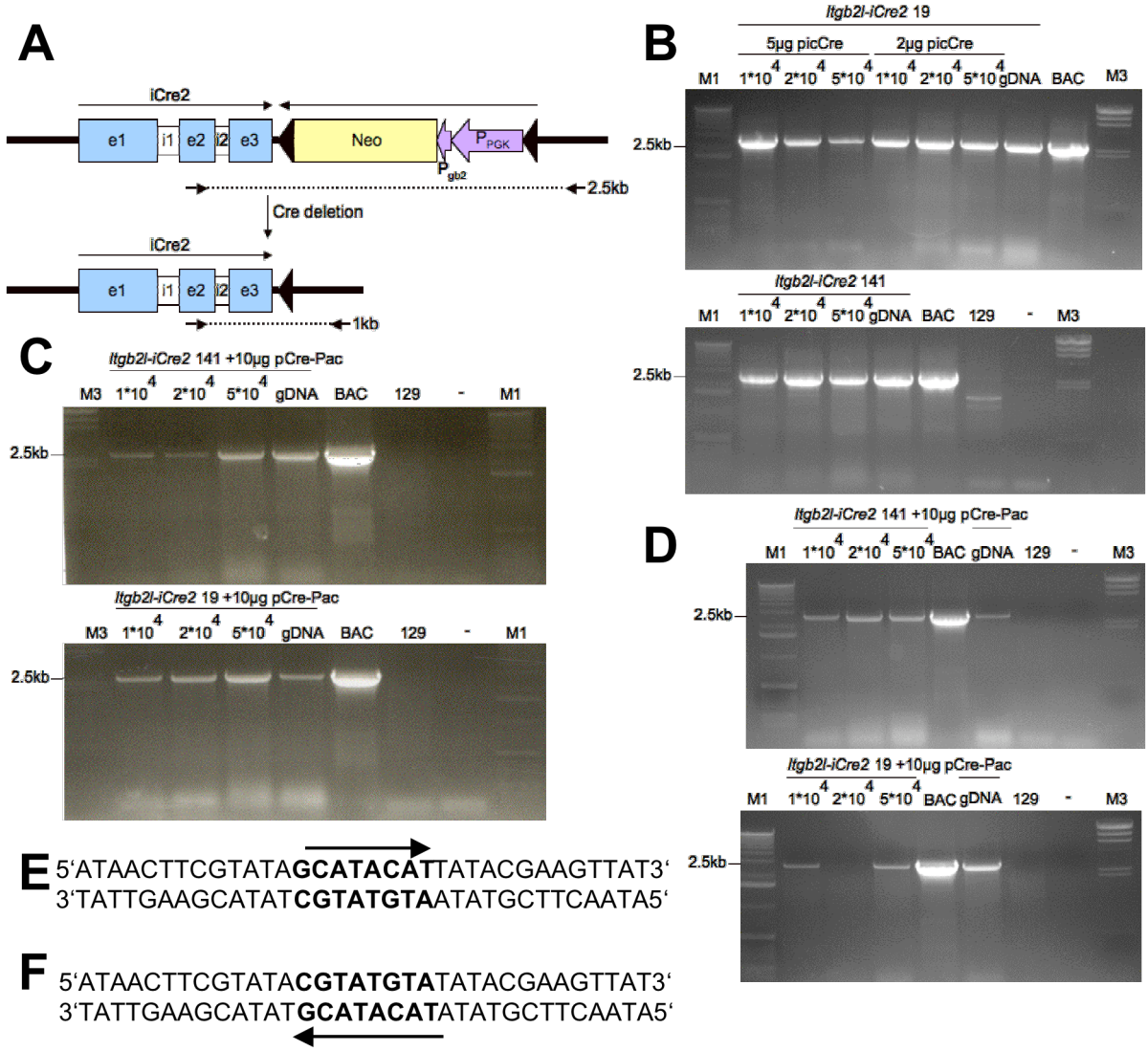


Figure 6.6 No Evidence for *Neo*^{R} Gene Excision
The *iCre2-loxP-Neo-loxP* construct before and after *Neo*^{R} deletion is illustrated in **A**. *iCre2* consists of 3 exons (e) and 2 heterologous introns (i). Arrows indicate the orientation of both genes. The *Neo* cassette has 2 promoters, one for prokaryotic (P_{gb2}) and one for eucaryotic (P_{PGK}) expression. In order to screen for successful deletion, a PCR primer pair (small arrows) flanking the *Neo*^{R} cassette, resulting in 1kb or 2.5kb products, was employed. PCRs were carried out using 10.000, 20.000 or 50.000 lysed mES cells per reaction as template. Positive and negative controls are same as before, except that clone specific genomic DNA (gDNA) isolated prior to transient transfection was used as an additional control. **B** to **D** shows clones 19 and 141 of *Itgb2l iCre2* after Cre mediated deletion with different Cre expression vectors. In **B** piCCre was used. **C** and **D** represent pCre-Pac without and with puromycin selection. Deletion of the *Neo*^{R} gene is not apparent in any sample. Sequencing of the *pSP72-iCre2-pAp-NeoLox* plasmid revealed the inverted orientation of the 5’ (**E**) and 3’ (**F**) loxP sites. The orientation-defining spacer region is bold. Sequencing was carried out using NeoLoxFSeq and a T7 standard primer.

The PCR exclusion study designed to discovers homologous recombination candidates by the absence of the BAC backbone and the presence of *iCre2*, worked very well, and successfully reduced the number of clones to analyze from 257 to 41 (Figure 6.2). This method has previously been described in the literature as being very effective in determining clones that warrant further screening (McDermott et al., 2004; Yang and Seed, 2003). On its own, however, it does not ascertain correct knock-in. A positive PCR result for *iCre2* and negative result for the BAC can also occur in random integrants that have lost part of the BAC backbone in a non-homologous recombination process. When looking at the fraction of candidates with favourable PCR results, *Itgb2l* and *Marco* have a high percentage with 10-24% and 29-33% depending on the targeting construct used. The *Itgb2l* and *Marco* targeting vectors also have the longest upstream homology arms with 10.7b and 16.7kb respectively. For *Msr1* the PCR positive fraction is only 6-8% coinciding with the shortest upstream homology arm of 5kb. There is no such correlation with the overall much longer downstream homology arms, the one in the *Marco* construct is actually shortest with 64kb whereas those in the *Itgb2l* and *Msr1* vectors are of similar sizes around 145kb. The presence of ER^{T2}, which extends the *iCre2* fragment to be inserted by around 2kb, but does not affect the homology arms, only had a noticeable influence in the *Itgb2l* vectors. With ER^{T2}, the fraction of candidate clones was about half as much (10%) than without (24%).

Achieving good results in the more conclusive Southern Blot aimed at verifying the upstream homology region in *Itgb2l* clones was difficult. Due to the length of the upstream homology arm, only an *XhoI/SalI* combination was a possible choice (Figure 6.3A), due to all other commonly used restriction enzymes cutting within the homology region. Whilst weak 13kb knock-in bands could eventually be obtained for some of the suspected recombinant samples, the 29kb wild-type band could not be clearly distinguished in any of the blots (Figure 6.4E).

In the meantime, the *BamHI* Southern Blot helped to exclude some of the candidate clones (Figures 6.4A&C). Whilst the method relies on measuring band intensities and is thus very dependent on blot quality, it allowed the exclusion of several samples that did either not exhibit the expected two bands (e.g. *Itgb2l iCre2 38*, 115 and *Itgb2l iCre2*ER^{T2} 8, 28, 29) or did significantly deviate from the band intensity ratio of 1 (e.g. *Itgb2l iCre2* 143, 147).

Finally, the long range PCR approach (Lay et al., 1998), amplifying across the entire 5' homology arm, proved the most successful method to assess homologous recombination (Figure 6.5). Four clones, *Itgb2l iCre2* 19, 107, 141 *Itgb2l iCre2*ER^{T2} 47 exhibited the PCR product agreeing with homologous recombination. An internal PCR control product amplifying both homologous recombinants and random integrants helped to avoid false negatives caused by PCR failure.

Overall, a combination of all screening methods (see Table 6.3) eventually allowed discriminating homologous recombinants from random integrants among the *Itgb2l* clones. Ultimately, 3 *Itgb2l iCre2* clones and 1 *Itgb2l iCre2ER^{T2}* clone could be identified as being knock-ins. This equals targeting frequencies of 8% and 1,5% respectively, much lower than the initial fraction of candidate clones listed in Table 6.1.

		HR Indicating Result in			
Targeting Construct	**Clones selected by PCR exclusion**	***XhoI/SalI* Southern Blot**	***BamHI* Southern Blot**	**Long Range PCR**	**Considered Knock-in Clone**
Itgb2l-iCre2	14	NDA	NA	NO	NO
	19	YES	YES	YES	YES
	38	NDA	NO	NA	NO
	107	YES	NDA	YES	YES
	115	NDA	NO	NA	NO
	125	NDA	YES	NA	NO
	141	YES	YES	YES	YES
	143	NDA	NO	NA	NO
	147	NDA	NO	NA	NO
Itgb2l-iCre2ER^{T2}	8	NDA	NO	NA	NO
	28	NDA	NO	NA	NO
	29	NDA	NO	NA	NO
	43	NDA	YES	NO	NO
	47	NDA	YES	YES	YES
	55	NDA	YES	NO	NO
	57	NDA	YES	NO	NO

Table 6.3 Identification of Homologous Recombinants among *Itgb2l* clones
The table lists the screening results for the *Itgb2l iCre2* and *Itgb2l iCre2ER^{T2}* samples that were selected as candidates by PCR exclusion and whether they were eventually considered to be knock-ins. NDA – no data available; NA – not assessed; HR Homologous Recombination

BAC vectors are not routinely used for gene targeting, therefore an assessment of targeting frequencies is difficult. In the first published study, a modified BAC was used to target two sites of the *Mll* (myeloid/lymphoid mixed lineage leukemia) gene, 43kb apart, with G418 and Hygromycin selection markers simultaneously. After double selection, a targeting frequency of 6% was obtained (Testa et al., 2003). A similar approach, aimed at concurrently disrupting the adjacent *Tec* (encoding tyrosine protein kinase Tec) and *Rlk* (encoding receptor like kinase) genes with G418 and blasticidin selection markers, reported 9% of clones being the desired homologous recombinants (Gomez-Rodriguez et al., 2008). Both groups observed that BACs electroporated into ES cells mainly integrated intact, but also high-lighted the possibility of BAC breakage resulting in difficult to monitor random integration of fragments within the genome or rearrangements occuring at the integration site (Heaney et al., 2004). A further high throughput approach using largely automated BAC targeting vector creation, reported an average targeting frequency of 3,8% across 200 genes (Valenzuela et al., 2003). The study also reported, that long BAC homology arms do not necessarily have a beneficial effect on the targeting frequency. For one gene, for example frequencies were around 6%, irrespective of

whether homology arms were long (80kb and 80kb) or short (5,5kb and 1,5kb). In a second example, frequencies could be increased from 2% to 6% with a BAC targeting vector (75kb and 35kb homology instead of 4,7kb and 0,5kb). It has been reported previously, however, that homology arms under 1kb have a detrimental effect on homologous recombination (Thomas et al., 1992). Thus far, the highest reported targeting frequency for BAC vectors is 15%, an average over 5 genes (Yang and Seed, 2003).

Collectively, BAC vectors have not yet been shown to achieve significantly higher targeting frequencies than conventional plasmid vectors. This supports the theory that targeting frequencies become saturated once homology arms exceed a length of 14kb (Deng and Capecchi, 1992). The confirmed frequencies in this study thus far, 8% for *Itgb2l iCre2* clones and 1,5% for *Itgb2l iCre2ER*^{T2}, fall well within the reported ranges. Frequencies do not appear to be significantly above those achieved with conventional vectors which typically range between 1-5% (Sorrell and Kolb, 2005).

The challenges of BAC targeting encountered in this work, the difficult Southern Blot Screening resulting from long homology arms, has also been discussed in the literature. Alternative screening protocols include qPCR, which analyses the copy numbers of disrupted loci or selection markers. qPCR has been described as highly accurate in excluding random integrants (Gomez-Rodriguez et al., 2008; Valenzuela et al., 2003). Another alternative is fluorescence in-situ hybridization (FISH), using the whole targeting BAC as a probe. FISH, however, has been linked to producing false positives but can be useful in monitoring partial random integrations of vector fragments (Gomez-Rodriguez et al., 2008; Yang and Seed, 2003). The gold standard for identifying targeted clones, however, is still Southern Blot. Therefore several groups have used BAC vectors with the modified regions sitting close to either end or "shaved" homology arms by recombineering to reduce their size (Testa et al., 2003). A combination of all of these additional methods could significantly improve efficiency if BAC targeting was to be repeated.

6.7 Conclusion

The failure to remove the G148 resistance marker from *Itgb2l iCre2* knock-ins revealed that all isolated ES cell clones contain inverse loxP sites. Because of that *Marco* and *Msr1* clones were not further assessed to identify homologous recombinants, but could potentially be used as transgenic lines to create "pseudo knock-in" mice (Sparwasser et al., 2004). This approach would be similar to models, in which faithful tissue-specific Cre expression was controlled from a randomly integrated BAC transgene, including IL-7Cre (Repass et al., 2009), Mcpt5-Cre

(Scholten et al., 2008) or CD11c-Cre (Caton et al., 2007). Further examples can be founds in Table 3.2.

The identified *Itgb2l iCre2* knock-ins could be used in an attempt to create knock-in mice as the presence of the resistance marker does not necessarily cause deleterious effects. The assessment, however, could only be made after a lot of time and resources had been invested in creating the mouse, making this approach rather risky. As a mouse model in which the Neo^R gene can be removed if necessary is preferable to avoid the creation of hypomorphic alleles or dysregulated expression (see Chapter 2.3), the *iCre2* targeting for all three loci was to be repeated. Work on the inducible *iCre2* ER^{T2} fusion constructs had to be discontinued due to lack of time and resources.

BAC knock-ins, due to their size, have proven to be too cumbersome to facilitate easy identification of knock-ins and have not delivered high enough homologous recombination frequencies to warrant continued use. As such, the vector construction strategy was re-designed to create shorter plasmid based targeting vectors of around 16.7kb total size with shorter homology arms of 4kb and 7kb respectively (see Chapter 7). Such retrieval vectors, where the insert intended for gene targeting is rescued from the engineered BAC, have been successfully used to generate knock-in ES cell lines and mice by other groups (Adams et al., 2005; Voehringer et al., 2009).

CHAPTER 7: CONSTRUCTING THE SECOND GENERATION *iCRE2* TARGETING VECTORS

Whilst knock-ins could be successfully identified after using the BAC targeting approach described in Chapter 6, the size of the vectors complicated the screening regime and an error in the reverse loxP site precluded removal of the G418 selection cassette in targeted embryonic stem cell lines. Therefore it was decided to re-construct the vectors and repeat the embryonic stem cell targeting.

First, the antiparallel loxP sites needed to be replaced by parallel loxP sites in the basic vector *pSP72-iCre2-pAp-NeoLox* to enable Cre mediated selection marker deletion in the final construct. The corrected plasmid served as template for the creation of second generation *iCre2* knock-in BACs by Red/ET recombineering. Shorter plasmid targeting vectors with homology arms of 4kb upstream and 7kb downstream were then generated by a second round of recombinogenic engineering in which the gene-specific knock-in BACs supply the starting template. While the sequence of the second generation targeting vectors was confirmed, a cryptic splice site in the *iCre2* coding sequence was discovered. This site leads to incorrect splicing and consequently interferes with protein expression. Removing the cryptic splice site was the final step in obtaining correct plasmid targeting vectors for murine embryonic stem cell transfection (Chapter 8).

7.1 Replacement of the Antiparallel loxP Site

A new vector containing *iCre2* and floxed Neo^R cassette was constructed by replacing the inverse 3' loxP site, as outlined in Figure 7.1. First, a fragment of Neo^R was amplified; the reverse PCR primer introduced the parallel loxP site. This PCR product was cloned into the *EagI/EcoRV* sites of the original vector replacing the antiparallel loxP sites. An initial screening selected correctly ligated plasmids for the loss (due to blunt end ligation) of the *EcoRV* site (data not shown). The loxP sites were further confirmed by sequencing.

7.2 Assembly of New BAC Maps

The creation of new knock-in BACs required the assembly of accurate maps. All maps were created by retrieving the BAC insert sequence from ENSEMBL (Adams et al., 2005; Xose et al., 2007). All sequences are based on the ENSEMBL *Mus musculus* assembly 47 of C57BL/6N, which is based on the NCBI assembly m37. Insert end points were derived from the sequencing trace file database as described in (Adams et al., 2005). The *Sau3AI* sites closest to the respective ends were considered to be the border points between mouse genomic DNA

insert and backbone pBACe3.6 (Frengen et al., 1999; Shizuya et al., 1992). The introns and exons of the respective target genes were annotated using the ENSEMBL Gene Report files and data described in the literature.

The respective inserts from a 129Sv background are deposited as bMQ203N9 for *Itgb2l*, bMQ239G13 for *Msr1* and bMQ294K23 for *Marco* in the ENSEMBL database (Adams et al., 2005). The translation start codons and exons for *Msr1* and *Marco* assigned in ENSEMBL differ from those described in (Aftring and Freeman, 1995) and (Kangas et al., 1999) respectively. Targeting primers were designed according to start codons identified in the literature by functional studies rather than those deposited in the database (also see 7.15.1). A summary of the data used for map generation is given in the following table.

	bMQ203N9	**bMQ239G13**	**bMQ294K23**
5' Trace sequence	bMQ203n09.p1kSP6	bMQ239g13.q1kT7	bMQ294k23.p1kSP6
3' Trace sequence	bMQ203n09.q1kT7	bMQ239g13.p1SP6	bMQ294k23.q1kT7
Gene Report	ENSMUSG00000000157	ENSMUSG00000025044	ENSMUSG00000026390
Total size in kb	166.5	157.4	89.5

Table 7.1 Targeting BAC Data

iCre2-loxP-Neo-loxP was positioned so that the Cre construct replaced the start codon of the respective target gene using the Red/ET Recombineering system (Angrand et al., 1999). An example for the *Itg b2l* BAC before and after targeting is shown in Figure 7.4A. Details of the individual experiments and confirmatory analyses can be found in the following sections.

7.3 Confirmation of BAC Identity and Structural Integrity by PCR

Prior to recombineering, a selection of short PCRs was employed to ensure the correct BACs were used for each loci. Also, this step helped to ensure that no major interfering re-arrangements had occurred in the area to be targeted. All 3 BACs (Figure 7.2) could be identified and the PCR product sizes were as expected. Those stock BACs were then individually electroporated into competent DH10TB1 *E. coli*. Colonies were grown on LB agar containing Chloramphenicol for BAC maintenance overnight at 37˚C and then kept at 4˚C before individual clones were used for recombineering.

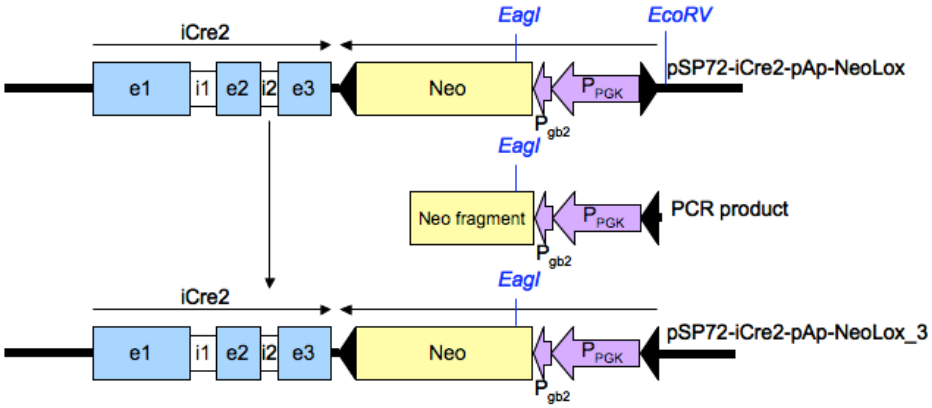
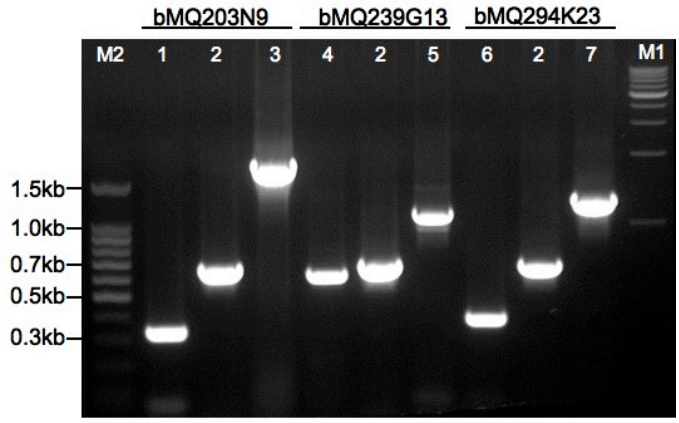


Figure 7.1 Generation of a Corrected *iCre2-pAp-loxP-Neo-loxP* Vector
The construct containing the inverse 3' loxP site (black triangles) is shown at the top. A PCR product, in which the new parallel loxP site is derived from the reverse primer was digested by *EagI* and cloned into the *EagI/EcoRV* sites of the original vector, resulting in the repaired vector (bottom) that was used for all further work.



No.	Primer pair	product size in bp
1	PactR + PactF	329
2	bckgrd3' + bckgrd5'	664
3	PactBamHISL + pact3check	1875
4	Msr1F + Msr1R	605
5	Msr1F2 + msr3check	1097
6	MaF + MaR	365
7	MaF2 + marco3check	1265

Figure 7.2 Confirmation of BAC Identity and Integrity
10pg of stock BAC was used per PCR amplification. Lanes 1, 4, 6 show amplifications of a short fragment spanning the insertion point (start codon) of the respective BAC. The same primer pairs were later used to identify knock-in BACs. Lane 2 shows a PCR product generated by amplifying a piece of the BAC backbone pBACe3.6 shared by all three vectors. Lanes 3, 5 and 7 represent longer fragments from the respective targeting regions to further assure BAC structure.
M2: 100bp marker; M1: 1kb marker

7.4 Amplification of the Targeting Fragment for bMQ203N9 *Itgb2l*

Red/ET recombineering requires a linear DNA fragment containing the sequence that is to be inserted flanked by 50nt long homology arms and is usually produced by PCR. The primer pair used to amplify the 3.1kb *iCre2 -loxP-Neo-loxP* targeting fragment is shown in Figure 7.3A. Primers consist of a 24nt annealing region and a 50nt homology region corresponding to the sequences immediately adjacent of the respective start codon. A 5'-3' proofreading polymerase (PrimeStar, Takara) was used to avoid the introduction of mutations. Curiously, Figure 7.3B shows that apart from the 3.1kb targeting band another band, approximately 1.6kb in size, was generated with a much higher efficiency. Various attempts at improving PCR specificity, i.e. by increasing the annealing temperature, did not prevent amplification of this product (data not shown).

A closer look at the amplified sequence and a restriction digest (data not shown) revealed that the employed primer pair amplifies different segments of the template due to insufficient length of the reverse primer (Figure 7.3C). The homology region of the reverse primer contains 24bp of the 3' loxP site. In the corrected vector (pSP72-iCre2-pAp-NeoLox_3), the loxP sites are, unlike the previously used inverted ones, direct repeats. The reverse primer is now able to anneal to the 5' loxP site as well, generating, with the forward targeting primer, a 1.6kb large fragment containing *iCre2-loxP*. It is also possible, that the reverse primer anneals to the 5' loxP site to create another 1.6kb large fragment containing *loxP-Neo-loxP* (Figure 7.3C). The primer annealing in such a manner, however, is not as specific due to a 6bp mismatch at the 5' loxP site's asymmetric central core. The sequence of a loxP site is given in Figure 7.3D for comparison. In order to avoid the production of multiple products in the future, the reverse targeting primer needs to be extended beyond the 3' loxP site to enhance annealing specificity.

7.5 New *iCre2* Knock-in in *Itgb2l* BAC bMQ203N9

A first round of Red/ET recombineering inserts *iCre2* into *bMQ203N9*, which carries the neutrophil-specific *Itgb2l* target gene. Bacteria are doubly selected for chlormaphenicol (BAC maintenance) and kanamycin (resistance obtained from incoming fragment). 151 bacterial colonies were obtained. A screening PCR employing primers flanking the start codon (Figure 7.4) indicated 7 out of 10 BAC clones had undergone recombination and showed the 3.3kb product size consistent with insertion of *iCre2-loxP-Neo-LoxP*. Several lanes, however, showed both the band resulting from insertion, and the smaller 329bp wild-type band indicating the presence of one or more copies of wild type BAC. In order to avoid carryover of unmodified BAC, only clones IV, VII and VIII, showing no wild-type band, were used for further work.

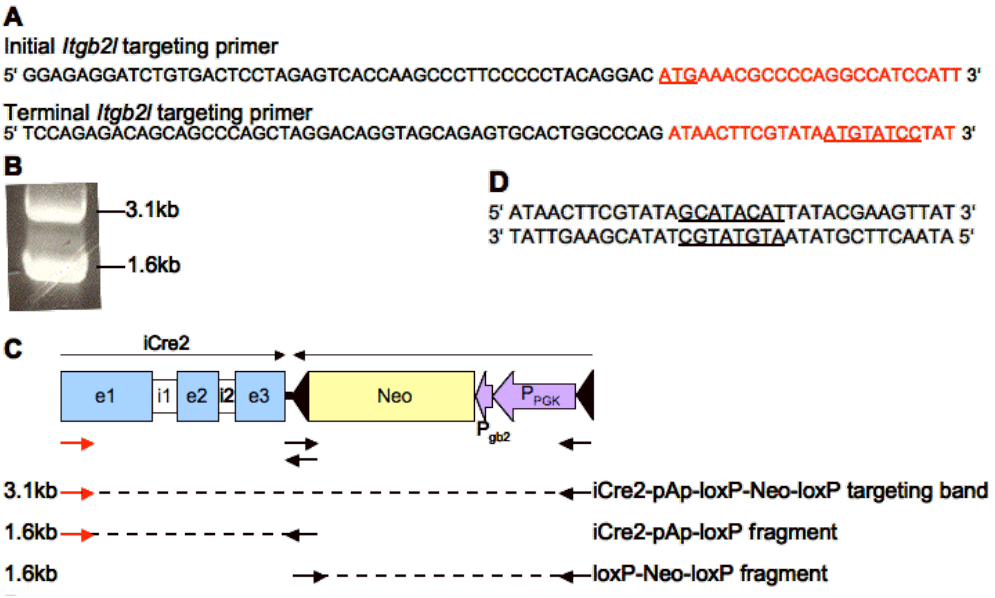


Figure 7.3 Amplification of the *Itgb2l* Targeting Fragment

The forward and reverse targeting primers are illustrated in **A**, the 50bp locus homology regions are shown in black, the annealing sequences for *iCre2* and loxP in red. The *iCre2* start codon in the forward primer and the loxP spacer in the reverse primer are underlined. The PCR, with 100pg of *pSP72-iCre2-pAp-NeoLox_3* as a template, resulted in a 3.1kb and 1.6kb fragment (**B**) due to the terminal primer annealing at both loxP sites. A schematic representation of generated products is shown in **C**, with the forward primer embodied by red; the reverse primer by black arrows. **D** illustrates the structure of a loxP site with 13bp inverted repeats and an underlined 8bp asymmetric core region.

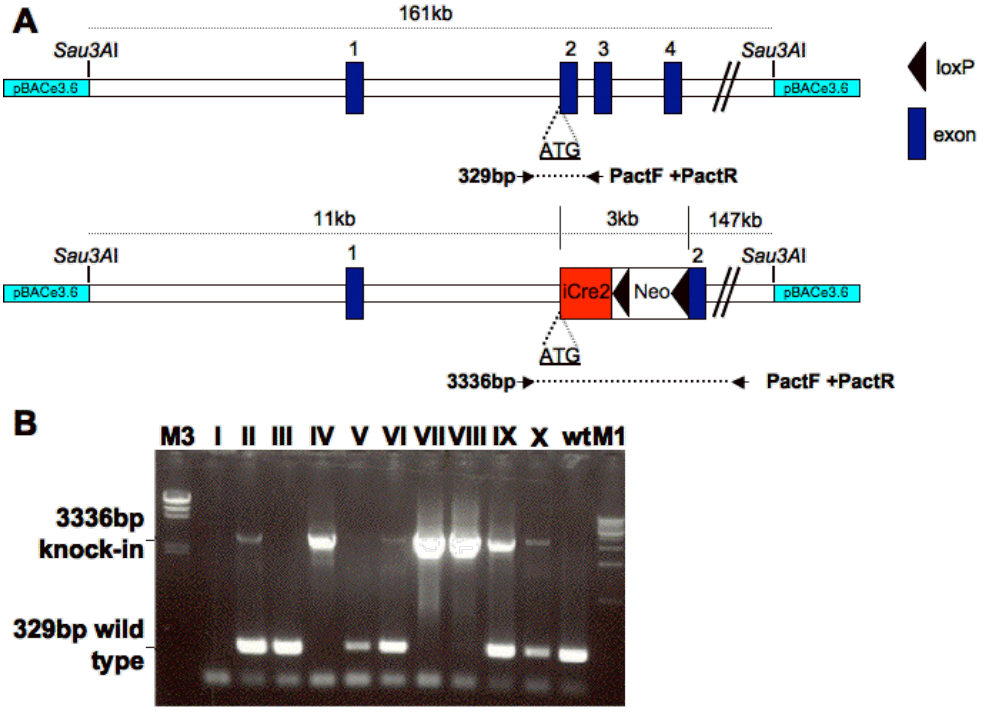


Figure 7.4 Generation of BACs bMQ203N9 *Itgb2l iCre2*

A Schematic representation of the BAC locus before and after recombination. Arrows and dotted lines indicate primer positions and lengths of screening PCR products. **B** The screening PCR, employing primer pair PactF & PactR, was carried out using 1% of a BAC preparation generated from 6ml of an overnight TB culture. Lanes I to X represent individual *E. coli* colonies. The wild type (wt) control used 100pg of the unmodified bMQ203N9 stock as a template.M1: 1kb marker; M3 *λHindIII* marker.

7.6 Creating a Short Plasmid Targeting Vector for *Itgb2l*

In a second round of recombineering, the *iCre2-loxP-Neo-loxP* insert flanked by 4kb and 7kb homology arms up- and downstream respectively, is transferred into a pUC19 plasmid to obtain a shorter targeting vector facilitating a more straightforward screening regime. To that end, the pUC19 vector was amplified with a proofreading polymerase. A primer pair (Figure 7.5A) introduced *Itgb2l* specific 50nt long homology arms, resulting in a linear DNA fragment that served as a template for recombineering. The insert is strategically placed into the multiple cloning site (MCS) of pUC19, so that a unique upstream *SalI* site can later be used for vector linearization. Bacteria are selected for being doubly resistant to kanamycin (resistance provided by insert) and ampicillin (resistance provided by pUC19 backbone). Recombineering between the pUC19 PCR product and BAC *bMQ203N9 iCre2-loxP-Neo-loxP* clone VII resulted in 800 colonies potentially harbouring correctly targeted plasmid vectors. An analytical *EcoRV* restriction digest (Figure 7.5B, see map in 7.5C) revealed 3 out of 20 tested clones (numbers 3, 5 and 7) as having a digest pattern of 6.3kb; 5.6kb and 4.8kb consistent with successful recombination.

7.7 Sequencing Reveals a Cryptic Splice Site in the *iCre2* Coding Sequence

In order to verify the vectors independently prior to starting the ES cell work, the pUC19 *Itgb2l iCre2* targeting plasmids were sequenced. Results exposed an inconsistency (Figure 7.6) in the reverse loxP site. All 3 clones, descendants of BAC knock-in clone VII, contained a 2bp TA deletion in the reverse loxP site. As the majority of the 5' loxP sequence is introduced by the reverse targeting primer, the error must have arisen from a faulty primer molecule in the initial amplification of *iCre2 -loxP-Neo-loxP*. Mutations of those particular nucleotides in loxP sites have not been described and are therefore likely to render the loxP site non functional (Missirlis et al., 2006).

New plasmid targeting vectors were then generated using BAC knock-in clones IV and VIII, assuming that the faulty primer molecules would only have been incorporated in a minority of targeted BACs. The process was as previously described (Chapter 7.6), and 600 and 550 resistant bacterial colonies were obtained respectively. 20 clones each were subjected to the diagnostic *EcoRV* restriction digest. 13 descendants from BAC VIII but only 1 from BAC IV showed the digestion pattern (data not shown) indicating successful homologous recombination. When 2 of the VIII derived vectors, clones p and i, were sequenced as before (Figure 7.7), it could be shown that the loxP site was indeed intact.

A

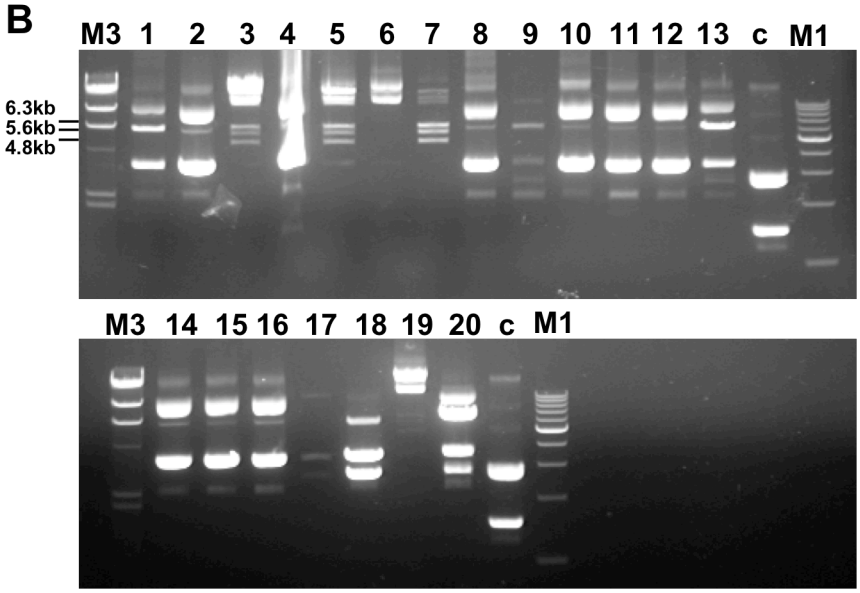
pUC19 *Itgb2l iCre2* forward primer

5‘ CCATAGTTAGGATATCACAGCTCCTATGTCCTCACCTGTAATTGGGCTAT CCTGTGTGAAATTGTTATCCGCTC 3‘

pUC19 Itgb2l *iCre2* reverse primer

5‘ ATATACTACACAGACATAATTGTACATGCACATAGACAAGGCATGTGCATCCTGCAGGTCGACTCTAGAGGATC 3‘

B



C

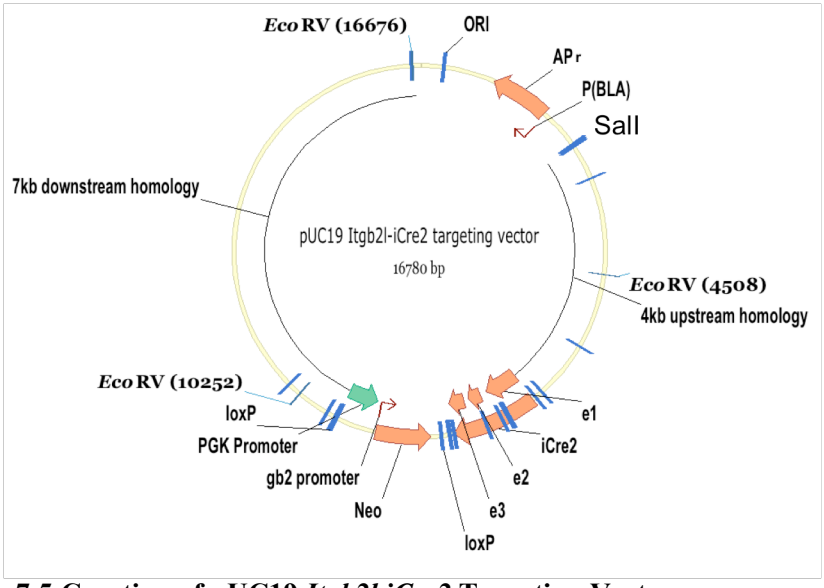


Figure 7.5 Creation of pUC19 *Itgb2l iCre2* Targeting Vectors

Targeting primers (**A**), consisting of 50bp long homology regions (black) and 24bp homologous to pUC19 (red) were used. Homology regions correspond to sequences 4kb upstream or 7kb downstream of the *Itgb2l* start codon. **B** Following recombineering, minipreps were carried out on 20 overnight TB-cultures. 25% of the DNA was subjected to an *EcoRV* digest and electrophoresed. In case of correct recombination, 3 fragments, 6.3kb, 5.6kb and 4.8kb (**C**), are expected. Clones 3, 5 and 7 showed this pattern and are thus considered to be correctly targeted vectors. Unmodified pUC19 plasmid DNA was used as a control (c).
M2: 1kb marker, M3 λ*HindIII* marker.

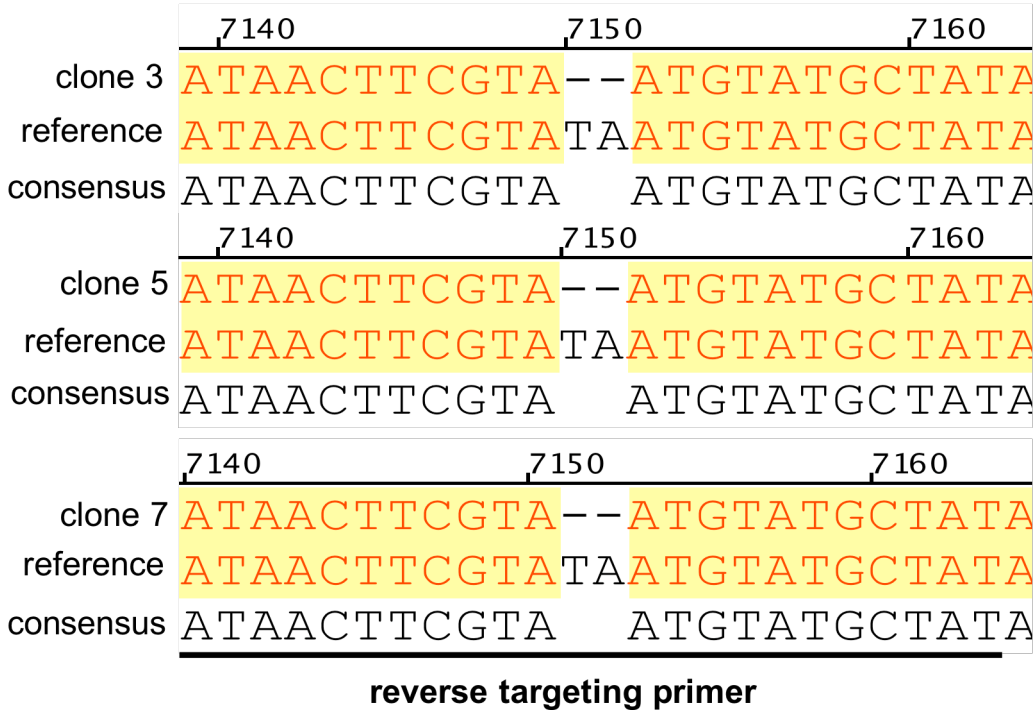


Figure 7.6 Sequence Alignment of the 3' loxP Site in BAC Clone VII Derived Vectors
All *3 Itgb2l iCre2* targeting vectors were sequenced using primer PactR. The sequencing results for the individual clones are given in comparison to the reference and consensus sequences. The DNA sequence introduced by the reverse targeting primer is underlined. Only part of the loxP site is shown.

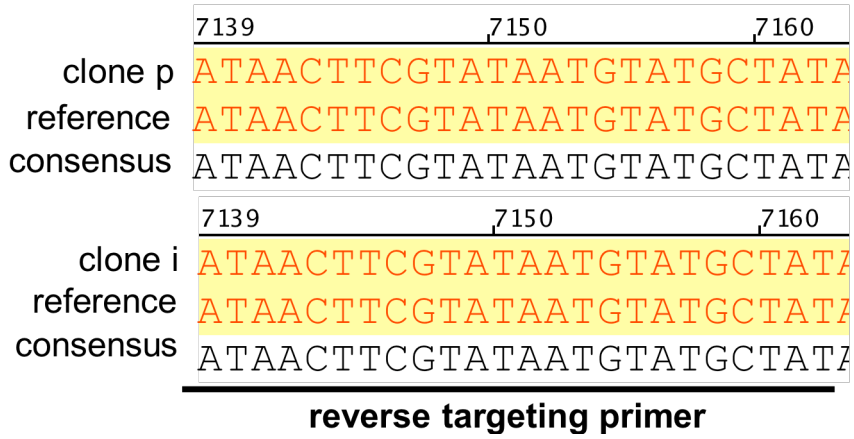


Figure 7.7 Sequence Alignment of the 3' loxP Site in BAC Clone VIII Derived Vectors
Two pUC19 *Itgb2l iCre2* targeting vectors, p and i, were sequenced using the primer PactR. Sequencing showed that descendants from clone VIII carry an intact loxP site. The DNA sequence introduced by the reverse targeting primer is underlined. Only part of the loxP site is shown.

Following verification of the loxP site, pUC19 *Itgb2l iCre2* targeting vectors were also checked for integrity of the *iCre2* coding sequence. It was discovered that all clones analyzed had 2 codons in Exon 1 of *iCre2* changed (CAA to CAG and CGG to CGT) in comparison to the reference sequence (Figure 7.8A). This mutation could eventually be traced back to the initial vector assembly. The CAG/CGT codons are consistent with the enhanced Cre wild type sequence designated Cre(x). Upon insertion of heterologous introns, however, a cryptic splice site was inadvertently created resulting in incorrect splicing of the Cre mRNA which deletes a fragment at the 5' end (Figure 7.8B). By introducing silent mutations (CAG to CAA and CGT to CGG), the splice site had been removed without impacting on the Cre amino acid sequence (Lacy-Hulbert et al., 2001).

The splice site-less vector was designated iCre2b. Sequencing of iCre2b during this work, however, revealed that it still carried the cryptic splice site. Since iCre2b had been used to generate all *iCre2* carrying vectors prior to the start of the project, the mutation was introduced into all descendants and could consequently also be found in all 4 sequenced pUC19 *Itgb2l iCre2* targeting vectors derived from BAC knock-in clone VIII. A vector designated mtiCre2, could finally be identified as having the splice site removed.

7.8 Repair of the Cryptic Splice Site

To rectify the cryptic splice, the plasmid vector carrying *iCre2 -loxP-Neo-loxP* was repaired as outlined in Figure 7.9A. *BamHI* and *ClaI* were used to excise a fragment containing the cryptic splice site. A fragment for replacement was amplified from vector mtiCre2. Following ligation of those two pieces, 20 clones were isolated and sequencing of three of those (Figure 7.9B) revealed successful removal of the cryptic splice site. The clone designated pSP72-iCre2*-pAp-NeoLox_20 was used for all further work. In this plasmid, the *iCre2* coding sequence, including pause and polyA sites, the Neo^R gene and its double promoter were re-sequenced. No further significant sequence discrepancies were found (data not shown).

7.9 Repair of the pUC19 *Itgb2l iCre2* Targeting Vector

In order to avoid repeating the entire recombineering process for the *pUC19 Itgb2l iCre2* targeting vectors, a cloning strategy (see Figure 7.10A) was devised to replace the cryptic splice site. In essence, the incorrect vector was digested with *AgeI* and *ClaI*. Three fragments were obtained, the smallest of which (957bp) contains the cryptic splice site. The same 957bp fragment was amplified from the vector mtiCre2 and re-ligated with the 1.8kb and 14kb pieces. Due to the low efficiency of 3 piece ligations and the relatively large size (16.7kb) of the targeting vector, only 2 bacterial colonies were obtained. Of those colonies, designated a and b,

only b could be expanded in liquid culture for DNA isolation. Sequencing (Figure 7.10B) and PCR (data not shown) revealed that ligation had indeed occurred in the right orientation and the cryptic splice site was corrected successfully.

7.10 Construction of *iCre2* knock-in BACs for *Msr1* and *Marco*

Using the corrected vector pSP72-iCre2*-pAp-NeoLox_20 as a template, an attempt was made to also create knock-in BACs for *Marco* and *Msr1*. To avoid the previously described hurdle (Figure 7.3B) of unintentionally amplifying 2 bands in the PCR producing the recombineering fragment, an elongated reverse primer, extending 6bp (total 90nt) beyond the loxP site was employed. Nevertheless, two bands were obtained (data not shown). This was most likely due to the 5'-3' nuclease activity of the utilized proofreading polymerase that cuts off the nucleotides from the 3' end of the reverse primer, thus reducing priming specificity. The obvious solution would be to extend the already 90nt long reverse primer even further or to place it outside the loxP site. Primer extension is limited by oligonucleotide synthesis. Whilst 120-140bp can be achieved, synthesis becomes less efficient beyond 50-100mers with more side products being created and purification is difficult resulting in significant cost. Placing the primer outside of the targeting area results in the introduction of bacterial DNA sequences into the final construct as amplification occurs from a plasmid vector. The presence of bacterial DNA in genetically modified animals has been linked to silencing effects (Chada et al., 1985; Clark et al., 1997).

Nonetheless a sufficient amount of the targeting fragments for recombineering could be obtained by gel purifying the larger band. 75 and 21 resistant *E. coli* colonies were generated for *Marco* and *Msr1* respectively. Colonies for *Marco,* however, could only be obtained after the third attempt, when the amount of the targeting fragment *iCre2-loxP-Neo-loxP* transformed into the *E.coli* strain harbouring BAC bMQ294K23 was quadrupled from the standard 50ng to 200ng. Colonies were small, and only 4 of 29 colonies that were inoculated could be expanded.

29 of the potential *Marco* knock-in clones and all 21 of the potential *Msr1* knock-in clones underwent PCR screening. Initial PCRs were carried out using *E.coli* colonies as template. The locus specific primer pairs and product sizes are shown in Figure 7.11A,B&E for *Marco* and *Msr1* respectively. This initial PCR (Figure 7.11C for *Marco*, Figure 7.11F for *Msr1*) revealed that the large 3.3-3.6kb band indicating successful recombination could not be observed in any of the samples with exception of the positive control. Shorter fragments using one locus- and one *iCre2*-specific primer, however, were amplified in several reactions, indicating potential BAC knock-ins. These clones were expanded, BAC DNA was purified and the PCR was repeated (Figure 7.11D&G). One knock-in BAC per target gene, clone 9 for *Marco* and clone 19 for *Msr1*, could be verified.

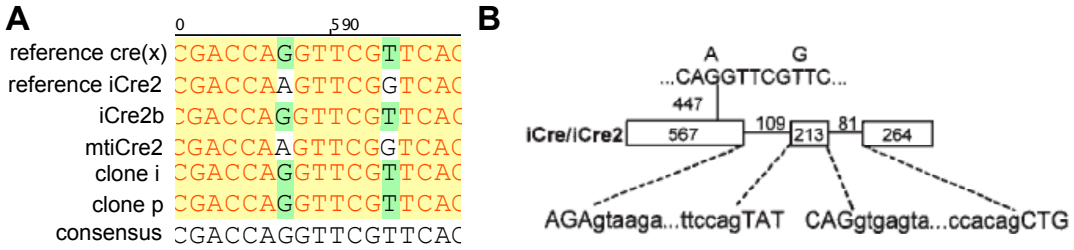


Figure 7.8 Cryptic Splices Sites
A The reference sequences for Cre(x) and *iCre2* were compared to sequencing results obtained from the plasmid vectors iCre2b, mtiCre2 and the BAC VIII derived pUC19 *Itgb2l iCre2* targeting vector clones i and p. Only mtiCre2 no longer carries the cryptic splice site. All sequencing reactions were carried out using primer iCrecheck2, which binds in exon 2. **B** shows the Cre coding sequence after the insertion of heterologous introns with boxes representing exons and numbers indicating the length of individual units. The nucleotide sequences of the new exon/intron boundaries are specified below; upper case letters indicate exons, lower case letters introns. The cryptic splice site activated by intron insertion is shown above together with the correcting point mutations. Figure 7.8B is taken from (Lacy-Hulbert et al., 2001).

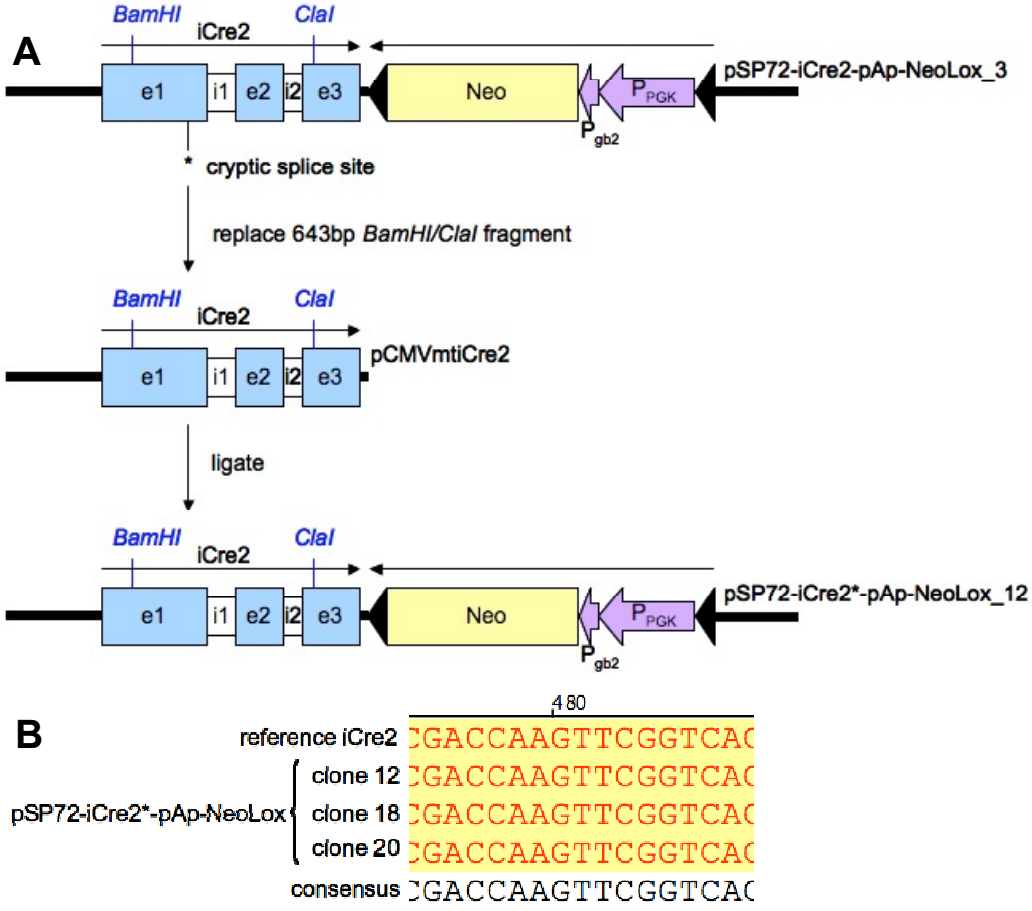


Figure 7.9 Removal of the Cryptic Splice Site
A details the strategy employed in repairing the vector carrying *iCre2-loxP-Neo-loxP*. **B** shows sequencing data for 3 isolated clones compared to the *iCre2* reference. Repair was successful in all clones. Sequencing was carried out using a standard SP6 primer.

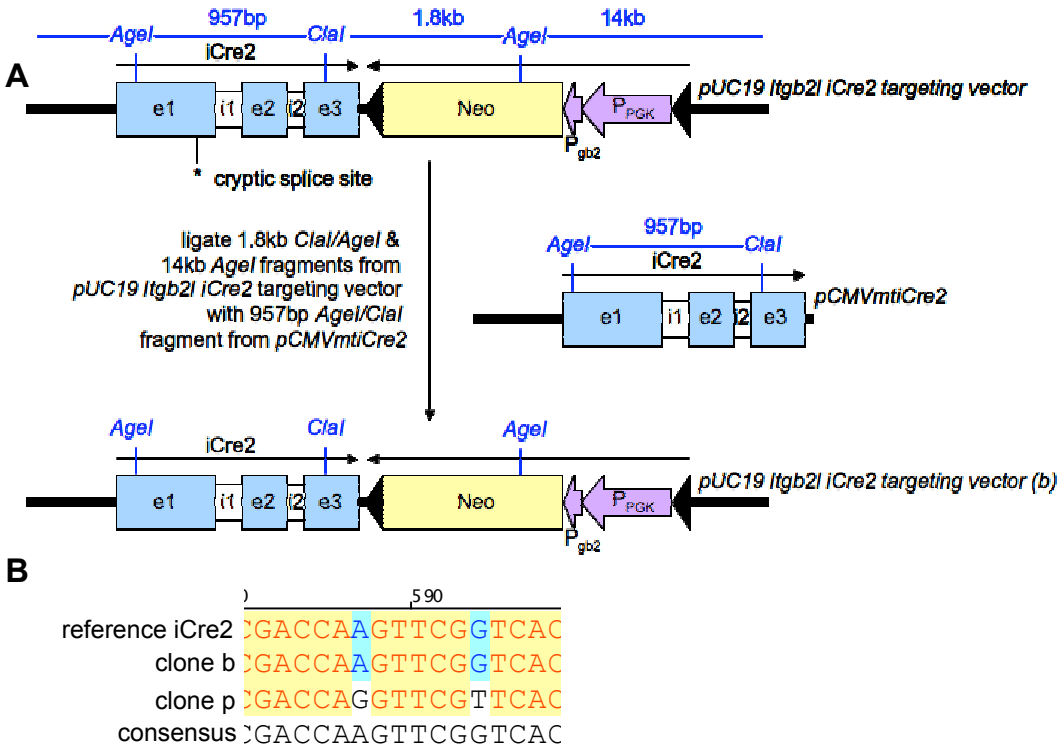


Figure 7.10 Repair of the Cryptic Splice Site
A illustrates the cloning strategy used for repairing the pUC19 *Itgb2l iCre2* targeting vector. **B** shows sequencing data comparing clone p (prior to repair) and clone b (following repair) to the *iCre2* reference sequence. Sequencing was carried out using primer iCrecheck2.

7.11 Construction of Plasmid Vectors from *Marco* and *Msr1* knock-in BACs

The two knock-in BACs were subjected to a second round of recombineering in order to insert the *iCre2-loxP-Neo-loxP* flanked by 4kb & 7kb gene specific homology arms into pUC19. Primer sequences are given in Figure 7.12A. The insertion points were chosen in a manner that unique restriction enzyme sites for linearization lie immediately upstream. For *Marco* this site is *SmaI*, for *Msr1 SalI*. PUC19 based targeting vectors are descendants of BAC knock-in clones *Marco iCre2* 9 and *Msr1 iCre2* 19. Several hundred resistant colonies were obtained for both target genes. 15 colonies were expanded and analyzed for the former, as were 10 colonies for the latter. Analytical restriction digests using *XbaI* (Figure 7.12B&D) and *BamHI* (Figure 7.12C&E) respectively, identified a number of correctly recombined targeting vectors. For *Marco* clones b, c, d, i and l show the correct restriction pattern of 8.5kb, 5.5kb and 2.8kb. For the other 10 clones digested plasmid amounts were too low to yield visible bands. In case of *Msr1,* clones 1, 2, 3, 5, 6, 7, 8 and 10 reveal expected product sizes (9.2kb, 4.4kb and 3.1kb) upon digestion. Two clones were chosen for further work: pUC19 *Marco iCre2* 9d and pUC19 *Msr1 iCre2* 19_10.

A

Forward *Marco-iCre2* targeting primer

5‘ TGGGCAGCACAGAAGACAGAGCCGATTTTGACCAAGCTATGTTCCCTGTG ATGAAACGCCCCAGGCCATCCATT 3‘

Reverse *Marco-iCre2* targeting primer

5‘ GCAACTGCATTACAAGGGAGTCATACTTTACCATTGATTTCGAAGGTCTC ATAACTTCGTATAATGTATGCTATACGAAGTTAT CTACCG 3‘

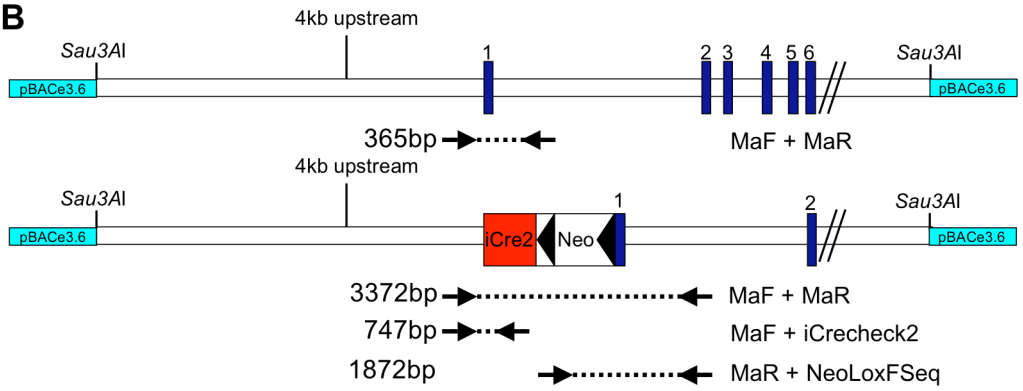
Forward *Msr1-iCre2* targeting primer

5‘ ATTGTAAACAGAGGGAAGTGGATAAATCAGTGCTGTCTTCTTTACCAGCA ATGAAACGCCCCAGGCCATCCATT 3‘

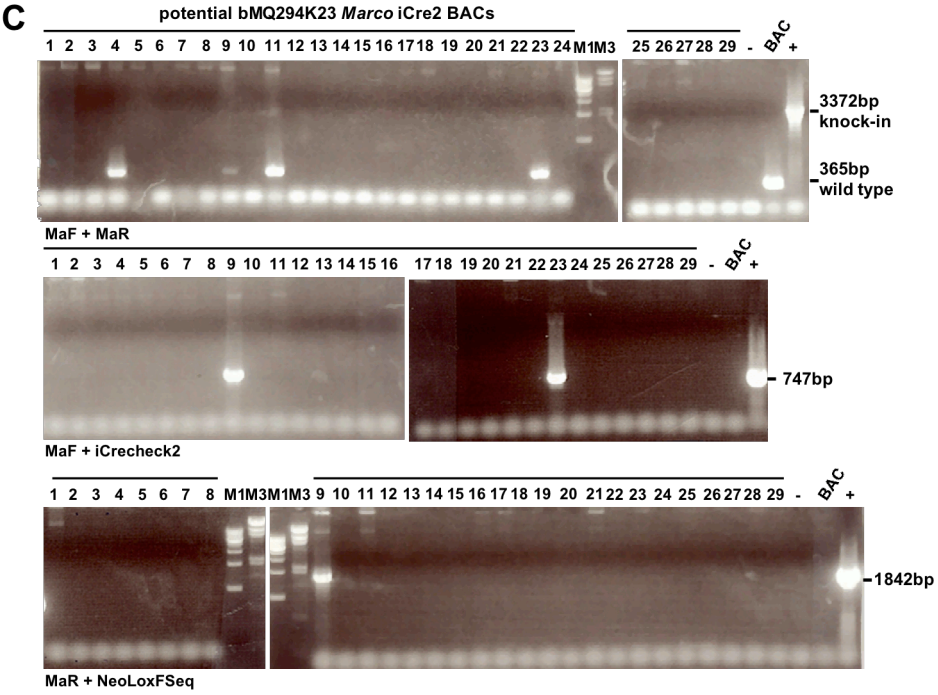
Reverse *Msr1-iCre2* targeting primer

5‘ TCAACATAAGTAATTTAAATAAGAAATAAATTAAACTATACCTCTTTTGT ATAACTTCGTATAATGTATGCTATACGAAGTTAT CTACCG 3‘

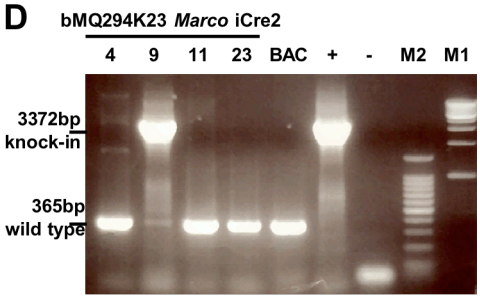
B



C



D



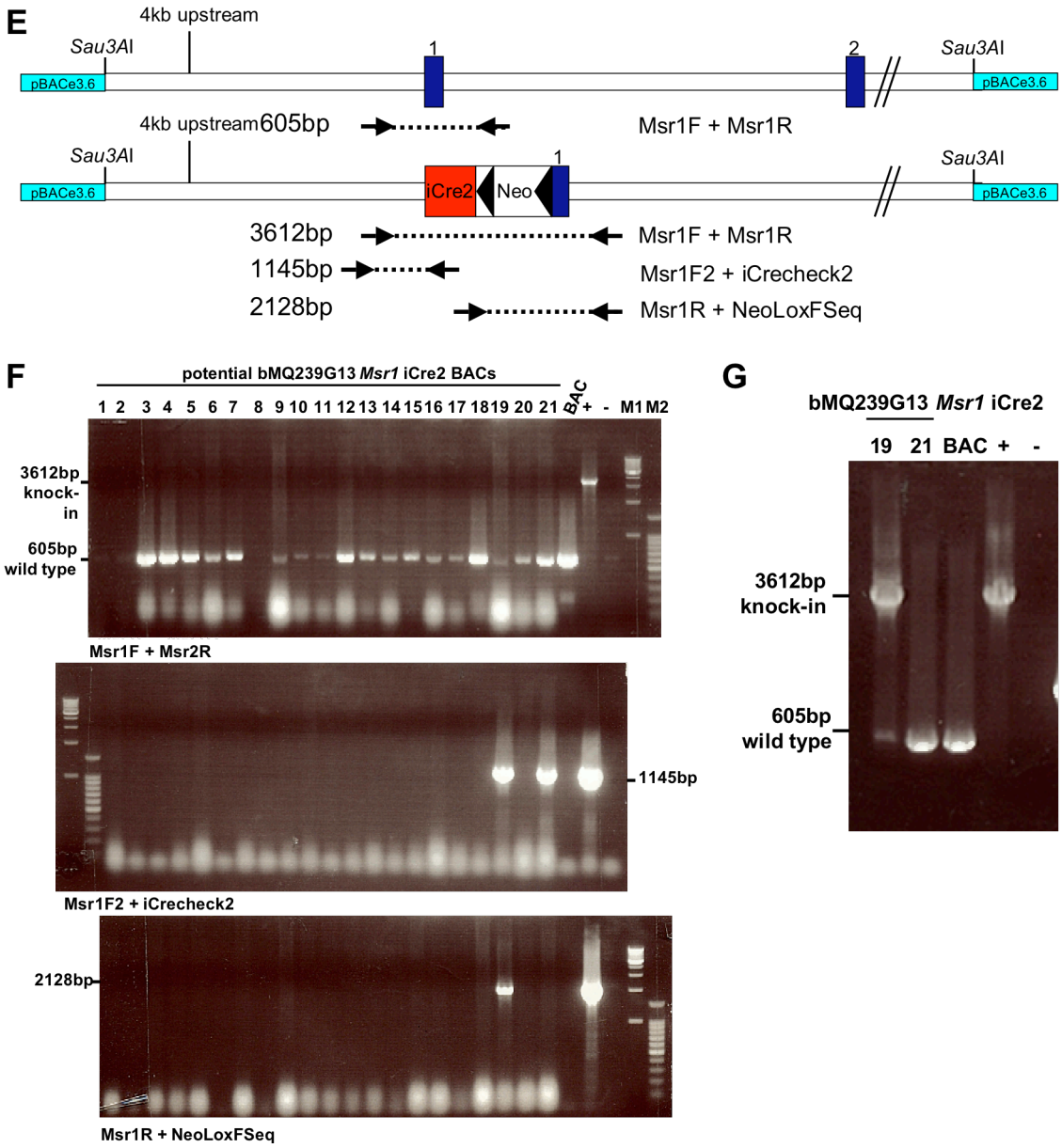


Figure 7.11 Generation of *Marco* & *Msr1* iCre2 Knock-in BACs
Targeting primer pairs (**A**) consisting of 50nt homology regions (black) corresponding to sequences immediately adjacent to the respective start codon are fused to annealing regions (red) complementary to either *iCre2* or the 3' loxP site. In an attempt to reduce non-specific priming, the terminal targeting primer was extended by 6nt into the PGK promoter. Successful recombineering occurs when the *iCre2-loxP-Neo-loxP* fragment replaces the start codon of either *Marco* (**B**) or *Msr1* (**E**). Three PCRs were carried out on bacterial colonies grown overnight to establish which ones harboured BAC knock-ins (**C** & **F**). Candidate colonies were expanded and 10% of the purified DNA isolated from a 6ml overnight TB culture was used for another PCR to verify recombination (**D** & **G**).
BAC: unmodified BAC; -: no template; +: previously generated, faulty knock-in BAC; M1: 1kb marker; M2: 100bp marker.

A

pUC19 *Marco-iCre2* forward primer

5‘ TAGAGAATCAGATGTCGTTCAGGTCTTTGCAGATAATGTGACCTTTCTAA CCTGTGTGAAATTGTTATCCGCTC 3‘

pUC19 *Marco-iCre2* reverse primer

5‘ TCTGATCTACTGGTCATCAAGTAGGCCCGGCCTACTGGCCAGTGAGCCTCCCTGCAGGTCGACTCTAGAGGATC3‘

pUC19 *Msr1-iCre2* forward primer

5‘ AGTTTCTGGTCCTCCCGGTTCCCTTCCCTTATCCCCTTGTACTGGTGGCACCTGTGTGAAATTGTTATCCGCTC3‘

pUC19 *Msr1-iCre2* reverse primer

5‘ ACTATATCTCTTCTGCCTTTAAACATGTTTTGACAGTGAGGGCTGCATACCCTGTGTGAAATTGTTATCCGCTC3‘

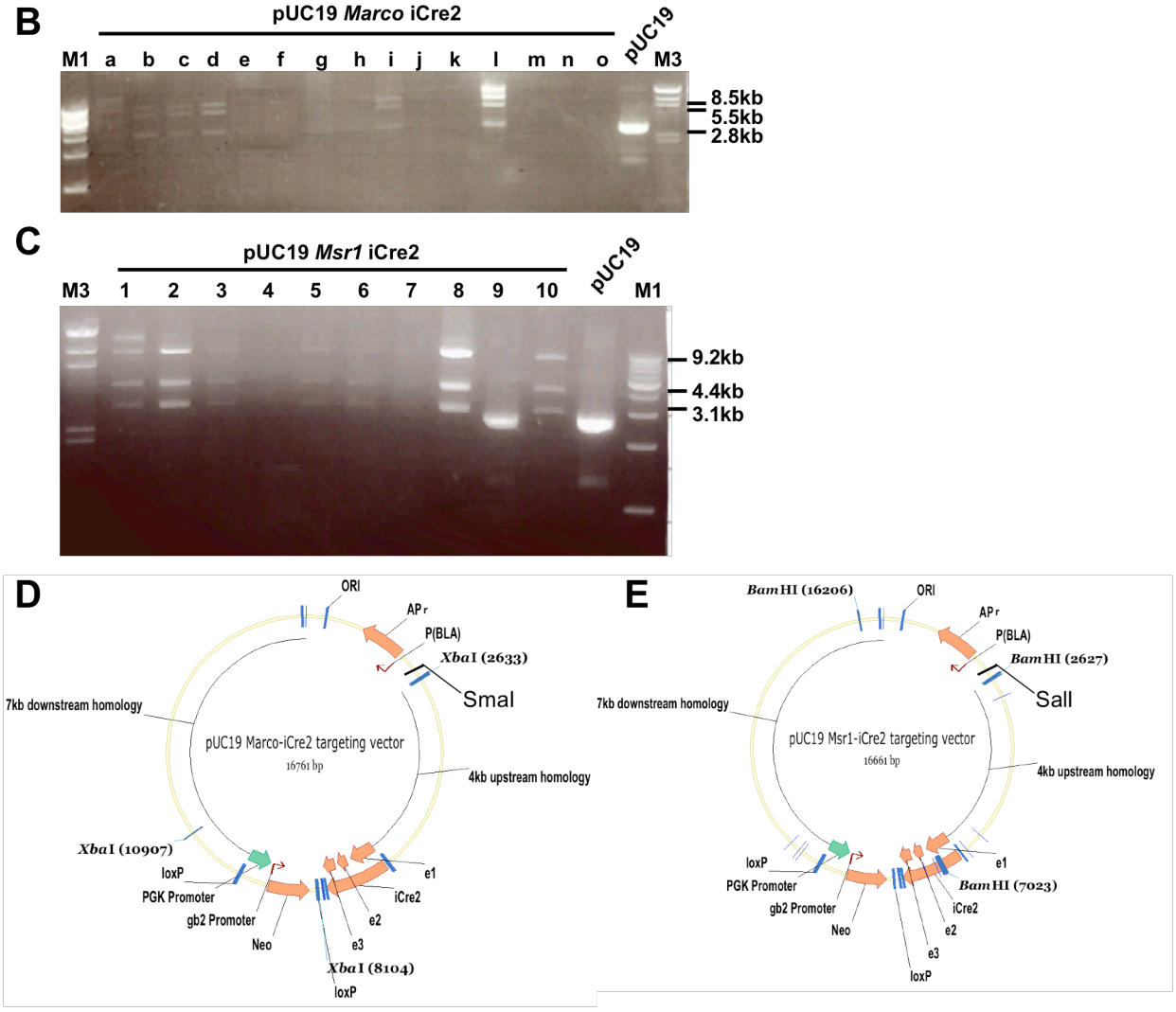


Figure 7.12 Generation of *Marco* & *Msr1* Plasmid Based Targeting Vectors

A Primer pairs contained 50nt homology arms (black) corresponding to sequences 4kb upstream and 7kb downstream of the respective start codon and 24bp pUC19 complementary sequence (red) bordering the insertion point. Colonies derived from the *Marco* (**B**) and *Msr1* (**C**) knock-in BACs were expanded, and isolated plasmid DNA was subjected to *XbaI* (**B** & **D**) or *BamHI* (**C** & **E**) digests. Patterns indicating successful recombination were 8.5kb, 5.5kb, 2.8kb for *Marco* and 9.2kb, 4.4kb, 3.1kb for *Msr1* respectively. Unmodified pUC19 served as a control.

7.12 Summary of Targeting Vector Construction

Target Gene	No. of generated BAC knock-ins/of those screened	No. of verified BAC knock-ins/in percent	Targeting vector based on BAC knock-in clone	No. of generated targeting vectors/of those screened	No. of verified targeting vectors/in percent	Clone used for ES cell transfection
Itgb2l	151/10	7/70%	VII IV VIII	800/20 600/20 550/20	3/15% 1/5% 13/65%	b*
Marco	75/29	1/4%	9	250/15	5/33%	9d
Msr1	21/21	1/5%	19	120/10	8/80%	19_10

* derived from BAC knock-in VIII

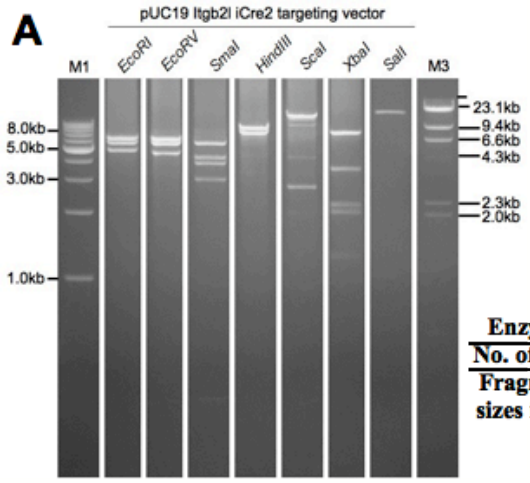
Table 7.2 Summary of Targeting Vector Construction
Summary of key steps during the targeting vector creation process, such as the total number of colonies generated, fractions shown to be correctly recombined BACs and plasmid targeting vectors, and the respective clones used in ES cell work (Chapter 8).

7.13 Comprehensive Restriction Digests of Targeting Vectors

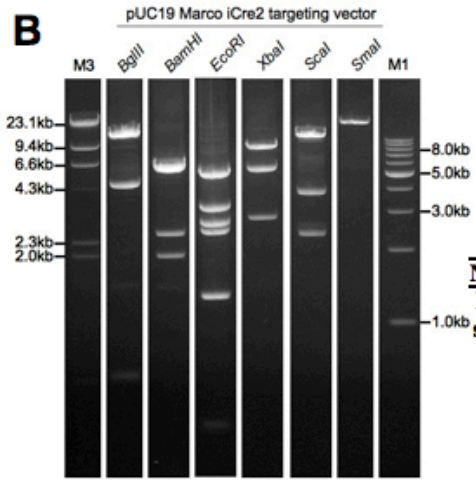
Comprehensive restriction digests were carried out following large scale preparation of all 3 pUC19 based targeting vectors to verify the overall structure of the insert and exclude the potential loss or re-arrangement of important sequences flanking *iCre2-loxP-Neo-loxP*. Figure 7.13A;B;C show the results for the targeting vectors for *Itgb2l, Marco* and *Msr1* respectively. Restriction patterns observed confirmed the correct structure of all targeting vectors.

7.14 Sequencing of Targeting Vectors

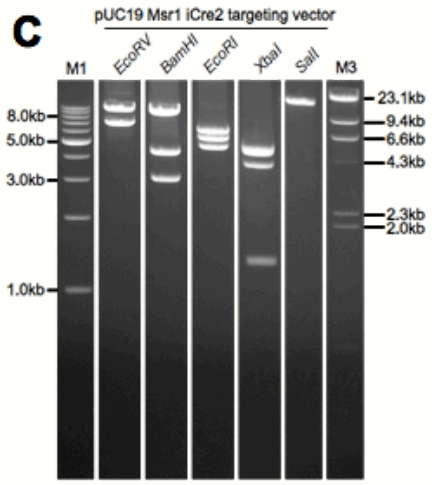
The targeting vectors were sequenced to ensure no significant mutations had been introduced during the recombineering process. The entire Cre coding sequence, the pause and polyA signals, the Neo^R gene with its promoters and flanking loxP sites as well as a short stretch of vector specific bordering DNA was sequenced. With 5 independent sequencing primers, 80-100% of the 3010bp large insert could be covered. An overview of the results is given in Figure 7.14A. The targeting vectors for *Itgb2l* and *Marco* have no significant mutations. The *Msr1* targeting vector, however, contains 2 potentially problematic sequence changes. A nucleotide (C) is deleted in the reverse loxP site at position 5 of the inverted repeat (Figure 7.14B). Similar to what was described in chapter 7.7, this is a mutation introduced by the reverse primer. There is also an additional mutation in exon 1 of the Cre coding sequence, changing codon 157 from ATG to ACG which results in an amino acid exchange from methionine to threonine (Figure 7.14C).



Enzyme	*EcoRI*	*EcoRV*	*SmaI*	*HindIII*	*ScaI*	*XbaI*	*SalI*
No. of sites	3	3	4	2	2	5	1
Fragment sizes in bp	6324	6424	5711	9051	14021	7757	16780
	5620	5744	4247	7729	2759	3551	
	4836	4612	3858			2211	
			2964			2031	
						1230	



Enzyme	*BglII*	*BamHI*	*EcoRI*	*XbaI*	*ScaI*	*SmaI*
No. of sites	4	5	7	3	3	1
Fragment sizes in bp	11661	6244	5323	8487	10620	16761
	4496	5962	3263	5471	3798	
	587	2475	2733	2803	2343	
	17	1970	2496			
		110	1328			
			1322			
			291			



Enzyme	*EcoRV*	*BamHI*	*EcoRI*	*XbaI*	*SalI*
No. of sites	2	3	3	5	1
Fragment sizes in bp	9551	9183	6331	5051	16661
	7110	4396	5570	4821	
		3082	4760	4005	
				1417	
				1364	

Figure 7.13 Comprehensive Restriction Digest of Targeting Vectors
Several restriction digests were carried out for each of the targeting vectors, results are shown in **A**, **B** and **C** for *Itgb2l*, *Marco* and *Msr1* respectively. The number of restriction sites per vector and the expected fragment sizes are given on the right. 1µg of plasmid DNA was digested per lane using 10U of the appropriate enzyme for one hour at 37˚C. Samples were electrophoresed on a 0.7% agarose gel for one hour before being photographed. Fragments smaller than 250bp are not visualized.
M1: 1kb marker; M3: *λHindIII* marker

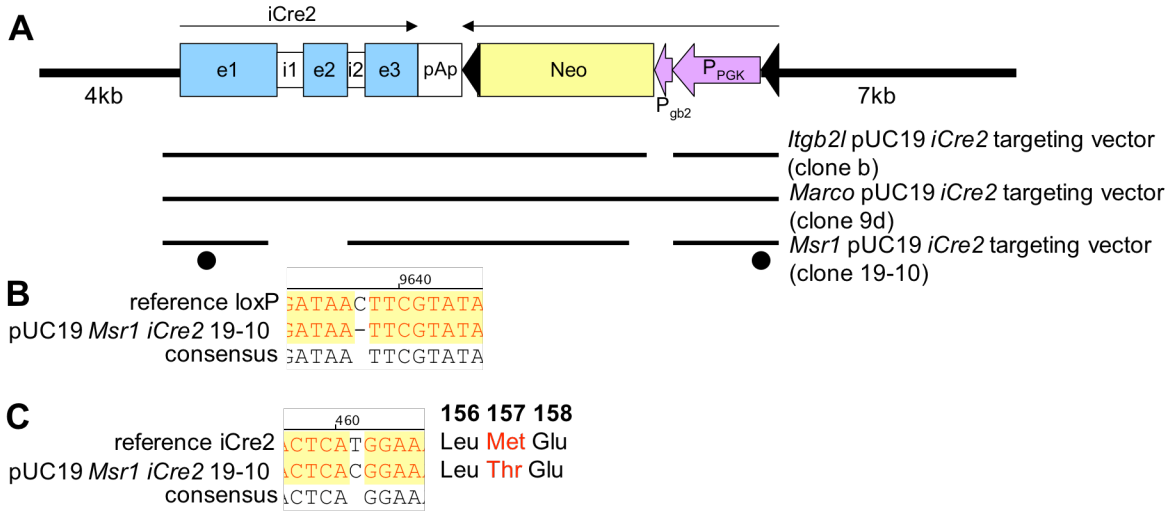


Figure 7.14 Illustration of Sequencing Coverage & Results
A shows the structure of the *iCre2-loxP-Neo-loxP* insert flanked by locus specific homology arms. The sequencing coverage is indicated by a black line for each individual vector. Sequence errors (compared to the reference) found are represented by black dots. **B** shows the fragment of the 3' loxP site carrying a 1bp deletion. **C** illustrates the sequence alignment for three codons of exon 1 of the Cre coding sequence (left), the 1bp exchange leads to a Thr at position 157 instead of a Methionine (right). Primers used for sequencing were PactF, MaF, Msr1F, iCrecheck2, NeoExcF, NeoLoxFSeq, PactR, MaR and Msr1R.

7.15 Discussion

7.15.1 The Choice of *iCre2* Insertion Points

Targeting experiments were designed such that the incoming *iCre2* sequence replaces the endogenous translation start codon to ensure minimal interference with the target gene's regulatory elements. Whereas *Itgb2l* has one identified start codon (Aftring and Freeman, 1995) that was consequently used as insertion point, both *Marco* and *Msr1* have two possible in-frame ATGs. For each gene, the most likely translation start codon was chosen, according to data published in the literature (see below).

7.15.1.1 Marco

Initial *Marco* cDNA analysis revealed two potential translation initiation codons (Figure 7.15) (Elomaa et al., 1995). Both are located towards the end of the first exon, approximately 10bp and 100bp downstream from a conserved TATA box required for transcription initiation. An additional TATA-like sequence is found further upstream. Both ATG codons agree with the Kozak consensus in positions -3 and +4, making them strong initiation sites (Kozak, 1984). The downstream ATG additionally exhibits a consensus at position -5. Primer extension analysis, S1 nuclease mapping and rapid amplification of 5' complementary DNA ends (RACE) have indicated the main transcription start point as being 27-28bp downstream of the TATA box (+1). A minority of clones exhibited transcription initiation at -63 to -66. As the main

transcription initiation start point is downstream from the first ATG, the second ATG was suggested as the main initiator of translation, whereas the more seldomly used upstream sites can give rise to alternative *Marco* variants with longer cytoplasmic domains (Kangas et al., 1999). In accordance with this data, the second translation initiation codon was selected as the insertion point for *iCre2*.

Figure 7.15 *Marco* 5' cDNA Nucleotide Sequences
Sequences upstream and downstream from the in-frame ATGs of *Marco* (top) are illustrated. The possible transcription start sites are indicated by asterisks with the main site being designated +1. Preceding TATA sequences are boxed. The two translation initiation codons are marked red, with nucleotides matching the Kozak consensus sequence (bottom) underlined. R: Purine nucleotide. Figure modified from (Kangas et al., 1999).

7.15.1.2 Msr1

Msr1 also carries two possible in-frame translation start codons, which are only 3 codons apart in the cDNA. The first is located towards the end of exon 1, the second at the beginning of exon 2. Upstream of either ATG, one cluster of transcription initiation sites lacking a TATA box but preceded by a GATA transcription factor binding side, has been identified (Figure 7.16) (Aftring and Freeman, 1995). The start codons have relatively weak Kozak sequences, both agreeing at -3 and the first ATG also at -5 with the consensus (Kozak, 1984). The in-frame ATG in exon 1, which was used as *iCre2* knock-in site, is only found in the mouse genome but not in the human, rabbit and bovine genes (Ashkenas et al., 1993), suggesting an extended murine gene product.

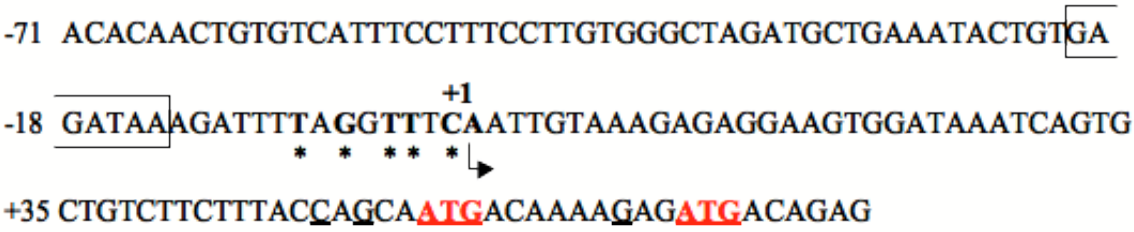


Figure 7.16 *Msr1* 5' cDNA Nucleotide Sequences
Sequences 5' upstream and downstream from the in-frame ATGs of *Msr1* are shown. The possible transcription start sites are indicated by asterisks with the main site being designated +1. A preceding GATA transcription factor binding site is boxed. The two translation initiation codons are marked red, with nucleotides matching the Kozak consensus sequence underlined. Figure adapted from (Aftring and Freeman, 1995).

7.15.2 Vector Creation and Verification

Overall, the application of the Red/ET recombineering system was very successful as both knock-in BACs and retrieval plasmid vectors could be generated for all three loci. Efficiency (Table 7.2), however, varied widely between target genes. The main factors influencing recombineering efficiency are both quality and purity of the PCR product and BAC; presence of repeats in the targeting region; secondary recombination and the used bacteria strain and its transformation competency.

The main issues encountered during this work were false positives and primer-induced mutations. False positives, drug resistant colonies not harbouring the modified vector, were observed at both stages (Table 7.2) of the vector creation process and are most likely due to the presence of traces of the respective supercoiled plasmids used in the PCR amplification of recombineering fragments. Whilst fragments were gel purified prior to electroporation, an inadvertent carry-over cannot be excluded. For future experiments, an additional *DpnI* digest of the PCR product, to remove traces of methylated template, is advisable.

Mutations in the final vector were another repeatedly encountered obstacle. Several sequence changes were introduced by the primers utilized in amplifying the targeting fragment (Figures 7.6 & 7.14). In both cases, single or double base pair deletions were observed in a part of the 3' loxP site that originates from the incoming PCR primer. Primers used for recombineering are relatively long (>74bp) and thus more likely to contain truncated or deleted side products after synthesis. Whilst primers used in this work were purified by capillary gel electrophoresis (CGE), achieving >90% purity (details provided by manufacturer), unintended sequence changes could not be avoided entirely. In case of *Itgb2l* (Figure 7.6), this was simply circumvented by using a different BAC knock-in clone that had incorporated a correct primer molecule. For *Msr1*, however, only one targeting was available carrying a mutation (Figure 7.14) located at position 5 of the loxP site's inverted repeat. Mutations of the first 5 bp of said

repeats have been described (Missirlis et al., 2006), so the site is likely to be functional. In addition, the *Msr1* targeting vector harbours a single base pair mutation in the Cre coding sequence caused by a PCR error that occurred despite using a proofreading polymerase with an average error rate of 4.8*10E-5 or 1 mutation every 20.000 synthesized base pairs (Cline et al., 1996). The mutation, however, causes an amino acid exchange outside of Cre's active site (Gopaul et al., 1998) and should therefore not interfere with protein function. Both sequence changes were considered too minor to justify repair, which would have necessitated a new round of BAC recombineering exacerbated by low PCR yields and knock-in frequencies.

7.15.3 Summary

BAC knock-ins and shorter plasmid targeting vectors were obtained for all three target genes *Itgb2l, Marco* and *Msr1* in two successful rounds of Red/ET recmbinogenic engineering. Discovered sequence discrepancies were corrected. Comprehensive restriction digests and sequencing ensured the integrity of the *iCre2-loxP-Neo-loxP* cassette as well as the absence of major re-arrangements in the final targeting vectors prior to the following embryonic stem cell work.

CHAPTER 8: CREATING 2ND GENERATION EMBRYONIC STEM CELL KNOCK-INS

The three newly created plasmid vectors targeting *iCre2* to *Itgb2l*, *Marco* and *Msr1* were used in a second round of ES cell transfection. G418 resistant clones were subjected to a screening regime similar to the one successfully applied in Chapter 6 in order to identify knock-ins. Briefly, the number of clones to be assessed was first reduced by a PCR covering the 5' homology arm, which allows the exclusion of random integrants. Candidates were confirmed as knock-ins by Southern Blots and further PCRs. Eventually, homologous recombinants could be successfully identified for *Itgb2l* and *Marco*, but not for *Msr1*. Subsequent Cre mediated Neo^R deletion was achieved, proving the functionality of the repaired loxP sites. Isolating individual Neo^R deleted clones, however, was associated with technical difficulties.

8.1 Transfection and Preliminary 5' PCR Screening

From two independent transfections, approximately 650 drug resistant colonies (summarized in Table 8.1) were isolated. Of those 48 each for *Itgb2l* and *Marco* and 24 for *Msr1* were analysed by PCR. The preliminary PCR screen equals the one described in Chapter 6.4. A gene specific external primer outside of the 4kb 5' homology region was paired with an *iCre2* specific primer (see maps in 8.1A & 8.2A). Only clones having undergone correct homologous recombination will amplify a product of the right size. As amplification occurs directly from the lysed cells of one half of a mES cell colony, the 5' PCR was optimized to detect the recombination event using low copy numbers (10.000 copies equalling 2pg of BAC) as a template (data not shown).

Results for *Itgb2l* and Marco are shown in Figures 8.1B and 8.2B. 5 potential *Itgb2l iCre2* knock-ins exhibited the 4.8kb band indicating homologous recombination: clones 8, 15, 17, 20 and 43. This gives a preliminary targeting efficiency of 10%. The band for *Itgb2l iCre2* 43, however, is very faint and needed further analysis. For suspected *Marco iCre2* recombinants, 10 clones: 10, 11, 23, 25, 29, 33, 37, 43, 44 and 48, resulted in the expected 4.8kb knock-in PCR band. If all of these clones were true knock-ins, this would correspond to a targeting frequency of 21%. Again, the bands for clones 11 and 43 were very weak, necessitating additional investigation. For *Msr1 iCre2,* 24 clones were assessed. Despite trying several primer pairs, polymerases and reaction conditions, no products, not even for the positive control knock-in BAC could be amplified (data not shown). Therefore, the following screenings focus on *Marco iCre2* and *Itgb2l iCre2*.

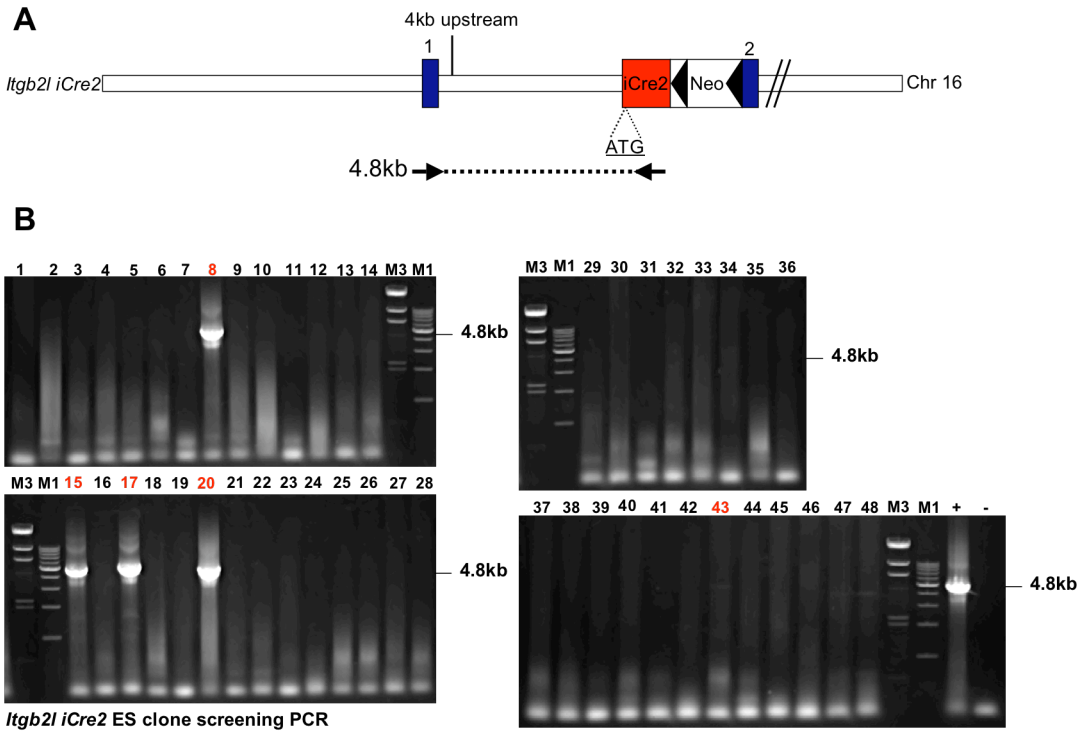


Figure 8.1 Diagnostic PCR Spanning the Upstream Homology Arm of *Itgb2l*

The structure of the correctly targeted locus for *Itgb2l iCre2* is illustrated in **A**, a forward primer (Paext) was placed just outside of the 4kb long upstream homology arm, the reverse primer (iCrecheck2) sits in the *iCre2* gene. Amplification is thus only expected if a knock-in was successful, resulting in a 4.8kb PCR product (**B**). Lanes where a product, indicating homologous recombination, was observed are labelled red.

M1: 1kb marker; M3: *λHindIII* marker; + *Itgb2l iCre2* knock-in BAC; - 129 DNA

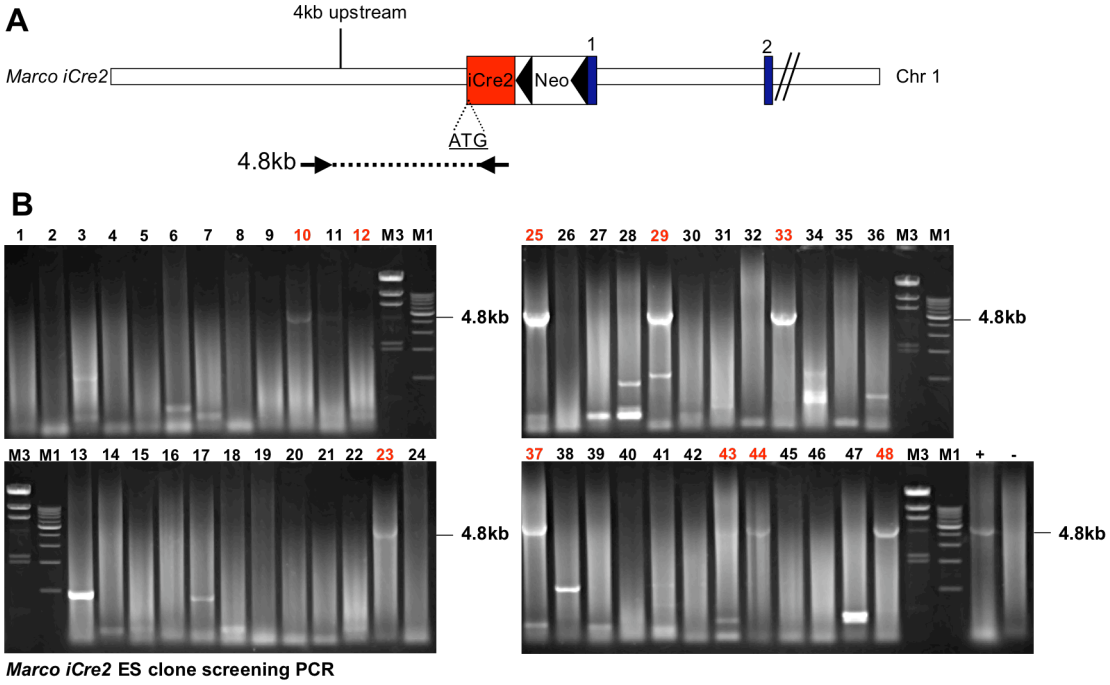


Figure 8.2 Diagnostic PCR Spanning the Upstream Homology Arm of *Marco*

The structure of the correctly targeted locus for *Marco iCre2* is illustrated in **A**, a forward primer (Maext) was placed just outside of the 4kb long upstream homology arm, the reverse primer (iCrecheck2) sat in the *iCre2* gene. Amplification is thus only possible if a knock-in was successful, resulting in a 4.8kb PCR product (**B**). Lanes where a product, indicating homologous recombination, was observed are labelled red.

M1: 1kb marker; M3: *λHindIII* marker; + *Marco iCre2* knock-in BAC; - 129 DNA

8.2 Secondary 3' PCR

After verifying the integrity of the upstream homology arm, the same PCR strategy was applied to the downstream area. The *Itgb2l iCre2* and *Marco iCre2* clones screened recombinant in the first PCR were re-assessed. A reverse primer was designed to bind just outside of the 7kb 3' arm, to amplify a ~7kb large PCR product when used in conjunction with a primer annealing to the Neo^R gene. Similar to the process described in 8.1, only correctly targeted loci will provide the template for a successful amplification (see maps in 8.3A&B). The length of the PCR product also indicates whether any large fragments were deleted during the recombination event. All of the 5 *Marco iCre2* knock-ins assessed showed a strong band corresponding to the expected 7.4kb fragment (8.3B&C). Working PCR conditions could not be established for *Itgb2l iCre2* (map shown in 8.3A, data not shown). Instead, a Southern Blot was used for those clones (see 8.3.2).

8.3 Southern Blots

Whilst the previously described PCR data is promising, a separate method was needed to verify homologous recombination. Therefore several Southern Blot strategies, covering both ends of the targeting zone, were designed to both confirm suspected knock-ins and exclude random integrants.

8.3.1 Southern Blot Analysis at the 5' End

Southern Blot strategies analyzing the 5' end of the targeted gene were devised for all 3 loci. Maps, chosen enzymes and expected fragments are illustrated in Figure 8.4A-C. Enzymes were selected for their ability to cut just outside of the 5' homology arm and the probe was also placed external to the targeted region. This method allows bands corresponding to either wild type or knock-in to be distinguished.

The *EcoRI* Southern Blot for *Itgb2l iCre2* is shown in Figure 8.5A and the *EcoRV/XhoI* Southern Blot for *Marco iCre2* in Figure 8.5B. Expected fragment sizes for *Itgb2l iCre2* were 9.6kb for the knock-in and 6.3kb for the wild-type locus. Clones 8, 15, 17 and 20 showed those bands in accordance with their PCR result. Clone 43, having shown a very weak PCR band, only had the wild type band and is thus not a knock-in. In case of *Marco iCre2*, all 8 assayed clones showed the correct combination of a 5.2kb wild type and a 6.2 knock-in band.

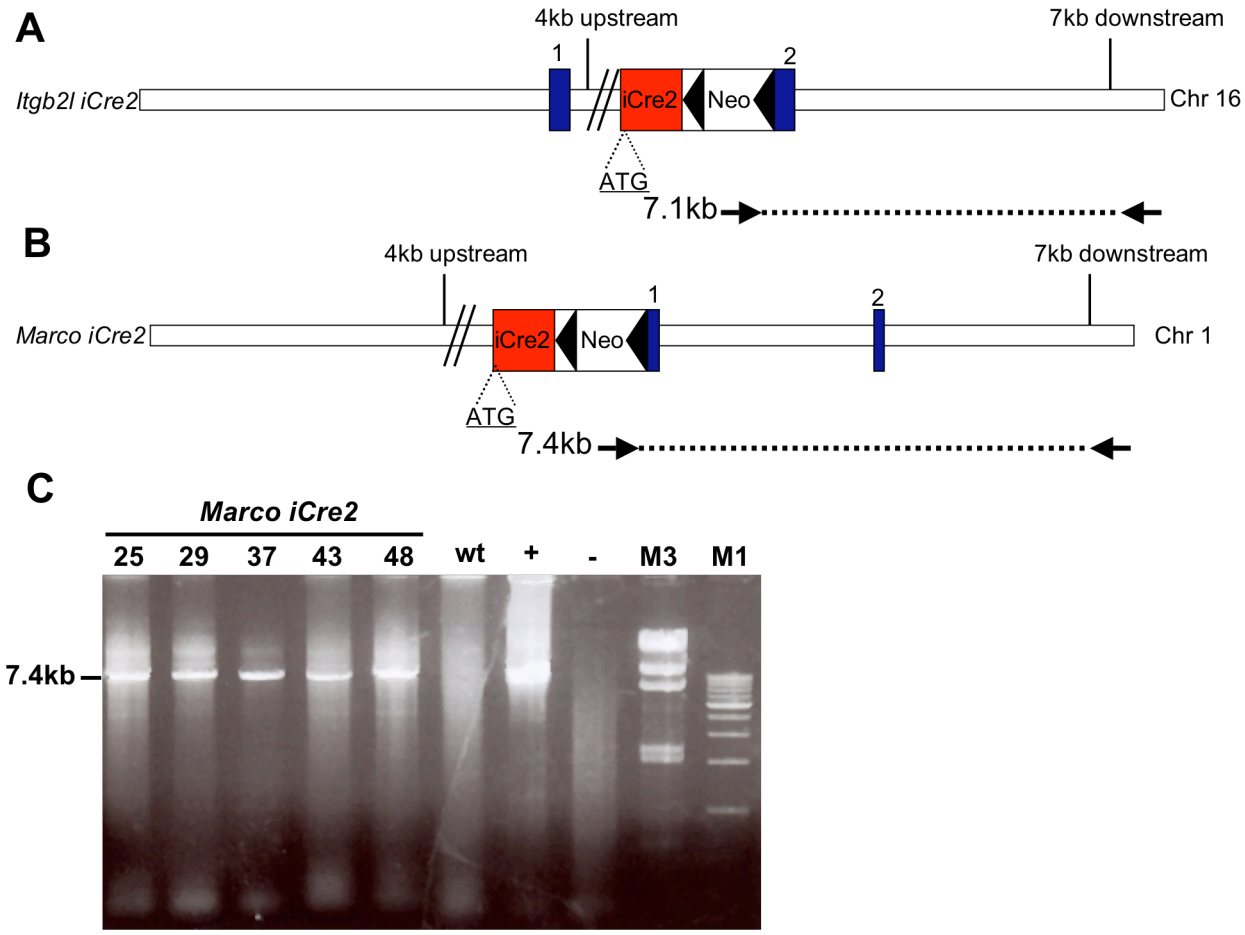


Figure 8.3 Diagnostic PCR Spanning the Downstream Homology of *Marco*
A and **B** show maps, primer locations and expected product sizes for the diagnostic 3' PCR for *Itgb2l iCre2* and *Marco iCre2* respectively. Working PCR conditions could only be established for *Marco iCre2* clones (**C**). 5 clones were tested; 50ng purified genomic DNA was amplified using primer pair Ma3'F/Ma3'B. Negative controls were 129 DNA (wt) or water (-). The *Marco* specific knock-in BAC was used as a positive control. (+)
M1: 1kb marker; M3: *λHindIII* marker

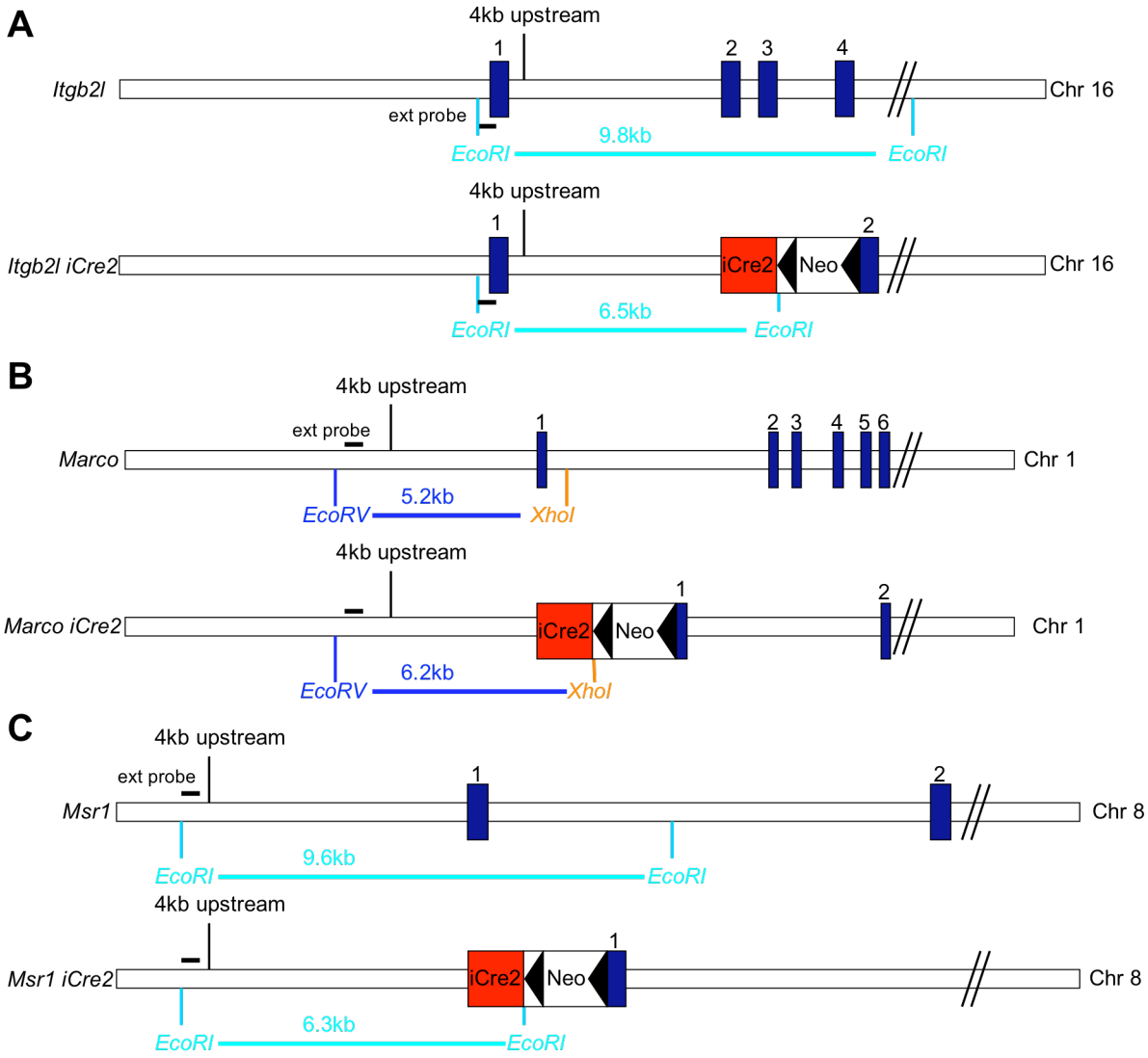


Figure 8.4 5' End Southern Blot Maps
Target loci for *Itgb2l* (**A**), *Marco* (**B**) and *Msr1* (**C**) are shown before (top) and after (bottom) *iCre2* knock-in. The border of the 4kb upstream homology arm is marked, bold black lines represent probes and the coloured lines indicate the expected product sizes. Probes were amplified from wild-type 129 genomic DNA using gene specific primer pairs and had a size of approximately 500bp.

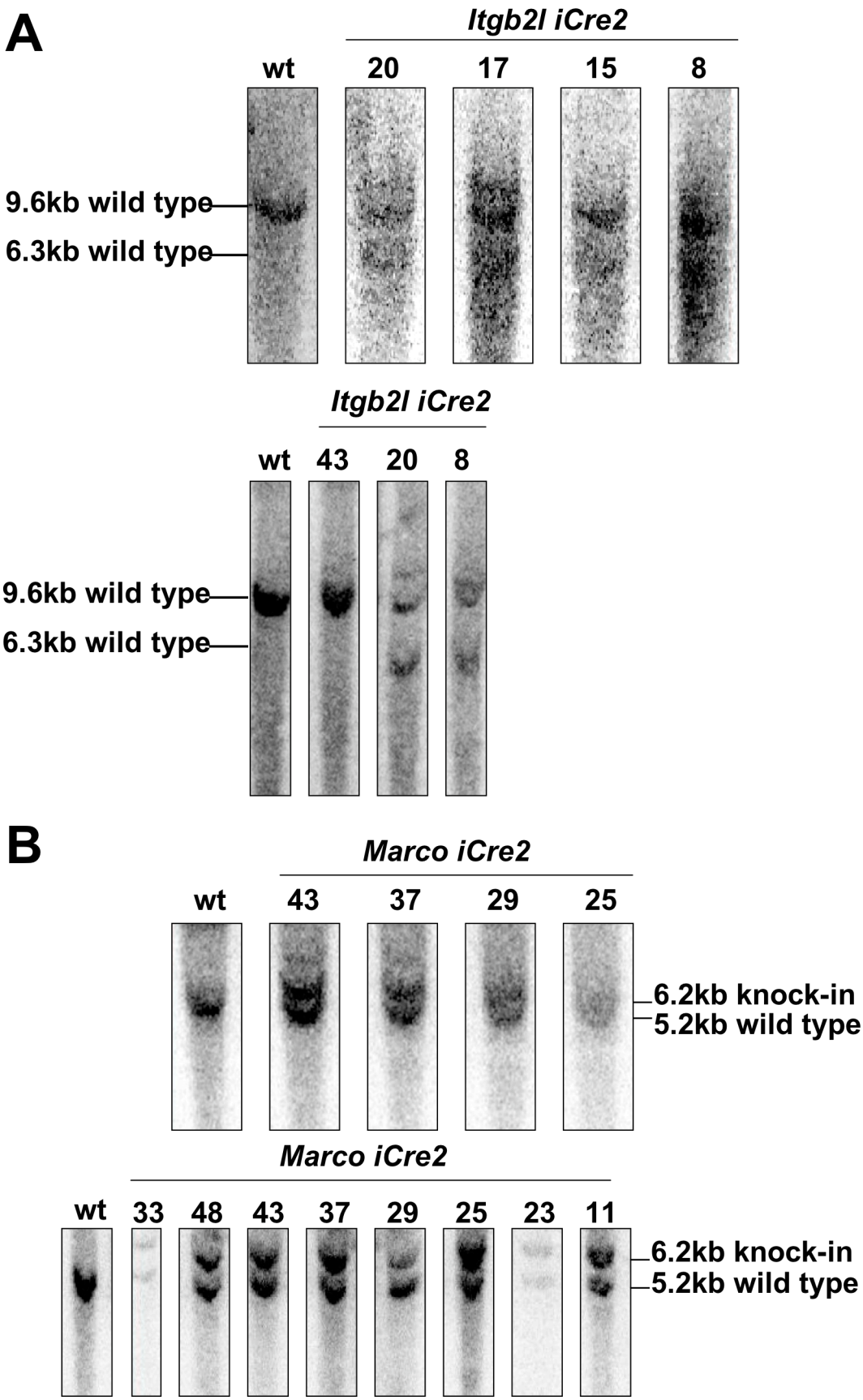


Figure 8.5 5' Southern Blot results for *Itgb2l iCre2* and *Marco iCre2*
Two separate Southern Blots are shown for both the *Itgb2l* (**A**) and *Marco* (**B**) locus. Probes, amplified from 129 wild type DNA, had sizes of 442bp and 444bp respectively. Wild type 129 genomic DNA served as a control. *Marco iCre2* clones 10 and 44 lost their ability to proliferate and could not be included in the Southern Blot.

8.3.2 Southern Blot Analysis at the 3' End

Additional Southern Blotting strategies were employed to probe the 3' end of the knock-in loci. Maps, chosen enzymes and expected fragment sizes are illustrated in Figure 8.6. Briefly, restriction enzymes that cut within the 7kb 3' homology arm and just beyond the border point were selected. The internal site was at least 3.5kb upstream of said point, which marks the boundaries of the targeting vector insert, in order to cover a significant piece of the 3' homology arm. Membranes were probed with both an internal and an external probe.

Figure 8.7A represents the result for the 4 *Itgb2l iCre2* knock-in clones hybridised with an external probe. Only one band, identical to the wild type, is visible. The same Southern Blot for *Marco iCre2* was unsuccessful (data not shown). No bands were visible, possibly due to low probe specificity. The PCR described in 8.2, however, produces equivalent data. Figure 8.7B and 8.7C show the outcomes of hybridization with an internal probe for *Itgb2l iCre2* and *Marco iCre2*, respectively. In both Southern Blots, only one band was visible; that of the wild type.

8.4 Overview of 2nd Generation Targeting

Target Genes	No. of G418 Resistant Clones Isolated*	No. of PCR Screened Clones**	No. of PCR Positive Clones	Of those Confirmed by Southern Blot	Targeting Frequency
Itgb2l	224	48	5	4	8%
Marco	224	48	10	5***	10%
Msr1	200	24	n/a	n/a	n/a

* of two independent transfections/target gene ** all derived from 1st transfection *** no data available for other 5 clones

Table 8.1 Distribution and Screening Results for 2nd Generation *iCre2* Knock-ins

Out of a total of ca. 650 resistant colonies, 120 clones were screened to identify 9 confirmed knock-ins; 4 for *Itgb2l iCre2* and 5 for *Marco iCre2*. This corresponds to total targeting frequencies of 8% and 10% respectively. *Msr1 iCre2* clones were not further assayed, as working conditions for the initial PCR screening could not be established.

8.5 *Neo*^{R} Deletion

In a final step of mES cell work, attempts were made to remove the floxed G418 selection marker in order to avoid deleterious influences of the expression unit on Cre function in the mouse model (see Chapter 2.3). To that end, several knock-in clones were transiently transfected with the Cre expressing vector pCre-Pac The procedure is identical to the one described in 6.5. In bulk cultures, the resistance gene could be deleted effectively, as assayed by PCR and Southern Blot. The isolation of individual clones, however, presented some technical challenges.

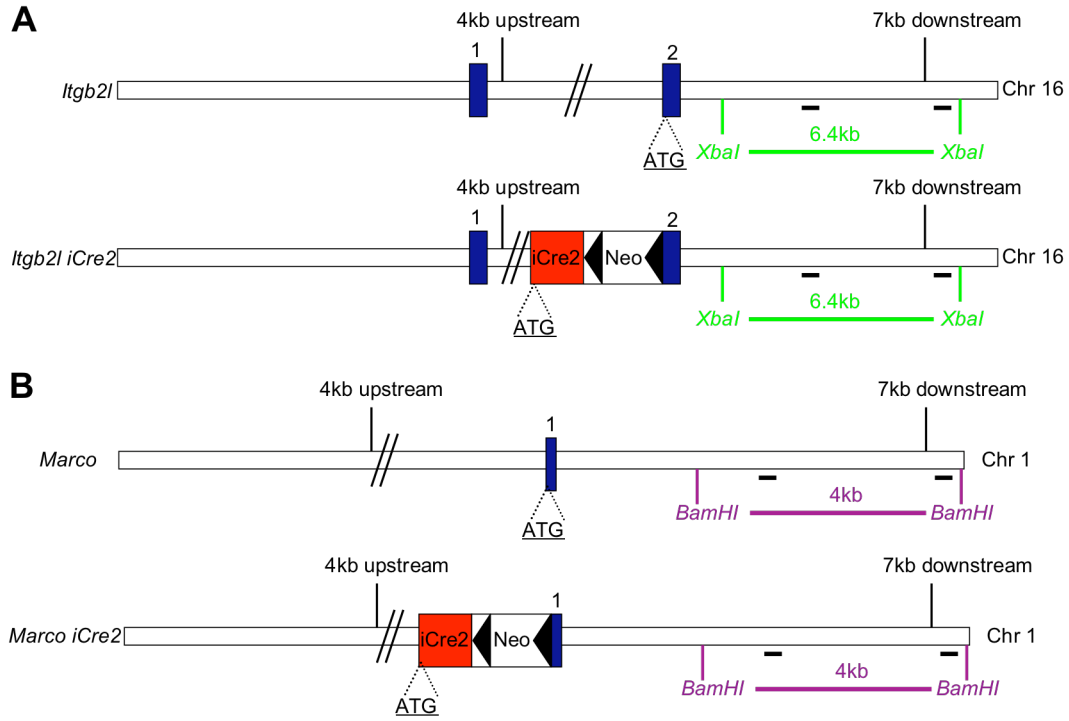


Figure 8.6 Southern Blot Strategies for the 3' Homology Arm
The *Itgb2l* (**A**) and *Marco* (**B**) loci are shown before (top) and after (bottom) targeting. Bold black lines represent probes and coloured lines indicate expected fragment sizes. Probes were amplified from wt 129 DNA using gene specific primer pairs.

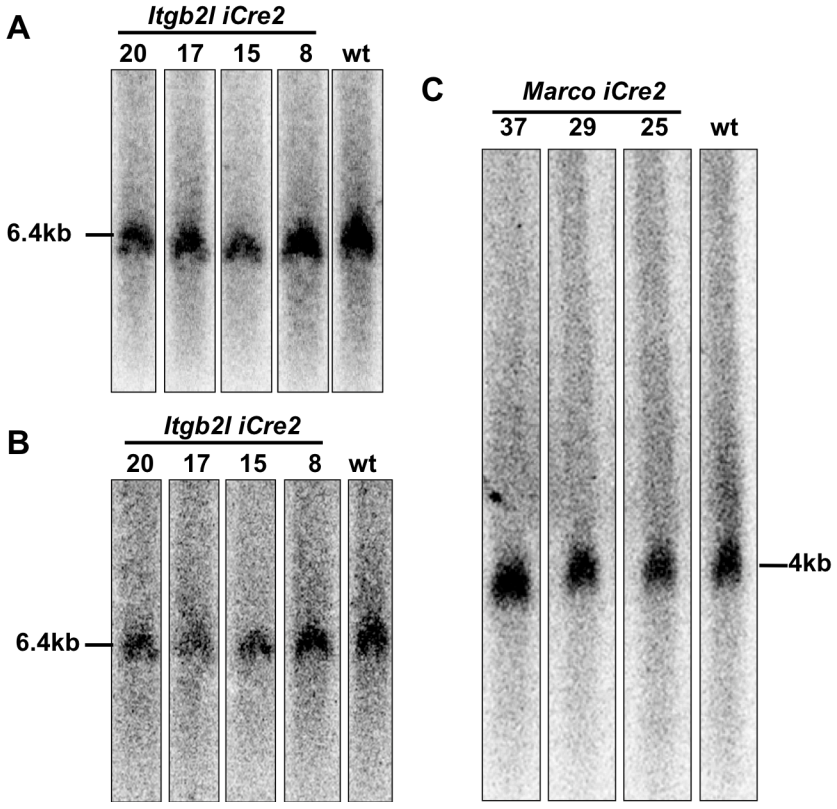


Figure 8.7 3' Southern Blot Results for *Itgb2l-iCre2* and *Marco-iCre2*
The *Itgb2l iCre2* clones were digested with *XbaI* and hybridized with an external probe (**A**) or, after stripping the same membrane, with an internal probe (**B**). Probes (884bp or 881bp respectively) were amplified from wild type DNA. **C** *Marco iCre2* clones were digested with *BamHI* and hybridized with an internal probe (844bp).

8.5.1 Successful *Neo*^{R} Deletion in Bulk Culture

Knock-in clones *Itgb2l iCre2* 17 and *Marco iCre2* 25 were transfected with 20µg pCre-Pac and DNA was isolated from bulk cultures after 48h. A diagnostic PCR was designed to generate a 2.5kb fragment for non-deleted DNA and a 1kb fragment in case *Neo*^{R} was excised successfully (Figure 8.8A). The bulk PCR (Figure 8.8B) showed the presence of both bands in either clone, indicating successful deletion in a fraction of the cells.

8.5.2 Isolating individual *ΔNeo*^{R} colonies

Following a positive result in the bulk PCR, transfected cells were plated at low densities (500 cells/ml) onto feeder layers to isolate individual subclones. Unfortunately the plating efficiency was very low, with less than 2% of the cells forming colonies. Colonies were very small and failed to proliferate after isolation. Another transfection, using less (2µg) pCre-Pac plasmid to exclude potential Cre toxicity, yielded no viable clones either. Nor did a new batch of growth serum (pre-tested for embryonic stem cells) improve embryonic stem cell survival. Eventually, the failure of mES cells to expand when plated at low density was traced to a protracted Mycoplasma infection, which was most likely transmitted from a batch of contaminated feeder cells in the initial stages of mES cell targeting. Cells were treated successfully with BM-Cyclin (Roche), and remain Mycoplasma-free to date.

Once individual colonies could be expanded, they were subjected to colony PCR (an example is shown in Figure 8.9). A summary of all pCre-Pac transfections, from which approximately 320 separate subclones were isolated, is given in Table 8.2. The efficiency of *Neo*^{R} excision, i.e. the fraction of isolated colonies showing the deletion PCR product, varied between 7 and 25%, depending on the amount of pCre-Pac used and the transfected clone.

Clone	Amount pCre-Pac	Clones deleted/Clones Isolated	Fraction	Cell Plating Density in cells/ml
Marco iCre2 25	20µg	5/33	15%	500
Marco iCre2 25	5µg	6/72	8%	200
Marco iCre2 29	5µg	3/48	6%	100
Marco iCre2 37	5µg	6/48	12.5%	50
Itgb2l iCre2 17	5µg	12/48	25%	500
Itgb2l iCre2 20	5µg	5/72	7%	200

Table 8.2 Overview of Transient Cre Transfection Experiments

Following identification by PCR, aliquots of several clones (descendants of *Marco iCre2* 29; *Marco iCre2* 25*; Itgb2l iCre2* 17 and *Itgb2l iCre2* 20) were subjected to G418 selection as a control. 1000 cells per clone were plated in 12 well (12w) plates on gelatin and selected with 250µg/ml G418 for 5 days. With removal of the *Neo*^{R} gene, cells should have become

susceptible to G418. Interestingly, the majority of tested clones were not killed by G418. Only one clone, designated *Marco iCre2* 29*ΔNeo*20, exhibited the expected behaviour and did not expand under these conditions.

To analyze the clones further, a Southern Blot was designed for both the *Itgb2l* and *Marco* loci. A *BamHI* (Figure 8.10A&B) digest liberates fragments differing in size depending on the presence or absence of the Neo^R gene. In case of *Itgb2l iCre2* fragment sizes were 4.5kb or 2.9kb, for *Marco iCre2* they were 6.2kb or 4.5kb. The Southern Blot revealed two surprising results. For the descendants of the two *Itgb2l iCre2* knock-in clones (8.10C), no subclone had a pure deleted genotype with only the 2.9kb band present. On the contrary, 6 of the assayed 9 subclones only showed the 4.5kb band indicating the presence of the Neo^R gene. Three subclones, two derived from *Itgb2l iCre2* 17 and one from *Itgb2l iCre2* 20, resulted in both bands, indicating partial deletion of about 75% of the population (Figure 8.10D) as evidenced by measuring the Southern Blot signal intensity.

Subclones descended from *Marco iCre2* knock-in clones 25 and 29, exhibited a similar result (Figure 8.10E). 3 out of 4 subclones had mixed deleted and non-deleted genotypes, with the deleted population encompassing about 25% (Figure 8.10F). Subclone *Marco iCre2* 29*ΔNe*o20, however, showed a pure deleted genotype, as expected from its susceptibility to G418. Curiously, the non-deleted control DNA isolated from *Marco iCre2* knock-ins expanded without G418 before pCre-Pac transfection, also presented both bands, indicating partial deletion.

8.6 Discussion

8.6.1 Identification of Homologous Recombinants

The previously designed *iCre2* vectors for *Itgb2l*, *Marco* and *Msr1* were used to target murine embryonic stem cells. From two independent transfections, around 200 G418 resistant colonies were isolated per gene (sum: 650), to ensure obtaining several homologous recombinants. Significantly more colonies were isolated than for the BAC targeting vectors used earlier, as the targeting frequencies of the new shorter plasmid vectors were unknown and could have been much lower than the 8% observed for *Itgb2l* in Chapter 6. The total number of drug resistant colonies generated for both independent *Itgb2l iCre2* and *Marco iCre2* transfections was roughly the same with about 300-500 per experiment. For the first *Msr1 iCre2* transfection, only around 50 colonies were obtained, in the second round, however, this increased to approximately 400.

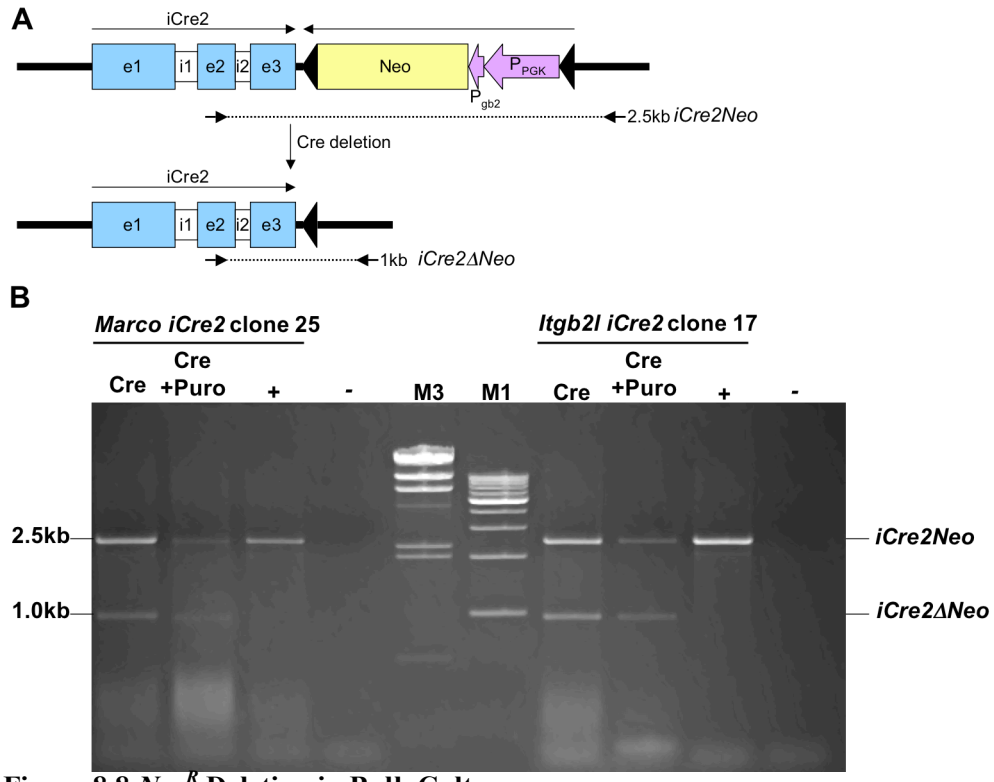


Figure 8.8 *Neo*^{R} Deletion in Bulk Culture

An *iCre2* specific forward primer binding in exon 2 (NeoExcF) was paired with a gene specific reverse primer (PaNeoExcB or MaNeoExcB) to enable screening for deletion. 2.5kb or 1kb PCR fragments (**A**) are generated depending on the locus structure. Bulk DNA from clones transiently transfected with 20µg pCre-Pac was subjected to PCR after 48h (**B**). Both bands are visible, suggesting the deletion of the floxed selection marker. A fraction of the cultures was subjected to a 24h transient puromycin (Puro) selection. DNA isolated from non-transfected knock-ins served as a positive control (+) and only showed the 2.5kb band. M1: 1kb marker; M2: *λHindIII* marker; - no template

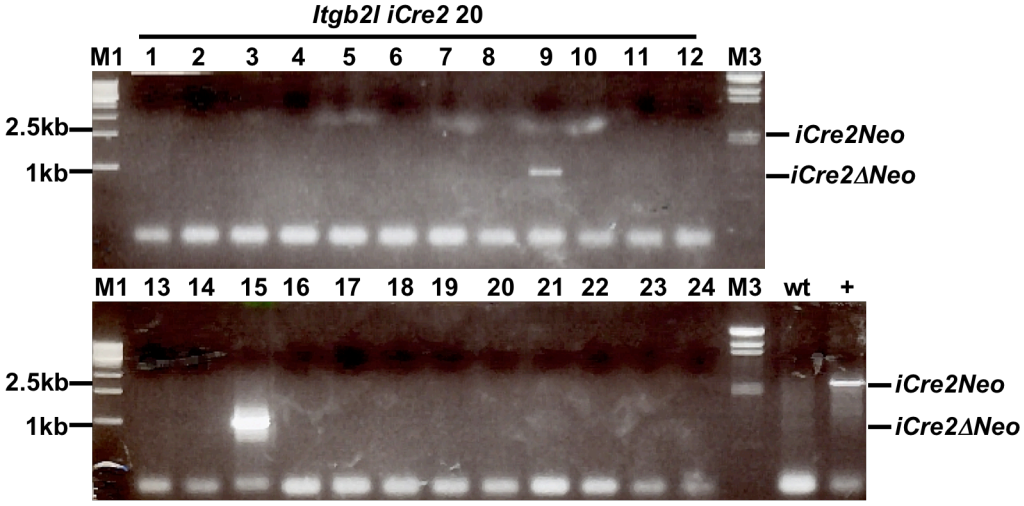


Figure 8.9 Colony PCR for *Neo*^{R} Deletion

This figure shows an example of the colony PCR employed to screen for *Neo*^{R} deletion. *Itgb2l iCre2* 20 had been transfected with 5µg pCre-Pac, plated at a density of 200 cells/ml for 10 days before 24 clones were subjected to PCR. DNA from non-transfected cells served as a positive control (+).

M1: 1kb marker; M3: *λHindIII* marker; - no template

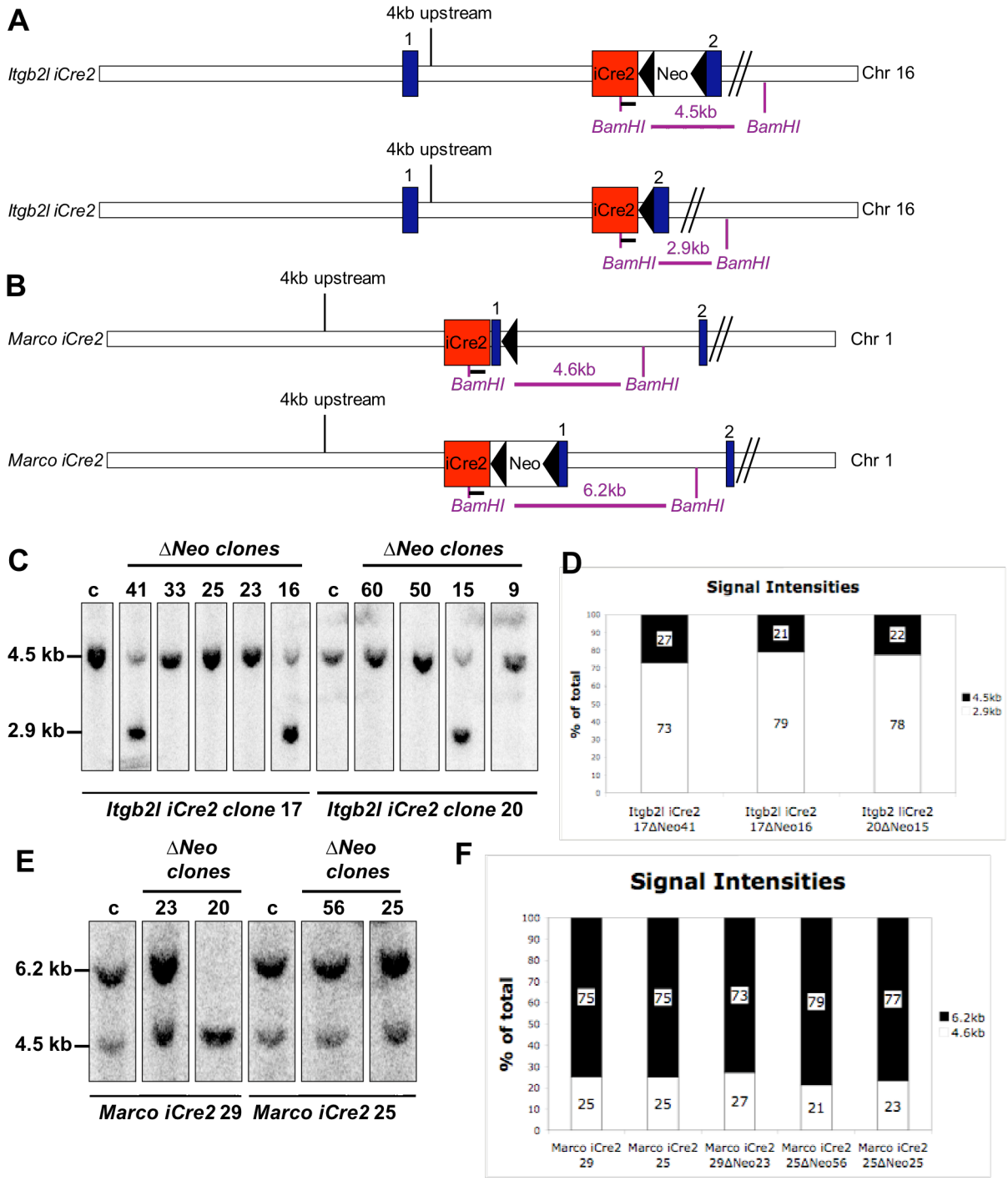


Figure 8.10 Southern Blot Analysis of *Neo*^{R} Deletion
Both *Itgb2l* (**A**) and *Marco* (**B**) knock-ins before (top) and after (bottom) Cre mediated deletion are shown. *BamHI* fragments probed with a Cre-specific PCR product were used to screen the potentially *Neo*^{R} deleted clones of *Itgb2l iCre2* (**C**) and *Marco iCre2* (**E**). c marks control DNA samples isolated prior to deletion. For lanes having two visible bands, signal intensities were quantified using ImageJ (**D&F**).

The shorter upstream homology arm (4kb) allowed skipping the PCR exclusion strategy applied in Chapter 6 and enabled the pre-selection of clones by a 5' PCR. For *Itgb2l iCre2* and *Marco iCre2*, the PCR was very efficient in selecting knock-in candidate clones (Figures 8.1 and 8.2). Consequently, it sufficed to assess just 96 of the total 448 clones. As working PCR conditions could not be established for the 24 tested *Msr1 iCre2* clones, all following work focused on *Marco* and *Itgb2l*. All *Msr1 iCre2* clones, however, are archived for future tests. Alternative options to identify homologous recombinants among *Msr1* include using different primers (so far three forward primers binding just outside the upstream homology arm were tested together with the Cre specific reverse primer iCrecheck2), using different polymerases (a Taq/Pfu combination 25:1 was used in all samples), performing the initial screening using a 3' PCR, narrowing down clone numbers by excluding those harbouring the vector backbone or skipping initial PCR analysis and focusing on Southern Blots (Figure 8.4C).

All *Itgb2l iCre2* and *Marco iCre2* knock-in candidates were subjected to a 5' Southern Blot with an external probe (Figure 8.5). The placement of the restriction enzyme sites and the utilization of a probe external to the targeting area ensures that only clones in which homologous recombination occurred are visualized. The shorter length of the upstream homology arm enabled a greater choice of restriction enzymes and simplified the screening considerably. All clones assessed, with the exception of *Itgb2l iCre2* 43, were found to be knock-ins with one allele of the respective target gene being modified and the other representing the wild type locus.

Gene targeting with sequence replacement vectors, such as those used herein, requires two reciprocal recombination events, one at each end of the targeting vector (Deng et al., 1993; Li et al., 2001; Sorrell and Kolb, 2005). Both crossing over incidents entail breaking and re-joining of DNA molecules. 5' PCR and 5' Southern Blot have assessed genetic integrity of the upstream homology arm, but further tests were needed to confirm the downstream homology arm. To that end, 3' Southern Blots and 3' PCRs were employed. In the 3' Southern Blot restriction enzymes cutting within the downstream homology region and just outside of the targeting area were utilized. Knock-in clones should, when hybridized with an external probe, only exhibit the wild type band, indicating that no gross re-arrangements or deletions have occurred in the downstream homology area. The blot for *Itgb2l iCre2* (Figure 8.7A) showed this very result, with none of the 4 knock-ins revealing any gross abnormalities. Results for *Marco iCre2* clones were inconclusive as, possibly due to low external probe specificity, bands could be not distinguished. In lieu thereof, the 3' PCR for *Marco iCre2* clones (Figure 8.3) serves to exclude the presence of major deletions.

The 3' Southern Blot, re-probed with an internal probe, also helped to verify the absence of additional random integration (Figure 8.7B&C). The presence of only one band, equivalent to the wild type, is the expected result in the absence of random integrants. Clones that have the vector integrated randomly within their genome carry the same upstream restriction site, but the closest downstream restriction site would be dependent on the random locus and likely result in additional bands of different sizes. Similarly, if vector concatemers had recombined into the locus additional bands of different sizes would occur. Neither *Itgb2l* nor *Marco* Southern Blots reveal any additional bands beyond the wild type. Insert duplication or additional random integration can thus be excluded. Those findings are consistent with reports in the literature that homologous recombination and random integration do not frequently happen in the same cell, due to either low probability of two such rare events occurring simultaneously (estimated at 10^{-9} per cell) (Waldman, 1992) or the cellular pathways involved in homologous recombination (double strand break repair; DSBR) and random integration (non-homologous end joining; NEJ) not being directly competitive (Ng and Baker, 1999; Reid et al., 1991).

One drawback of the 3' Southern is that, due to the absence of suitable restriction sites, it does not cover the entire 3' homology arm. Also, though there is no indication of additional random integration, the employed technique cannot distinguish random bands of the same size as the wild type. Alternatives in excluding random integrations are further quantitative Southern Blots that allow establishing the knock-in gene copy number. Options include the use of restriction sites and probes internal to the incoming sequences to allow the quantitation of band intensities (as has been done in Chapter 6) or utilization of a copy number standard (by including the vector).

When all screenings were taken together, homologous recombinants could be identified for both the *Itgb2l* and *Marco* loci, the results are summarized in the following Table 8.3.

8.6.2 Targeting Frequency

The targeting frequency for the *Itgb2l* locus was 8%, identical to the value observed in Chapter 6, when the *Itgb2l iCre2* BAC vector was utilized. For *Marco*, a 10% targeting frequency was established. Several candidate clones, however, lost their ability to proliferate in culture before all screenings could be performed. If all 8 clones assessed as homologous recombinant during the 5' Southern Blot were actually knock-ins, the frequency would be a high 17%.

Targeting Construct	Clones selected by 5' PCR	HR Confirming Result in			Considered Knock-in Clone
		3' PCR	5' Southern Blot	3' Southern Blot	
Itgb2l iCre2	8	NDA	YES	YES	YES
	15	NDA	YES	YES	YES
	17	NDA	YES	YES	YES
	20	NDA	YES	YES	YES
	43	NDA	NO	NA	NO
Marco iCre2	10	NDA*	NDA*	NDA*	NO**
	11	NDA*	YES	NDA*	NO**
	23	NDA*	YES	NDA*	NO**
	25	YES	YES	YES	YES
	29	YES	YES	YES	YES
	33	NDA*	YES	NDA*	NO**
	37	YES	YES	YES	YES
	43	YES	YES	NDA*	NO**
	44	NDA*	NDA*	YES	YES
	48	YES	YES	YES	YES

Table 8.3 Identification of Knock-ins for *Itgb2l* and *Marco*
The table lists the screening results for the *Itgb2l iCre2* and *Marco iCre2* samples that were selected as candidates by 5' PCR and whether they were eventually considered to be homologous recombinants. NDA – no data available; NA – not assessed; NDA* – no data available due to clones loosing ability to proliferate in culture; NO** – not considered knock-ins as not all screenings could be performed, HR Homologous Recombination

Targeting frequencies depend on the locus (Hasty et al., 1994), the length of total homology (Hasty et al., 1991) and the selection strategy applied (Mansour et al., 1988). The presented work used a total homology of 11kb and a simple positive selection approach. Table 8.4 lists several similar experiments, lengths of homologies utilized and the corresponding targeting frequencies. Overall, frequencies observed in this work are in the same (8% for *Itgb2l*) or slightly higher (10-17% for *Marco*) range, which can be explained by using homology arms almost two times longer than those listed. The results indicate that the *Marco* locus is more amenable for homologous recombination than *Itgb2l*. Targeting frequencies could have been further increased by using an additional negative selection marker (HSVtk), which typically provides a 2-5 fold enrichment for correctly targeted clones (Sedivy and Dutriaux, 1999).

Targeted Locus	Sum Homology	Upstream	Downstream	Targeting Frequency	Reference
Various	5-8kb	n/a	n/a	1-5%	(Sorrell and Kolb, 2005)
Fgr (src kinase)	6kb	2kb	4kb	5%	(Hasty et al., 1994)
Fah (fumarylaceto-acetate hydrolase)	6kb	1.4kb	3.6kb	7.5%	

Table 8.4 Overview of Targeting Efficiencies
Summary of targeting frequencies obtained when autosomal mouse genes were targeted with sequence replacement vectors carrying single positive selection markers.

8.6.3 Technical Obstacles in Removing the G418 Resistance Marker

In bulk culture, deletion of the *Neo*^{R} gene by transient Cre expression could be verified for all six assessed knock-in clones (Figure 8.8). Genomic DNA isolated from such cells amplified deleted and non-deleted products. The non-deleted band appeared stronger, which coincides well with the assumption that only a fraction of cells is successfully transfected by electroporation. In an earlier experiment, using an EGFP expressing reporter plasmid, the transfection efficiency was around 15% (data not shown). Subclones assessed by colony PCR (Figure 8.9) established the fraction of deleted colonies to be in the same range (Table 8.2), corresponding to values between 2-15% reported in the literature (Abuin and Bradley, 1996; Sauer and Henderson, 1989).

Subclones screened as deleted, however, were not killed by G418 selection. Whilst massive cell death was observed around day 4, a significant fraction of the cells continued to thrive. Unmodified embryonic stem, grown under the same conditions, died within 7 days. A Southern Blot (Figure 8.10) revealed that the majority of samples exhibited a mixed genotype of *Neo*^{R} containing and *ΔNeo* genomic DNA. There are two possibilities to explain these results:

1. Isolated colonies derive from more than one cell and are not actually clonal, but mixed populations containing both cells successfully transfected by pCre-Pac and those that were not.
2. Colonies are derived from single cells, but transfected pCre-Pac became active during or after cell division, which led to an asymmetric distribution of *Neo*^{R} deletion.

The second option appears plausible in the transfections of *Marco iCre2* 25 and *Itgb2l iCre2* 17, as cells were diluted and plated for clonal expansion immediately after electroporation, when considering the short doubling times of murine embryonic stem cells of around 12h (Udy et al., 1997). For all other transfections (Table 8.2), however, cells were plated as bulk for 48h before being cloned. After two days recombination should be complete, and since the enzymatic equilibrium favours the deletion of *Neo*^{R}, the process is irreversible.

The first option, colonies not being of clonal origin, appears more likely. Embryonic stem cells tend to clump together, despite taking great care to plate dilute single cell suspensions. The number of cells plated per 10cm feeder plate was stepwise reduced from 5000 to 1000. Those numbers resulted in a low density of colonies evenly spaced across the entire dish allowing easy isolation. However, as no selection is applied and pCre-Pac transfection is relatively inefficient, the possibility of colonies deriving from more than one cell and harbouring different genotypes cannot be excluded. Subclone *Marco iCre2* 29*ΔNe*o20, which has a pure deleted genotype, shows that the method's general principle is sound.

The PCR utilized to identify deleted subclones (Figure 8.8) inadvertently encouraged the expansion of mixed colonies. Whilst faithfully amplifying the 1kb deleted band; the larger 2.5kb non-deleted band could not be obtained reliably thus largely precluding the identification of mixed populations at this stage. As one lysed half of a picked colony served as template, this is likely due to cellular debris and low template copy number inhibiting polymerase amplification. The complete Neo^R cassette spans about 1.5kb, so placing the primer pair slightly closer together, for example by choosing a forward primer that aligns within exon 3 of *iCre2* instead of exon 2 (Figure 8.8A), could have improved this PCR screen, though the final product size cannot be reduced much beyond 2kb.

Re-cloning of mixed cell lines at a lower density (500 cells per 10cm plate yielding a few dozen colonies) and scoring isolates by PCR, Southern Blot and G418 selection largely alleviated the problem for *Itgb2l.* Whilst some samples remained mixed populations, several colonies of a pure deleted genotype were obtained. Subcloning of *Marco* samples, however, only resulted in G418 resistant colonies. Colony PCRs only exhibited the 2.5kb Neo^R containing bands. The reason for the apparent loss of the deleted cell fraction is unclear, but may be related to its low proportion of just one quarter among the whole population (Figure 8.10D). For *Itgb2l*, this fraction was more than three quarters (Figure 8.10F), which seems to indicate that the locus is more accessible for Cre mediated recombination.

By and large, the problem of mixed genotypes is mainly caused by low transient Cre transfection efficiency. Increasing the frequency of Cre mediated recombination and thus the proportion of the *ΔNeo* population would help to reduce the appearance of mixed genotype cell lines, even for non-clonal colonies arising from several cells. Utilizing alternative methods to ensure a higher efficiency of Cre delivery to ES cells *in vitro* would be a sensible choice in future experiments. Adenoviral Cre, for example, successfully infected and recombined 91% of an embryonic stem cell population. This high fraction meant that the isolation of clones was not necessary. Mixtures of deleted and non-deleted cells were used directly for blastocyst injection 20h after infection, allowing significant time savings. Chimeras reliably transmitted the deleted genotype to their offspring (Shui and Tan, 2004). Similarly, varieties of cell permeant Cre protein have been used to treat cultured cells, including mES cells. Depending on the chosen Cre protein, cell line and recombination target, between 20% and 80% of all cells were recombined successfully (Jo et al., 2003; Lin et al., 2004; Patsch and Edenhofer, 2007).

In conclusion, *ΔNe*o subclones were eventually isolated for both *Marco* and *Itgb2l* knock-ins. Due to the hurdles encountered, however, embryonic stem cells were kept in culture for an extended period of time and manipulated frequently. The stock of ES cells used in this work was passage 12. Resistance marker containing knock-in clones are around passage 20, whereas

confirmed deleted clones are between passages 35-40. A high number of passages have been reported to reduce mES ability to contribute to the mouse germ line, likely due to an accumulation of genetic and epigenetic changes influencing pluripotency (Fedorov et al., 1997; Kondoh et al., 1999; Liu et al., 1997). Therefore, it may be prudent to use earlier passage Neo^R containing knock-ins for blastocyst injection and remove the resistance marker *in vivo*. Possible methods are discussed in Chapter 10.

8.6.4 Basal *iCre2* Expression in *Marco* Knock-in Clones

The Southern Blot aimed at confirming Neo^R deletion, also revealed the presence of low level Cre activity within *Marco* knock-ins. Control DNA of both tested clones 25 and 29, grown without G418, exhibited deletion of the resistance marker in the absence of transiently expressed Cre (Figure 8.10E) in 25% of the population. Therefore the activity must have arisen from within those cells through *iCre2* expression from the *Marco* locus. Whilst this shows principle functionality of the *iCre2* coding sequence and protein, it also indicates promoter leakiness as *Marco* has thus far only been described as active during macrophage development (see Chapter 3.2.3). Contrariwise *Itgb2l*, the candidate for neutrophil specific Cre expression (see Chapter 3.2.1), showed no such behaviour in undifferentiated embryonic stem cells (Figure 8.10C). The observed activity could have several reasons:

1. Low level *Marco* activity is a part of basal embryonic stem cell gene expression.
2. *Marco* plays a role in embryonic development that is yet to be described.
3. *Marco,* and consequently *iCre2* expression, are dysregulated by the presence of Neo^R within the locus.

The finding that embryonic stem cells express 50% more genes than differentiated adult cells supports the first theory (Golan-Mashiach et al., 2005). Similarly, genome wide gene trapping in embryonic stem cells has shown that around 2/3 of all mouse genes are expressed to a high enough level to be trapped by promoterless vectors (Nord et al., 2006; Schnutgen et al., 2008; Skarnes et al., 2004). It has been suggested that embryonic stem cells maintain a broad basal level of gene expression as a preface for cell fate determination. Major transcriptional changes occur during differentiation, a large number of genes are down-regulated, whereas tissue specific expression is increased (Fathi et al., 2009; Ivanova et al., 2002; Sharova et al., 2007). Thus gene expression in the undifferentiated state *in vitro* is not necessarily an accurate model for assessing tissue specific gene activity in the adult *in vivo*.

A recently discovered link between pluripotent stem cells and macrophages sustains the second assumption. Murine embryonic stem cells were found to share macrophage abilities, such as phagocytosis – a process in which *Marco* is involved. In addition, peritoneal macrophages and

embryonic stem cells were shown to have remarkable similarities within their transcriptome (Charriere et al., 2006). Likewise, a multipotential subpopulation of peripheral macrophages has been discovered. These cells can, if stimulated with appropriate growth factors, differentiate into neuronal, hepatic, endothelial, epithelial and lymphocytic cells and thus resemble embryonic stem cells (Kuwana et al., 2006; Ruhnke et al., 2005; Zhao et al., 2003).

Thirdly, wide spread expressional dysregulation by the selection cassette has been reported. The Neo^R gene, due to its procaryotic origin, carries numerous cryptic splice sites that can cause aberrant splicing (Ren et al., 2002). Also, a high CpG content can adversely affect expression *in cis* (Jin and Mann, 2005). The strong promoter (PGK1) has the potential to influence gene expression across several 100kb (Olson et al., 1996; Pham et al., 1996) (Chapter 2.3).

On the whole, *iCre2* expression in undifferentiated ES cells could be detrimental to the envisaged macrophage specific conditional mouse model if the activity persists throughout embryonic development at a high enough level to mediate recombination *in vivo*. Partial recombination in the blastocyst would lead to a mosaic adult genotype, negating the objective of tissue specificity. Nevertheless, the actual *iCre2* expression pattern can only be verified in the mouse model *in vivo*.

8.6.5 Summary

In conclusion, the herein reported findings confirm that the use of homology arms of 4kb upstream and 7kb downstream is a good compromise between obtaining high targeting frequencies and facilitating straightforward screening. The utilized shortened plasmid vectors are therefore superior to the BAC vectors employed in Chapter 6. *iCre2* knock-ins with no obvious chromosomal rearrangements or additional random insertions were generated for both *Itgb2l* and *Marco* loci. The number of individual knock-in clones (4 for *Itgb2l* and 5 for *Marco*) should be sufficient. General recommendations are that, when working within the 129 background, 3 independent clones per target gene should be injected to obtain sufficient numbers of high-level coat colour chimeric mice to ensure germ line transmission (Ware et al., 2003). The problematic positive selection cassette removal suggests the exploration of alternative methods to accomplish this feat *in vitro* or moving it to a later stage of the mouse production process *in vivo*. The general expressibility and protein functionality has been shown in *Marco* knock-ins as the Southern Blot designed to detect selection marker removal indicated deletion in the absence of extraneous Cre recombinase. However, as it also raises the question of potential *Marco* promoter leakiness, *Itgb2l iCre2* clones may be the first choice for producing mice at this stage.

CHAPTER 9: GENERATING EMBRYONIC STEM CELL DERIVED MACROPHAGES

In a final part of the project, an attempt was made to assess *iCre2* expression specificity *in vitro*. To that end, a protocol was adapted to differentiate knock-in embryonic stem cells along the haematopoietic pathway. As macrophages are easier to obtain than neutrophils, the primary focus lay on *Marco iCre2* knock-in lines. Differentiated cells were identified using specific surface markers; expression of *Marco* and i*Cre2* was analyzed on the total RNA level and with the help of a newly created reporter cell line able to switch on GFP expression after Cre mediated recombination. Whereas macrophages could be created successfully, expression of *iCre2* could not be verified.

9.1 Conditions for Haematopoietic Development

Initially, conditions were established that permit haematopoietic differentiation of IB10 murine embryonic stem cells. The three-dimensional embryoid body (EB) method was applied to induce early mesodermal differentiation (Keller et al., 1993; Wiles and Keller, 1991). Three approaches were used to form EBs: culture in hanging drops (Wang and Yang, 2008), dilute culture in suspension and methyl cellulose based semisolid culture (Keller, 1995).

The suspension culture protocol involved dilute suspensions of ES cells (100-500 cells/ml) plated onto bacteriological grade dishes. Within a week, small EBs became apparent. Efficiencies were relatively low. On average, only about 10% of all cells formed EBs in this culture system. Further growth of such EBs was also hampered by their strong tendency to adhere to all tested types of culture dishes. Early adherence and spreading of EBs significantly reduces the development of haematopoietic and other tissues by hindering orderly three-dimensional differentiation (Bautch et al., 1996; Dang et al., 2002).

Semi-solid culture was attempted by plating the dilute ES cell suspension (100 cells/ml) into differentiation medium containing 1% methylcellulose. With this method, however, EB formation took almost two weeks and the formation frequency was lower than 25%. In addition EBs tended to adhere, as was observed in suspension culture, and the gel like quality of the medium hampered the removal of grown EBs without damage.

For the hanging drop culture 20µl drops containing 100 or 1000 cells per drop were plated into the lid of 10cm bacteriological grade culture dishes. After two days of culture, one embryoid body was typically formed per drop with a frequency of 63±8% for 100 cells/drop and 85±6% for 1000 cells/drop. On day 3, individual embryoid bodies contained 1050±210 cells or

6800±450 cells respectively (Figure 9.1). Thus, owing to its ease of use and high EB formation efficiency, the hanging drop method (1000cells/drop) was eventually adapted for all haematopoietic differentiation assays.

After 48h in hanging drop culture, EBs were transferred into suspension culture containing the appropriate growth factors to induce macrophage development (see below) (Keller et al., 1993; Wiles and Keller, 1991). In cases where excessive adherence of EBs occurred, non-adherent embryoid bodies were transferred onto a fresh dish. With a plating density of 30-40 EB/ml, the medium needed replacing every 2-3 days. Stages of development observed are described in the next section.

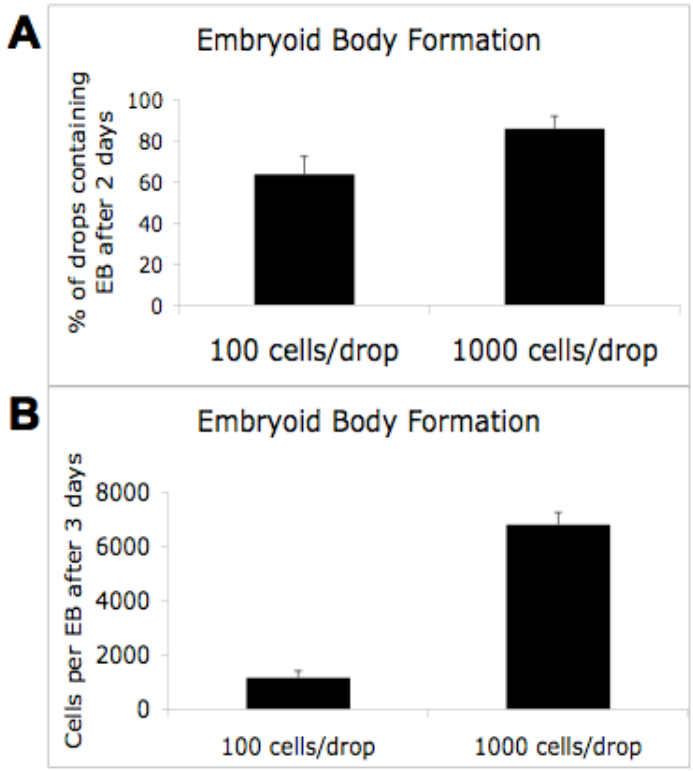


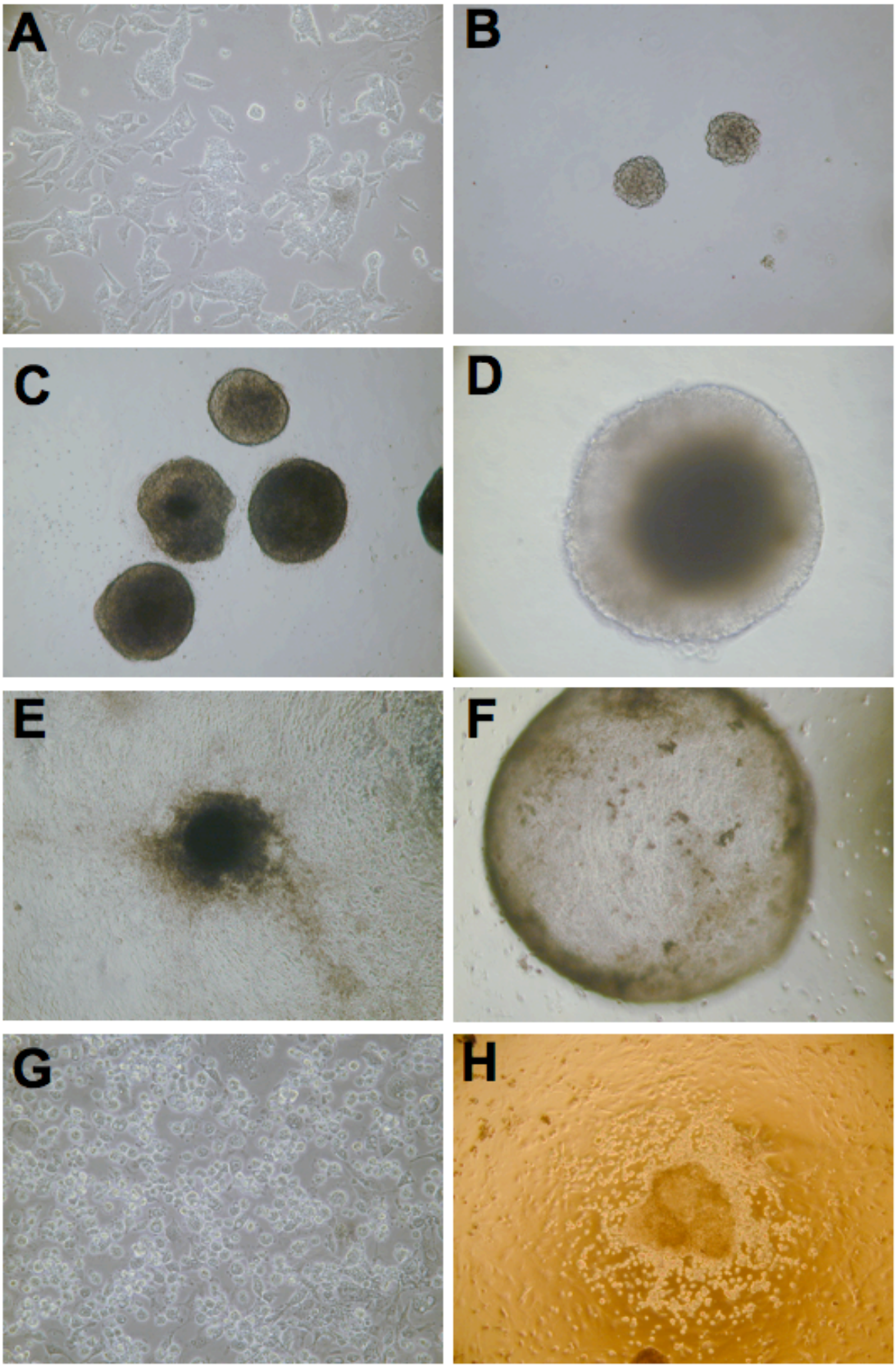
Figure 9.1 Embryoid Body Formation in Hanging Drop Culture
Two cell densities, 100 and 1000 cells/drop were tested for the frequency of EB formation after 48h (**A**). The cell number per EB after 3 days (**B**) was measured by pooling a defined number of EBs, trypsinization and counting in a haemacytometer. Values given represent 5 individual experiments±standard deviation of the median.

9.2 Stages of Development in Differentiation Cultures

Undifferentiated ES cells (Figure 9.2A) can be induced to form early embryoid bodies (Figure 9.2B) within two days, which recapitulates the stages of early embryonic development (see Chapter 4). Three-dimensional development is necessary to induce haematopoietic commitment. The initial stages of differentiation, up to the formation of mesodermal precursors, are growth

factor independent (Keller et al., 1999). Further haematopoietic development, however, is dependent on the presence of appropriate cytokines. Cultures were consequently supplemented with 5ng/nl MCSF and 1ng/ml IL-3 from day 2 onwards. Was this supplementation performed too late (i.e. after day 10), development of detectable amounts of embryonic macrophages did not occur (data not shown). EBs, transferred from hanging drop into suspension culture, grow in volume and the majority undergo globinization, the development of primitive and definitive erythrocytes, from day 7 onwards (Figure 9.2C&D). Early myeloid precursors are thought to be present by day 6 (Wiles, 1993). When suspension EBs were transferred into tissue culture plates, they attached within 48h (Figure 9.2E) and spread, forming several poorly defined tissues. Left in suspension culture, EBs kept growing and began to shed cells, under supplemented conditions the majority of these cells are believed to be macrophages (Wiles, 1993). By day 15, a large proportion of EBs had transformed into liquid-filled cystic embryoid bodies reminiscent of the *in vivo* blastocyst stage (Figure 9.2F). When suspension EBs (no earlier than day 7) were disrupted using trypsin or collagenase IV and plated onto tissue culture dishes, clusters of embryonic macrophages began appearing after about 5 days, indicating the presence of haematopoietic precursors in the three-dimensional structures. Survival of these precursors and formation of macrophages, however, varied greatly as disruption of EBs required long protease incubation times and strong physical shear force. Attached whole EBs also began forming embryonic macrophages after about 5 days, which typically appear as halos around the individual colonies (Figure 9.2G).

Figure 9.2 Stages of Development (see following page)
Undifferentiated mES cells were grown on gelatin in the presence of Leukemia Inhibitory Factor (LIF) (**A**) before being subjected to EB formation in hanging drops for 48h (**B**). By day 7 the majority of EBs have turned red at the centre due to globinization (**C&D**). When left to attach to tissue culture plastic, several tissues appear within a few days, **E** shows such an EB on day 13. EBs left in suspension culture begin to turn into liquid filled cystic embryoid bodies from day 10, such a large structure, on day 15, is shown in **F**. Embryonic macrophages (**G**) can be derived from EBs de-aggregated from day 7 onwards, here EBs were broken apart on day 12 and left to grow for 6 days. Embryonic macrophages can also appear as halos (**H**) around EBs left to adhere and spread for 5-7 days. Magnification: 40x (B; C; E; F; H); 200x (A; D; G)


A
B
C
D
E
F
G
H

9.3 The Appearance of Macrophage Differentiation Markers

CD11b and F4/80 were used as markers for murine macrophages (see Chapter 4.1) (Moore et al., 1998). Cells were analyzed by flow cytometry at different stages of the differentiation process. The gating strategy applied to all samples is shown in Figure 9.3B. In order to avoid cellular debris and clumps of cells, only events of medium forward scatter and low to medium side scatter were gated. Debris and clumps in the samples could not be avoided entirely due to the physical force (i.e. prolonged protease treatment and passaging through a needle) needed to break apart embryoid bodies or differentiated cells prior to staining.

Figure 9.3A details the differentiation protocols assessed in the pilot experiment. EBs were either kept in suspension culture beyond day 9 or were, from then onwards, attached to tissue culture plastic as whole structures (4-6EB/well of a 12w plate) or single cell suspension (10E5 cells/well of a 12w plate). In addition, EBs were grown in semisolid culture for 9 days before attaching them to tissue culture plates as a whole. Samples for staining were taken on days 9; 14; 17 and 21.

CD11b/Mac-1 was used as the first indicator for monocyte/macrophage development. On day 9 in suspension culture, only trace expression of CD11b could be observed (Figure 9.3C). By day 14, independent of culture method, about 11% of all cells expressed this marker. Beyond that, for both suspension and attached cultures, the value fell to about 4% on day 17 and 0.9% or 2.6% respectively on day 21. For de-aggregated cells, expression levels peaked later, on day 17 with a value of 14%, before falling to 5% on day 21. In semisolid culture, 5% of all cells stain positive for CD11b on day 17 (only data point available).

Similar data was obtained for the second macrophage marker F4/80 (Figure 9.3D). No trace expression could be picked up in suspension culture on day 9, but up to 10% of all cells cultured as attached EBs express F4/80 after 14 days. As with CD11b, the expression was reduced after that in suspension and attached cultures. For the de-aggregated culture method, expression peaked at day 17 with about 13% before being reduced to 5% on the last assayed day 21. In semisolid culture, about 2% of all cells expressed F4/80 on day 17.

Overall, the combination of forming EBs in hanging drops, further growth in suspension and attached de-aggregated single cell culture proved to be most efficient and straightforward in generating embryonic macrophages. Technical problems encountered were primarily related to breaking apart the embryoid bodies. Prolonged incubation of up to 15min in 0.1% trypsin resulted in poor viability (less than 50% of the cells, by Trypan Blue) and excessive force was needed to generate single cell suspensions.

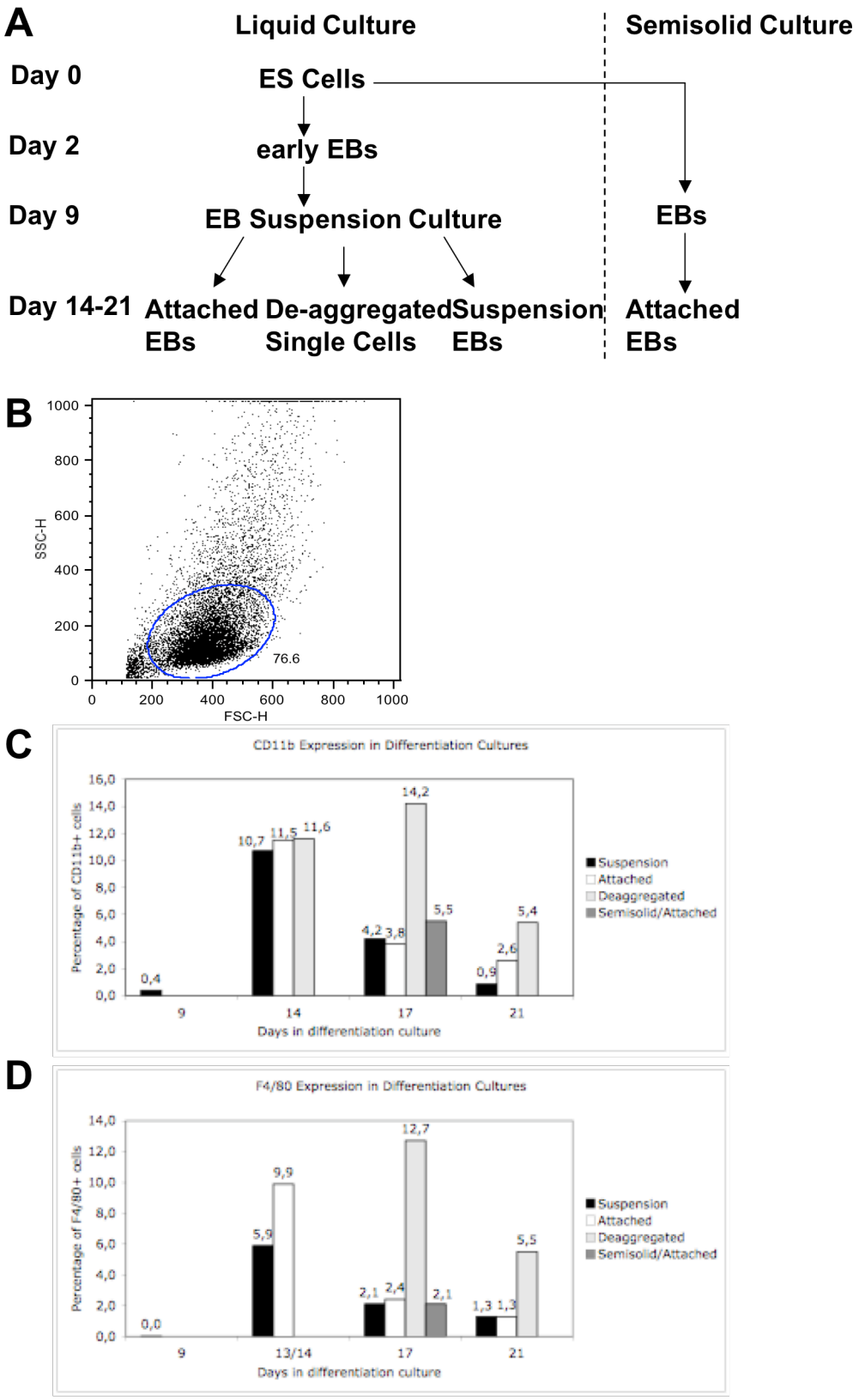


Figure 9.3 CD11b and F4/80 Expression in Pilot Differentiation Cultures
A illustrates the differentiation protocols applied to the ES cells. Cells of medium forward and low to medium side scatter were gated to avoid debris and clumps of cells (**B**). CD11b (**C**) and F4/80 (**D**) expression is plotted as the fraction of cells expressing the respective marker in the total sample depending on the applied culture method. Expression levels of parallel experiments varied greatly. Antibodies used were CD11b-PE and F4/80-FITC.

Consequently, few of the cells attached and differentiated further. Switching to collagenase IV largely alleviated the problem. Collagenase is not inhibited by serum proteins and could be added (at 0,2%) straight to the medium. After a 30min incubation with pipetting in 10min intervals and finally passing the cells through a needle, the majority of cells was in single cell suspension and viability was greater than 90%.

After establishing the general parameters, the differentiation process was repeated using the selected suspension/de-aggregation method. EBs were disrupted slightly earlier on day 7 (as opposed to 9) and cultured as single cells until day 15. Earlier time points were included in the analysis, days 0; 4; 7 (Figure 9.4A). Day 0 represents undifferentiated ES cells, cultured in the presence of LIF, which are CD11b and F4/80 negative. As before, CD11b expression appears initially on day 7 (with approx. 3.5% of all cells), slightly earlier than in the previous experiment. The fraction of cells expressing CD11b increases to 11% on day 10 and 15% on day 15. F4/80 cell surface expression appeared later than CD11b, with 1.6% of cells expressing the marker after 10 days and 12.5% of all cells staining positive after 15 days. The double staining shows that the majority of stained cells (12.5%) expressed both markers after about 2 weeks (Figure 9.4B). Isotype matched controls (Figure 9.4C&D) did not indicate any significant non-specific staining.

Levels of CD11b and F4/80 expression varied greatly between experiments (i.e. compare to wild type differentiation in 9.3), but 10-15% embryonic macrophages in the total cell population after two weeks of differentiation were considered to be sufficient to attempt detection of *iCre2* expression. Several trials were made to expand embryonic macrophages beyond days 15-17. Upon harvesting and re-plating of the cells, no further expansion could be achieved. Cells generally failed to attach and propagate further. Again, detaching differentiated cells from tissue culture plastic needed long proteinase incubation times and excessive force.

9.4 Further Markers Assessed in Wild Type Differentiation Cultures

To further dissect the process of differentiation, several other markers were used for staining. Those include an endothelial (CD31), a haematopoietic (CD117), a B cell lineage (CD45R/B220) and a neutrophil (Gr1) marker. Culture conditions were as described before. Following EB formation, cells were kept in suspension culture until day 7 before being plated as single cells until day 15. The majority of undifferentiated ES cells (day 0; Figure 9.5A) expressed CD31 when they were attached to gelatin (Figure 9.2A). CD31 expression levels continuously fell over the course of haematopoietic differentiation, from 98% on day 0 to less than 10% on day 15. The majority of undifferentiated cells (84%) also expressed CD117 (Figure 9.5B).

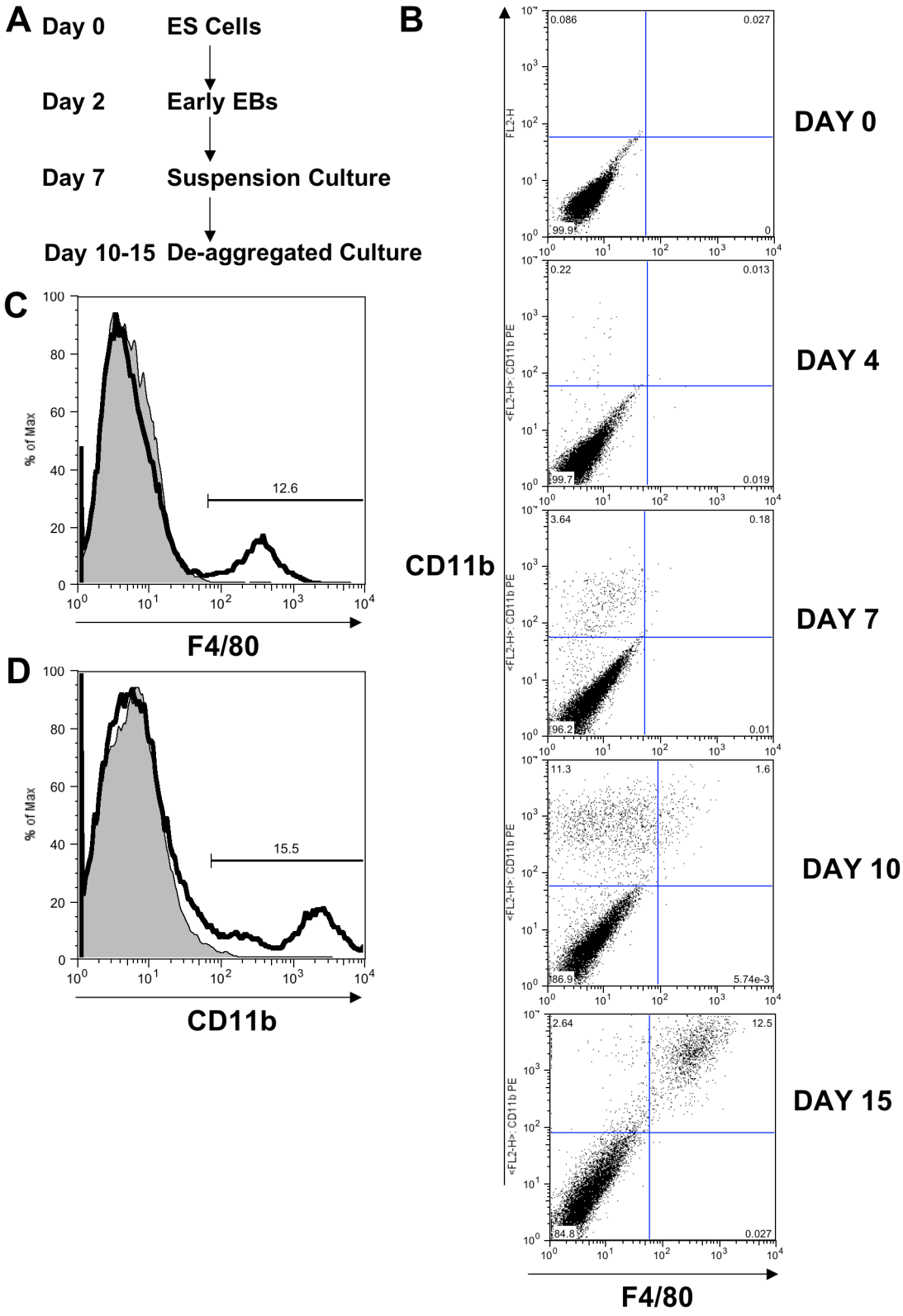


Figure 9.4 Macrophage Development in Wild Type Cultures Using the Suspension/de-aggregation Method

Wild-type mES cells were differentiated for 15 days (**A**) and stained for CD11b/F4/80 expression at regular intervals (**B**). Isotype matched controls (tinted) for F4/80 (**C**) and CD11b (**D**) showed no significant non-specific staining when compared to their respective antibodies (bold). Antibodies used were CD11b-PE and F4/80-FITC.

CD117 expression was reduced almost 10 fold in suspension culture EBs by day 7. De-aggregated and re-attached cells showed a slight increase by day 15. Expression of the neutrophil marker Gr1 and the B cell marker CD45/B220 could not be observed at any stage of the haematopoietic development process nor in undifferentiated ES cells (data not shown).

9.5 Generation of a Reporter Cell Line Based on *Marco iCre2*

With macrophage differentiation conditions having been established, a system allowing the live read-out of *iCre2* activity had to be set up. The read-out is based on the activation of GFP expression by Cre mediated recombination – both an appropriate vector and reporter cell lines were thus created.

9.5.1 Creation of a Cre-inducible GFP Reporter Vector

In order to obtain a Cre-inducible version of the vector pEGFPN1_int (an intron improved version EGFP termed iGFP) (Lacy-Hulbert et al., 2001), the *XhoI* fragment from pS3 containing a floxed stop sequence (FLST) was cloned into aforementioned vector (Figure 9.6A). FLST contains a pause signal and polyA site flanked by loxP sites and is able to suppress expression when placed between a promoter and the gene to be blocked. Out of 50 screened colonies 3 (9, 46 and 47) contained FLST as evidenced by a *SalI/NotI* digest (Figure 9.6B). Two of those (46 and 47) carry FLST in the same orientation as iGFP. The resulting pEGFPN1-int-FLST (FLST-iGFP) can be used as reporter construct, in which *iCre2* mediated FLST deletion switches on iGFP expression from the CMV promoter.

9.5.2 Creation of Stable CMV-FLST-iGFP Transfectants

The *AflIII* linearized reporter plasmid was used to generate stable transfectants from knock-in clone *Marco iCre2* 29*ΔNeo*20. To establish reporter functionality before differentiation, a transient Cre expression was performed. Five resistant clones were analysed at 24h and 48h post electroporation. Three of the clones tested showed a small (2%), but distinct, population of green fluorescing cells (Figure 9.7), with no background activity observable in the controls. Transfection of an iGFP expressing plasmid served as a control. Reporter clone 12 was used for subsequent differentiation assays as it had the highest and brightest fraction of green fluorescing cells after 24h (2.05%). After 48h, 1.79% of cells were still fluorescent.

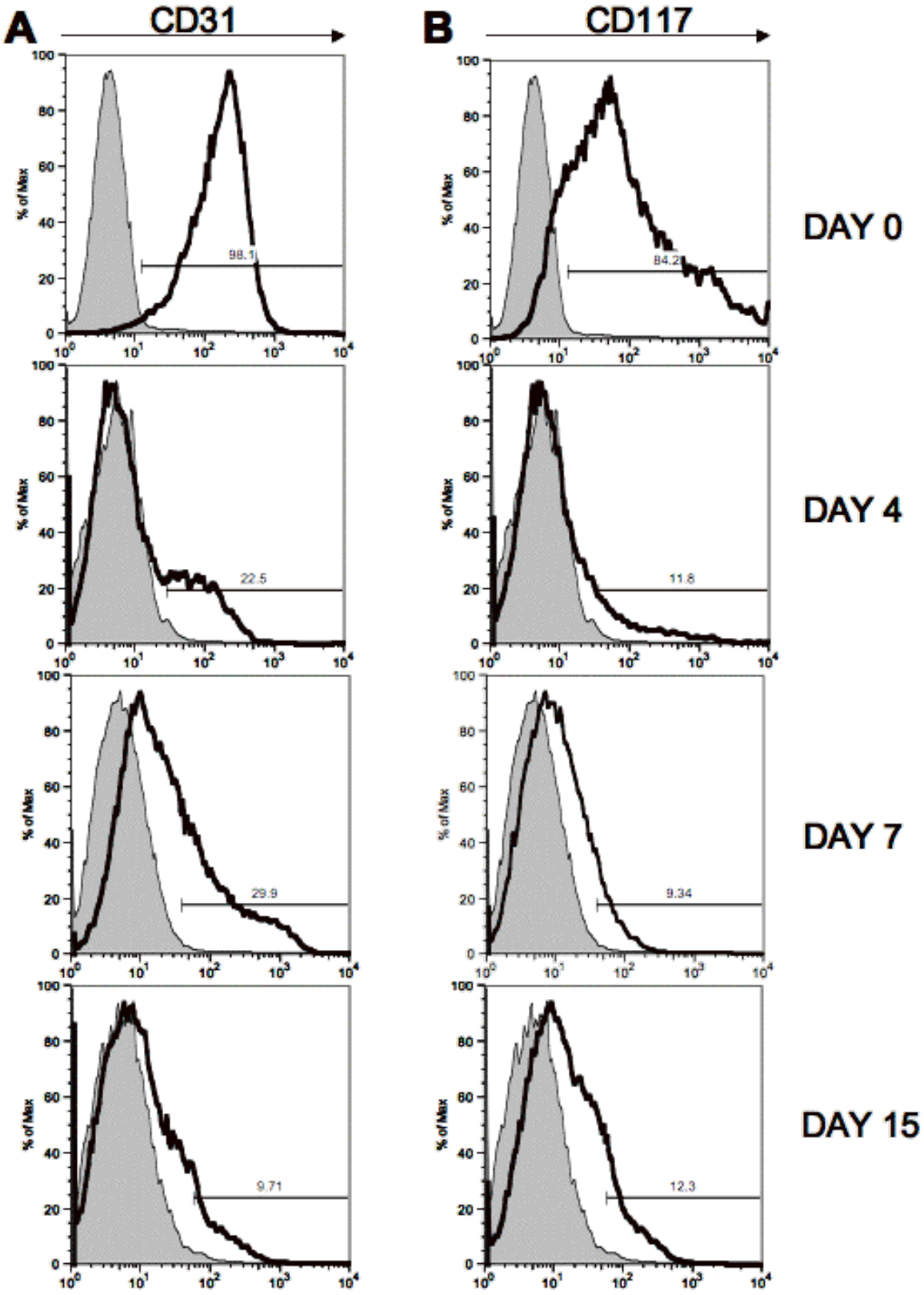


Figure 9.5 CD31 and CD117 Expression in Wild Type Differentiation Cultures
Fractions of CD31+ (**A**) and CD117+ cells (**B**) are shown as bold lines compared to the isotype matched controls (tinted) over the differentiation time period of 15 days.
Antibodies used were CD31-PE and CD117-PE.

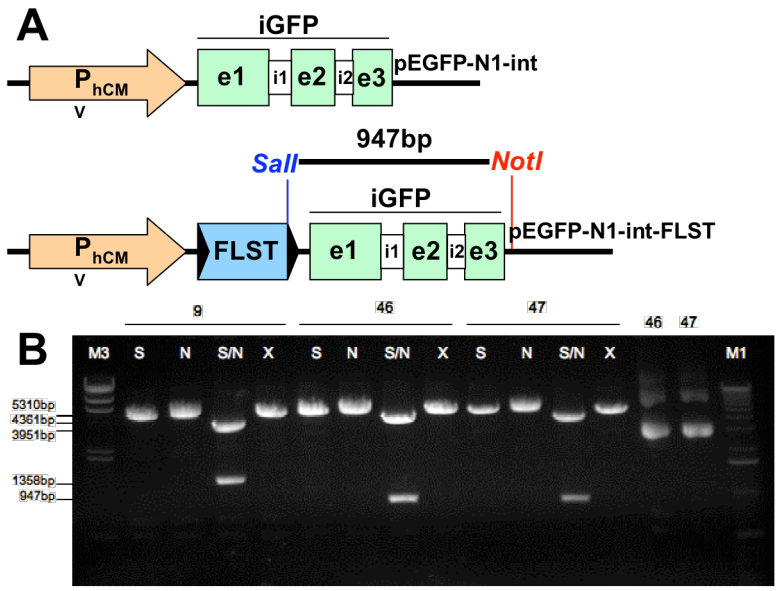


Figure 9.6 Production of Cre-inducible Reporter Vector
A illustrates the structure of *iGFP* before (top) and after (bottom) FLST insertion. As unidirectional cloning was used with only one restriction enzyme (*XhoI*), FLST can insert in both orientations. A *SalI/NotI* digest identified clones carrying FLST and the orientation (**B**). *SalI* (S), *NotI* (N) and *XbaI* (X) linearize the vector (5.3kb). *SalI* and *NotI* create fragment sizes of 947bp and 4361bp or 1358bp and 3951bp respectively, depending on the orientation of FLST. M1: 1kb Marker; M3: *λHindIII* Marker

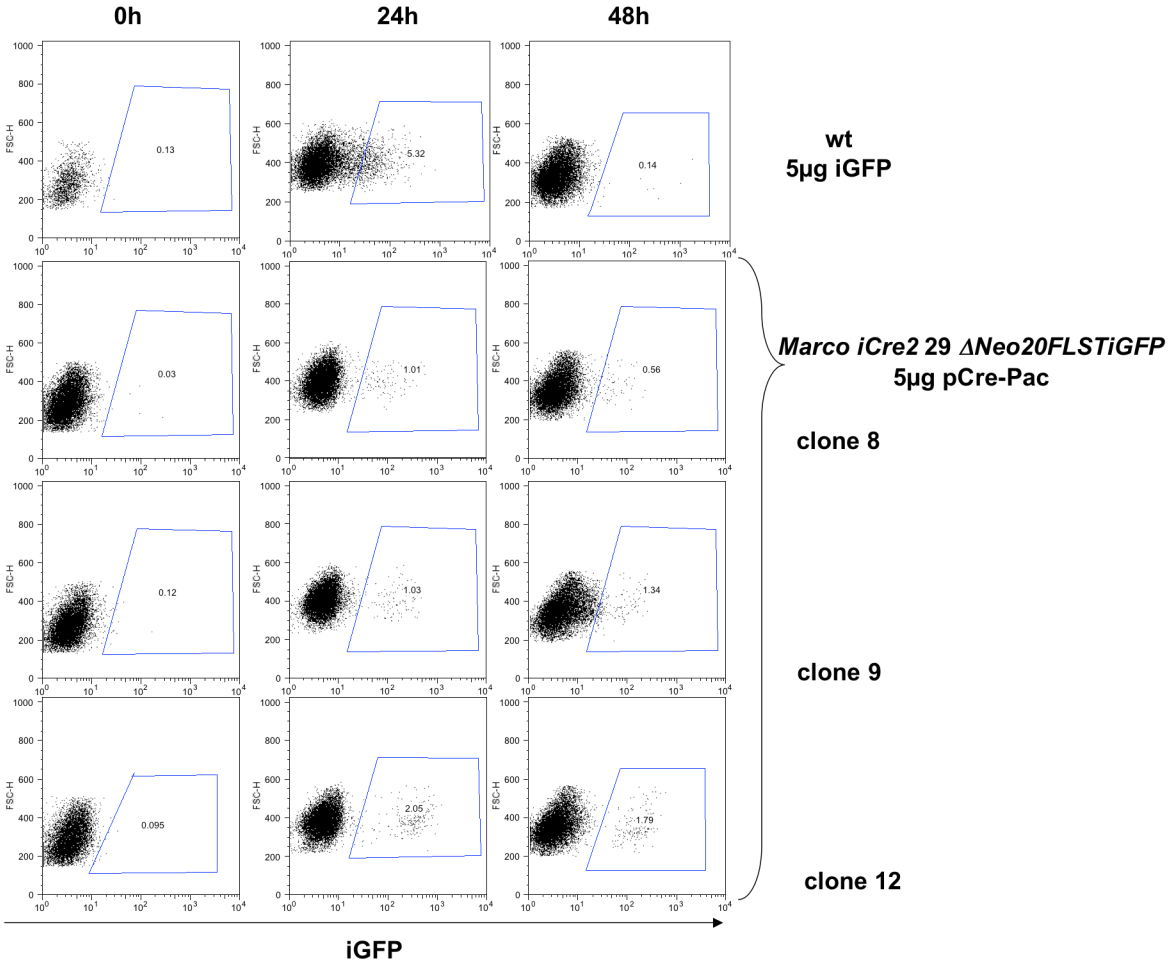


Figure 9.7 Cre-mediated GFP Expression in Reporter Cell Lines
G148 resistant clones were electroporated with 5µg pCre-Pac. Cells were analyzed before (0h, left) and 24h (middle) and 48h (right) after transfection. Wild-type ES cells, transiently transfected with 5µg pEGFPN1-int (top) served as a positive control for GFP expression

9.6 Haematopoietic Differentiation of *Marco iCre2* Knock-ins

Thus far, all differentiations had been carried out using wild type mES cells. In order to analyze *iCre2* expression, these methods needed to be reproduced for the *Marco iCre2* knock-ins. Several of the *Marco iCre2* knock-in clones were consequently subjected to the previously described culture regime. Clones assayed were *Marco iCre2* 29; *Marco iCre2* 29 *CMVFLSTiGFP12* (the reporter line) and *Marco iCre2* 48. Various attempts at differentiating the first 2 clones did not result in any CD11b or F4/80 expression and thus no macrophage production (see Figure 9.8), even though wild type cells assayed at the same time differentiated into embryonic macrophages.

The *CMVFLSTiGFP* reporter clone also showed no GFP expression at any stage of the differentiation cycle (data not shown). Morphologically, however, the differentiation process appeared to be similar to that observed with wild type mES cells. EB formation frequencies were around 80% and EBs turned red. The only difference was that the EB size for *Marco-iCre2* 29 knock-in clones was consistently lower than for the wild type. This is reflected in a lower cell number per EB. On day 7, wild type EBs contained about 12180±1731 cells per EB; compared to 8180±841 for *Marco iCre2* 29. Extending the time in suspension culture to 10 days before generating single cell suspensions, gave *Marco iCre2* 29 EBs the chance to grow further (*Marco iCre2* 29 EB: 9480±1099; wild type EB: 11700±1421), but did not improve the formation of macrophages

Following the attachment of EB-derived single cells, the typical morphology of ES macrophages (Figure 9.2G) did not appear. Instead, some clusters of cells began looking more like undifferentiated ES cells (Figure 9.2A) again. CD31 and CD117 expression throughout the differentiation (Figure 9.9) was similar to that observed in wild type cells (Figure 9.5), until day 15 – macrophages should have been present by then – when both markers were up-regulated.

In order to establish whether the failure to produce embryonic macrophages was a feature of these particular clones or of knock-ins in general, another *Marco iCre2* clone, no. 48, was differentiated in the same manner. From this particular clone, macrophages could finally be generated (Figure 9.10A). Again, the EBs appeared to be smaller after a week in suspension culture when compared to the wild type, but on day 14 circa 4% of all gated cells expressed both CD11b and F4/80. This fraction is lower than before, however, in this particular experiment, the wild type also only yielded a distinct embryonic macrophage population of about 3%. At this stage, cells were also stained for Marco, the receptor whose gene served as the *iCre2* knock-in target (Figure 9.10B). Interestingly the majority of cells, more than 75% in case of *Marco iCre2* 48, expressed the marker.

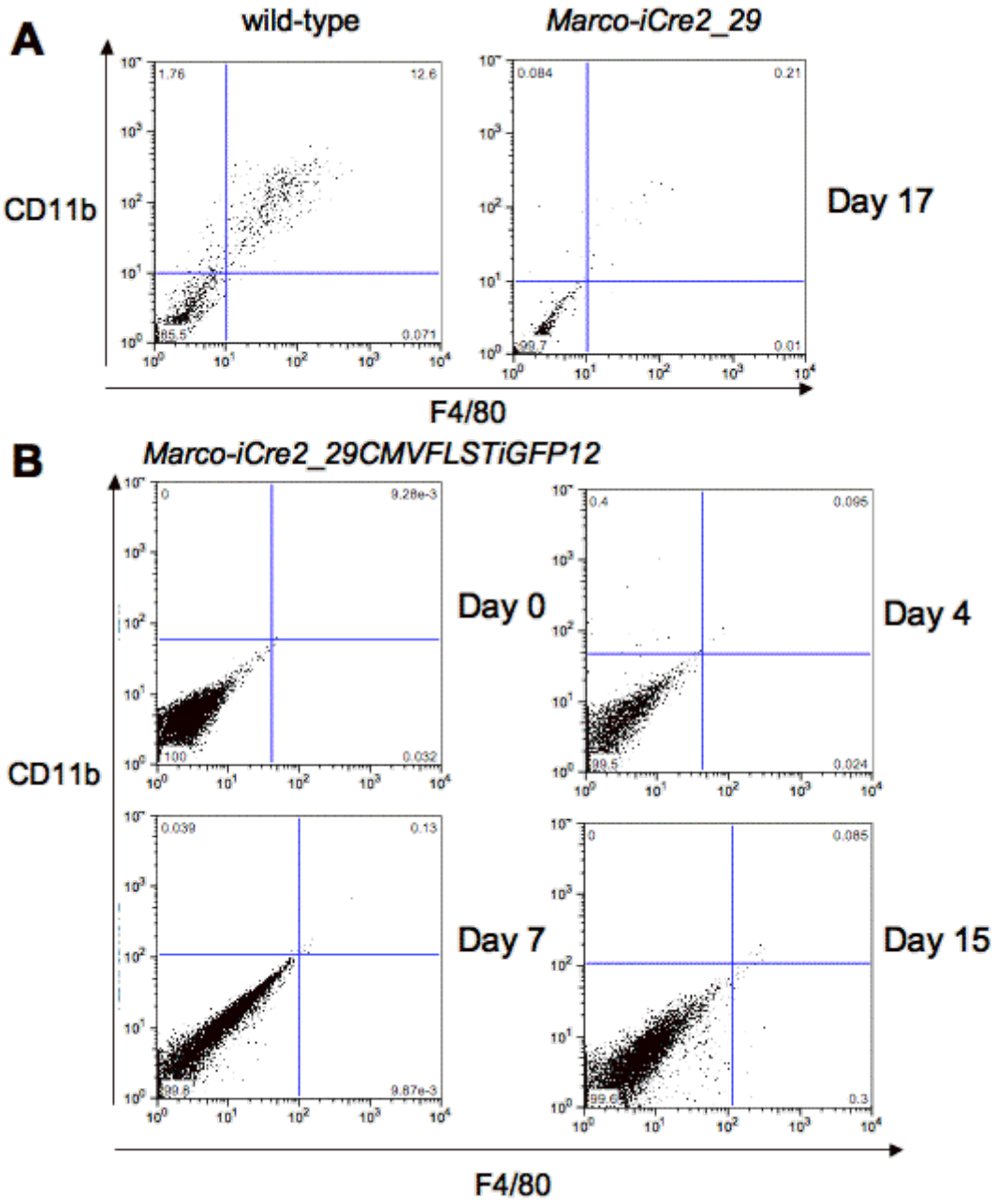


Figure 9.8 F4/80 and CD11b Expression in *Marco iCre2* 29 and Reporter Differentiation Cultures

A shows CD11b/F4/80 double stainings for wild type and *Marco iCre2* 29 cells differentiated according to the protocol described in Figure 9.3A on day 17. **B** represents the same staining data, but for the *Marco iCre2* 29 reporter cell line from the ES cell stage through to day 15. For the parallel wild type staining, please see Figure 9.4B.

Antibodies were CD11b-PE and F4/80-FITC in **A** and CD11b-PE and F4/80-Bio/CyC in **B**.

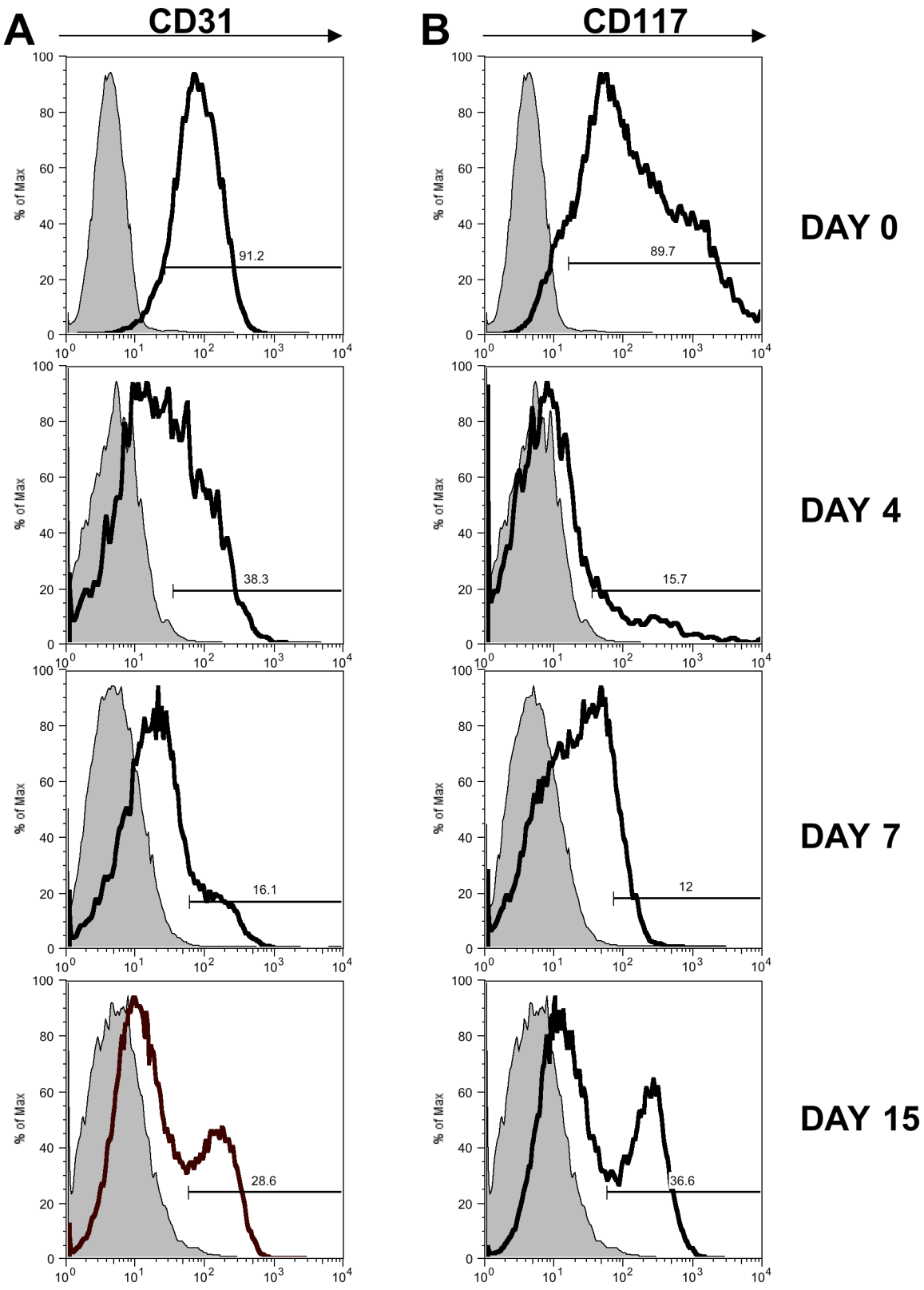


Figure 9.9 CD31 and CD117 Expression in *Marco iCre2* Reporter Differentiation Cultures
Fractions of CD31 (**A**) and CD117 (**B**) are shown as bold lines compared to the isotype matched controls (tinted). Antibodies used were CD31-PE and CD117-PE. Equivalent stainings for the wild type are found in Figure 9.5.

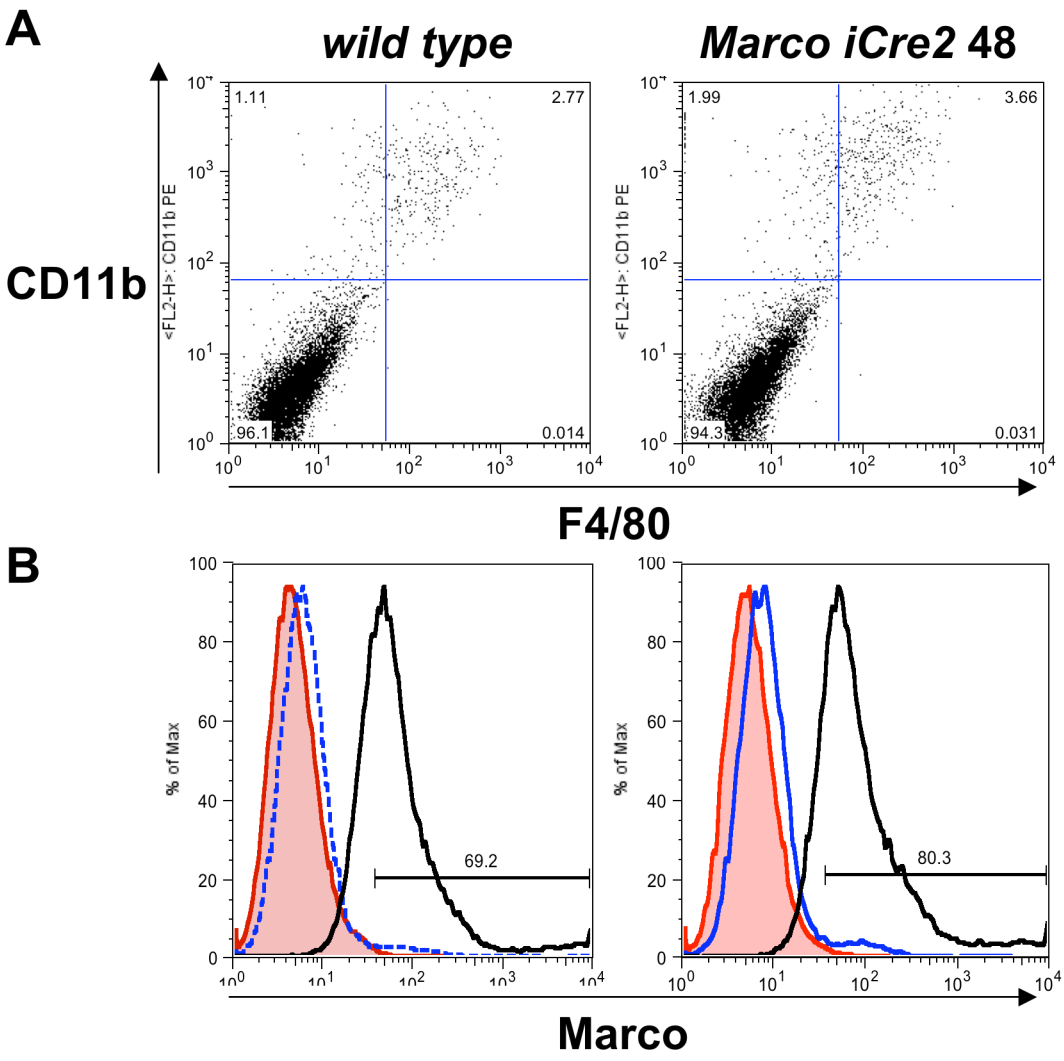


Figure 9.10 Expression of Macrophage Markers on Day 14 in *Marco iCre2* 48 Differentiation Cultures

Marco iCre2 48 and wild type mES cells were differentiated as previously described. On day 14, cells were stained for CD11b and F4/80 (**A**). Separately, cells were labelled with a Marco-specific antibody and a fluorescent 2nd AB (**B**). Unstained cells are tinted red, 2nd AB only labelled cells are blue, cells labelled with both antibodies are black.

Antibodies used are F4/80-FITC, CD11b-PE, Marco and Star69-FITC.

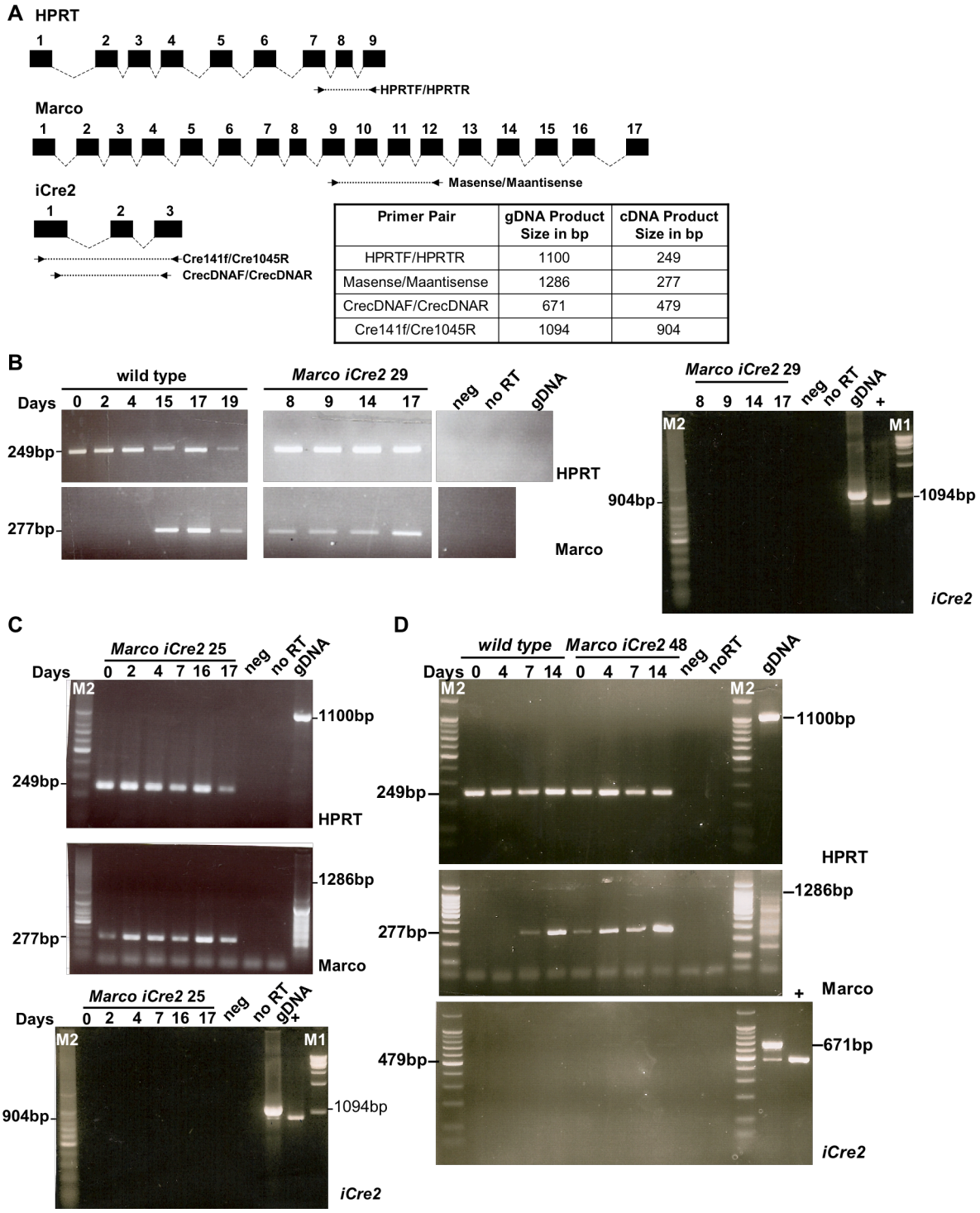
9.7 RT PCR for *Marco* and *iCre2* Expression

A RT PCR strategy was designed to analyse the expression of *Marco*, *iCre2* and, as internal standard, *HPRT* in the macrophage differentiation cultures on the RNA level. Cell samples for RNA isolation, reverse transcription and PCR had been taken at regular intervals during the differentiation of *Marco iCre2* 29 (Figure 9.11B); *Marco iCre2* 25 (Figure 9.11C), *Marco iCre2* 48 (Figure 9.11D) and wild type controls. All samples were assessed, whether or not macrophage markers could be identified by flow cytometry. Product sizes, for genomic and cDNA, and primer locations are shown in Figure 9.10A. For the *HPRT* internal standard, previously published primers (Kieusseian et al., 2006) were used. *Marco* primers were newly designed and amplify across exons 9 to 12. For *iCre2,* two primer pairs were utilized. Both amplify across exons 1 to 3 but produce different product lengths. The first, Cre141f/Cre1045R, was previously described and applied in 9.11B and 9.11C (Lacy-Hulbert et al., 2001). The second, novel, set of primers, CrecDNAF/CrecDNAR, was used in 9.10D.

As expected, expression of the housekeeping gene *HPRT* could be detected in all samples tested. The expression pattern for *Marco* varied between wild type cells and *Marco iCre2* knock-ins.

For wild type cells *Marco* could only be detected after differentiation had taken place for at least 7 days – in both independent differentiations (Figure 9.10B&D) assayed. Curiously, for all *Marco iCre2* knock-ins (clones 25; 29 and 48; Figure 9.10BDC) analysed, *Marco* expression was detectable from the undifferentiated ES cell level (day 0) onwards. Unfortunately, no *iCre2* expression could be detected in any of the samples, even though the positive control shows that cDNA synthesis and PCR themselves worked. For *Marco iCre2* clones 25 and 29 this may be explained by the absence of macrophages in the differentiation cultures (data not shown and Figure 9.6). *Marco iCre2* clone 48, however, had shown a small fraction of embryonic macrophages after 2 weeks of differentiation, and even here no PCR product corresponding to Cre cDNA/RNA was detected.

The Cre coding sequence contained in *Marco iCre2* clones 25 and 37 was recently re-sequenced. No mutations could be identified in the *iCre2* exon-intron structure and several adjacent 100bp up- and downstream. The apparent lack of *iCre2* expression cannot be explained at this stage.



Primer Pair	gDNA Product Size in bp	cDNA Product Size in bp
HPRTF/HPRTR	1100	249
Masense/Maantisense	1286	277
CrecDNAF/CrecDNAR	671	479
Cre141f/Cre1045R	1094	904

Figure 9.11 RT PCR Data for 3 Differentiated *Marco iCre2* Knock-in Clones
A illustrates the gene structure (exons as black squares, introns as dotted lines), primer pairs and product sizes for the genes assessed. Three different *Marco iCre2* knock-ins were differentiated, together with wild type controls. RT PCR results are shown for wild type, *Marco iCre2* 25 (**C**); *Marco iCre2* 29 (**B**) and *Marco iCre2* 48 (**D**).
no RT: non reverse transcriptase treated sample, gDNA: genomic DNA of respective clone; +: RNA isolated from wild type mES transiently transfected with 5µg pCre-Pac and harvested after 48h.

9.8 Discussion

The goal of this chapter was the development of an embryonic stem cell differentiation procedure that, in conjunction with flow cytometry, RNA analysis and a reporter system, allows tracking tissue specific *iCre2* expression from the *Marco* locus *in vitro*. The approach is essentially similar to the one presented in (Faust et al., 1994), where *in vitro* differentiation enabled tracing the activity of a chicken lysozyme transgene in murine embryonic macrophages. The ideal model system provides a mixture of easily identifiable differentiated tissues (macrophages vs. non-macrophages) that closely resemble those of the adult knock-in mouse model. Ultimately, *iCre2* expression could not be verified – possible explanations are discussed in the following sections. Two issues are key when considering the applied experimental approach: the suitability of the differentiation culture method and the utilized reporter systems.

9.8.1 IL-3 and MCSF Are Sufficient to Obtain Macrophages from IB10 ES Cells

Initially, a system needed to be developed, which allowed the straightforward production of embryonic macrophages from the murine ES cell line IB10 that had thus far never been used for differentiation. A minimal protocol (Figure 9.4A), based on embryoid body formation in hanging drops and expansion in the presence of IL-3 and MCSF modelled after (Keller, 1995; Wassarman and Keller, 2003; Wiles, 1993), was found to be suitable, if occasionally variable.

The main advantages of the adapted differentiation protocol are that it is relatively quick (2 weeks), cheap (relies only on two recombinant cytokines) and creates a mixture of differentiated tissues – containing sufficient amounts of the desired cell type (10-15% macrophages) but also other cell types, which is ideal for the assessment of a promoter's specificity. Disadvantages include the variability of macrophage yield, the limited amount of differentiated cells obtained and the possibility that cells differentiated *in vitro* have different characteristics than their adult counterparts.

Though there are currently no accepted benchmarks for EB formation, observations during this work agree well with published reports for other murine embryonic stem cell lines. EBs formed with high efficiency from drops of 1000 cells (Figure 9.1) and reached cell numbers comparable to reported figures. On day 3, IB10 EBs contained 6800 cells on average, which corresponds to values of 6000 cells observed for the CCE line (Dang et al., 2002). Later stage cell numbers on day 10 were slightly lower with 11700 cells/IB10 EB compared to around 20000 cells/CCE EB (Dang et al., 2002). Differences in EB formation capacity and overall differentiation potential between murine embryonic stem cell lines have been documented (Wiles and Keller, 1991).

Common milestones of EB development, such as globinization (Doetschman et al., 1985) (Figure 9.2D) – regarded as a success criterion for haematopoietic potential – formation of cystic cavities (Figure 9.2F) or the appearance of beating cardiomyocytes (Keller, 1995), were all met during the first two weeks of differentiation.

Targeted expansion of embryonic macrophages, defined by the appearance of CD11b and F4/80 in the presence of IL-3 and MCSF, was successful. The pilot experiment (Figure 9.3) revealed, that macrophage marker expression is, in line with early mesodermal development and the appearance of myeloid pre-cursors, induced after about 9 days in suspension culture. Marker expression is maintained irrespective of culture method, but reaches highest values when individual EBs are broken apart and cultured as single cells. Marker levels eventually taper off for all culture methods, likely caused by reduced cellular survival due to terminal differentiation or the lack of further developmental cues (Odegaard et al., 2007). Subsequent experiments (Figure 9.4) were then optimized by more frequent media changes, avoiding the early attachment of EBs by replating, and the use of Collagenase IV, overall increasing cell survival and proliferation. Two weeks sufficed to obtain around 15% macrophages expressing both CD11b and F4/80 in the total differentiated cell population.

The CD11b marker appears first, followed by F4/80 (Figure 9.4B), resulting in a distinct doubly stained population once cells become terminally differentiated. The stepwise marker emergence mirrors macrophage maturation *in vivo*, where CD11b is a broader spectrum label for myeloid cells, including, but not restricted to, monocytes, macrophages, granulocytes and dendritic cells, possibly appearing as early as on the clonogenic myeloid progenitor found in the fetal liver or adult bone marrow (Akashi et al., 2000; Leenen et al., 1994; Traver et al., 2001). Similarly, F4/80 is recognized as a later marker mainly being expressed on mature macrophages (Anderson et al., 1999; Leenen et al., 1994; Lloyd et al., 2008). Differentiation cultures did not show neutrophilic Gr-1 or lymphoid CD45R/B220 expression at any stage, reflecting both the later induction of those cell types *in vivo* and the lack of necessary growth factors in the herein utilized culture conditions (Cho and Zuniga-Pflucker, 2003; Lieber et al., 2004).

In general, the yield of macrophages obtained from wild type IB10 ES cells was variable (compare Figures 9.4B and 9.8A to Figure 9.10A). Three main factors influencing the reproducibility of differentiation attempts include the utilized serum (Bruce et al., 2007) and growth factors, the culture method and the quality of the ES cells. Analyses have shown that serum free conditions can increase reproducibility and benefit differentiation as the medium composition can be defined exactly (Keller, 2005; Kubo et al., 2004; Ng et al., 2005). The utilized culture method may also have contributed to the variability. While embryoid bodies all have the same size and developmental status after hanging drop culture, transfer in suspension

culture causes de-synchronisation of differentiation. This is primarily due to EBs randomly fusing together creating a heterogeneous population. When EBs grow larger they are prone to necrosis and diminished differentiation potential (Dang et al., 2002) and EB size has been associated with varying myeloid potential (Ng et al., 2005). One way to avoid EB fusion and maintain synchronous growth is the use of methylcellulose medium. In this work, it was observed that IB10 ES had a low plating and EB formation efficiency in semisolid medium. Handling was problematic. However, forming EBs in hanging drops first before transferring them into methylcellulose medium, may improve performance.

The method described herein was geared towards allowing straightforward macrophage production. While it works satisfactorily, there are several options to increase both yield and specificity of macrophage formation. These either target the initial EB growth period or the expansion of myeloid progenitors. Recent advances in EB production include the use of polymeric non-adherent or hydrophobic substrates and matrices other than methylcellulose (Fathi et al., 2009; Koike et al., 2005; Konno et al., 2005; Valamehr et al., 2008), rotary cell culture (Carpenedo et al., 2007; Wang et al., 2006), ES cell encapsulation in alginate beads (Rohani et al., 2008) and growth in lithographic microwells (Moeller et al., 2008) or microfluidic chambers (Torisawa et al., 2007) – all aimed at enabling synchronized growth and reduced attachment.

Additional cytokines – together with IL-3 and MCSF – can improve the expansion and differentiation of myeloid precursors. IL-1, for example, can boost macrophage yield up to two fold (Wiles and Keller, 1991). IL-11 and the kit ligand (KL) increase the overall number of haematopoietic progenitors (Keller et al., 1993). Vascular endothelial growth factor (VEGF) and stem cell factor (SCF) are also beneficial for early haematopoietic development (Nakayama et al., 2000; Orlovskaya et al., 2008).

Other improvements may allow the long term production of embryonic macrophages. The most common approach uses EBs grown for at least 10 days, which are then attached to tissue culture plastic. In the presence of appropriate cytokines, myeloid cells can be harvested from the supernatant after two to three weeks for several weeks running (Karlsson et al., 2008; Moore et al., 1998). Such floating cells were not readily observed in this work – possibly due to the shorter culture periods. Another innovative experiment incorporated the overexpression of the oncoprotein HoxA9 to immortalize myeloid progenitors isolated from EBs, that could then be differentiated into macrophages indefinitely (Odegaard et al., 2007).

A way to influence macrophage purity is to isolate haematopoietic progenitors early on in differentiation through FACS or MACS, followed by selective maturation. Three suitable

purification markers are Flk-1 (fetal liver kinase), CD34 and CD45, representing stepwise development from the early mesodermal through to early haematopoietic stages respectively (Faloon et al., 2000; Fehling et al., 2003; Kabrun et al., 1997; McKinney-Freeman et al., 2009; Nakayama et al., 2000; Wang et al., 2005).

Lastly, the question remains whether *in vitro* produced macrophages are an accurate model to predict *iCre2* expression from the *Marco* locus in adult mouse macrophages. Haematopoietic differentiation from embryonic stem cells essentially recapitulates yolk-sac haematopoiesis and is thus a process separate from adult bone marrow haematopoiesis (Keller, 2005; Murry and Keller, 2008). Notwithstanding, no gross variances have been found in either morphology or cellular metabolism between embryonic and adult macrophages to date. Marker expression (CD11b, F4/80, CD68, CD36, CD14, scavenger receptors) (Odegaard et al., 2007), cellular processes such as phagocytosis or cytokine secretion upon stimulation (Clarke et al., 2000; Moore et al., 1998) and gene expression profiles (Lindmark et al., 2004) have all been found to be very similar. One group, however, found that fetal/embryonic macrophages, both those generated *in vivo* as well as those differentiated *in vitro* had lower expression levels of a chicken lysozyme transgene than their adult counterparts derived from the bone marrow, spleen or peritoneal cavity due to differential promoter activity (Faust et al., 1999; Huber et al., 1997).

Overall, macrophages generated from *Marco iCre2* knock-in cells should be a good model for the adult mouse. It has to be noted, however, that the adult macrophage population is very diverse (Lloyd et al., 2008). Any *in vitro* differentiation attempt is therefore likely to only model a fraction of adult cells.

9.8.2 *Marco iCre2* Knock-in Lines Show Reduced Differentiation Potential

When the optimized differentiation protocol was applied to *Marco iCre2* knock-in cells, macrophages failed to develop from clones 25; 29 and the reporter line (based on 29), as judged by marker expression and general morphologic appearance. For clone 25, used in pilot experiments (data not shown) this is, retrospectively, likely due to growth factor supplementation occuring too late (after day 10) to promote significant macrophage development.

However, clone 29 and its GFP-reporter descendant, also consistently failed to produce macrophages expressing CD11b and F4/80, unlike wild type cells differentiated in parallel under the same conditions (Figure 9.8). While there were no apparent differences in plating efficiency, EB formation or globinization; EB size and marker expression varied between clone 29 and wild type cells. Wild type EBs had reached their maximum cell number of around

12.000 cells on day 7; whereas clone 29 EBs were 33% smaller with around 8.000 cells. Three days later, knock-in EBs still had 22% less cells (9.500) than wild type structures (12.000).

The expression of surface markers CD31 and CD117 during late myeloid differentiation also varied between knock-in cells (*Marco iCre2* clone 29; Figure 9.9) and wild type (Figure 9.5). CD31 (Pecam-1; platelet endothelial cell adhesion molecule 1) is a widely used murine marker, primarily found on endothelial cells of the vascular system, but also on certain haematopoietic cells such as platelets, neutrophils, monocytes and selected T cells (Ilan and Madri, 2003). CD31 was intended to enable the identification of endothelial cells within the differentiation culture in order to, in conjunction with macrophage markers, test the tissue specificity of *iCre2* expression. More than 90% of all undifferentiated embryonic stem cells, whether *iCre2* knock-ins or wild type, express this marker (day 0 Figures 9.5A and 9.9A). Other studies found similar expression levels, primarily located at the cell-cell junctions and designated the presence of CD31 as a constitutive feature of undifferentiated embryonic stem cells, which reflects *in vivo* CD31 appearance in the inner cell mass of pre-implantation embryos (Li et al., 2005; Redick and Bautch, 1999; Robson et al., 2001). Furthermore, CD31 expression has been positively correlated with pluripotency as such cells show higher levels of pluripotency markers Oct3/4 and Nanog and greater differentiation potential (Furusawa et al., 2006). During differentiation, CD31 levels are sharply down regulated and become restricted to distinct endothelial lineages and subsets of haematopoietic cells (Li et al., 2005; Redick and Bautch, 1999). In this work, such reduction could only be observed for wild type cells (days 4 to 15, Figure 9.5A). *Marco iCre2* knock-in clone 29 showed an up-regulation of CD31 expression on day 15 (Figure 9.9A).

CD117 (Figures 9.5B and 9.9B) expression levels, follow a similar pattern. CD117 (c-kit) is a widely used stem cell marker involved in haematopoiesis, pigmentation and fertility (Fleischman, 1993). It is broadly expressed in adult tissues and promotes cell survival, particularly for pluri- and multipotent stem cells (Ashman, 1999). It has been found on embryonic stem cells, where it is essential to avoid apoptosis after differentiation is induced *in vitro*, but not *in vivo* (Bashamboo et al., 2006). Accordingly, the majority of both undifferentiated wild type and knock-in cells (day 0; Figures 9.5B and 9.9B) express the marker. As CD117 is linked to the maintenance of pluripotency, it also becomes rapidly down-regulated during differentiation *in vitro,* under appropriate growth conditions CD117 is eventually restricted to many types of stem cells including those facilitating haematopoiesis (McKinney-Freeman et al., 2009; Palmqvist et al., 2005). The wild type cells (days 4-15; Figure 9.5B) accurately match these observations whereas CD117 in the knock-in clone (Figure 9.9B) is up regulated on day 15.

The high levels of CD31 and CD117 observed on both wild-type and knock-in undifferentiated cells support that these cultures were in good condition when differentiation was induced. The increase of both markers in the knock-in cells at the end of the differentiation period – when macrophages should have been present – seems to suggest that this particular clone either reverts to a less differentiated state or predominantly forms cell types expressing CD31 or CD117. The former seems unlikely, as LIF withdrawal has been shown to result in a near complete loss of pluripotency after 72h (Palmqvist et al., 2005). The latter would be surprising, as apart from serum, IL-3 and MCSF, there were no other growth factors present that would favour either endothelial or stem cells in the differentiation culture. Another possibility is, as marker expression is measured in the whole cell population, relatively higher survival of CD31 and CD117 expressing cells. Though why other cell types (i.e. myeloid lineages) would not be supported in the knock-in cultures remains unexplained.

The final differentiation experiment seems to suggest that the failure to produce macrophages is a phenomenon restricted to *Marco iCre2* knock-in clone 29. Clone 48 (Figure 9.10A) produced approximately the same amount of macrophages after 2 weeks as wild-type cells – though these amounts were lower than observed in other experiments. Again, EBs appeared smaller, though in this case this did not seem to interfere with myeloid potential.

At this stage it can only be speculated about the reasons of clone 29 not being able to produce macrophages. Several options are conceivable, like the epigenetic state or age/quality of that particular clone. Further experiments are needed to establish whether clone 29 is less pluripotent – despite its CD117/CD31 profile and its ability to form EBs. It may be wise to focus on the other *Marco iCre2* knock-in clones for injection. Knock-in EBs reaching a smaller size than their wild type counterparts can also not be easily explained at present. It may also be a feature of embryonic stem cell age/passage number, but could theoretically mean that *Marco* exerts an unknown dose-dependent role during development, as there is only one active copy in the *iCre2* knock-in cells.

9.8.3 Results from the Reporter GFP System Remain Inconclusive

In line with the previous findings, the failure to detect iGFP activity (Figure 9.8B) induced by recombination in differentiated cells can potentially be attributed to the inherent inability of clone 29 to form macrophages and thus not switching on *iCre2* expression.

The reporter system has generally been shown to work in a transient transfection assay (Figure 9.7). Nevertheless there may be room for improvement. One of the most important features in a reporter construct is the promoter utilized. The herein used reporter vector (Figure 9.6A) is

based on pEGFPN1 (Clonetech) and is driven by a standard cytomegalovirus (CMV) promoter. The CMV promoter has long been widely used to drive reporter expression in a wide variety of tissues and cells, both *in vivo* and *in vitro*, and is generally considered to be constitutively active (Makrides, 1999). In the system presented here, it is essential that the promoter driving the reporter is active during the undifferentiated ES cell stage to distinguish leakiness of *iCre2* expression, during the EB differentiation process to detect when *iCre2* expression is switched on, and finally in terminally differentiated embryonic macrophages.

Whilst the CMV promoter has been successfully used in haematopoietic cells in general (Keating et al., 1990) and macrophages that were either monocyte (He et al., 2006) or embryonic stem cell derived (Stevenson et al., 2000) in particular, its suitability to drive gene expression in undifferentiated and differentiating embryonic stem cells has been controversially discussed. It has been reported, active in transient expression systems by several groups (Kim et al., 2007; Wang et al., 2008b; Ward and Stern, 2002), but inactive in mouse ES cells and EBs by others (Chung et al., 2002; Kawabata et al., 2005). In stably transfected mouse ES cells, CMV promoter activity can usually be seen in ES cells and throughout development, including in differentiated cells such as vascular progenitors, cardiomyocytes, mesodermal and neuronal cells and fibroblasts (Alexopoulou et al., 2008; Bagchi et al., 2006; Hong et al., 2007).

Some studies noted a variability of expression, with the CMV promoter being dynamically switched on and off throughout development (Stevenson et al., 2000), down regulated after longer culture periods (Wang et al., 2008b; Xia et al., 2007) or specifically activated (Chung et al., 2002; Hong et al., 2007) or de-activated (Bagchi et al., 2006) in certain terminally differentiated tissues such as neurons. These widespread differences have been attributed to varying vector delivery methods (i.e. virus versus electroporation), the utilized ES cell line, vector design (i.e. use of an internal ribosomal entry site) and chromosomal location of stably transfected reporters. Taken as a whole, it would appear that the CMV promoter should provide sufficient activity for the herein designed reporter system.

Several studies, however, have suggested that other promoters may provide stronger, more sustainable reporter expression, especially in undifferentiated ES cells and during the EB formation process, though not necessarily in terminally differentiated cells. Those are in particular the elongation factor 1 α (EF), the phosphoglycerate kinase (PGK) and the hybrid CMV chicken β actin promoters – though the latter has often been reported as only marginally more active than the CMV promoter (Chung et al., 2002; Kim et al., 2007; Wang et al., 2008b). Whilst the alternative promoters also have been associated with reporter silencing (Xia et al., 2007) and variable expression levels (Hong et al., 2007) and therefore do not guarantee better

expression, they may be worth testing if the herein described reporter system was to be improved.

One surprising result is that none of the reporter lines show any significant activation of iGFP expression in undifferentiated ES cells (Figure 9.8; 0h). The basal level of *iCre2* expression noted in Chapter 8.6.4 does not translate into reporter activation. It is conceivable, however, that basal expression levels are too low to mediate efficient recombination of the randomly integrated reporter construct at its particular chromosomal location or that the reporter gets silenced rapidly.

9.8.4 *iCre2* Expression Cannot Be Observed; *Marco* Appears to Be Dysregulated

The utilized RT PCR failed to detect *iCre2* expression in all three assayed clones (Figure 9.11). While the result is understandable for *Marco iCre2* clones 25 and 29, which failed to develop measurable amounts of macrophages, clone 48 was expected to switch on expression in the differentiated macrophage subpopulation.

Even more surprising is, that *Marco* expression was both detected by RT-PCR and by flow cytometry (Figure 9.10A). Wild type cells showed *Marco* mRNA from day 7 onwards, when macrophage markers also began appearing, underlining the expected tissue specificity. In knock-in cells, however, *Marco* was constitutively expressed even at the undifferentiated cell level indicating a degree of dysregulation. As all assessed clones still harbour the resistance gene, this may well be due to Neo^R disturbing expression across the locus, i.e. through its effect on the endogenous promoter. To further illuminate this, *Marco* mRNA expression would need to be assessed in a *ΔNeo* knock-in cell line. The one that is available to date had not been tested as it consistently failed to produce macrophages.

When differentiated cells were stained with a *Marco* specific antibody on day 14, a much larger population of differentiated cells (70%), for both knock-in and wild type, show the marker than would have been expected from the level of CD11b+/F4/80+ macrophages (3%). If this staining was specific as suggested by unstained controls and cells stained with the secondary antibody only, it would indicate that *Marco* is actually not exclusively restricted to macrophages in the differentiation culture. In depth analysis of *Marco* expression through the entire developmental process is needed to clarify this.

Due to its knock-in status the recombinase should be mirroring the host gene's expression. The failure to detect amplification of *iCre2* cDNA when *Marco* mRNA is clearly present is therefore

puzzling. The reasons for this finding are speculative at this point, though several options are imaginable:

1. PCR and or cDNA synthesis failed: This scenario appears unlikely as both the housekeeping gene control (HPRT) and the Cre positive control consistently provided PCR products. All shown PCRs (Figure 9.11) were carried out after total RNA had been reverse transcribed using a oligodT primer. The utilization of random primers did not facilitate *iCre2* amplification either (data not shown).

2. *iCre2* mRNA copy number too low: PCR failure due to low copy number is imaginable as the Cre control utilizes mRNA from a transient transfection that may be more abundant than those expressed from the single copy endogenous locus and the RT PCR has not been quantified. *iCre2*, however, should be expressed at the same level/copy number as *Marco* mRNA as it utilizes the same promoter – and therefore produce roughly the same number of transcripts/RT PCR templates. The amount of macrophages among the total cell population was quite low (3% in the *Marco iCre2 48* sample) and therefore restricting the template amount if expression is specific. Increasing the number of macrophages in the cultures or cDNA synthesis using an *iCre2* specific primer may help to increase DNA copy numbers and sensitivity of the assay. It is, however, also possible that *iCre2* mRNA, for unknown reasons, suffers from transcript instability.

3. *iCre2* is not expressed at all: *iCre2* has thus far been functionally tested in CHO and NIH/3T3 cells *in vitro* and the rat brain *in vivo*, expression in murine embryonic stem cells should therefore be possible as well (Lacy-Hulbert et al., 2001). As potential expression failure is not due to any mutations in the coding sequence (data not shown), two scenarios are conceivable. The first is epigenetic silencing during culture, i.e. by methylation, which could be illuminated by carrying out a methylation specific PCR. Secondly, *iCre2* may have been inserted in the wrong place. As discussed in Chapter 7.15, *Marco* contains two potential translational start codons (Figure 7.15) and *iCre2* was inserted into the one identified as the main translation initiation point in macrophages (Kangas et al., 1999). It may be possible that this data is not accurate and that the exact location of the translation start codon either needs re-assessing or *iCre2* has to be inserted to replace the second ATG to function. On the other hand, it seems surprising that there should be a complete exclusion of translation from start codons only 100bp apart when known mRNA species would support protein synthesis from either one.

9.8.5 Summary and Conclusion

Collectively, this chapter establishes a framework for analyzing the expression characteristics of the recombinant Cre recombinase before investing resources into creating a mouse model. For the pilot study, macrophages and therefore the expression of *Marco iCre2* were chosen, as it is relatively straightforward to differentiate them *in vitro* compared to protocols for the generation of neutrophils required for the assessment of *Itgb2l iCre2*. The ability of the tested embryonic stem cell lines to form embryoid bodies, beating cardiomyocytes and the expression patterns of CD31 and CD117 support they are indeed pluripotent. When it came to the selective expansion of myeloid progenitors, however, only one *Marco iCre2* knock-in was able to produce measurable numbers of macrophages under the experimental conditions applied. It may be necessary to further optimise haematopoietic growth conditions. Non-differentiating clones may have acquired genetic aberrations during culture that reduce their pluripotency – the ultimate test for this will be their ability to differentiate *in vivo*, i.e. the generation of chimeric mice. The failure to detect *iCre2* expression remains unexplained as the coding sequence is intact. Considering the many unknown factors and evidence of expressional dysregulation pertaining to *Marco iCre2* clones, it may be prudent to focus the mouse creation process on *Itgb2l iCre2* knock-in clones until the *Marco* samples can be scrutinized further.

GENERAL DISCUSSION AND FUTURE WORK

CHAPTER 10: FUTURE WORK AND THE APPLICATION OF MACROPHAGE AND NEUTROPHIL SPECIFIC *iCre2* KNOCK-IN MOUSE MODELS

With *Itgb2l* and *Marco iCre2* knock-in cell lines having been established and verified, the next step is to create the Cre mice by either blastocyst injection or morula aggregation. In this context, alternative ways for removing the G418 selection marker during the mouse production process are discussed. Also, current methods for assessing *iCre2* tissue specificity and expression levels are recommended, as the mouse models will need to be thoroughly tested before they can become useful tools in immunology. Finally, as the envisaged mouse models are based on 129, the challenges of choosing an appropriate genetic background are explored and several possibilities for the long term application of the novel myeloid *iCre2* mice in studying innate immunity in health and disease are suggested.

10.1 Future Work

10.1.1 The G418 Selection Marker Can Be Removed During the Mouse Creation Process

As described in Chapter 8.6.2, the G418 resistance marker could not be removed from some of the knock-in embryonic stem cell lines due to difficulties in obtaining ES cell subclones. There are, however, several methods to enable marker deletion with high efficiency *in vivo* during or after the mouse production process.

The first is removal of Neo^R at the zygote/embryo stage, which requires an additional mouse generation and is technically complex; but helps maintain the mice's genetic background. In one study 16 cell stage morulae were isolated, infected with adenoviral Cre, cultured *in vitro* until the early blastula stage and re-implanted into foster mothers. The majority of offspring were mosaic for the deleted marker and several were able to transmit the deletion to the next generation. Genomic integration of the virus, and therefore the potential of insertional mutagenesis, was not observed (Kaartinen and Nagy, 2001).

Alternatively, injection can deliver a Cre expressing plasmid directly to the pro-nucleus of fertilized eggs. Two studies, where a floxed Neo^R marker was removed after homologous recombination, reported 1 or 3 correctly deleted offspring per 160 or 176 (Sunaga et al., 1997; Xu et al., 2001b) injected zygotes respectively. In an experiment where the same method was used to eliminate a floxed sequence from a strongly expressed transgene, correct genotypes were obtained in 18 offspring after the injection of 206 zygotes (Araki et al., 1995). Similarly, *in vitro* transcribed Cre mRNA has been injected into the zygote cytoplasm to reduce the

number of floxed transgene copies in a highly efficient manner (de Wit et al., 1998). Most recently, zygote injection of a maltose binding protein (MBP) Cre fusion protein (Kolb and Siddell, 1996) resulted in efficient marker removal depending on protein concentration. The deletion was transmitted to the offspring (Luckow et al., 2009). The use of protein or mRNA is advantageous as there is no risk of insertional mutagenesis caused by accidental genome integration of plasmid or adenoviral vectors, an event that is not routinely screened for but can affect the phenotype.

Secondly, the marker can be removed *in vivo* by mating the proposed *iCre2* knock-in mouse to a deleter strain. This method does not require sophisticated embryo manipulation, but needs at least two generations and may result in mixed genetic backgrounds. It can, however, be easily incorporated into a breeding programme. Deleter strains carry either ubiquitously active Cre – often controlled by a viral promoter – or germline specific Cre. A variety of choices are listed in Table 10.1.

Two possible transgenic Cre strains designed for ubiquitous expression are EIIa-Cre (Lakso et al., 1996) and Meu40Cre (Leneuve et al., 2003). In the former, recombinase expression occurs from an adenoviral promoter whereas the latter utilizes a minimal human cytomegalovirus (CMV) promoter. Both strains facilitate recombination during early embryonic development. F1 offspring are either completely deleted or mosaic for the floxed target gene. EIIa-Cre produced 50% complete deletions in F1 when crossed to a mouse carrying a single copy loxP-Neo-loxP targeted insert in the immunoglobulin light chain kappa constant region locus (Lakso et al., 1996). When the desired total deletion cannot be obtained in F1, further crossing of individuals exhibiting strong mosaicism reliably creates such individuals in F2. Owing to strong expression of EIIa in oocytes, deletion is most efficient when Cre is inherited from the mother. Meu40Cre is similarly effective and recombination levels do not differ between maternally and paternally transmitted Cre (Holzenberger et al., 2000a).

Numerous alternative ubiquitous Cre models exist, including, other CMV promoter controlled strains (Schwenk et al., 1995; Su et al., 2002; Zinyk et al., 1998), β-actin-Cre (Lewandoski and Martin, 1997), c-kit-Cre (Bergqvist et al., 1998) as well as a strain expressing a GFP-Cre fusion transgene from a prion promoter (Scheel et al., 2003).

Germline-specific elimination of the resistance marker is sufficient to transmit the *Δneo* genotype to the offspring. Various transgenic models direct Cre expression to spermatocytes, for example Sycp1-Cre (Chung et al., 2004) or Prm-Cre (O'Gorman et al., 1997). In the transgenic Syn-Cre model, originally produced for neuronal-specific recombination, efficient floxed target gene deletion was recently discovered in the testes during spermatogenesis,

ultimately resulting in knock-out phenotypes for male progeny (Rempe et al., 2006). Equally, there are several Cre systems expressing the recombinase in the female germline, such as Zp3-Cre, GDF-9-iCre and Msx2-Cre, all of which are active during different stages of oogenesis (Lan et al., 2004).

To summarize, there are a number of alternative approaches that allow the removal of the resistance marker from the germ line. The suitability of using a particular injection method or mouse strain needs to be established experimentally. For an optimal choice, the desired target genetic background needs to be considered.

Strain Name	Promoter or Locus/Type	Genetic Background(s)	Activity	Reference
Ubiquitous Cre				
EIIa-Cre	Adenoviral EIIa Promoter/Transgene	C57BL/6 129/Sv FVB/N	From oocyte through, pre-implantation embryo, maternal imprinting	(Lakso et al., 1996)
MeuCre40	Human CMV Promoter/Transgene	C57BL/6 129/Sv	From morula stage until birth	(Leneuve et al., 2003)
Deleter	Human CMV Promoter/Transgene	C57BL/6J	Pre-implantation embryo, X-linked transmission	(Schwenk et al., 1995)
CMV-Cre	Human CMV Promoter/Transgene	C57BL/6	Pre-implantation embryo 2-8 cell stage	(Zinyk et al., 1998)
X-linked CMV Cre	Human CMV Promoter/Knock-in *hprt* locus	129S5/SvEvBRd	Early embryonic development, X-linked transmission	(Su et al., 2002)
Prion-GFPCre	Prion Promoter/ Transgene	129S6/SvEvTac	Early embryonic development	(Scheel et al., 2003)
c-kit-Cre	c-kit Promoter/Transgene	C57BL/6	Early embryonic development	(Bergqvist et al., 1998)
Germline Cre				
Sycp1-Cre	Sycp1 Promoter/Transgene	C57BL/6	Spermatogenesis, paternal imprinting	(Chung et al., 2004)
Syn-Cre	Rat Synapsin I Promoter/Transgene	C56BL/6	Neurons, during spermatogenesis	(Zhu et al., 2001)
Prm-Cre	Protamine 1 Promoter/Transgene	129/SvJae	During spermatogenesis	(O'Gorman et al., 1997)
Zp3-Cre	Zona pellucida protein 3 Promoter/Transgene	C57BL/6J	Postnatal oocytes	(de Vries et al., 2000)
GDF-9-iCre	Mouse Growth Differentiation Factor 9 Promoter/Transgene	C57BL/6SJ	Postnatal oocytes	(Lan et al., 2004)
Msx2-Cre	Msh Homeobox 2 Promoter/Transgene	C57BL/6	Postnatal oocytes	(Sun et al., 2000)

Table 10.1 Overview of Possible Cre Strains for Selection Marker Deletion *in vivo*

10.1.2 Verification of Tissue-Specific Expression

If *iCre2* expressing mouse models were created from the verified knock-in mES cell clones described in this work, the tissue specificity and expression levels, as well as potential leakiness of the Cre recombinase would need to be assessed before the mouse can be a useful tool in immunological research.

In recent years, reporter mice have become popular tools for the exploration of these characteristics. A range of available mice is summarized in Table 10.2. Such animals are genetically engineered to express a reporter, such as β-galactosidase, a fluorescent protein or, most recently, luciferase, following Cre mediated recombination. The Cre strain is crossed to the reporter strain and the offspring are assessed, tissue by tissue, for reporter gene activity. Some systems rely on only one reporter, which is activated by the deletion of a floxed stop cassette placed between the coding sequence and cooperating promoter (Constien et al., 2001; Kawamoto et al., 2000; Luche et al., 2007; Mao et al., 2001; Soriano, 1999). This approach is similar to the GFP reporter construct created in this work aimed at establishing *iCre2* expression in differentiated embryonic stem cells (Figures 9.6&7). Such systems have the disadvantage, that there is no way of knowing whether the absence of the reporter reliably reflects Cre expression or is instead due to the reporter not functioning properly in the particular tissue.

To circumvent this potential problem, models allowing a binary read-out of two reporters have been developed. The first floxed reporter is expressed independently of Cre and gives an indication of tissues in which its presence is measurable. Recombinase catalysed deletion switches on the second reporter and provides an estimate of Cre expression. Various combinations of reporters have been established, ranging from β-galactosidase or β-geo and EGFP (Novak et al., 2000; Yamamoto et al., 2009) to various colour fluorescent proteins (De Gasperi et al., 2008; Muzumdar et al., 2007).

A reporter mouse would ideally allow visualization of *iCre2* activity in all tissues. As these models are genetically engineered, chromosomal positioning effects, instability of multiple transgenes and epigenetic modulation can complicate this analysis. Strong, ubiquitous expression of the reporter requires an appropriate promoter. Two strategies dominate the available systems – the use of the heterologous CAG[10] promoter (Niwa et al., 1991) in a transgene or the endogenous ROSA26 locus[11] (Soriano, 1999) as a site for reporter knock-in.

[10] The CAG promoter combines the CMV early enhancer with the β actin promoter for strong ubiquitous expression.
[11] ROSA26 is a mouse locus, ubiquitously expressed throughout development and in adult tissues, encoding three non-coding transcripts of unknown function.

Despite this, reporter expression varies among the strains. The RA/EG strain (Constien et al., 2001), for example, was reported non-functional in the brain and liver and only partially active in the spleen, whereas Z/EG (Novak et al., 2000) showed no activity in the lung. Several strains are unable to report Cre expression in erythrocytes, including Z/AP (Lobe et al., 1999) and CAG-CAT-EGFP (Kawamoto et al., 2000). Recently, it was found that Z/AP, Z/EG and ROSA26-EYFP were all equally effective during early embryonic development. When it came to assessing Cre activity in adult haematopoietic cells however, both Z/AP and Z/EG reporters showed a lower fraction of reporter positive cells than ROSA26-EYFP. These observations were made with the LysMCre mouse, a granulocyte specific strain similar to the novel models proposed in this work (see Chapter 3.3) (Long and Rossi, 2009). ROSA26-EYFP labelled 85% of peripheral granulocytes in the double transgenic animal, compared to only 57% or 36% with Z/AP and Z/EG respectively. This effect was traced to transgene methylation and incorporation into heterochromatin, silencing the expression of the reporter and inhibiting Cre mediated deletion. Similar effects were reported for other adult tissues, such as kidney, liver, lung and retina (Jullien et al., 2007; Zhang et al., 2005).

Alternatively, recombination specificities and levels can be established by Southern Blot. To that end, the new Cre mouse is crossed to a floxed model of choice and tissues are assessed by quantitative Southern Blot, similar to what has been done in this work to estimate the copy number of the knock-in target locus (Figure 6.4). This technique was used to establish the recombination frequencies in the original publication of the LysMCre mouse (Clausen et al., 1999). Southern Blot and fluorescence measurements, have been reported to be sometimes conflicting. In the assessment of the functionality of the CD11b-Cre transgene, a monocyte targeted Cre and thus similar to the macrophage Cre presented here, Southern Blot analysis showed close to 100% recombination in peritoneal macrophages but only 38% for the reporter EGFP (Ferron and Vacher, 2005). These experiments were, however, carried out using the Z/EG reporter, which has since been identified as being of limited use for haematopoietic cells (Long and Rossi, 2009).

Collectively, these experiences show that the choice of reporter is a crucial factor in assessing the usefulness of the proposed macrophage and neutrophil specific *iCre2* lines. First, the reporter must have sufficient activity in the haematopoietic lineage. The ROSA26 knock-in constructs appear to be the most promising candidates. Second, in order to get an accurate impression of potential leakiness, several reporter strains may be necessary to guarantee truthful reporter read-out across all tissues. Thirdly, recombination levels deduced from reporter gene expression should be verified by an independent method, i.e. Southern Blot. Lastly, as the recombination efficiency depends on the chromosomal location of the loxP sites, levels

established in the reporter strain may not always be an accurate representation for all potential floxed target genes.

Strain Name	Reporter	Locus or Promoter	Activa-table by	Reporter Activity Verified in	Reference
Z/AP	β-geo, Alkaline phosphatase	Transgene, CAG promoter	Cre	Embryo, most adult tissues	(Lobe et al., 1999)
ROSA26-βgeo	β-geo	Gene trapped ROSA26	Cre	Early embryo, haematopoietic tissues, germ line	(Mao et al., 1999)
R26R	β-geo	Gene trapped ROSA26	Cre	Embryo	(Soriano, 1999)
CAG-CAT-EGFP	EGFP	Transgene, CAG promoter	Cre	Most adult tissues	(Kawamoto et al., 2000)
Z/EG	β-gal, EGFP	Transgene, CAG promoter	Cre	Most adult tissues	(Novak et al., 2000)
RA/EG	EGFP	Knock-in RAGE	Cre	Embryo, most adult tissues	(Constien et al., 2001)
ROSA26-EGFP	EGFP	Gene trapped ROSA26	Cre	Embryo, haematopoietic tissues	(Mao et al., 2001)
ROSA26-EYFP ROSA26-ECFP	EYFP ECFP	Knock-in ROSA26	Cre	Embryo, brain	(Srinivas et al., 2001)
Z/RED	RFP	Transgene, CAG promoter	Cre	Embryo, ES cells, most adult tissues	(Vintersten et al., 2004)
mT/mG	Tomato, EGFP	Knock-in ROSA26 & CAG promoter	Cre	Most adult tissues	(Muzumdar et al., 2007)
ROSA26-RFP	RFP	Knock-in ROSA26	Cre	Most adult tissues, T lymphocytes	(Luche et al., 2007)
GNZ	GFP/β-gal fusion	Knock-in ROSA26	Cre	Embryo	(Stoller et al., 2008)
IRG	RFP, EGFP	Transgene, CAG promoter	Cre	Most adult tissues	(De Gasperi et al., 2008)
Tg(CAG-luc)	Luciferase	Transgene, CAG promoter	Cre	Induced gliomal and lung tumors	(Woolfenden et al., 2009)
$R26^{NZG}$	β-gal, EGFP	Knock-in ROSA26 & CAG promoter	Cre, Flp	Most adult tissues	(Yamamoto et al., 2009)

Table 10.2 Reporter Mice for the *in vivo* Assessment of Site Specific Recombination

10.1.3 Considering the Genetic Background of Mouse Models

10.1.3.1 129 or C57BL/6 in Immunology

Inbred mouse strains have initially been generated to achieve certain phenotypes. As such, they differ significantly and also lack the genetic diversity found in wild derived mice (Salcedo et al., 2007; Yoshiki and Moriwaki, 2006). Therefore the genetic background is a crucial factor that needs to be considered when designing experiments. 129 mice, for example, have lower breeding efficiencies, and show significant differences in immune response (McVicar et al., 2002) and behaviour (Crawley et al., 1997) when compared to another commonly used strain C57BL/6. Furthermore, the 129 inbred strain has a complex and poorly documented genetic history. Deliberate and accidental out crossings have created several distinctive substrains and high levels of genetic variation (Simpson et al., 1997; Threadgill et al., 1997).

Nevertheless, the first mouse embryonic stem cell lines were isolated from the 129 background (Evans and Kaufman, 1981; Martin, 1981) and have become widely used. They are robust in culture, exhibit good homologous recombination efficiency, chimera formation and germline transmission frequencies. Consequently and in spite of the associated drawbacks; 129 ES cells remain a major resource for generating mice and numerous models established in the past, particularly Cre producer and responder strains, are based on this background.

129P2 ES cells were also chosen as the starting point for the project presented here because they matched the background of a floxed responder strains previously generated in the lab (Cazac and Roes, 2000). Thus, when both models are crossed to obtain novel conditional knock-outs, there is no mixing of the genetic background. Mixed backgrounds can be detrimental to phenotypic analysis for reasons described below. However, even though the targeting vectors created in this work are derived from a 129 BAC library (more specifically 129S7/SvEvBrd-*Hprt*$^{b\text{-}m2}$) (Adams et al., 2005), they could easily be used to target C57BL/6 ES cells, as there are no known SNPs in either the 4kb upstream or 7kb downstream homology arms[12].

Which mouse strain is best suited for experimental models remains a controversial issue. In immunology, in recent years the pure C57BL/6 background has been established as the de-facto gold standard (Rivera and Tessarollo, 2008). Notably, C57BL/6N embryonic stem cell lines have also been chosen as the base for recently established high-throughput mouse mutagenesis programmes (see Chapter 2.6) and C57BL/6 is also the reference strain of the mouse genome project (Waterston et al., 2002).

[12] As ascertained by carrying out a SNP query comparing the respective sequences between 129/Sv and C57BL/6 using the Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine. World Wide Web (URL: http://www.informatics.jax.org). (March 2010)

The shift from 129 to C57BL/6 has primarily been facilitated by the recent expansion of the ES cell repertoire. It is now possible to generate mice directly from C57BL/6 lines, thus avoiding lengthy periods of backcrossings onto the target genetic background (Auerbach et al., 2000; Kontgen et al., 1993; Pettitt et al., 2009; Schuster-Gossler et al., 2001). It should be noted that in general C57BL/6 embryonic stem cell lines are less robust in culture than 129 lines. For example, Bruce4 ES cell clones are four times more likely to be aneuploid than those of the 129 line E14Tg2a.4. Aneuploidy reduces the ability to re-populate the mouse germ line (Hughes et al., 2007). C57BL/6 ES cells also showed a 40% diminished ability to form chimeras as judged by coat colour when injected into BALB/c blastocysts compared to 129 ES cells injected into C57BL/6. Five independent clones needed to be injected, as opposed to three, to obtain the same number of chimera (Ware et al., 2003). Lower germ line transmission frequencies have also been reported (Auerbach et al., 2000).

Conversely there are published findings in which C57BL/6 embryonic stem cell lines have been shown to be as efficient as those derived from 129. A newly isolated line from C57BL/6NHsd mice, for instance, displayed only insignificantly lower targeting and chimera formation frequencies than an established 129SvJ line (GSI-1), apart from exhibiting slower growth. In terms of germline transmission, the C57BL/6 line was almost 3 times more efficient when considering the necessary chimera breeding time (Keskintepe et al., 2007). Similarly, isogenic BAC vectors targeted to *Ap3b2* and *Lmna* loci showed comparable levels of homologous recombination in both C57BL/6 (Bruce4 line) and 129 (CJ7 and W9.5 lines). Though the proportion of chimeras born from C57BL/6 ES cells was about 50% lower, all tested chimera transmitted to the germline, compared to only 30% from the 129 background (Seong et al., 2004). Better C57BL/6 lines are in development and may replace 129 in the future (Pettitt et al., 2009).

There are, however, limitations of rigidly restricting immunological research to one inbred background. C57BL/6 does possess some features that preclude the analysis of certain questions, for example a low tumor incidence, a skewing towards a T helper 1 (Th1) responses (O'Neill et al., 2000) or the inability to model human asthma accurately (Herz et al., 2004). It is also unwise to try and develop models of complex human disease in just one inbred mouse strain, given that human populations display extreme genetic heterogeneity (Rivera and Tessarollo, 2008).

This problem may be overcome by the ever increasing number of available embryonic stem cell lines. Lines derived from DBA, C3H, FVB and BALB sub strains have been isolated (Schoonjans et al., 2003). Most recently, a line originating from MSM/Ms, an inbred wild-derived strain phylogenetically separated from the common laboratory mouse by about 1

million years, has joined the repertoire (Araki et al., 2009). The rapidly increasing selection should enable the creation of mouse models in any desired genetic background in the near future.

10.1.3.2 Mixed Backgrounds and Appropriate Controls

Should it become necessary to cross the herein proposed 129 *iCre2* producer mice onto C57BL/6 responder mice, the loss of the congenic background means that appropriate controls are needed. Due to linkage, sequences flanking the modified target gene will always reflect the donor ES cell strain even after 10 or more backcrosses achieving a more than 99.9% identity with the desired recipient genome (Figure 10.1A). Linked loci are only segregated following recombination, which is a stochastically unlikely event (Crusio, 2004). Linked regions can be very large in size and have been reported to influence the phenotype. For example, a knock-out of *Cd38* carried out in 129 cells, backcrossed onto C57BL/6 for 10 generations, still carried about 40Mb of linked 129-dervied DNA, which is equivalent to about 400 genes (Leiter, 2002).

Optimal control animals in mixed background phenotypic analyses are those that are identical at all loci. If the introduced modification is dominant, a co-isogenic[13] 129 heterozygote (created by mating the chimera to the strain from which the ES cells were derived) can be crossed to C57BL/6 wild type mice (Figure 10.1B). Control crosses are more complicated if the modified locus is recessive (Figure 10.1C), a co-isogenic 129 heterozygote needs to be crossed to a congenic[14] C57BL/6 homozygote created by at least 10 backcrosses. Offspring will be either homozygous or heterozygous for the targeted loci and flanking genes (Wolfer et al., 2002).

Attempts have been made to reduce the time needed for backcrossing. Recently created mouse SNP and micro satellite databases are useful tools in order to identify the origin of linked flanking genes and locate those possibly impacting on the mutant phenotype (Ridgway et al., 2007). In conventional set-ups, ten backcrosses take 2-4 years on average. Speed congenics uses genome-wide polymorphic markers (microsatellites or SNPs) to identify offspring with minimal linked gene content that are selected for continued breeding. In several studies, the number of backcrosses required to achieve more than 99% congenicity could be halved to 4-5 generations. This method, however, comes with the need for extensive genotyping of all offspring, beyond just the traditional test for the presence of the engineered locus, which creates its own significant cost (Armstrong et al., 2006; Collins et al., 2003; Wong, 2002). Lately, speed congenics were combined with speed mating. Spermatids were isolated from immature mixed background males at 22-25 days of age and injected into C57BL/6 donor oocytes.

[13] Genetically identical to the parent inbred strain except for the mutation in one defined gene.
[14] Genetically identical to the parent inbred strain except for a limited genetic region, i.e. the mutated gene and flanking alleles.

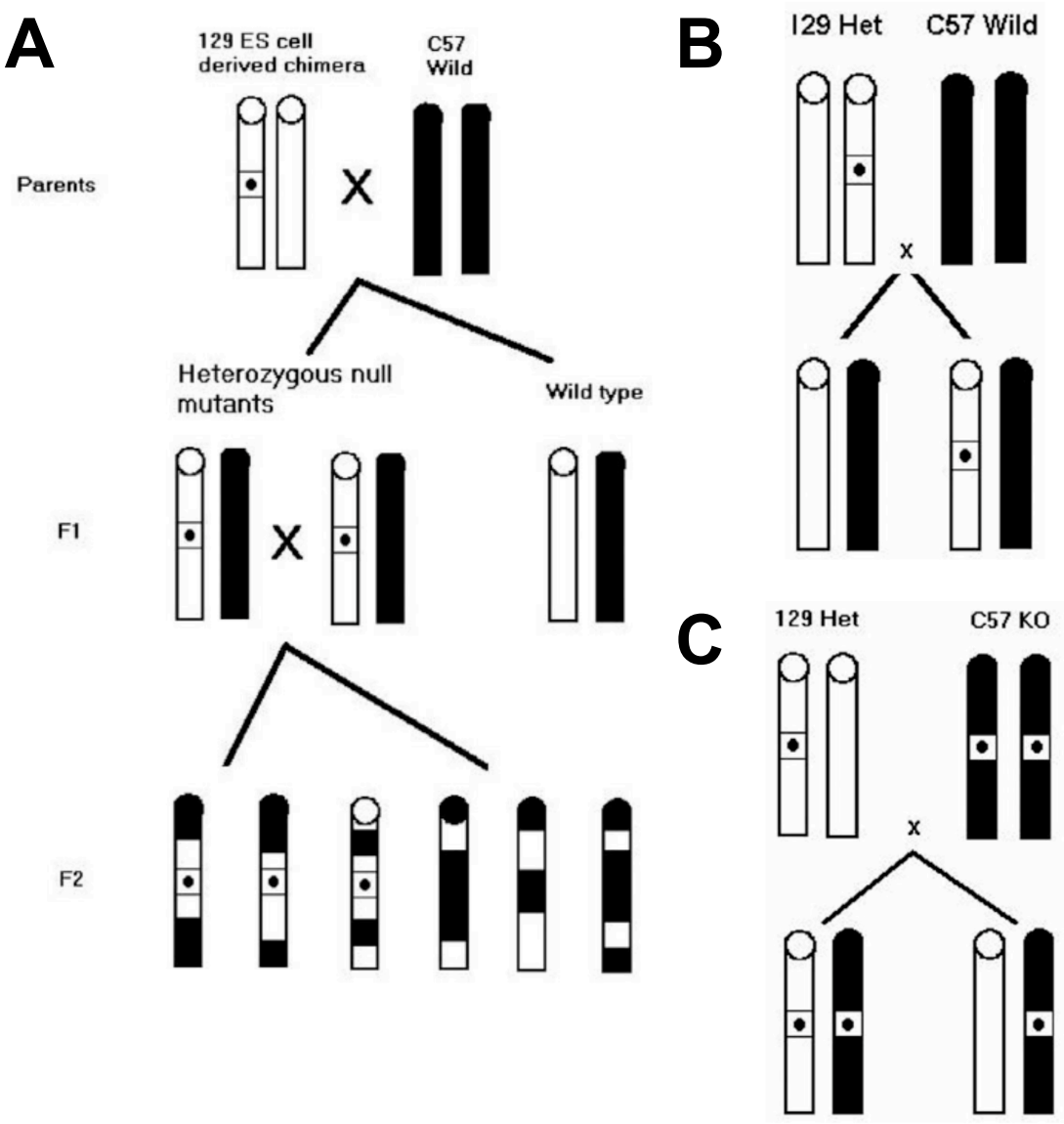


Figure 10.1 Linked Genes and Controls
A illustrates the chromosomal makeup of several generations of crosses between gene-targeted (black dot) 129 mice and pure C57BL/6 animals. By F2 chromosomes will be of mixed origin, genes linked to the modification (black lines), however, will always represent the 129 donor strain. In order to exclude the phenotype arising from linked genes, control animals are necessary. For dominant modifications **(B)**, a co-isogenic 129 heterozygote can be crossed to a wild type pure C57BL/6 mouse. Offspring are either homozygous wild type or heterozygous for the modification. Any observed phenotype must derive from the modification as the animals are otherwise genetically identical. In case of recessive modifications **(C)**, heterozygous co-isogenic targeted 129 mice are crossed to homozygous congenic C57BL/6 mutants. Offspring, whether homozygous or heterozygous, is identical at all loci, including the flanking genes of the targeted locus, thus influence from linked alleles can be eliminated.
Adapted from (Crusio, 2004).

After selecting appropriate offspring based on genome markers, congenicity could be achieved after approximately 6 months with 4-5 accelerated generations (Ogonuki et al., 2009).

In general, there seems to be no ideal genetic background choice for the introduction of targeted modifications (Frankel, 1998). All available inbred strains have certain disadvantageous phenotypic traits due to a lack of genetic variability (Linder, 2006). The mixing of backgrounds can further confound the analysis of observed phenotypes and should either be avoided by using ES cells co-isogenic to the inbred strain chosen for analysis or by carefully monitoring with the appropriate controls. Ideally mutant phenotypes should be analyzed in different, well-characterized, genetic backgrounds. In many cases this labour and cost-intensive approach may not be feasible, though it could be beneficial in dissecting complex phenotypes originating from the function of numerous genes.

In conditional knock-out experiments, an additional Cre-only control – a mouse carrying the recombinase but not the target of choice – should be considered. Most Cre donor strains appear phenotypically normal, though some researchers consider this a surprising feat in light of the ongoing potential of Cre toxicity. It has been postulated that Cre mice, due to developmental adaptation, possess a more active DNA repair machinery, which is particularly relevant if genes involved in DNA repair are to be assessed (Schmidt-Supprian and Rajewsky, 2007). There are also Cre producers whose phenotypic abnormalities have complicated the investigation of the conditional knock-out. The RIP-Cre model, for example, was eventually shown to be glucose intolerant – problematic in a model primarily used for diabetes research (Lee et al., 2006). Therefore, the inclusion of a Cre control in all experiments ensures that observed variances, especially subtle ones, can indeed be attributed to the gene investigated.

10.2 Application of the Envisaged *iCre2* Models

10.2.1 Dissecting the Function of the TGF-β Receptor in the Myeloid System

The TGF-β cytokine system plays roles in tissue homeostasis, disease and development. It is involved in diverse cellular processes, such as differentiation, proliferation and apoptosis (Kang et al., 2009). As conventional TβR knock-outs are lethal (Oshima et al., 1996) and organism wide deletion of the receptor's agonist isoforms results in complex, difficult to dissect and potentially also lethal phenotypes (Dunker and Krieglstein, 2000); conditional models are the best way to examine transforming growth factor β receptor type II (TβR) function in individual immune cells. TGF-β function is considered to be of major regulatory importance in the immune system in general and a large body of research focuses on its anti-inflammatory impact

as a potential treatment route for immune disorders (Moore et al., 2008; Schmidt-Weber and Blaser, 2006; Taylor, 2009; Wahl et al., 2006).

One of the aims of this thesis is to utilize the novel *iCre2* mice for the myeloid specific in-activation of TβR as relatively little is known about the role of the TGF-β system in neutrophils and macrophages and prior research has been predominantly focused on creating conditional knock-outs in either B cells or in the entire haematopoietic system.

A mouse model where loxP sites flank exon 3 of the gene encoding the TGF-β receptor's ligand binding domain has been created in the lab (Cazac and Roes, 2000). Previous use of the floxed TβR mouse, in conjunction with CD19-Cre, enabled the B cell specific ablation of TβR, which identified important roles for TGFβ in B cell homeostasis, maintenance of an appropriate immune response level and the control of IgA production (Cazac and Roes, 2000). Furthermore, TGF-β receptor induced inhibition of antigen and Toll-like receptor signalling, as well as increased calcium flux and suppression of apoptotic factors, were shown to be main elements in avoiding autoimmunity (Roes et al., 2003).

A similar mouse model, in which exon 4 of the TβR gene is flanked by loxP sites, is available. Deletion resulted in receptor inactivation as exon 4 encodes the majority of the receptor kinase and the entire transmembrane domain (Leveen et al., 2002). A widespread conditional knock-out in the haematopoietic system, achieved with the interferon inducible Mx1-Cre strain (Kuhn et al., 1995), caused an inflammatory disorder characterised by multifocal tissue leukocyte infiltration that typically was lethal after 8-10 weeks (Leveen et al., 2002) and mirrored the conventional knock-out phenotype (Oshima et al., 1996). The same model was used to show that TβR deficiency does not influence thymic T cell differentiation, though there was an increase in CD8+ T cell proliferation (Leveen et al., 2005).

10.2.1.1 TGF-β and Neutrophils

Neutrophils, like most tissues in the body, express TGF-β receptors and are capable of storing and secreting TGF-β (Fava et al., 1991). The effect of TGF-β signalling on neutrophil recruitment and activation *in vivo* remains unclear. On the one hand, TGF-β can act as a very potent chemoattractant for neutrophils *in vitro* (Brandes et al., 1991; Reibman et al., 1991) as well as *in vivo* after being injected into rat knees resulting in an arthritis like phenotype (Allen et al., 1990; Fava et al., 1991) or applied via the intra-bronchial route of rats experiencing *E.coli* induced pneumonia (Cui et al., 2003). These findings are confirmed by reduced neutrophil migration in mice where the TGF-β signalling molecule Smad3 is knocked-out (Yang et al., 1999). On the other hand, TGF-β has been demonstrated to impair IL-8 dependent neutrophil

transendothelial migration (Smith et al., 1996), reduces neutrophil adhesion to endothelial cells isolated from the umbilical vein (Gamble and Vadas, 1988), inhibits neutrophil response when co-injected with LPS into rat trachea (Ulich et al., 1991) and also impairs neutrophil migration to the peritoneum after stimulation with thioglycollate (Gresham et al., 1991). One study found an increased neutrophil influx into the lungs when TGF-β function was suppressed by an antibody in a guinea pig model of tuberculosis, even though an adequate immune response failed to develop (Allen et al., 2008).

Many of the downstream signalling targets relevant to TGF-β chemotaxis in neutrophils are poorly characterised. Weak activation of the p38 MAP kinase (Hannigan et al., 1998) and a unique subset of G proteins (Haines et al., 1993) are involved in signalling, as is the modulation of L-Selectin expression (Malipiero et al., 2006). Adhesion and migration in response to TGF-β is dependent on fibronectin deposition in the extracellular matrix (Parekh et al., 1994). TGF-β signalling in conjunction with other inflammatory stimuli such as LPS, does not, however, activate neutrophil processes such as the respiratory burst or de-granulation (Haines et al., 1993; Shen et al., 2007), though one study infers such activation when neutrophils are attached to fibrinogen *in vitro* (Balazovich et al., 1996)

Recent findings suggest that TGF-β receptor induced neutrophil suppression can have adverse effects on disease outcome. In a mouse model of meningitis, the inactivation of TβR on leukocytes promoted host defense and reduced brain damage and was accompanied by an increased number of neutrophils in the central nervous system (Malipiero et al., 2006). Similarly, tumor infiltrating neutrophils assumed capabilities that enabled them to arrest tumor growth and kill tumor cells in conjunction with CD8+ T cells when the TβR kinase was inhibited by a small molecule (Fridlender et al., 2009), The exact mechanisms of these processes are unknown, but could provide novel treatment options.

10.2.1.2 TGF-β and Monocytes/Macrophages

As with neutrophils, the knowledge about TGF-β function in monocytes and macrophages is limited. Resting monocytes constitutively express TGF-β receptors and react to their stimulation by chemotaxis (Wahl et al., 1987). Activation and differentiation of monocytes results in a marked down-regulation of cell surface receptor concentration which reduces the sensitivity to TGF-β (McCartney-Francis and Wahl, 1994).

The role of TGF-β signalling in the macrophage context is somewhat unclear. Pro-inflammatory effects include the recruitment to sites of injury, secretion of cytokines, for example IL-1β and TNF-α (Wahl et al., 1987), expression of macrophage integrins for endothelial adhesion and

matrix metalloproteinases (MMP) for the dissolution of vascular basement membranes (Wahl et al., 1993), induction of Fc gamma receptor 3 (FcγRIII) to recognize bound IgG and stimulate phagocytosis (Welch et al., 1990) and priming of bone marrow derived macrophages to produce more nitric oxide in response to some parasites (Lin et al., 1995). *In vivo* studies support this pro-inflammatory impact. Blocking TGF-β receptor associated kinase function, for example, lowered macrophage TNF-α production and reduced levels of tissue damage and lethality in mouse models of septic shock (Chen et al., 2008). In activated human macrophages, TGF-β stimulation resulted in a pattern of inflammatory gene activation similar to that found in macrophages associated with atherosclerotic lesions (Gratchev et al., 2008).

Yet, TGF-β receptor signalling in macrophages also has powerful anti-inflammatory effects. These include the deactivation of macrophages, i.e. terminating the production and release of antimicrobial substances such as reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) and inflammatory cytokines (Bogdan and Nathan, 1993; Ding et al., 1990; Langermans et al., 2001; Tsunawaki et al., 1988) or the suppression of pro-coagulant activity (Jungi et al., 1994). Macrophages produce TGF-β after phosphatidylserine receptor mediated ingestion of apoptotic cells (McDonald et al., 1999) and downregulate themselves in an autocrine manner. Inflammatory cytokine production is reduced through the sequential activation of TβR and ERK and ultimately inhibition of p38 MAPK (Xiao et al., 2002). The same process, though meant to prevent excessive inflammation, is also associated with pathological fibrosis (Nacu et al., 2008). In general, the dampening effects of TGF-β on macrophages are believed to be essential to resolve inflammation and prevent tissue damage.

A link between TGF-β signalling in macrophages and cancer has been established, similar to that found with neutrophils, though the findings appear to be contradictory. One study observed that reduced TGF-β receptor activity in advanced colon cancer is accompanied by a higher fraction of infiltrating tumor associated macrophages and worsened clinical prognosis (Bacman et al., 2007). TGF-β receptor suppression by a dominant negative mutant, however, rendered the murine macrophage cell line RAW264.7 more cytotoxic towards co-cultured carcinoma cell lines due to the increased production of TNF-α, IFN-γ and induced nitric oxide synthase (iNOS). This study postulated that TGF-β resistant macrophages have a higher tumour killing ability, though this comes at the price of increased systemic autoimmunity (Lee et al., 2007).

10.2.1.3 Summary

An overall view is emerging that TGF-β function in the immune system in general, and in neutrophils and macrophages in particular, is of bipolar nature (summarized in Figure 10.3). Whether TGF-β receptor signalling will be pro- or anti-inflammatory seems to depend on the

cellular differentiation status, local levels of TGF-β, its receptor and other cytokines as well as the stage of the inflammatory process (Ashcroft, 1999; Wahl, 2007). The role of TGF-β appears to be the establishment of an adequate immune response early on in inflammation, but also the resolution of the late inflammatory process. The overzealous dampening of immune excitability by TGF-β, however, plays an important role in disease.

Much of the current knowledge about TGF-β function in innate immunity has been obtained by systemic blockade using anti-TGF-β or anti TGF-β receptor antibodies, TGF-β decoy molecules or TGF-β serine/threonine kinase inhibitors (Malipiero et al., 2007). All these strategies affect multiple tissues and, due to the widespread influence of TGF-β, bear the risk of creating complex phenotypes that may preclude discovering effects on individual immune cell types. The herein proposed neutrophil and macrophage specific *iCre2* deleter strains, however, are ideally suited to produce superior models in which receptor function is only switched off in the murine myeloid compartment. Combining such novel conditional knock-outs with murine disease models may help to clarify the role of TGF-β in the innate immune system.

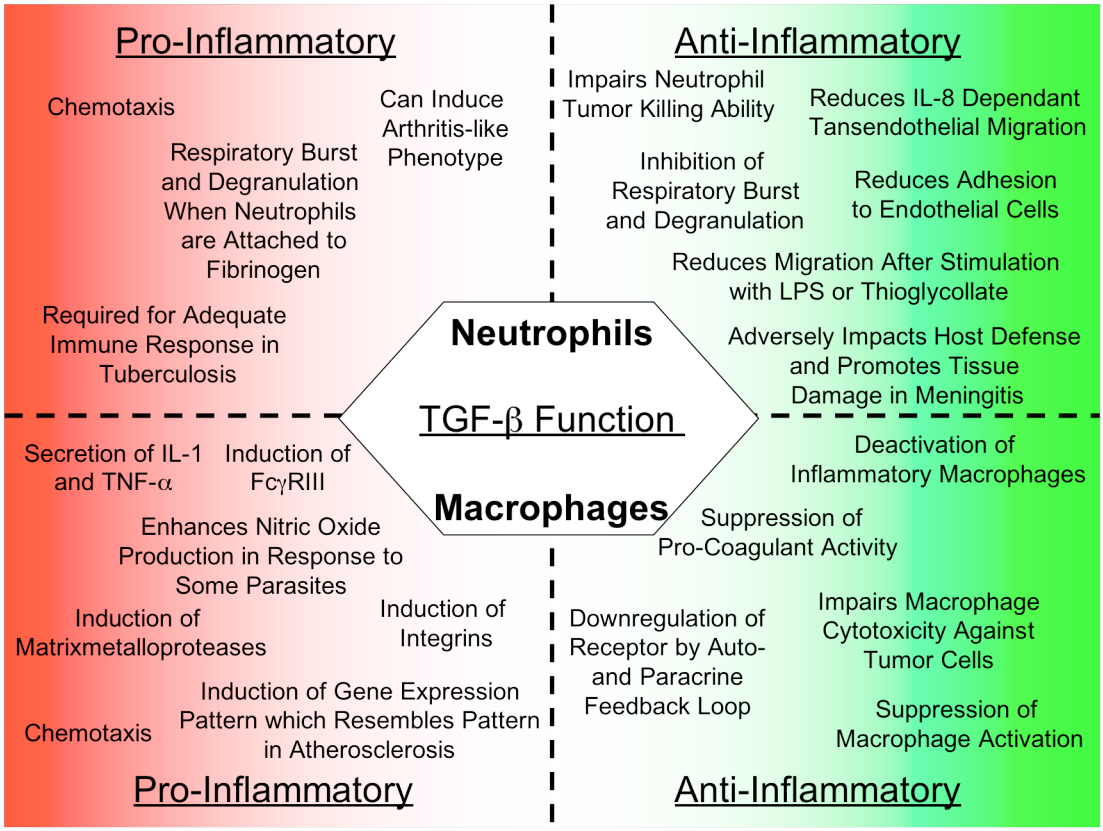


Figure 10.2 Multiple Effects of TGF-β on Neutrophils and Macrophages
TGF-β receptor function exerts varied influences neutrophils (top) and macrophages (bottom), which are often of bipolar nature and cause cells to react either pro- (left) or anti-inflammatory (right).

10.2.2 Assessing the Role of Macrophages and Neutrophils in Health and Disease

Despite being primarily intended to create conditional TGF-β receptor knock-outs, the proposed *iCre2* models can be applied to facilitate the tissue specific ablation of any gene of interest for which floxed mouse models are either already available or can be produced. As such they will significantly expand the toolbox available for immunological research by allowing the somatic mutagenesis of widely expressed genes to elucidate their role in health and disease (Roes, 2007). This concluding section is aimed at suggesting an overview of areas for which the novel *iCre2* mice may be useful.

The *Msr1 iCre2* mouse is anticipated to mediate deletion in all populations of murine macrophages. As such, it would be ideally suited to study gene function in conditions characterized by systemic dysregulation of monocyte/macrophage activation processes. One such disease is rheumatoid arthritis (RA), a multisystemic autoinflammatory syndrome that results in the destruction of synovial tissues (Drexler et al., 2008). RA is a highly complex disease with virtually all signalling pathways, soluble mediators and cell surface proteins affecting macrophages being altered and contributing to pathogenesis (Kinne et al., 2007). Therefore, a virtually endless array of genes that have been implicated in RA, may be suitable for conditional knock-out to help and understand disease etiology or identify novel drug targets. These may include transcription factors of the fos and jun families (Zenz et al., 2008), whose conventional knock-outs largely exhibit lethal phenotypes (Hilberg et al., 1993; Karreth et al., 2004; Schorpp-Kistner et al., 1999; Schreiber et al., 2000); cytokines, their receptors and downstream signalling molecules considered to be novel drug targets such as IL-15, IL-17 or IL-18 (Pickens et al., 2010; Ruckert et al., 2009; Shao et al., 2009); or the macrophage matrix metalloprotease MMP12 (Chen, 2004; Liu et al., 2004) co-responsible for observed tissue damage.

The *Marco iCre2* strain, with its more restricted expression pattern and its expected ability to catalyse recombination in alveolar macrophages in particular, will be ideally suited to investigate murine models of airway disease. The role of alveolar macrophage (AM) function in asthma, a condition characterized by chronic inflammation and the narrowing of bronchi, is generally considered to be the suppression of airway hyperresponsiveness. How AM control this process, whether cell-cell contacts or soluble mediators are involved, and how AM behaviour is altered in the disease state is not understood (Peters-Golden, 2004). Murine models in which candidate mediators or their up- and downstream targets are inactivated in an AM specific manner will help to comprehend disease progression. Currently discussed candidates include IL-17 (Song et al., 2008), IL-33 (Kurowska-Stolarska et al., 2009), nitric oxide

(Esposito and Cuzzocrea, 2007; Mulrennan and Redington, 2004) and Prostaglandine E2 (Huynh et al., 2005).

Finally, the envisaged *Itgb2l iCre2* strain, with its anticipated pan-neutrophil expression pattern, may help to shed light on neutrophil involvement in chronic illness. In cancer, for example, neutrophils have recently been found to play key, but not well understood, roles in tumor growth and metastasis. Neutrophils can have pro- and antitumor effects, though what regulates this switch is not known (Fridlender et al., 2009; Mantovani, 2009). Effector molecules and pathways connected to this recently identified behaviour, and therefore prime candidates for conditional knock-outs, are soluble mediators and their receptors IL-1, IL-8, IFN-β, MMP-9 as well as transcription factors STAT-3 and c-myc (De Larco et al., 2004; Jablonska et al., 2010).

CONCLUSION

The main aim of this thesis was to establish novel myeloid specific Cre mice for the application in conditional mutagenesis. To that end, three murine target genes, *Itgb2l*, *Marco* and *Msr1*, with predicted expression specificities in neutrophils or macrophages respectively, had been chosen and BAC knock-in vectors harbouring the improved recombinase *iCre2* had been created by Red/ET recombinogenic engineering prior to the start of this project. These vectors were used to directly target 129P2 embryonic stem cells as the long BAC homology arms were believed to have a beneficial influence on the targeting frequency. However, the length of the homology significantly complicated the screening regime without actually improving the targeting efficiency. When the discovery of antiparallel loxP sites in the original targeting vectors precluded the removal of the G418 selection marker in all targeted cell lines, the project was restarted with an overhauled vector design. In the second generation vector construction process, after re-creating BAC knock-ins, an additional round of Red/ET recombineering was applied to transfer the *iCre2-Neo*^{R} cassette flanked by a total homology of 11kb into plasmid based vectors. Several additional sequence inconsistencies, including a cryptic splice site in the *iCre2* coding sequence causing aberrant splicing and errors introduced by PCR amplifications, were found and repaired. The second generation targeting vectors were then used to obtain a total of 650 G418 resistant clones. Nine knock-ins for *Itgb2l* and *Marco*, with targeting frequencies of 8 and 10% respectively, could be confirmed. The plasmid vectors did indeed facilitate a straightforward screening regime whilst maintaining good targeting frequencies. The repaired loxP sites were functional, as indicated by the successful removal of the selection marker. Some technical difficulties were encountered in producing pure deleted cell populations, which may partially be due to the low efficiency of transiently delivering Cre expression plasmids to the knock-in lines by electroporation.

A pilot study was carried out to analyse tissue specific *iCre2* expression *in vitro*, before investing significant resources in the production of a mouse. To that end, the ability of embryonic stem cells to differentiate into virtually any tissue can be utilized to assess promoter specificity using a reporter system or RNA analysis. Macrophages were differentiated relatively quickly with a minimal protocol based on three dimensional aggregation of ES cells and selective outgrowth of myeloid progenitors in the presence of IL-3 and MCSF. The read-out of *iCre2* expression, however, was hampered by the reduced ability of knock-in cell lines to produce macrophages and a certain level of dysregulation in the *Marco* locus was observed. The question remains whether this is a locus specific feature or due to the presence of *Neo*^{R}. If the former is true, *Marco* may not be as accurate driving *iCre2* expression *in vivo* as initially hoped. The established procedure could be applied to the *Itgb2l* knock-ins to test expression *in vitro* in the future, though the generation of neutrophils requires a more time and resource intensive

protocol. The differentiation approach may also provide a useful alternative for studying aspects of myeloid cell biology *in vitro*, as genetically engineered ES cells can be turned into neutrophils or macrophages without having to produce a mouse model.

In essence, this project provides the resource to generate neutrophil and macrophage specific *iCre2* mice in the near future, which will prove useful in light of the tens of thousands new floxed cell lines and mouse models currently being generated by high throughout mutagenesis programmes. Retrospectively, as a lot of time was required to correct the sequence inconsistencies encountered in the course of this project, it may have been prudent to advance the mouse generation process in parallel with the differentiation experiments, and remove the Neo^R selection marker *in vivo* instead of focusing on *in vitro* deletion. Ultimately, only the completed mouse model will accurately predict how useful the new Cre lines are going to be. With absolute tissue restricted expression patterns in Cre mice being the exception rather than the rule, specificity will need to be very carefully assessed. Compared to conventional Cre, *iCre2* should provide higher, more consistent, expression and therefore recombination levels. In addition, the knock-in loci are genes of the immune system, which opens up the option to induce inflammatory processes to further increase expression levels *in vivo*.

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APPENDICES

APPENDIX A: PRIMER SEQUENCES

Name	Sequence (5'- 3')	Function
BAC bckgrd 5'	TAC CCA CTA GTC AAT TCG GGA GG	5' BAC backbone PCR
BAC bckgrd 3'	TAC CTG CGC TGT TGT CAG GCT TTG	
PactF	AGA CAT TGG TGC CTG ACT TGT ATG G	*Itgb2l* specific Primer for Cre PCR
MarcoF	TTG TAT GCA TCT CCA GCT AGC TGC	*Marco* specific Primer for Cre PCR
Msr1F2	AGC CTC CTT TAG TCC ACA TGG TCC	*Msr1* specific Primer for Cre PCR
iCrecheck	AAA TCA GTG CGT TCG AAC GCT AGA GC	Cre specific Primer for Cre PCR
Pact1	AGT CGA CTA TTT TCC AGT TCA AAG GG	PCR to amplify *Itgb2l* external Southern Probe
Pact2	TGC TCT GTA GGC TTT AAG GCC TGG	
PactBamHISR	GTG TAA ATG TCT GCT GAG GGT AGA	PCR to amplify *Itgb2l* internal Southern Probe
PactBamHISL	ATT TCC AGT GTT GTG TAG GGA CTT	
PactTarg	TCT CCA AAC AAA GGC TGT ACA CAG ATG TGC C	External primer for Long Range PCR of *Itgb2l*
Pactin2	AGG AGG ACT CAA TTA GGC TAC GGT AAC CTC C	Internal primer for Long Range PCR of *Itgb2l*
iCrecheck2	ATC CTG GCA ATT TCG GCT ATA CGT AAC AGG	Cre primer for Long Range PCR
PactNeoExcF	ACT TAG CCT GGG GGT AAC TAA ACT	PCR to screen for Neo deletion
PactNeoExcB	GTC CTT GGT ACA CAA CTG AGA CAG	
NeoloxF seq	GTA CCG AAC ATA CGC TCT CCA	Sequencing of pSP72-iCre2-pAp-NeoLox
NewNeoLoxF	CAT TGC ATC AGC CAT GAT GGA TAC TTT CTC	Primers to repair pSP72-iCre2-pAp-NeoLox
NewNeoLoxR	AGT ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA TCT ACC GGG TAG GGG AGG CGC TTT T	
Pactinitialtarg	GGA GAG GAT CTG TGA CTC CTA GAG TCA CCA AGC CCT TCC CCC TAC AGG ACA TGA AAC GCC CCA GGC CAT CCA TT	Targeting primers for bMQ203N9
Pactterminaltar	TCC AGA GAC AGC AGC CCA GCT AGG ACA GGT AGC AGA GTG CAC TGG CCC AGA TAA CTT CTG ATA ATG TAT GCT AT	
Pactpucresf	CCA TAG TTA GGA TAT CAC AGC TCC TAT GTC CTC ACC TGT AAT TGG GCT ATC CTG TGT GAA ATT GTT ATC CGC TC	Targeting primers for Rescuing the BAC insert of bMQ203N9-iCre2
Pactpucresrev	ATA TAC TAC ACA GAC ATA ATT GTA CAT GCA CAT AGA CAA GGC ATG TGC ATC CTG CAG GTC GAC TCT AGA GGA TC	
Msr1F	ACT TGT GCA TTG AAG AGA AGG C	Alternative *Msr1* forward primer
Msr1R	ATG GCA AGG AAA CAC ACT AAG C	*Msr1* reverse primer

Name	Sequence (5'- 3')	Function
Msr3check	TCG GCA GCC ATT CAC TCT CTA AGC	Alternative *Msr1* reverse primer
Pact3check	TGA GAC TAT GAC ACA TCA TCA GGC	Alternative *Itgb2l* reverse primer
MaR	AAG TCC AGA TGG ACA GAG TCT AGC	*Marco* reverse primer
Marco3check	AAG CTG GCT TGG CAC TCC TCA TCC	Alternative *Marco* reverse primer
Maintarg	ATT GTA AAC AGA GGG AAG TGG ATA AAT CAG TGC TGT CTT CTT TAC CAG CAA TGA AAC GCC CCA GGC CAT CCA TT	Targeting primers for bMQ239G13
Matermtarg	TCA ACA TAA GTA ATT TAA ATA AGA AAT AAA TTA AAC TAT ACC TCT TTT GTA TAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT CTA CCG	
Maresf	AGT TTC TGG TCC TCC CGG TTC CCT TCC CTT ATC CCC TTG TAC TGG TGG CAC CTG TGT GAA ATT GTT ATC CGC TC	Targeting primers for Rescuing the BAC insert of bMQ239G13-iCre2
Maresr	ACT ATA TCT CTT CTG CCT TTA AAC ATG TTT TGA CAG TGA GGG CTG CAT ACC CTG TGT GAA ATT GTT ATC CGC TC	
Msr1intarg	TGG GCA GCA CAG AAG ACA GAG CCG ATT TTG ACC AAG CTA TGT TCC CTG TGA TGA AAC GCC CCA GGC CAT CCA TT	Targeting primers for bMQ294K23
Msr1termtarg	GCA ACT GCA TTA CAA GGG AGT CAT ACT TTA CCA TTG ATT TCG AAG GTC TCA TAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT CTA CCG	
Msr1resf	TAG AGA ATC AGA TGT CGT TCA GGT CTT TGC AGA TAA TGT GAC CTT TCT AAC CTG TGT GAA ATT GTT ATC CGC TC	Targeting primers for Rescuing the BAC insert of bMQ294K23-iCre2
Msr1resr	TCT GAT CT CTG GTC ATC AAG TAG GCC CGG CCT ACT GGC CAG TGA GCC TCC CTG CAG GTC GAC TCT AGA GGA TC	
Paext	ATA CCC ATC AAG TAG AGG CAC CAT GAA ACC	Forward primer for 5' *Itgb2l* PCR
Maext	GAG TGC TTC TTT TAC AGA TGT GCC ACG AAC	Forward primer for 5'Marco PCR
Msr1ext	CAA AAT GTG GAA TTT GGA GTT XAA GTC AGG	Forward primer for 5' *Msr1* PCR
Msr1ext2	AAT GCA TGT AGG TGC AAT ATC TCT GGA CAA C	Alternative forward primer for 5' *Msr1* PCR
Pact3'F	TAG TGA GAC GTG CTA CTT CCA TTT GTC ACG	Primer pair for 3' *Itgb2l* PCR
Pact3'B	GTG ATG CTT TCC ACA GAC AGC AAA GAA TTT	
Pa3'F2	CTA AAG CGC ATG CTC CAG ACT GCC TTG G	Alternative primer pair for 3' *Itgb2l* PCR
Pa3'B2	CCC TCT ACA ACT CTA GGA GGA GGG GGA CTG	

Name	Sequence (5'- 3')	Function
Ma3'F	GGG GAA CTT CCT GAC TAG GGG AGG AGT G	Primer pair for 3' *Marco* PCR
Ma3'B	AAT GTG GTT AGT GCC CAA GGA ATG ACA CTT	
Itgb2lSF2	CCT ACC TTG TAG CCG CTG AG	Primer pair to amplify *Itgb2l* 5' Southern Blot external probe
Itgb2lSR2	AGG GTT CTG CCT GAA GGT TT	
MaSF2	AGG CAC ACA GGC AAC TTT TT	Primer pair to amplify *Marco* 5' Southern Blot external probe
MaSR2	TGC CCA TCT GTT GTG AGT GT	
Itgb2l 3' ext F	TTC TTT GTG CAT GCT TCT GG	Primer pair to amplify *Itgb2l* 3' Southern Blot external probe
Itgb2l 3' ext R	GTC AGC TTG GGG ATG AAT GT	
Itgb2l 3' int F	ATG CAA GCC AAA GCA GAA CT	Primer pair to amplify *Itgb2l* 3' Southern Blot internal probe
Itgb2l 3' int R	AAC GAT GTC AGG GGA GTG AC	
Ma 3' ext F	GCA CGC GAG TGT GAG AGA TA	Primer pair to amplify *Marco* 3' Southern Blot external probe
Ma 3' ext R	CTT CCC TGG TGA AGA GCC TA	
Ma 3' int F	TGA GCA CCG AGA GAC CTT TT	Primer pair to amplify *Marco* 3' Southern Blot internal probe
Ma 3' int R	ACT GGG CTG AGA TGA TGG TC	
Cre Probe F	CGT ACT GAC GGT GGG AGA AT	Primer pair to amplify probe for *ΔNeo* Southern Blot
Cre Probe R	TTG CCC CTG TTT CAC TAT CC	
HPRT_F	CAC AGG ACT AGA ACA CCT GC	Primer pair to amplify *HPRT* cDNA
HPRT_R	GCT GGT GAA AAG GAC CTC T	
Cre141f	ATA CCT GGA AAA TGC TTC TGT CCG	Primer pair to amplify *iCre2* cDNA
Cre1045R	ATC TTC CAC CAG GCG CAC CAT TGC	
CrecDNAF	GAT CCG AAA AGA AAA CGT TGA TGC	Alternative primer pair to amplify *iCre2* cDNA
CrecDNAR	AGT CAT CCT TAG CGC CGT AAA TAC	
Ma sense	GAA ACA AAG GGG ACA TGG G	Primer pair to amplify *Marco* cDNA
Ma antisense	TTC ACA CCT GCA ATC CCT G	

Table A: Overview of Primer Sequences