Tetracycline-inducible RNAi Knockdown of SCL (Stem Cell Leukaemia) in Mice

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Abstract

RNAi (RNA interference) is a powerful technology for sequence-specific targeting of mRNAs. This thesis was aimed at establishing conditions for conditional RNAi-mediated silencing first *in vitro* and subsequently also in transgenic mice. As a target the basic helix-loop-helix transcription factor encoding gene SCL (stem cell leukaemia also known as Tal-1 or TCL5) was used. SCL is a key regulator for haematopoietic development and ectopic expression of SCL is correlated with acute T-lymphoblastic leukaemias.

Loss of SCL function studies demonstrated that *ab initio* deletion of SCL resulted in embryonic lethality around day E9 in gestation. To be able to conditionally inactivate SCL, RNAi technology was combined with the tetracycline-dependent regulatory system. This strategy allowed to exogenously control the induction of RNAi in a reversible fashion and consequently the generation of a completely switchable RNAi knockdown.

First a suitable vector allowing for co-expression of tetracycline-controlled shRNAs (small hairpin RNAs) and constitutively active EGFP (enhanced green fluorescent protein) was generated. This novel vector, pRNAi-EGFP, was then evaluated for EGFP expression and tetracycline-mediated expression of shRNAs. Four sequences targeting different regions within the SCL mRNA were tested for their efficiency to specifically knockdown SCL. These experiments were performed in M1 murine leukaemia cells and subsequently in the HEK 293 cell line, expressing an engineered HA-tagged SCL protein. The second assay provided a solid experimental method for determining the efficiency of different SCL-siRNA knockdown constructs in tissue culture. Western blotting analyses revealed a down regulation of SCL protein for all four tested SCL-specific target sequences albeit with different knockdown efficiencies (between 25% and 100%). Furthermore, stringent tetracycline-dependent switchability of shRNA expression was confirmed by co-transfecting the SCL-specific pRNAi-EGFP vector (SCL-siRNA) together with the HA-tagged SCL expression plasmid into the HEK 293TR /T-REx cell line constitutively expressing the tetracycline repressor (TetR). These series of experiments demonstrated tight regulation of siRNA expression without background activity.

To be able to control the SCL knockdown *in vivo* and especially to circumvent any possible embryonic lethality a transgenic mouse line with general expression of a tetracycline repressor was needed. Two alternative methods were used to generate TetR mice. The first approach was to co-inject the tetracycline-regulated RNAi vector together with a

commercially available and here specifically modified T-REx expression vector (SCL-siRNA T-REx FRT LoxP mouse line). The second method involved the generation of a TetR expressor mouse line, which was then used for donating TetR-positive oocytes for pronuclear injection of the RNAi vector (SCL-siRNA T-REx mouse line).

As expected, and in agreement with data from conditional *Cre*-controlled adult SCL knockout mice, post-transcriptional silencing of SCL by RNAi caused a shift in the maturation of red blood cell populations. This was shown in the bone marrow and peripheral blood by FACS analysis with the red blood cell-specific TER119 and CD71 markers which can be used to define erythrocyte differentiation (Lodish plot technique).

In conclusion this study established conditions for effective SCL RNAi-mediated silencing *in vitro* and *in vivo* providing an important tool for further investigations into the role of SCL and, more generally, of its *in vivo* function in haematopoiesis and leukaemia. Most importantly, the here acquired knowledge will now allow the establishment of other completely conditional and reversible knockdown phenotypes in mice.

Zusammenfassung

RNAi (RNA interference) ist eine wirkungsvolle Technologie, welche die sequenzspezifische Degradation von mRNA erlaubt. Ziel dieser Doktorarbeit war es, die notwendigen experimentellen Bedingungen für die schaltbare RNAi-vermittelte Degradation spezifischer RNAs zuerst *in vitro* und dann in transgenen Mäusen zu etablieren. Als Zielgen zur Degradation wurde der basische Helix-Loop-Helix Transkriptionsfaktor SCL (Stem Cell Leukaemia, auch Tal-1 oder TCL5 genannt) verwendet. SCL ist ein Schlüsselregulator für die Bildung von Blutzellen, und ungewollte Expression von SCL wurde auch bei akuten lymphoblastischen Leukämien gefunden.

Klassische knockout Versuche zeigten, dass der *ab initio* Verlust von SCL embryonal lethal um den Tag E9 war. Um die Funktion von SCL exogen schaltbar inaktivieren zu können, wurde in dieser Arbeit RNAi Technologie mit dem durch Tetrazyklin schaltbaren tet on/off System kombiniert. Diese Vorgehensweise erlaubt die exogen-regulierbare und reversible Kontrolle der RNAi Induktion und ermöglicht somit die Schaltbarkeit des knockdown Phänotyps.

Im Rahmen der hier vorgestellten Doktorarbeit wurde zuerst ein geeigneter Vektor hergestellt, welcher sowohl die durch Tetrazyklin kontrollierte Transkription von so genannten shRNAs (kurze, haarnadelförmige RNAs oder hairpin RNAs) erlaubt und gleichzeitig permanent EGFP (enhanced green fluorescent protein) exprimiert. Dieser neue Vektor, pRNAi-EGFP, wurde sowohl auf Expression von EGFP als auch auf kontrollierte Tetrazyklin-vermittelte Expression von shRNAs getestet. Vier verschiedene Zielsequenzen, welche die SCL mRNA erkennen, wurden auf ihre Wirksamkeit getestet, spezifisch SCL zu inaktivieren. Hierfür wurden zuerst leukämische M1 Mauszellen verwendet und dann später die HEK 293 Zelllinie eingesetzt, welche ein speziell für diesen Zweck hergestelltes, mit einer HA-Erkennungssequenz versehenes, SCL Protein exprimierte. Western blot Analysen zeigten, dass rekombinantes SCL-HA Protein durch alle vier RNAi Sequenzen herunterreguliert wurde – allerdings mit unterschiedlicher Stärke (zwischen 25% und 100% knockdown Raten). Darüber hinaus wurde die Tetrazyklin-abhängige Regulierbarkeit der Expression von shRNAs mit Hilfe von Ko-transfektionsexperimenten mit SCL-spezifischem pRNAi-EGFP Plasmid (SCL-siRNA) und dem SCL-HA Expressionsvektor in der HEK 293TR /T-REx Zelllinie bestätigt, die konstitutiv den Tetrazyklin-Repressor

exprimiert (TetR). Diese Versuchsreihen bewiesen die strikte Regulierbarkeit der shRNA Expression ohne Hintergrundaktivität.

Um den SCL knockdown *in vivo* kontrollieren zu können und um eine mögliche embryonale Letalität zu vermeiden, wird eine transgene Mauslinie benötigt in welcher der Tetrazyklin-repressor, TetR, ubiquitär exprimiert wird. Hier wurden zwei alternative Methoden verwendet, um solche TetR Mäuse zu generieren. In einer ersten Versuchsreihe wurde der Tetrazyklin-regulierbare RNAi Vektor zusammen mit dem kommerziell erwerblichen, aber hier speziell modifizierten, T-REx Expressionsvektor zur Pronukleusinjektion verwendet (SCL-siRNA T-REx FRT LoxP Mausvariante). Die zweite Methode bestand darin, dass zuerst eine konstitutiv T-REx exprimierende transgene Mauslinie etabliert wurde, welche dann als Eidonor zur Pronukleusinjektion mit dem siRNA knockdown Konstrukt verwendet wurde (SCL-siRNA T-REx Mausvariante).

Wie antizipiert und in guter Übereinstimmung mit den verfügbaren Daten von *Cre*-induzierbaren, konditionalen SCL knockout Mäusen, resultierte die post-transkriptionelle RNAi-vermittelte Inaktivierung von SCL in einer Verschiebung der Anzahl der reifen zur Anzahl der unreifen roten Blutzellen. Dieser „shift“ konnte mit Hilfe von FACS Analysen durch spezifische Oberflächenerkennung mit den für rote Blutzellen spezifischen Markern Ter119 und CD71 sowohl im Knochenmark als auch im peripheren Blut nachgewiesen werden (Lodisch Plot Technik).

In der vorliegenden Arbeit wurden die Grundlagen zur konditionalen, mittels siRNA vermittelten, *in vitro* und *in vivo* Inaktivierung von SCL etabliert. Diese Grundlagen sind ein wichtiges Werkzeug für die darauf aufbauende Forschung zur Aufklärung der Rolle von SCL und seiner *in vivo* Bedeutung für die Blutzellbildung und für Leukämien im Allgemeinen. Der wichtigste Aspekt dieser Arbeit ist jedoch, dass das hier etablierte exemplarische Wissen die Herstellung von komplett konditional regulierbaren und reversiblen knockdown Phänotypen in der Maus ermöglichen wird.

Declaration

I hereby declare that the submitted dissertation was completed by myself and no other.

Moreover I declare that the following dissertation has not been submitted further in this form or any other form, and has not been used to obtain any other equivalent qualifications at any other organisation/institution. Additionally, I have not applied for, nor will I attempt to apply for any other degree or qualification in relation to this work.

Louise J. Griffin

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List of abbreviations

A absorbance
α alpha
aa amino acid
mAb monoclonal antibody
ATG translation start codon
ATCC American type culture collection
ATP adenosine triphosphate
bp base pair
ß beta
cDNA complementary DNA
CFU colony forming units
Ci Curie
CMV cytomegalovirus
Cre Cre recombinase enzyme
ddH_2O double distilled water (millipore)
DMEM Dulbecco's Modification of Eagle's medium
DNA deoxyribonucleic acid
DNA deoxy nucleic acid
dsRNA double-stranded RNA
E. coli Escherichia coli
ES (cells) embryonic stem cells
FACS fluorescence activated cell sorting
FCS fetal calf serum
FITC fluorescein isothiocyanate
GFP green fluorescent protein
g gram
h hour/hours
HSC haematopoietic stem cell
kb kilo base
kDa kilo dalton
kJ kilo joule
kV kilo volts
KO knockout
L Liter
LoxP locus of X-over (target sequence for the *Cre* recombinase)
µ micro
µg micro gram
µl micro liter
µm micro meter
min minute/minutes
mA milli amps
ml milli liter
mM milli molar
miRNA micro RNA
M1 (cells) murine myeloid leukaemia cells
mRNA messenger ribo nucleic acid

nm nano meter
Ω ohm
OD optical density
O/N overnight
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffer salt solution
PCR polymerase chain reaction
pH pH scale (the concentration of hydrogen ions in a solution)
pM pico molar
PNI pronuclear injection
polyA polyadenylation signal
RNA ribonucleic acid
RNAi RNA interference
rpm rotations per minute
RT room temperature
RT PCR reverse transcription PCR
sec second/s
SCL stem cell leukaemia
shRNA short hairpin RNA
siRNA short interfering RNA
SV40 Simian virus 40
T-ALL T-cell acute lymphoblastic leukaemia
TF transcription factor
TG transgenic mouse
U units
UV ultra violet light
W watts
w/v weight per volume
WT wildtype mouse

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1. INTRODUCTION

1.1 RNA interference (RNAi)

RNA interference (RNAi) has been extensively used for sequence-specific silencing of gene function in mammalian cells. Recently several groups have demonstrated that RNA polymerase III expression constructs can be used for producing transgenic knockdown mice and rats (Bockamp *et al.*, 2002; Prawitt *et al.*, 2004; Spankuch and Strebhardt, 2005). However, at the beginning of this study it was not feasible to reversibly express shRNAs in transgenic animals. Using a tet on/off strategy it has been shown that the exogenous control of shRNAs expression in tissue culture was possible (van de Wetering *et al.*, 2003). To control shRNA expression in the tissue culture system an inducible form of the RNA polymerase III H1 promoter was used in combination with the tetracycline-dependent repressor. Based on these results we it was planned to generate a transgenic mouse line constitutively expressing the doxycycline-dependent tetracycline repressor (TetR). This mouse line could then be used for producing transgenic mice expressing, in addition to the tetracycline-repressor, a conditionally inducible TetR-dependent short hairpin RNAi (shRNA).

For our biological readout we have chosen the SCL gene, which is involved principally in embryogenesis, haematopoiesis and is also known in an aberrant form in childhood leukaemia (T-ALL). However the mechanism of SCL's involvement in T-ALL is not fully understood and that SCL null mice are embryonic lethal at E9.5 which makes it difficult to characterize. Based on this knowledge, the aim of this study was to establish a novel reversible *in vivo* system that would allow for the dose- and time-dependent induction of any shRNA knockdown. Therefore, we have incorporated the tetracycline system for the RNAi induced knockdown of SCL in the mouse and this will be the basis for further work to elucidate the distinct functions of the transcription factor SCL.

1.1.1 A historical perspective

RNA interference (RNAi) is a conserved biological mechanism for silencing genes in a sequence-specific manner at both a transcriptional and post-transcriptional level. RNAi is probably a conserved defence mechanism against foreign double-stranded

RNA (dsRNA) produced by replicating RNA viruses and endogenous repetitive sequences know as transposons, which can disrupt the integrity of the genome. Interestingly, only the presence of dsRNA-species triggers gene-silencing effects and results in the loss of the corresponding messenger RNA (mRNA). The first indication of RNAi silencing was observed already in 1928. Tobacco plants infected with a tobacco ring spot virus, showed a remarkable resistance to the virus in their new shoots (Wingard *et al.*, 1928). However, for these studies the underlying silencing mechanism was not elucidated. Later it was discovered by Richard Jorgensen that in transgenic purple petunias plants, while enhancing of a pigment-producing gene, an unexpected variegation in pigmentation intensity was observed, with some plants lacking the pigment altogether (Jorgensen, 1990). The transgenes were found to induce silencing of the homologous endogenous genes leading to a mechanism called co-suppression (Napoli *et al.*, 1990; Matzke *et al.*, 2001). In the fungus *Neurospora crassa* amplification of the carotenoid pigment gene, also resulted in suppression of the endogenous gene which was termed quelling (Cogoni and Macino, 1997).

More evidence for RNAi silencing was provided by Guo and Kemphus when they investigated the function of the protease-activated receptor-1 (par-1) gene, in the nematode worm *Caenorhabditis elegans* (*C. elegans*). This gene is involved in germline development and embryonic polarity. They injected small synthesized anti-sense strands of RNA complementary to a sequence in par-1 mRNA into the worm. Injection of the anti-sense RNA resulted in a lethal phenotype, but injection of a sense strand (par-1 mRNA) also gave the same lethal phenotype (Guo and Kemphues, 1995). However, the functional siRNA silencing mechanism by dsRNA was first shown by in Fire and colleagues in *C. elegans.* When they determined the synergy of sense and anti-sense RNAs, they showed the mixture containing dsRNA was at least tenfold more potent at silencing genes then the sense or anti-sense RNA strands alone (Fire *et al.*, 1998).

Later studies provided evidence that dsRNA molecules (30 bp or more) could provoke the anti-viral interferon response, through interference with the RNA-dependent protein kinase (PKR) pathway, which responds to dsRNA by phosphorylating EIF-2a and this results in the non-specific shutting down of gene translation (McManus and Sharp, 2002). However, in a later study it was shown that shorter dsRNAs, which

were 19-22 nucleotides in length, could also sequence specifically silence mRNAs without inducing the interferon pathway. These short dsRNAs were termed small interfering RNAs (siRNAs) which can bypass the interferon response in mammalian cells (Elbashir *et al.*, 2001; Elbashir *et al.*, 2001; Caplen, 2003). It was proposed that co-suppression in plants, quelling in fungi, RNAi in nematodes and mammals all share a common silencing mechanism, which contributed to the organisms defence against viruses (Colbere-Garapin *et al.*, 2005). It was also postulated that this mechanism is in charge of silencing aberrantly expressed transgenes through mRNA degradation and translation inhibition (Mello and Conte, 2004). Additionally, RNAi silencing is thought to play a role in guarding the genome from disruption by transposons (Volpe *et al.*, 2002; Sijen and Plasterk, 2003; Hodgetts, 2004; Kato and Kakutani, 2004; Nolan *et al.*, 2005).

1.1.2 The mechanism of RNAi gene silencing

Accumulating biochemical and genetic evidence has provided an increasing understanding of the RNAi-mediated gene silencing mechanisms. The RNAi process is triggered by the presence of double-stranded RNAs (dsRNAs) in the form of aberrantly expressed transgenes, RNA viruses, transposons, plasmid-based short hairpin RNAs (shRNAs) or endogenous hairpin micro-RNAs (miRNAs), which all target messenger RNA (mRNA). This was first reported by Hamiltion and Baulcomb, showing that a small 25 nucleotide dsRNA derived from the target mRNA sequence was involved in post-transcriptional gene silencing (PTGS) (Hamilton and Baulcombe, 1999). The major breakthrough of the underlying mechanism was the biochemical analysis of RNAi using *Drosophila* embryo or Drosophila S2 cell extracts. This experiments lead to the isolation of an enzyme complex known as RNA-induced silencing complex (RISC). By using dsRNA molecules to 'knockdown' genes in *C. elegans* this complex was shown to process long dsRNA substrates into 21-22 nucleotide fragments (Tuschl *et al.*, 1999; Hammond *et al.*, 2000; Zamore *et al.*, 2000). Analysis of these RNAs showed similarities to RNase III enzyme products, which are also double-stranded and have two nucleotide 3′-overhangs and 5′-phosphorylated termini (Zamore *et al.*, 2000; Bernstein *et al.*, 2001; Elbashir *et al.*, 2001; Schwarz *et al.*, 2002). This observation triggered the assumption that RNase III enzymes were needed to initiate RNAi response *in vitro*. The required RNase III enzyme is now appropriately named Dicer and acts as a dimeric protein, which

cleaves/dices an unusual size fragment of 22-nucleotide compared to the normal cleavage frequency of 9–11 nucleotide for other RNase III family enzymes (Bernstein *et al.*, 2001). Dicer contains a N-terminal RNA helicase domain, a Piwi, Argonaute, Zwille/Pinhead (PAZ) domain, two RNase III domains, and a C-terminal dsRNA-binding motif (Bernstein *et al.*, 2001) used to process dsRNA into siRNA duplexes. The PAZ domain is also present in Argonaute1 (Ago1) and Argonaute2 (Ago2) proteins, two of the five Argonaute proteins found in *Drosophila melanogaster* (Carmell *et al.*, 2002). In mice and humans (Bernstein *et al.*, 2003; Sasaki *et al.*, 2003; Liu *et al.*, 2004) Ago2 was shown to be important in formulation of the Dicer-mRNA-RISC endonuclease complex, which targets the mRNA (Williams and Rubin, 2002; Liu *et al.*, 2004; Rand *et al.*, 2004; Hammond, 2005; Rivas *et al.*, 2005; Sen and Blau, 2005).

RISC uses the unwound siRNA for guidance to the target mRNA. SiRNAs are double-stranded duplexes with two-nucleotide 3′-overhangs (Bernstein *et al.*, 2001; Schwarz *et al.*, 2002) and 5′-phosphate termini (Zamore *et al.*, 2000; Elbashir *et al.*, 2001). This structure was shown to be critical for its incorporation into the RISC complexes. First siRNA duplexes are incorporated into a siRNA containing ribonucleoprotein complex (siRNP), which subsequently becomes RISC activated on addition of ATP. This complex then targets homologous mRNAs for degradation (Elbashir *et al.*, 2001; Nykanen *et al.*, 2001; Martinez *et al.*, 2002). Two Dicers, Dicer-1, and Dicer-2, were found in *Drosophila*. The Dicer-2/R2D2 complex, but not Dicer-1, is the main Dicer enzyme responsible for siRNA production in *Drosophila* cells. R2D2 acts by stabilizing Dicer-2 and regulates siRNA production by loading siRNA onto the RISC complex (Liu *et al.*, 2003). R2D2 tends to bind the more stable 5′-phosphate end of the siRNA duplex and enables the siRNA duplex to unwind from the less stable end, allowing siRNA single strand to assemble with the RISC's core protein Ago 2 (Tomari *et al.*, 2004). In *D. melanogaster* Schneider 2 (S2) cells siRNAs are incorporated into the multi-component enzyme RISC complex by the interaction between Dicer and Ago2 PAZ. RISC must be activated from a latent form (250K), containing a double-stranded siRNA to an active form (100K), upon the addition of ATP to the system (Zamore *et al.*, 2000), which initiates unwinding of the duplex siRNA into single-stranded siRNA (Nykanen *et al.*, 2001; Tomari *et al.*, 2004). However, the full mechanism of RNAi is not yet completely understood and

current studies show a wide variation among organisms containing different Dicer-RISC homologues and/or associated proteins (Hutvagner, 2005).

Although dsRNAs are thought to be the main protagonists of RNAi, siRNA, shRNA and miRNA induce RNAi through common biochemical pathways. All are involved in sequence-specific post-transcriptional regulation of gene expression and utilise the same underlying RNAi mechanisms. Interestingly, miRNAs suppress the expression of partially complementary target mRNAs by translation inhibition rather than mRNA degradation (Doench *et al.*, 2003; Doench and Sharp, 2004). In comparison the miRNA biogenesis pathway is initiated in the nucleus and unlike siRNA, it includes three distinct RNA intermediates. The initial pri-miRNA transcript is processed by the RNase II enzyme Drosha (found in *Drosophila* and humans), into a pre-miRNA hairpin which is then exported from the nucleus into the cytoplasm by exportin-5 (Lund *et al.*, 2004). The multi-domain RNase III enzyme Dicer dices the short precursor miRNAs with a stem-loop structure into miRNAs as compared to dicing of the dsRNA into siRNAs. The siRNA and miRNA are incorporated into related RNA-induced silencing complexes (RISCs), termed siRISC and miRISC, respectively. The current model argues that siRISC and miRISC are functionally inter-changeable as they target specific mRNAs either for cleavage or for translational repression. Emerging evidence indicates, however, that siRISC and miRISC are distinct complexes that regulate mRNA stability and translation (Tang, 2005).

The anti-sense strand of a siRNA can direct the cleavage of a corresponding sense RNA target, and vice versa. RNA with a perfect match to a target mRNA behaves like an siRNA and results in mRNA degradation, whereas an RNA with a partial match functions as an miRNA and causes translational repression as with miRNAs (Hutvagner, 2005). Many endogenous miRNAs and their RISCs are genetically programmed to regulate gene expression and are assumed to be important for such basic features as embryogenesis, oncogenesis and haematopoiesis in mice and humans (Benard and Douc-Rasy, 2005; Chen and Lodish, 2005; Croce and Calin, 2005). In contrast, siRNAs are a first line of defence against any viral infection or transposon interference in the genome (Hutvagner, 2005; Kanellopoulou *et al.*, 2005).

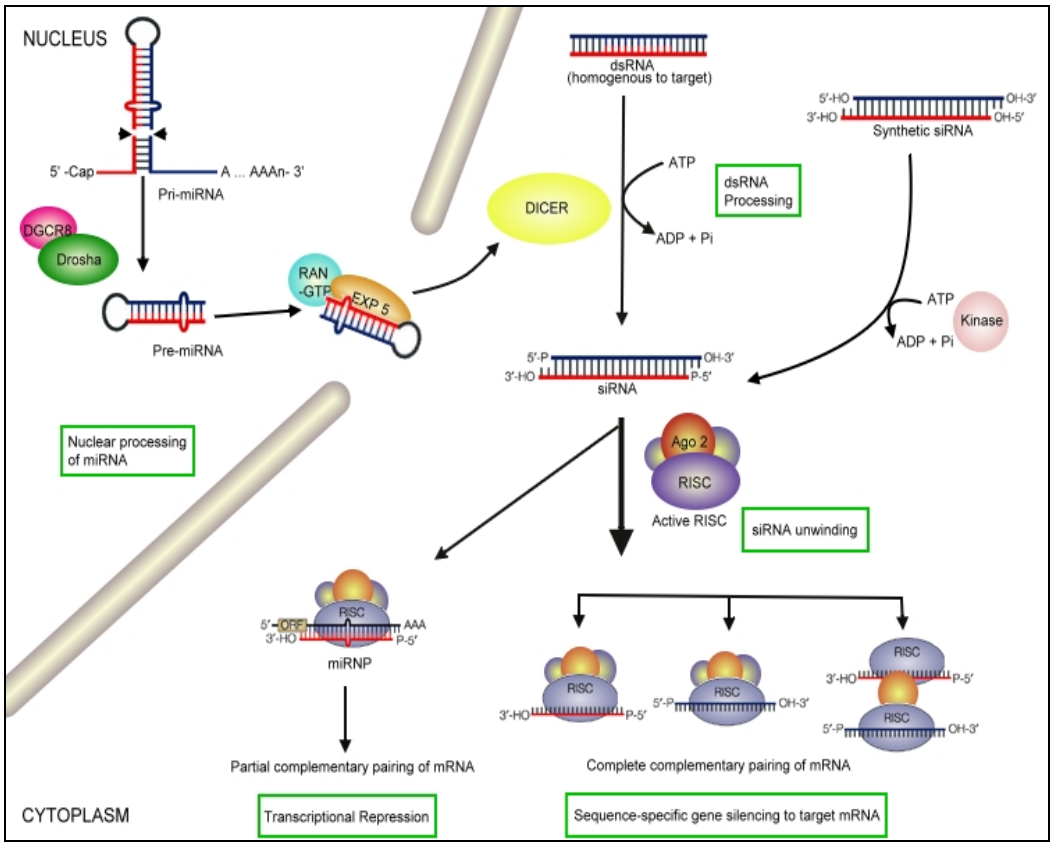


Figure 1: A model for the mechanism of RNAi

Silencing is triggered by double-stranded RNA (dsRNA), which maybe presented in the cell as synthetic RNAs, replicating viruses or may be transcribed from nuclear genes. The processing of long dsRNAs, hairpin microRNAs (miRNAs) or plasmid-synthesized short hairpin RNAs (shRNAs) by Dicer (presented in yellow) leads to the formation of small interfering RNAs (siRNAs). Exogenously provided synthetic siRNAs are converted into active functional siRNAs by an endogenous kinase that provides 5'-phosphate groups (Pi) in the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). The duplex siRNAs are passed to RISC (RNA-induced silencing complex, in purple), a multi-component complex, containing a helicase complex (purple) that unwinds the duplex siRNA in an ATP-dependent manner. Then Argonaute 2 protein (Ago2, in orange) cleaves the target mRNA. The activated RISC complexes can regulate gene expression by promoting RNA degradation by siRNA targeting and translational inhibition by miRNA targeting of mRNA. Each of the siRNA strands (sense, anti-sense or sense and anti-sense) can be incorporated into RISC to regulate gene expression by complete complementary pairing to the target mRNA leads to mRNA degradation and gene silencing. MiRNA is processed into a hairpin transcript in the nucleus, Drosha shortens the pri-miRNA transcript to a short pre-miRNA hairpin transcript, which is transported out of the nucleus by exportin 5 (Exp-5) and is processed in the cytoplasm by Dicer. Endogenous miRNA is processed by Dicer into a form of siRNA duplex which is incorporated into a RISC-like complex miRNP and inhibits translation by partial complementary paring of the 3´-untranslated region (3´-UTR) of the mRNA by a still unclear mechanism.

In summary, the RNAi pathway can be structured into an initiation and an effector step. First, the ribonuclease Dicer generates siRNA or miRNA from long dsRNA or short hairpin RNA (pre-miRNA). Second, the siRNA is incorporated into the RISC complex for sequence-specific mRNA degradation, whereas the miRNA is assembled into the miRNP complex to repress translation of target mRNA. In conclusion, both miRNA and siRNA play a crucial role for regulating and safe guarding multi-cellular organisms. It is also expected that the use of miRNAs and siRNAs will revolutionize many areas in biomedical research.

1.2 Expression of inducible siRNA using the tetracycline system

The post-transcriptional mechanisms of synthetic siRNAs and of vector-based shRNAs are a powerful tools in loss-of-function phenotypic studies. Since RNAi has become an excellent strategy for gene silencing, it is tempting to apply this technology to 'knockdown' gene expression in living animals. Several publications have demonstrate the power of shRNA mediated knockdown in animals (Hasuwa *et al.*, 2002; Carmell *et al.*, 2003; Prawitt *et al.*, 2004). In some cases, knockdown mice were phenotypically identical to those that carried a null mutation for the gene (Kunath *et al.*, 2003).

There are two main approaches for expressing siRNA:

1) The sense and anti-sense strands constituting the siRNA duplex can be directly transfected into a cell or injected into an animal or

2) An expression vector can be used to express siRNAs as stem-loop structures that give rise to shRNAs.

Initially, approaches for knocking down a specific mRNA in mammalian cell lines used transfection of synthetic siRNA duplexes, identical in structure to miRNA duplex intermediates (Elbashir *et al.*, 2001). This approach efficiently down regulated target mRNAs but had the disadvantage of being both transient and expensive. It was then demonstrated that expression of siRNA in the form of shRNAs, which bear a fold-back stem-loop structure mimicking endogenous miRNAs induced gene silencing in mammalian cells (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul

et al., 2002; Sui *et al.*, 2002). Small interfering RNA (siRNA) duplexes of 21–23-nucleotides containing a 19-nt duplexed region, symmetric 2–3-nt 3' overhangs, and 5'-phosphate (P) and 3'-hydroxyl (OH) groups mimicking the Dicer substrates were shown to be functional. The 5'-phosphate termini are not essential because endogenous kinase activity will phosphorylate the 5' ends of synthetic siRNA duplexes. Stable expression of siRNAs is facilitated by using plasmid-based short-hairpin RNA (shRNA) expression vectors that recapitulate the endogenous pre-miRNA hairpin transcript.

These shRNAs can be used for knocking down gene function in mammalian cell and mice. The constitutive expression of plasmid-based shRNAs by RNA polymerase III (pol III) U6 (Zhang *et al.*, 2004; Anderson and Akkina, 2005; Cao *et al.*, 2005; Ren *et al.*, 2005; Seibler *et al.*, 2005; Wooddell *et al.*, 2005) and H1 (Scherr *et al.*, 2003; Hasuwa and Okabe, 2004; Nishitsuji *et al.*, 2004; Anderson and Akkina, 2005; Cao *et al.*, 2005; Fu *et al.*, 2005; Wooddell *et al.*, 2005) promoters and RNA-pol-II-based CMV (cytomegalovirus) promoters using miRNA (Dickins *et al.*, 2005) has been used successfully to obtain stable and efficient suppression of target genes in mice. These shRNA expression vectors can be used to establish stable RNAi responses in culture or in transgenic mice. Constitutive delivery of specific shRNA is possible by inserting DNA templates for siRNAs into RNA polymerase III (pol III) transcription units, which are based on the sequences of the natural transcription units of the small nuclear RNA U6 or the human RNase P RNA H1 genes. Plasmid-based expression of short hairpin loops, which give rise to siRNAs, is normally achieved by utilizing the polymerase III promoter of H1 gene (human RNase P) thereby driving the transcription of a 19bp-loop-19bp RNA hairpin. RNA transcription is terminated by a polythymidine (TTTTTT/T5) termination signal for polymerase III (pol III). The insertion of siRNA expression cassettes into retro- (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002), lenti- (Rubinson *et al.*, 2003; Anderson and Akkina, 2005; Chang *et al.*, 2005; Raoul *et al.*, 2005) and adeno-viruses (Kuninger *et al.*, 2004; McCown, 2005; Rodriguez-Lebron *et al.*, 2005) is a complementary approach to plasmid based vectors. In addition, viral vectors will also enable the efficient targeting of primary cells, which are difficult to transfect with plasmid DNA.

Several groups have developed inducible siRNA systems, the expression of which is controlled by tetracycline (Tet) or Tet analogues doxycycline (Dox) (Chen *et al.*, 2003; Czauderna *et al.*, 2003; van de Wetering *et al.*, 2003; Chang *et al.*, 2004; Coumoul *et al.*, 2004; Hosono *et al.*, 2004). Regulation of the Tet-responsive pol III U6 or H1 promoters, is mediated by binding of the repressor (TetR) protein binding to its Tet operator (*tetO)* anchor sequence. All inducible RNAi systems use a modified form of the tetracycline controlled gene expression system first described by (Gossen and Bujard, 1992; Gossen and Bujard, 1995). The system is designed so that expression of a shRNAs can be induced by Tet or Dox. Induction of the shRNA results in suppression of the targeted mRNA by the shRNA through RNAi. Thus, the system allows for tight regulation of the expression of a shRNA in response to Tet or Dox. The Tet on/off strategy relies on two components: a Tet-repressor (TetR) and a Tet-responsive promoter, the activity of which is regulated by binding or release of the TetR. In the absence of the tetracycline repressor (TetR), the *tetO* sites in the tetracycline responsive promoter are bound by the TetR and transcription of the shRNA is repressed. Conversely, in the presence of Dox, TetR is released TetR from the *tetO* sites, allowing transcription of shRNA and thus the mRNA-specific knockdown to proceed.

Tetracycline responsive expression systems for conditional shRNA expression normally use a H1 or U6 promoter that drives expression of the shRNA. In addition they contain *tetO* binding sites interplayed between the promoter of the transcription start site to provide a binding surface for the Dox-dependent TetR (Chen *et al.*, 2003; Czauderna *et al.*, 2003; van de Wetering *et al.*, 2003; Coumoul *et al.*, 2004).

Two tetracycline repressor systems have been chosen for this study to provide tetracycline-induciblity. T-REx (Tetracycline regulated expression system, invitrogen pcDNA6/TR) (Yao *et al.*, 1998) and tetracycline-controlled transcriptional suppressor (tTS) (Freundlieb *et al.*, 1999). The second repressor tTS, is a fusion of the Tet repressor protein (TetR) and the KRAB-AB silencing domain of the Kid-1 protein (SD^{Kid-1}). This fusion protein has been shown to act as a strong suppressor for pol III and pol II promoters (Freundlieb *et al.*, 1999; Mallo *et al.*, 2003). In the absence of Tet or Dox, tTS tightly binds the *tetO* sequences similar to the T-REx only the additional KRAB-AB domain is not only believed to act as a physical road block but

in also is thought to act as a potent suppressor of transcription to the *tetO* responsive promoter. The tTS protein has been shown to actively suppresses polymerase II activity (Freundlieb *et al.*, 1999) but to date no evidence has been provided the tTS also acts upon pol III dependent promoters. It is however, expected to be the case since the KRAB silencing domain is thought to be an epigenetic silencer leading to chromatin modifications. This epigenetic activity of the tTS silencer might be an additional benefit besides its function as a physical road block. Inducible RNAi systems, therefore allow for the tightly regulated expression of functional shRNAs in mammalian cells, thus facilitating the conditional silencing of any target genes in an exogenously controllable manner Figure 2.

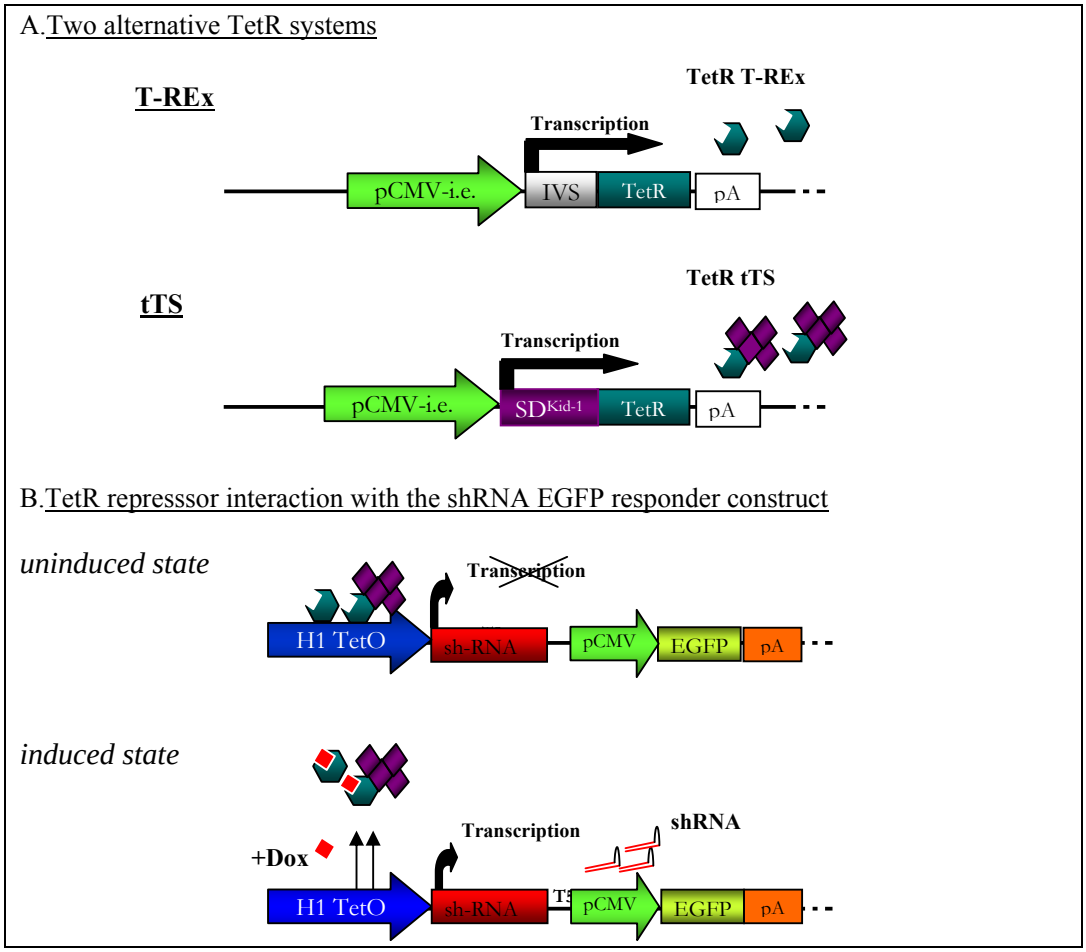


Figure 2: Inducible shRNA utilising the tetracycline system

A. The T-REx protein is an enhanced wildtype TetR protein also containing an additional intronic sequence (IVS) to enhance the stability of the mRNA. The Tet controlled repressor (tTS) is a fusion of the wildtype Tet repressor protein (TetR) to the KRAB-AB silencing domain of the Kid-1 protein (SD^{Kid-1}) B. The TetR domain binds to the

tetracycline operator (*tetO*) sequence in the absence of doxycycline (Dox) (uninduced state), alternatively, conformational changes and binding characteristics in the presence of Dox lead to a release from the *tetO* binding site (induced state). As a consequence adding Dox induces transcription of short hairpin RNA (shRNA). An enhanced green fluorescent protein (EGFP) expression is also contained in the shRNA construct reporter.

Inducible RNAi systems are very useful in cases where suppression of a gene may be lethal or toxic which might prevent its analysis in a constitutive expression system. The current hypothesis as to how bound TetR blocks shRNA transcription is that it acts as a "road block" between the RNA pol III dependent promoter element and the TATA box/ the transcriptional start site of the shRNA. Therefore, in the current study the shRNA expression cassette contains the *tetO* binding site between the H1 promoter and the transcription start site and has also been shown to function in cell culture (van de Wetering *et al.*, 2003). Current knockdown strategies utilize RNAi transcription, of RNA Pol II or Pol III promoters or use synthetic miRNA transcripts, both result in the production of siRNAs (Cullen, 2004). In a recent report a substantial RNAi knockdown phenotype was observed when sh/miRNAs were transcribed by a Pol II dependent promoters (Cullen, 2004). Silva and collaborators compared the knockdown efficiency of RNAi induced by siRNAs derived either from simple, Pol III shRNA expression vectors or by processing of artificial pri-miR-30 derivatives, also transcribed by Pol III.

By examining a range of different siRNAs, it was shown that pri-miR-30 based vectors gave a higher level of siRNA expression and a more effective knockdown of target genes (Silva *et al.*, 2005). The most comprehensively studied miRNA in humans is the miR-30. Interestingly, pri-miR-30 precursors can be designed to allow for the expression of artificial miRNAs or siRNAs. In particular, the stem of the pri-miR-30 stem-loop, which contains the mature miR-30 sequence, can be substituted with heterologous sequences, as long as the helical nature of the stem is maintained, which does not interfere with the miRNA function. Therefore any target mRNA sequence, transcribed by Pol II or Pol III, can be targeted by the RNAi chain of events (Zeng *et al.*, 2002). In a later experiment, Dickins and collaborators analyzed the ability of a similar retroviral siRNA expression vector designed to knockdown gene expression of the Trp53 gene (Dickins *et al.*, 2005). In this study a retroviral vectors were employed that utilized tetracycline-regulation of Pol II promoters to drive the

expression of pri-miR-30–based siRNAs. Using these vectors, the authors showed that the *Trp53*-specific siRNA was indeed expressed was also targeting p53. Expression of siRNAs could be regulated both in culture and in mice by changing the level of Dox, thereby promoting or inhibiting tumour growth. This effect was then confirmed by Stegmeier and collaborators, who showed that a lentiviral, pri-miR-30–based siRNA expression vector, containing a Tet-responsive Pol II promoter, can be used to tightly regulate the knockdown of target genes in transfected cells (Stegmeier *et al.*, 2005).

From a practical perspective, these papers describe expression systems that allow for potent, regulated RNAi expression *in vitro* and *in vivo*. Furthermore, these systems also allow for large scale loss-of-function screens in tissue culture, including murine ES cells, and subsequently in mice. Perhaps the most important implication of these findings is that it should now be possible to use RNAi to induce gene knockdown phenotypes that are identical or at least very similar to the classical knockout phenotype (Silva *et al.*, 2005). Also, the fact that these pri-miR-30 based siRNA expression constructs are transcribed by Pol II means that it will be possible to carry out RNAi in a tissue-specific manner, which are also regulated by the tetracycline system (Dickins *et al.*, 2005; Stegmeier *et al.*, 2005). These studies illustrated the ease at which RNAi "gene knockdowns" can be used as a potential tool to create a full range of mild to severe phenotypes, which can be used for time- and drug-dependent studies as developing stem cell-based and other therapeutic strategies.

1.3 RNAi gene silencing application in therapeutic intervention

Human immuno-deficiency virus (HIV) is thought to be one of the main viral targets of RNAi. Several groups have shown that siRNAs can inhibit HIV replication effectively in cell culture. HIV infection can also be blocked by targeting either viral genes (for example, gag, rev, tat and env) or human genes (for example, CD4) or cytokine co-receptors that are involved in the HIV life cycle (Anderson *et al.*, 2003; Arteaga *et al.*, 2003; Park *et al.*, 2003; Chiu *et al.*, 2005). SiRNA targeting against the tat and rev regulatory proteins of HIV-1, or CCR5, the human cellular co-receptor for HIV-1, specifically block the function of these viral genes without which HIV cannot replicate. When expressed in human T-cell lines and primary lymphocytes, these siRNAs effectively block HIV replication (Cullen, 2002). An alternative approach

would be to knockdown the HIV-1 regulatory genes. For example siRNA-producing retroviral vectors could be used to transfect primary T-lymphocytes as well as CD34+ haematopoietic stem and progenitor cells. In one study CD34+ stem cells transfected with anti-HIV-1 siRNA expression constructs were transplanted into immuno-deficient (SCID-hu) mice. siRNAs expressed in both primary T-lymphocytes and in CD34+ derived monocytes made these cells resistant to HIV-1 infection (Li *et al.*, 2003). Inhibition of HIV-1 infection in human T cells by the lentivirus-mediated delivery of siRNA against HIV surface co-receptor CCR5 (the chemokine (C-C motif) receptor 5) has generated tremendous excitement owing to the potential therapeutic application of virus-mediated RNAi delivery (Cullen, 2005). Lentivirus-mediated delivery has a crucial advantage, lent viruses can efficiently integrate into the genome of non-dividing cells, such as stem cells or terminally differentiated cells, which are refractory to conventional retroviral infection. Recently, however, retrovirus-mediated gene therapy for treatment for X-linked severe combined immunodeficiency (X-SCID) resulted in the development of T-cell leukaemia in two children owing to the insertional mutagenesis of the vector in the oncogene LMO 2 (LIM-domain-only 2) (Wu and Burgess, 2004). Possible dangers of any gene therapy have therefore to be considered before treatment is initiated.

The primary hurdle that must first be overcome before RNAi might become a useful therapy is the invention of clinically practical methods that can efficiently deliver siRNAs to the targeted gene or cell populations. This might be done using retroviral vectors, as in other forms of gene therapy, or with chemical modification of siRNA that prevent its degradation and turn these molecules into stable drugs. Finally, shRNAs could be combined with existing high-efficiency gene delivery vehicles to create suitable RNAi-based therapeutics. Successful delivery of shRNAs from replication deficient retroviruses has already allowed clinical applications of *ex vivo* manipulated of stem cells followed by bone marrow stem cells transplantation. These studies showed that the progeny derived from the *ex vivo* transduced stem cells were resistant to the HIV infection (Spankuch and Strebhardt, 2005).

Song's group injected siRNA targeting *Fas* intravenously into two models of autoimmune hepatitis in mice. This decreased *Fas* mRNA and protein levels in hepatocytes and protected the cells against liver injury from apoptosis in mice with

hepatitis autoimmune disease, even when siRNA was administered after the induction of injury (Song *et al.*, 2003). These studies clearly indicate a potential use of siRNA for treating autoimmune disease.

Gregory Hannon and colleagues have used RNAi to silence expression of the tumour suppressor gene p53, by introducing several p53-targeting shRNAs into stem cells and observed a p53-specific knockdown effect in a mouse model lymphoma. The shRNAs produced a wide range of clinical effects, ranging from benign to malignant tumours, the severity and type of which correlated with the extent to which the shRNA has silenced p53. The potential specificity of RNAi may make it possible to silence a disease-causing mutant allele specifically, such as an activated oncogene, without affecting the normal allele. In leukaemia's and lymphomas oncogene activation frequently occurs through chromosomal translocations. These translocations lead to juxtaposition of gene segments usually found on different chromosomes, and the creation of a composite gene sequence. The prototype of such a translocation is for example the generation of the Philadelphia chromosome, by the translocation of the long arms of chromosomes 9 and 22 [t(9;22)] in patients with chronic myelogenous leukaemia and acute lymphoblastic leukaemia. The translocation fuses the BCR gene from chromosome 22 and ABL gene from chromosome 9, creating an oncogenic BCR-ABL gene. The BCR-ABL fusion protein increases the tyrosine kinase activity, compared to the normal ABL protein, leading to phosphorylation of several downstream molecules. The kinase activities of both BCR-ABL and ABL can be inhibited by specific synthetic tyrosine kinase inhibitors, which are now used for the effective treatment of BCR-ABL-positive leukaemia. RNAi was also used to target the BCR-ABL mRNA, comparison between these two different approaches showed that the RNAi mediated approach was as effective as the specific inhibition by the STI 571 drug used in normal treatment (Wilda *et al.*, 2002).

With respect to future medical applications, siRNAs were recently directed against a mutated mRNA associated with the spinobulbular muscular atrophy (SBMA) in tissue culture. SBMA, together with Huntington Disease, belongs to a growing group of neurodegenerative disorders caused by the expansion of trinucleotide repeats. Targeting the CAG-expanded mRNA transcript with RNAi may be an alternative to other therapeutic strategies that, only treat the symptoms of the disease (Caplen *et al.*,

2002; Harper *et al.*, 2005). Collectively, RNAi holds great promise for treating human disease, not only in the laboratory, but also in a clinical setting.

1.4 Basic helix-loop-helix transcription factor SCL as a target of RNAi

To define the stage at which SCL becomes necessary for erythroid development, transgenic mice should be generated which express Tet-regulated shRNA to knockdown SCL. The screening for the different maturation of erythroblast sub-populations by the "Lodish plot" technique was envisaged as a rapid immuno-phenotyping method for detecting SCL mRNA knockdown *in vivo*. This would not only allow for a switchable, reversible system of SCL siRNA regulation but will give a biological feedback to the Dox induced system showing functional analysis of the system.

1.4.1 The role of the SCL gene in haematopoiesis

The SCL (named also TCL-5 or TAL1) protein belongs to the class II basic helix-loop-helix (bHLH) transcription factor family. These factors display a tissue-restricted expression and are known to often play critical roles in regulating differentiation of many cell types (Green *et al.*, 1991; Green and Begley, 1992). SCL is expressed in the vascular and in the haematopoietic system, especially in haematopoietic progenitors and in erythroid, megakaryocytic, and mast cell precursors, where it has been implicated during different stages of development and differentiation (Kallianpur *et al.*, 1994). SCL participates in embryonic haematopoietic development. SCL null embryos fail to develop any haematopoietic cells and this failure can be rescued by expressing SCL (Robb *et al.*, 1995; Shivdasani *et al.*, 1995). SCL is also important for the establishment of definitive haematopoiesis since SCL null mouse embryonic stem (ES) cells injected into blastocysts do not participate in any adult haematopoietic lineage, whereas their contribution to other tissues of the mouse body is normal (Porcher *et al.*, 1996). The role of SCL in adult haematopoiesis has recently been investigated using a conditional knockout model (Hall *et al.*, 2005). Interestingly, deletion of SCL in adult bone marrow (BM) haematopoietic cells did not interfere with the reconstitution properties of haematopoietic stem cells and early progenitors such as colony-forming unit-spleen (CFU-S) in transplantation but impaired their

erythrocytic and megakaryocytic differentiation capacities. The absence of SCL protein demonstrated that SCL is required for the normal functioning in short term repopulation of haematopoietic stem cell population (Curtis *et al.*, 2004).

1.4.2 SCL gene involvement in T-cell acute lymphoblastic leukaemia (T-ALL)

Activation of the basic-helix-loop-helix (bHLH) gene *TAL1* (or *SCL*) is a frequent gain-of-function mutation in T cell acute lymphoblastic leukaemia (T-ALL). T cell acute lymphoblastic leukaemia (T-ALL) accounts for 10%–15% of paediatric and 25% of adult T-ALL cases (Ferrando *et al.*, 2002). Activation of the basic-helix-loop-helix SCL gene occurs by chromosomal translocation, interstitial deletion, or mutation in over 60% of children and adults with T-ALL (Bash *et al.*, 1995). In contrast to T-ALL induced by other oncogenes such as HOX1-1 or MLL-ENL, patients with SCL activation respond poorly to therapy, with only 50% of patients surviving for five years (Ferrando *et al.*, 2002).

SCL heterodimerizes with class I or A bHLH proteins including E12, E47, HEB and E2-2 (Hsu *et al.*, 1991; O'Neil *et al.*, 2001) and in haematopoietic cells is part of a large transcriptional complex that includes GATA-1 and the LIM-only proteins LMO2 and Ldb-1 (Larson *et al.*, 1996; Rabbitts *et al.*, 1997). Mice deficient for tal1/scl have no primitive or definitive haematopoiesis and exhibit angiogenic defects (Shivdasani *et al.*, 1995; Visvader *et al.*, 1998). Surprisingly, conditional inactivation of SCL in adult mice does not result in haematopoietic defects, suggesting that SCL is critical for the genesis of the haematopoietic stem cell (HSC), but not required for its maintenance (Mikkola *et al.*, 2003). The function of SCL in haematopoietic stem cells has suggested that tal1/scl activation in leukaemia may stimulate the activation of genes important in stem cell expansion and/or self-renewal.

In mouse SCL tumours and in Jurkat cells, a human leukaemia cell line that expresses SCL, stable SCL/E47 and SCL/HEB heterodimers are readily detected (Hsu *et al.*, 1991; O'Neil *et al.*, 2001), as well as the related bHLH proteins LYL-1 and bHLH-1. These complexes may contribute to leukaemia by interfering with E protein function(s). Consistent with this idea, a percentage of surviving E2A-deficient mice develop T cell leukaemia/lymphoma (Bain et al. 1997 and Yan et al. 1997). Disruption of E2A function is also believed to be the consequence of chromosomal

translocations involving the LIM-only domain proteins, LMO1 and LMO2, recently also found to be activated in gene therapy-induced T-ALL (O'Neil *et al.*, 2001). However the mechanism of how LMO1/2 inhibits E2A function and contributes to leukaemia remains unclear. In some leukaemia patients, LMO2 and SCL are co-expressed (Ferrando *et al.*, 2002), suggesting that leukaemia transformation is dependent on the expression of both the SCL and LMO2 proteins. Consistent with this idea, a SCL/LMO2/E2A complex is detected in mice (Chervinsky *et al.*, 1999), and leukemogenesis is observed in mice that express SCL and LMO2 (Larson *et al.*, 1996). However, not all human T-ALL patients who express SCL also express LMO1 or LMO2 (Ferrando *et al.*, 2002).

SCL induces leukaemia by interfering with E47 and HEB, when expressed in an E2A or HEB heterozygous background (O'Neil *et al.*, 2004). The SCL/E2A$^{+/-}$ and SCL*/HEB*$^{+/-}$ mice, exhibit thymocyte differentiation arrest and disease acceleration in due to repression of the E47/HEB target genes. These experiments also revealed the repression of several genes. For example, E47/HEB target genes, Rag 2 and Pre-Tα, which are important for thymocyte differentiation in premalignant SCL cells, are repressed. SCL mediates gene repression by depleting the E47/HEB heterodimer and by recruiting the mSin3A/HDAC1 co-repressor complex to target loci bound to the CD4 enhancer, whereas an E47/HEB/p300 complex is detected in wild-type thymocytes (Huang and Brandt, 2000). The results of this study demonstrate that SCL, like the leukemogenic fusion protein AML-1/ETO, contributes to leukaemia by repressing gene expression and inducing differentiation arrest. Also SCL tumours are sensitive to pharmacologic inhibition of HDAC (O'Neil *et al.*, 2004). These data demonstrate that SCL induces leukaemia by repressing E47/HEB and suggest that HDAC inhibitors may prove more efficient in T-ALL patients with an underlying SCL disregulation.

Although frequently activated in human T-ALL patients, the mechanism(s) by which SCL contributes to leukaemia remains unclear. Studies suggest that SCL may transactivate the expression of novel target genes in leukaemia (Hsu *et al.*, 1994) and that the LIM-only protein LMO2 is required for SCL induced leukaemia (Larson *et al.*, 1996). Absence of LMO2 activation by inducing differentiation arrest and by interfering with E47/HEB function was shown in SCL expressing mouse T cells.

E47/HEB heterodimers appear to regulate CD4 expression, in part, by recruiting the coactivator p300 to the enhancer. This observation provides a new mechanism to explain how SCL contributes to leukaemia. Rather than operating like an Id dominant negative bHLH partner and inhibiting the ability of E proteins to bind DNA, SCL may repress gene transcription by recruiting co-repressor complexes to the E47/HEB target gene, CD4 or others (O'Neil *et al.*, 2004). It seems likely that decreased expression of the other E47/HEB target genes, including TCR α and β, CD5, rag 2, and pre-Tα, may also be mSin3A/HDAC1-mediated SCL repressive effects. These effects may not be limited to E47/HEB target genes that regulate thymocyte differentiation, but may include other genes that regulate proliferation and survival. It was suggested that thymocyte differentiation arrest induced by SCL expression predisposes thymocytes to leukaemia by additional mutations occurring during the DN3 arrest, where the thymocyte precursor undergoes extensive cell division. Consistent with this idea, increased cell cycling is observed in E2A-deficient DN3 precursors, suggesting that E2A proteins function as cell cycle inhibitors in thymic precursors (Engel and Murre, 2004; O'Neil *et al.*, 2004).

In addition to aberrant cell cycling, survival pathways may also be activated during the differentiation arrest and maintained throughout leukaemia progression. The anti-apoptotic transcription factor NF-κB is activated in SCL thymocytes, and NF-κB activity is maintained in mouse SCL tumours, and observed in a majority of human T-ALL samples (O'Neil *et al.*, 2003). Identification of the anti-apoptotic NF-κB target genes in *tal1/scl* leukaemia cells will be the focus of future work.

The biologic activity of SCL relies on two important domains in the protein. The helix-loop-helix (HLH) domain is common to the HLH transcription factor family and allows its members to homo- or heterodimerize. Hence, this domain is crucial for the heterodimerization of SCL with the members of the ubiquitously expressed E2A proteins (i.e., E47, E12, and HEB), which are the main partners of SCL (Hsu *et al.*, 1991) The basic domain is present in many HLH proteins where it confers binding to DNA on the E-box consensus sequence CANNTG and further transactivation of target genes (Hsu *et al.*, 1994). Mutations and deletions of these two domains and studies of the mutated SCL proteins in functional assays have indicated that the DNA-binding domain, but not the HLH dimerization domain, is dispensable for some SCL

properties (O'Neil *et al.*, 2001). The SCL protein lacking its capacity to bind to DNA can rescue some of the primitive and definitive haematopoietic potentials of SCL null mouse ES cells. (Porcher *et al.*, 1999; Mikkola *et al.*, 2003).

Induced SCL down-regulation using conditional siRNA strategies might therefore shed light on the mechanism which leads to the development of leukaemia and/or the differentiation of pre-T-cells to a more mature T-cell population.

1.4.3 SCL knockout and partial loss-of-function studies in mice

SCL null mice lacked yolk sac haematopoiesis (Robb *et al.*, 1995; Shivdasani *et al.*, 1995) but initially were reported to develop morphologically normal endothelium. Defects in brain development were also not reported. Analysis at earlier time points demonstrated that homozygous null embryos were dying around embryonic day 9.5. These embryos were pale, oedematous, and markedly growth retarded after embryonic day 8.75. The results implicated SCL as a crucial regulator of early haematopoiesis.

Aplan and colleagues demonstrated that transgenic mice in which inappropriately expressed SCL protein, driven by regulatory elements of the upstream SIL gene, developed aggressive T-cell malignancies in collaboration with a misexpressed LMO1 protein, thus recapitulating the situation seen in a subset of human T-cell ALL. This study also demonstrated that inappropriately expressed SCL can interfere with the development of other tissues derived from the mesoderm. The authors also provided evidence that an SCL construct lacking the SCL transactivation domain induced misexpression of LMO1, suggesting that the SCL transactivation domain is dispensable for oncogenesis (Aplan *et al.*, 1997).

In an other study, the mechanism involved in SCL induced leukemogenesis where further elucidated (O'Neil *et al.*, 2001). Transgenic mice were generated expressing a DNA binding mutant of SCL. Surprisingly, these mice developed disease, demonstrating that the DNA binding properties of SCL are not required to induce leukaemia/lymphoma in mice. However, wild type SCL and the DNA binding mutant both formed stable complexes with E2A proteins. In addition, SCL was shown to stimulate differentiation of CD8-single positive thymocytes but to inhibit development of CD4-single positive cells, these effects were also observed in E2A-

deficient mice. The results suggest that the bHLH protein SCL contributes to leukaemia by interfering with E2A protein function and the loss of DNA binding domain was not important for leukaemia induction.

Loss-of-function studies had showed that SCL is essential for the formation of haematopoietic stem cells, subsequent erythroid development, and yolk sac angiogenesis (Robb *et al.*, 1995; Porcher *et al.*, 1999; Hall *et al.*, 2003). To identify control and regulatory elements necessary for SCL expression in erythroid cells, Sinclair and colleagues used $SCL^{-/-}$ mice. They demonstrated that a 130-kb YAC containing the human SCL locus completely rescued the embryonic lethal phenotype of $SCL^{-/-}$ mice. YAC-rescued $SCL^{-/-}$ mice were born normal, and exhibited no detectable abnormalities in the yolk sac, fetal liver, or adult haematopoiesis. The deletion of SCL which is lethal in the embryo was shown to be rescued the by human SCL locus. The results also demonstrated that the human SCL YAC contains the chromosome domain necessary to direct the expression to the erythroid lineage and to all other tissues in which SCL performs an essential function (Sinclair *et al.*, 2002).

In order to carry out genetic analysis of the function of SCL for adult mouse haematopoiesis, a conditional knockout of SCL was generated using Cre/LoxP technology and an interferon-inducible Cre transgenic mouse. With this it was able to study SCL loss-of-function in the mouse (Shivdasani *et al.*, 1995; Hall *et al.*, 2003; Mikkola *et al.*, 2003). Deletion of SCL in adult mice disturbed megakaryopoiesis and erythropoiesis with the loss of early progenitor cells in both lineages. Immature progenitor cells (day 12 CFUs in the spleen) with multilineage capacity were still present in the SCL-null bone marrow, but these progenitors no longer had the capacity to generate erythroid and megakaryocyte cells, and colonies were composed of only myeloid cells. These results showed that the SCL is required for megakaryopoiesis and erythropoiesis, but is dispensable for production of myeloid cells during adult haematopoiesis (Shivdasani *et al.*, 1995; Hall *et al.*, 2003).

SCL is initially expressed in mesodermal cells that give rise to embryonic blood cells, and continues to be expressed in fetal and adult haematopoietic stem cells (HSCs). However, SCL is not required for the maintenance of established long-term repopulating (LTR) HSCs in the adult (Mikkola *et al.*, 2003; Curtis *et al.*, 2004; Gothert *et al.*, 2005; Zhang *et al.*, 2005). The time point at which HSC development

becomes SCL-independent has not yet been defined. Tie2 expression appears in heamogenic and vasculogenic sites shortly after SCL. Therefore the Tie2-*Cre* mouse was used to inactivate SCL early during embryonic and fetal haematopoiesis. Tie2-*Cre* completely inactivated SCL in the yolk sac, AGM (aorta-gonad-mesonephros-region) and fetal liver haematopoietic cells and circulating blood cells (Schlaeger *et al.*, 2005). These experiments once again highlight the importance of SCL for blood and development.

Gene targeting studies have shown that the transcription factor SCL is required for embryonic haematopoiesis, but the early lethality of SCL-null mice and the genetic analysis of this function in the adult showed that SCL is also important during haematopoietic cell repopulation (Hall *et al.*, 2005).

In the current approach it was planned to use a different strategy, a conditional SCL knockdown which also facilitates the *in vivo* study of SCL function. As the knockdown strategy uses a tet on/off technique it was envisaged that loss of SCL function could be studied in a reversible fashion. Which allows for the study of a knockdown phenotype at different time points in development and later in the adult mouse.

Finally, as the knockdown phenotype is to be expected to result in complete loss of function, the effect of graded gene inactivation from epi-allelic series might also be studied using the current strategy.

1.5 Lodish plot as a technique for FACS analysis of a SCL-knockdown phenotype

Conditional knockout studies in mice demonstrated that SCL is required for proper red blood cell maturation (Shivdasani *et al.*, 1995; Hall *et al.*, 2003; Mikkola *et al.*, 2003). To test siRNA mediated knockdown of SCL an appropriate readout was needed. Lodish and colleagues established a flow cytometry assay that allows for quantitative evaluation of erythroid differentiation in neonatal and adult haematopoietic tissues (Socolovsky *et al.*, 2001). On the basis of the expression of the erythroid-specific TER119 and red blood cell marker CD71 (transferrin receptor), erythroid cells are sorted into four populations that correspond with their maturation

stages ranging from proerythroblasts to mature erythroblasts. During erythroid differentiation, erythroblasts decrease in cell size, condense chromosomal DNA, increase cellular haemoglobin concentration, decrease surface expression level of the EpoR and exit the cell cycle permanently. Since the Lodish plot technique allows for quantifying immature versus mature red blood cells, this technique was initially envisaged as a quantitative analysis for siRNA mediated knockdown efficiencies *in vivo*.

Figure 3, shows a Lodish blot from the Lodish group (Socolovsky *et al.*, 2001). To examine the four populations of cells corresponding to erythroblasts at different developmental stages, red blood cells were first sorted by FACS, double labelled with CD71 and TER119 (Figure 3, left panel) and each population region was numbered R1-R5 depending on the expression of TER119. Sorted cells from the different regions were analyzed by colony assays, histologic staining and surface expression levels of EpoR. Region one (R1) cells contained a high frequency of cells containing pre-erythrocytes, R2 contained only a few maturing erythrocytes positive cells whereas R3 to R4 contained no erythroid progenitors only mature erythrocytes. These results indicated that immature red blood cells were contained in R1 and R2. When R1 to R4 cells were processed by May-Grunwald Giemsa stains (Figure 3, right panel), their morphologies resembled erythroblasts at different developmental stages (Zhang *et al.*, 2003), with R1 being the least and R4 being the most differentiated. The morphologic characteristics generally corresponded to primitive progenitor cells and proerythroblasts in the $CD71^{high}TER119^{med}$ population (R1); proerythroblasts and early basophilic erythroblasts in the $CD71^{high}TER119^{high}$ population (R2); early and late basophilic erythroblasts in the $CD71^{med}TER119^{high}$ population (R3); chromatophilic and orthochromatophilic erythroblasts in the $CD71^{low/\ med}$ $TER119^{high}$ population and late orthochromatophilic erythroblasts and reticulocytes in the $CD71^{low}TER119^{high}$ population (R4). These results were consistent with those obtained from benzidine-Giemsa–stained R1 to R5 cells (Figure 3, right panel).

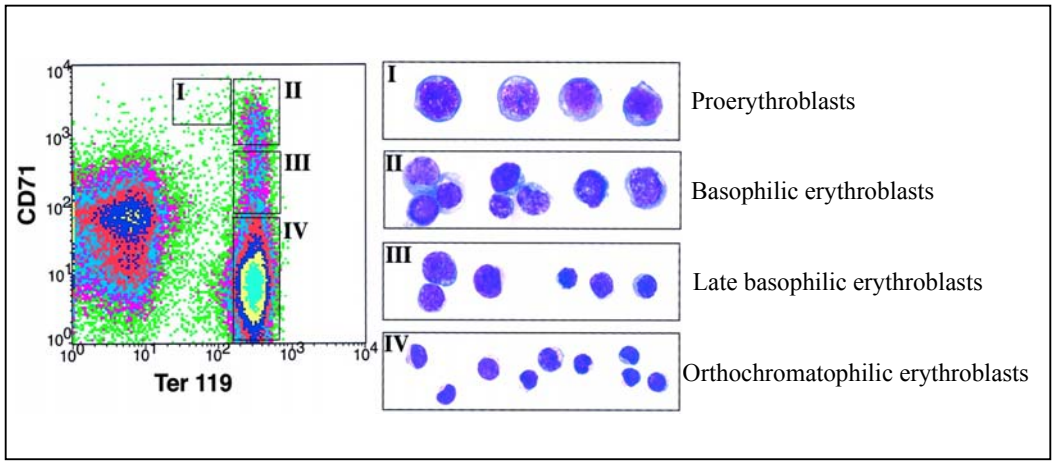


Figure 3: Lodish plot analysis

Bone marrow (BM) FACS based analysis of erythropoiesis. Mouse BM cells double labelled with anti-CD71 monoclonal antibody (mAb) and a PE-conjugated anti-TER119 mAb. The left panel illustrates a density plot of all viable cells. Regions R1 to R4 are defined by characteristic staining pattern of cells, including $CD71^{high}TER119^{med}$(proerythroblasts), $CD71^{high}TER119^{high}$ (basophilic erythroblasts), $CD71^{med}TER119^{high}$(late basophilic erythroblasts), and $CD71^{low}TER119^{low}$ (orthochromatophilic erythroblasts) respectively (middle panel). These populations were reconfirmed by Giemsa staining (right panel). Adapted from (Socolovsky *et al.*, 2001; Zhang *et al.*, 2003)

In the present study the Lodish plot technique was used for determining the biological effect of SCL knockdown in mice by shRNAs. We reasoned that if shRNA expression results in knockdown of SCL, we would be able to detect a shift towards immature red blood cell precursors due to the requirement of SCL for erythroblast formation.

1.5.1 Final remarks

The fascination for RNAi has become contagious within the research community. RNA Interference (RNAi) is one of the most exciting discoveries of the past decade in functional genomics and proteomics. While first recognized in nematodes as a response to exogenously introduced long double-stranded RNA, it is now clear that RNAi is utilized by most eukaryotes *in vivo* for anti-viral defence, transposon activity and functional gene regulation, and is rapidly becoming an important research tool for gene silencing. The absence of any immune response to shRNA also circumvents the known difficulties when protein-based therapeutic agents are used for clinical treatments of human diseases.

Collectively, RNAi represents not only an excellent tool for studying gene function but holds the clear promise for efficiently treating malignancies. It is also to be expected that RNAi-mediated strategies are not restricted to mammalian systems but will also find crucial applications in agriculture, animal husbandry, prevention of plagues and defence against infectious agents.

2. MATERIALS AND METHODS

2.1 General Materials

2.1.1 Reagents

Acetic acid	Merck, (Darmstadt, D)
Acrylamide(30%)	Roth (Karlsruhe, D)
Agar	Life Technologies, (Paisley, UK)
Agarose	Invitrogen (Karlsruhe, D)
Ammonium peroxydisulfate (ACS)	Sigma (Deisenhofen, D)
Ampicillin	Sigma (Deisenhofen, D)
Bacto-peptone	Invitrogen (Karlsruhe, D)
Bacto-agar	Roth (Karlsruhe, D)
Bovine serum albumin (BSA)	Sigma (Deisenhofen, D)
Bradford-reagent (Roti Quant)	Roth (Karlsruhe, D)
Bromophenol blue	Sigma (Deisenhofen, D)
Bovine serum albumin (BSA)	Sigma (Deisenhofen, D)
ß-Mercaptoethanol (ß-ME)	Roth (Karlsruhe, D)
Chloroform	Roth (Karlsruhe, D)
Coomassie-Blue R-250	Life Technologies (Paisley, UK)
Deoxyribonucleotide triphosphate (dNTP)	Fermentas (St. Leon-Rot, D)
Diethyl pyrocarbonate (DEPC)	Merck (Darmstadt, D)
Dulbeccos Modified Eagles Medium (DMEM)	Gibco (Eggenstein, D)
Dimethyl sulfoxide (DMSO)	Sigma (Deisenhofen, D)
Ethanol (EtOH)	Merck (Darmstadt, D)
Ethidium bromide (10mg/ml)	Roth (Karlsruhe, D)
Ethylenediamine tetra-acetic acid-disodium salt (EDTA)	Sigma (Deisenhofen, D)
Fetal calf serum (FCS)	Greiner (Frickenhausen, D)
Formaldehyde	Sigma (Deisenhofen, D)
Formamide	Sigma (Deisenhofen, D)
L-Glutamine (x100)	Invitrogen (Karlsruhe, D)
Glycerol	Sigma (Deisenhofen, D)
Glycine	Merck (Darmstadt, D)

N-2-hydroxyethyl piperazine-N-2-ethane-sulphonic acid (HEPES)	Sigma (Deisenhofen, D)
Hydrochloric acid (HCl)	Roth (Karlsruhe, D)
Isopropanol	Roth (Karlsruhe, D)
6x loading buffer	Invitrogen (Karlsruhe, D)
Potassium dihydrogen phosphate	Roth (Karlsruhe, D)
Potassium chloride	Roth (Karlsruhe, D)
Kanamycin Agar	Fermentas (St. Leon-Rot, D)
Methanol	Merck (Darmstadt, D)
Magnesium chloride ($MgCl_2$)	Sigma (Deisenhofen, D)
3-(N-morpholino)-propane sulfonic acid (MOPS)	Sigma (Deisenhofen, D)
Sodium acetate (Na_2^+)	Roth (Karlsruhe, D)
Sodium chloride (NaCl)	Roth (Karlsruhe, D)
Sodium hydrogen phosphate	Riedel de Haen (Seelze, D)
Sodium hydroxide	Roth (Karlsruhe, D)
Nitrocellulose Protran 2x membrane	Schleicher & Schüll (Dassel, D)
Nitrocellulose Hybond N+ membrane	Pall life sciences (Dreieich, D)
Non fat dry milk	Sigma (Disenhofen, D)
OptiMEM	Gibco (Eggenstein, D)
Penicillin-Streptomycin (10000 units/ml)	Invitrogen (Karlsruhe, D)
Phenol	Roth (Karlsruhe, D)
Phosphoric acid	Roth (Karlsruhe, D)
Phosphate buffered saline (PBS)	PAN Biotech (Aidenbach, D)
Ponceau S solution	Sigma (Deisenhofen, D)
Potassium acetate (KAc)	Sigma (Deisenhofen, D)
Potassium chloride (KCl)	Sigma (Deisenhofen, D)
Potassium dihydro-phosphate (KH_2PO4)	Merck (Darmstadt, D)
Proteinase Inhibitors	Roche (Mannheim, D)
Proteinase K	Sigma (Deisenhofen, D)
Propidium iodide (1mg/ml)	Sigma (Deisenhofen, D)
Protein marker (SDS-7B)	Fermentas (St. Leon-Rot, D)
Ribonuclease A (Typ I-AS)	Sigma (Deisenhofen, D)
RNase Inhibitor	Sigma (Deisenhofen, D)

RNA sample buffer	Sigma (Deisenhofen, D)
RPMI 1640	Life Technologies, (Paisley, UK)
Sucrose	Roth (Karlsruhe, D)
Sodium chloride	Merck, (Darmstadt, D)
Sodium dodecyl sulfate (SDS)	Sigma (Deisenhofen, D)
Sodium citrate	Merck (Darmstadt, D)
Sodium dihydrogen phosphate (NaH2PO4)	Merck (Darmstadt, D)
Sodium hydrogen phosphate (NaHPO4)	Merck (Darmstadt, D)
Sodium hydroxide (NaOH)	Sigma (Deisenhofen, D)
Tetracycline free fetal calf serum	BD Clontech (Heidelberg, D)
N, N, N', N'- Tetraethylethylenediamine (TEMED)	Sigma (Deisenhofen, D)
TRIS-(hydroxymethyl)-aminomethane (TRIS)	Roth (Karlsruhe, D)
Triton-X100	Sigma (Deisenhofen, D)
TRIzol Reagent	Invitrogen (Karlsruhe, D)
Trypan blue	Sigma (Deisenhofen, D)
Trypsin/EDTA	PAN Biotech (Aidenbach, D)
Tween-20	Sigma (Deisenhofen, D)
Whatman 3MM Paper	Schleicher & Schüll (Dassel, D)
X-ray film	Amersham-Buchler
Xylene cyanol	Sigma (Disenhofen, D)
Yeast Extract	Invitrogen (Karlsruhe, D)

2.1.2 Radioactive substances

α-^{32}P-dCTP	Amersham (Braunschweig, D)

2.1.3 Enzymes

Calf intestinal phosphatase (CIP) (1U/µl)	Roche (Mannheim, D)
DNA Polymerase Large Fragment (Klenow) (5U)	NEB (Frankfurt a. M., D)
Restriction enzymes	Fermentas (St. Leon-Rot, D)
	NEB (Frankfurt a. M., D)
RNase A (1U/µl)	Roche (Mannheim, D)
T4 DNA Polymerase (5U/µl)	Fermentas (St. Leon-Rot, D)
T4 Ligase (5U/µl)	Fermentas (St. Leon-Rot, D)
	NEB (Frankfurt a. M., D)

2.1.4 Kits

ECL Western blotting detection reagent	Amersham (Braunschweig, D)
Lipofectamine 2000	Invitrogen (Karlsruhe, D)
Prime-It Random Primer Labelling kit	Stratagene (Heidelberg, D)
Protein estimation kit	Biorad (Munich, D)
RNeasy kit	Qiagen (Hilden, D)
Superscript^{TM} one step RT-PCR	Invitrogen (Karlsruhe, D)
TOPO TA Cloning kit	Invitrogen (Karlsruhe, D)
QIAEX II Gel Extraction kit	Qiagen (Hilden, D)
QIAGEN Plasmid kit (Midi, Maxi)	Qiagen (Hilden, D)
QIAquick PCR purification kit	Qiagen (Hilden, D)
QIAquick nucleotide removal kit	Qiagen (Hilden, D)

2.1.5 Consumables

Biomax MS Film	Kodak (Rochester, USA)
Corex II Rotor	DuPond (Bad Homburg, D)
Cling film	MiniMAL Handelsmarkt (Mainz, D)
Disposable plastic cuvettes	Braun (Melsungen, D)
Elektroporation cuvette (2 mm)	Peqlab (Erlangen , D)
Eppendorf tubes (1,5 and 2 ml safe-lock)	Eppendorf (Hamburg, D)
Glas woll	Merck (Darmstadt, D)
Neubauer counting chamber	Braun (Melsungen, D)
Pasteur pipettes	Roth (Karlsruhe, D)
PCR-Reaction tubes (0,2 ml Safe-Lock)	Eppendorf (Hamburg, D)
Petri dish (steril, 82 mm)	Greiner (Frickenhausen, D)
Falcon tubes (sterile, 5 ml, 15 ml and 50 ml)	Greiner (Frickenhausen, D)
S&S-Rotrand-1x-Sterile filter (0,22 µm)	Schleicher&Schuell (Dassel, D)
Spin X costar 8160 column	Corning Inc. (Massachusetts USA)
Sterile tips	Henke Sass Wolf (Tuttlingen, D)
Cell culture plates (6-well, 9 cm culture plates)	Greiner (Frickenhausen, D)

2.1.6 Equipment

Autoclave	KSG Steriliser (Olching, D)

Bacteria incubator	Heraeus B 6200 (Hanau, D)
Bacteria shaker	Certomat BS-T, B. Braun Biotech (Melsungen, D)
Bench centrifuge 5417 C	Eppendorf (Hamburg, D)
Cell culture incubator	Nunc (Wiesbaden, D)
Cell culture microscope	Carl Zeiss (Jena, D)
Cell culture hood HLB2472	BIO-FLOW Technik
Computer	Power Macintosh G3 (Apple Macintosh, D) ASUS Laptop M2400N (ASUSTech, Taiwan)
Deep freezer (-80°C)	Snijders (Tilburg, NL)
Stratalinker linker Amplirad	Genetic research instruments Ltd (Essex, U.K)
Developing cassettes	Dr. goods supreme GmbH (Heidelberg, D)
Developing machine	Kodak (Rochester, USA)
Electrophoresis power supply	E844, Consort
Electrophoresis unit,	Work shop, Johannes Gutenberg-Universität (Mainz,D)
Electroporator	Gene Pulser II Bio Rad (München, D)
FACS sorter	FACS Calibur™, Ventage SE™ Becton Dickenson (Heidelberg, D)
Fluorescence microscope	Olympus 1x70 (Olympus Optical Co., LTD, Japan)
Heating block	Dri-Block DB-3A Techne (Cambridge, UK)
Hybridiser	WTB Binder (Tuttlingen, D)
Lab centrifuge 400 R	Haraeus (Hanau, D)
Laboratory shaker	UNIMAX 2010 Heidolph (Kehlheim, D)

Liquid nitrogen tank	Air Liquide Kryotechnik GmbH (Düsseldorf, D)
Millipore H_2O-prduction unit	Milli-Q plus Millipore GmbH (Eschborn, D)
Power supply	Braun (Melsungen, D)
pH-Meter	Multical Typ 538 (Weilheim, D)
Pipettes (p-20, p-200, p-1000)	Gilson (Bad Camberg, D)
Pipetman pipetus-akku	Hirschmann laboratory instrument (Eberstadt, D)
Scale BP2100S, BP310S	Sartorius
Scintilation counter (Radioactivity)	Tri-carb 2100 TR, Canberra-Packard (Dreieich, D)
Spectrophotometer UV-Vis GeneSys 5	Spectronic Instruments (USA)
Typ gradient thermocycler	Eppendorf (Hamburg, D)
Typ UNO 96 thermocycler	Biometra (Göttingen, D)
UV-Transluminator and video system	LTF Labortechnik (Wasserburg, D)
Vortex	Heidolph (Kehlheim, D)
Water bath	Grant Science Services (München, D)
Ultra centrifuge	Sorvall Superspeed RC2-B DuPond (Bad Homburg, D)

2.1.7 Software

DNA-Strider 1.2	CEA (Gif-sur-Yvette, F)
EditView 1.0.1	Perkin Elmer (USA)
Mac-Plasmap 2.1	CGC Scientific Ltd. (St. Louis, USA)
Sequencer Demo Version	Gene Codes Corporation (Ann Harbor, USA)
TILLvisION v 4.00	TILL Photonics GmbH, (Munich, D)

2.1.8 Buffers

Buffers provided with the kits were used in the case of plasmid isolation, DNA gel extraction, protein expression, RNA isolation, and PCR reactions including RT-PCR. All other buffers used are listed below.

2.1.8.1 Bacterial medium and DNA buffers

Annealing buffer	100mM potassium acetate, 30mM HEPES-KOH pH 7.4, 2mM Mg-acetate.
DNA gel loading buffer (Blue marker)	0.25% bromo-phenol blue, 0.25% xylencyanol , 15% glycerol add 10 ml ddH2O.
EDTA 0,5 M	14.6 g EDTA, add 100 ml ddH_2O, pH 8.0. Autoclaved sterile.
LB (Luria-Bertani) medium	10 g/l bacto-tryptone, 10 g /l NaCl, 5 g yeast extract, pH to 7.5. For plates, add 15 g bacto-agar
Sodium acetate 3M	24.61 g sodium acetate, add 100 ml ddH_2O, pH 4.8. Autoclave sterile.
RNase-A buffer	100 mg RNase in 10 ml, 0.01 M sodium acetate pH 5.2, heat to 100^0C for 15 min, add 1 ml 1 M TRIS chloride pH 7.4, aliquot, store at -20^0C.
Lysis buffer for mouse tails	50 mM TRIS pH 8.0, 100 mM NaCl, 100 mM EDTA pH 7.4, 1% SDS, 1 mg/ml proteinase K.
10 x TBE- buffer	108 g TRIS base, 55 g boric acid, 40 ml 0.5 M EDTA pH 8.0, add 1 l ddH_2O, pH 8.0. Autoclave sterile.
1 M TRIS base	121.1 g TRIS base, add 1 l ddH_2O, pH 7.4
2 x TY medium	16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl, pH to 7.4 add 1 l ddH_2O. Autoclave sterile. For plates, add 15 g bacto-agar

STET buffer	0.1 M NaCl, 20 mM TRIS pH 7.4, 10 mM EDTA pH 8.0, 8% sucrose, 5% tween-20.
HEPES buffer	100 mM HEPES, in 1 l ddH_2O, pH 7.0. Autoclave sterile.
HEPES-glycerol buffer	10% glycerol in HEPES buffer, pH 7.0

2.1.8.2 RNA buffers

DEPC-H_2O	100 µl DEPC to 100 ml ddH_2O, leave 12 h. Autoclaving sterile.
10x MOPS buffer	0.2 M MOPS, 0.05M sodium acetate, 0.001 M EDTA, pH 7.0. Filter sterile.
Pre- and hybridisation buffer	7% SDS, 0.5 M phosphate buffer pH 7.2, 1 mM EDTA.
Phosphate buffer	89 g $NaH_2PO_4.2H_2O$, 4 ml 85% phosphoric acid add 1 l DEPC H_2O, pH 7.2. Autoclave sterile.
10% SDS	50 g SDS added to 500 ml dd H_2O. Heat to 68°C.
20 x SSC	3 M NaCl, 0.3 N Tri-sodium citrate-dihytrate, pH 7.8. Autoclave sterile.
Washing buffer 1	2 x SSC, 0.1% SDS.
Washing buffer 2	1 x SSC, 0.1% SDS.
Washing buffer 3	0.1 x SSC, 0.1% SDS.

2.1.8.3 Protein analysis buffers

1st Antibody dilution buffer	10% BSA in 1 x PBS.
10% ACS	1g in 10 ml H_2O.
Blotting buffer	39 mM glycine, 48 mM TRIS, 0.037% SDS, 20% methanol.
Blocking buffer	5% of non-fat dry milk in 1 x PBST.
Destaining solution	30% methanol, 10% acetic acid.
Electrophoresis buffer (for SDS-PAGE)	25 mM TRIS, pH 8.3, 250 mM glycine, 0.1% SDS.

2x Laemmli buffer	125 mM TRIS pH 6.8, 5% SDS, 10% ß-mercaptoethanol, 2% glycerol, 0.5% bromophenol blue
6x Laemmli buffer	125 mM TRIS pH 6.8, 5% SDS, 10% ß-mercaptoethanol, 6% glycerol, 0.5% 0.5% bromophenol blue
1x PBS	8 g sodium chloride, 0.2 g potassium chloride, 1.44 g phosphate, 0.24 g potassium hydrogen phosphate, add 1 l ddH_2O, pH 7.4. Autoclave sterile.
PBST	1 x PBS, add 0.05% tween-20
Running buffer (SDS-PAGE)	25 mM TRIS, 250 mM glycine, 0.1% SDS
Staining solution	30% methanol, 10% acetic acid, 0.25% coomassie-Blue R-250
Stripping buffer	0.1 M ß-mercaptoethanol, 2% SDS, 1 M TRIS pH 6.8, 50 mM TRIS, pH 8.0, 1 mM EDTA, 0.05% trypsin, 0.02% EDTA in PBS.
Tissue lysis buffer	2% SDS, 50 mM TRIS pH 7.5. One table proteinase inhibitors (Roche)/ 10 ml.
1M TRIS	121.1g TRIS in 1l H_2O, pH 6.8
1.5M TRIS	181.65g TRIS in 1l H_2O, pH 8.8

2.1.9 Cell culture materials

2.1.9.1 Eukaryotic cell lines

M1	Mouse myeloid cell line (ATCC CL-1.4)
HEK 293	Human embryonic kidney cells (ATCC), a gift from Carsten Weiss, Institute of Toxicology, Johannes Gutenberg -Universität Mainz
HEK 293TR (T-REx)	HEK 293 expressing the T-REx repressor (Invitrogen), a gift from Marc van de Wetering

HEK 293 tTS	HEK 293 expressing the KRAB silencer tTS (Clontech #630927)

2.1.9.2 Culture media

Cell lines were maintained in DMEM or RPMI 1640 media supplemented with 10% FCS, 1% L-Glutamine, 1% penicillin-streptomycin (P/S).

2.1.9.3 Cell freezing medium

70% complete DMEM or RPMI 1640 media (10% FCS, P/S), 10% DMSO.

2.1.9.4 FACS buffer

5% FCS in PBS

2.1.10 Antibiotics

Ampicillin	500 mg ampicillin in 10 ml ddH_2O, filter sterile, aliquot, store at -20^0 C.
Doxycycline in sucrose (Dox)	10 mg/ml Dox in 1% sucrose autoclaved drinking water.
Doxycycline stock (Dox)	2 mg in 10 ml ddH_20, filter sterile aliquot, store at -20^0C. Working solution 5 µl/ml

2.1.11 Bacterial strains

DH5α	supE44deltalacU169(ϕ80lacZdeltaM15) hsdR17recA1endA1gyrA96 thi-1 relA1 Institute of Toxicology, Johannes Gutenberg-Universität Mainz
Top10	deoR+, endA1-, recA1-, lacZdelta M15+, hsdRMS+, delta (mrv-mcr BC). Invitrogen™ used for TOPO T/A cloning

2.1.12 Animals

Specific pathogen-free mice (4 weeks old) were purchased from Charles Rivers (Sulzberg, D). Animal colonies are kept in pathogen free conditions in individual

ventilated cages (IVC) at the animal facility of the Johannes Gutenberg-Universität Mainz. For micro injection and generation of the transgenic mouse lines, the FVB mice strains were used with NMRI mice supplementing as pseudo-mothers.

tTS KRAB mice were kindly provided by Dr. Moises Mallo (Mallo *et al.*, 2003) containing the human ß actin gene promoter (Ng *et a*l., 1985, MCB 5, 2720-2732 (Ng *et al.*, 1985) and the tTS gene containing the a TetR sequence plus the KRAB domain of Kox1 (Deuschle *et al.*, 1995).

2.1.13 DNA expression vectors

Table 1: cDNA libraries and plasmid DNA

Plasmid	Index number	Functional sequence	Reference
CMB TetR B/E - HDAC4	1464	CMV immediate enhancer (CMV-i.e.) ß-actin promoter, Tet R (B/E)-HDAC-4 expression vector.	Dr. Cerstin Christel, Institute of Toxicology, Johannes Gutenberg-Universität Mainz
pTER	1533	H1 promoter driven RNAi expression vector	Marc van de Wetering *et al.*, 2003, *EMBO Reports* 4, 6:609-15
pEGFP-C3	1547	CMV promoter driven EGFP expression vector	Clontech, 6082-1; U57607
TOPO TA EGFP	1558	TOPO cloning of an EGFP cassette	A PCR product amplifying the EGFP cassette of vector # 1547 was cloned into a TOPO TA vector (Invitrogen)
pRNAi EGFP	1551	H1 RNAi expression vector containing in addition an EGFP expression cassette	Oligos #161 and #162 were used for the amplification of an EGFP cassette from vector 1547 which was cloned into *Xba I* and *Spe I* restriction sites of vector 1533
pSCL-siRNA 1	1555	SCL H1 RNAi expression vector	Using vector 1551, RNAi oligos #111 and #115 were annealed and cloned into *Bgl II* and *Hind III* sites
pSCL-siRNA 2	1556	SCL H1 RNAi expression vector	Using vector 1551, RNAi oligos #112 and #116 were annealed and cloned into *Bgl II* and *HindIII* sites
pSCL-siRNA 3	1557	SCL H1 RNAi expression vector	Using vector 1551, RNAi oligos #113 and #117 were annealed and cloned into *Bgl II* and *Hind III* sites
pSCL-siRNA 4	1558	SCL H1 RNAi expression vector	Using vector 1551, RNAi oligos #114 and #118 were

			annealed and cloned into *Bgl II* and *Hind III* sites
T-REx Invitrogen	1543	TetR Repressor expressing the pcDNA6/TR from Invitrogen	Marc Van de Wetering *et al.*, 2003, *EMBO Reports* 4, 6:609-15
TOPO TA T-TEx	1546	TOPO cloning of the T-REx cassette	A PCR product amplifying the T-REx cassette of vector # 1543 was cloned into a TOPO TA vector (Invitrogen)
T-REx	1553	CMV immediate enhancer (i.e.) ß-actin promoter, TetR Repressor (1543) expression vector.	Cloning of TetR repressor (1543) into *Alf II* and *NotI* restriction sites of vector 1464
T-REx FRT LoxP	1567	Vector 1553 TetR repressor containing a single FRT site and two LoxP sites flanking the TetR expression unit	Oligos #203 and #204 containing the FRT and LoxP sites cloned into *Afl III* and *Not I* digested 1533.
TOPO T/A	Invitrogen™ Kit	For T/A nucleotide cloning of PCR insert	Invitrogen™
pcDNA3.1 HA-tag	1577	pcDNA3.1 backbone with additional HA tag sequence	A gift from Dr. Carsten Weiss, Institute of Toxicology, Johannes Gutenberg -Universität Mainz
SCL HA-tag	1578	SCL cDNA insert coding sequence with in frame N-terminal expressed with HA tag	SCL coding sequence from vector pBi SCL EGFP vector 1568. Dr. Marko Maringer Institute of Toxicology, Johannes Gutenberg -Universität Mainz was cloned into *EcoR V* and *Xba I* sites of vector 1577

Abbreviations: CMB, surnames Christel Maringer Bockamp of contributors of the cloning of the CMB promoter; Oligos, oligonucleotides; EGFP, enhanced green fluorescent protein; FRT, recognition site for *Flp* recombinase; HA, influenza A virus heamaglutinin; LoxP, recognition site for *Cre* recombinase; SCL, stem cell leukaemia ;TetR, tetracycline repressor

2.1.14 Oligonucleotides

All used oligonucleotides were obtained from Metabion or OPERON Biotechnologies and are indicated in table 2 and 3.

Table 2: RNAi-specific oligonucleotides

Name	Number	Position	Sequence 5´-3´	Application
siRNA1	111 115	SCL cDNA Exon 6*	gaccttctacaggacgtgct	SCL mRNA knockdown
siRNA2	112 116	SCL cDNA Exon 6*	ggcacccagcgtgccaagc	SCL mRNA knockdown
siRNA3	113 117	SCL cDNA Exon 6*	cagccgggaacgatggagg	SCL mRNA knockdown
siRNA4	114 118	SCL cDNA Exon 6*	gtagtgcggcgcatcttca	SCL mRNA knockdown

*SCL mouse cDNA accession nr.(NM011527); (Begley *et al.*, 1994).

Table 3: Oligonucleotides used for PCR for direct cloning and genotyping

Name	Number	Sequence 5´-3´	Application
GAPDH-1	63	CGTCTTCACCACCATGGAGA	Internal control for genotyping
GAPDH-2	64	CGGCCATCACGCCACCGTTT	HK control for genotyping
Hygromycin c	125	ATGAAAAAGCCTGAACTCACCG	PCR for genotyping T-REx mice. Vectors 1553 and 1567
Hygromycin nc	126	CTATTCCTTTGCCCTCGGACG	PCR for genotyping T-REx mice. Vectors 1553 and 1567
pEGFP-c3 *Xba l* nc	161	GCTCTAGAATTCTTGTACAGCTCGTC	PCR CL for generation of plasmid 1551
T-REx c1	159	GCAGAGCTCTCTGGCTAACTAG	Vector 1553
T-REx nc 1	160	AGAGGTCACCTTAATAGGATCTGAATTCCCGGGATCCGC	Vector 1553
pEGFP-CMV *Spe I* c	162	GACTAGTTATCCCCTGATTCTGTGGATAACCG	PCR CL for generation of plasmid 1551
RT-PCR siRNA SCL c	195	CGCTGCTCTATAGCCTTAGCCA	RT-PCR; for detection of endogenous SCL expression
RT-PCR siRNA SCLnc	196	CTTTGGTGTGAGGACATCAGAAATC	RT-PCR; For detection of SCL expression
T-REx LoxP FRT c	203	GGCCGCATAACTTCGTATAGCATACATTATACGAAGTTATCCGCTCGAGCGG CCGCCGGCGTATTGAAGCATATCGTATGTAATATGCTTCAATAGGCGAGCTC GCCG	CL for generation of vector 1567

T-REx LoxP nc	204	GAGCTCCTAGCTAGCTAGGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC CTCGAGGATCGATCGATCCTTCAAGGATAAGAGATCTTTCATATCCTTGAAG	CL for generation of vector 1567
PD c	221	ATTCGGGGAACCTCAACAC	Internal control for genotyping
PD nc	222	CCCACAGCATACATGCATT	Internal control for genotyping
T-REx c	224	GTACATTTAGGTACACGGCC	PCR for genotyping T-REx mice. Vectors 1553 and 1567
SV40pA nc	188	GGACATGTCGACCAGACATGATAAGATACATTG	PCR for genotyping T-Rex mice. Vectors 1553 and 1567
Tet B/E-H4 c	218	GATAGAGCCCGATTGCATAT	PCR for genotyping TetR (B/E) HDAC-4 mice. Vector 1464
Tet B/E-H4 nc	219	CTGCCTCTTCCTCATCGT	PCR for genotyping TetR (B/E) HDAC-4 mice. Vector 1464
hKRAB c	241	GGAGGGCATGGATGCTAAG	PCR for genotyping KRAB (tTS) mice
hKRAB nc	242	GAAACTGATGATTTGATTTCAAATG	PCR for genotyping KRAB (tTS) mice
Zeocin c	269	ATGGCCAAGTTGACCAGTGC	PCR for genotyping SCL-siRNA mice. Vectors 1556 and 1557
Zeocin nc	272	GTCCCGGAAAACGATTCCGA	PCR for genotyping SCL-siRNA mice. Vectors 1556 and 1557

Abbreviations : CL, Cloning; c, coding; nc, non-coding; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); SCL, mouse mRNA (LOC212429); PCR, polymerase chain reaction; PD, porphobilinogen deaminase; RT-PCR, semi-quantitative RT-PCR

2.1.15 Antibodies used for FACS Analysis of transgenic mice.

Table 4: Antibodies used for FACS Analysis of transgenic mice

Antigen	Conjugate	Tissue	Background	Company	Dilution
CD3ε	PE	pan T-cells	α-mouse	BD	1/100
CD4	PE	T_H-cells	α-mouse	BD	1/100
CD8	APC	T_K-cells	α-mouse	BD	1/100
CD11c	PE	Dendritic cells	α-mouse	Caltag	1/100
CD19	PE	B-cells	α-mouse	BD	1/200
CD23	PE	Mast Cells	α-mouse	Southern	1/100
CD31	PE	Endothelial, megakaryocytes, platelets, macrophages	α-mouse	BD	1/200
CD41	FITC	Megakaryocytes, platelets	α-mouse	BD	1/100
CD45	PE	Lymphocytes	α-mouse	BD	1/100
CD71	Biotin	Erythroid precursors	α-mouse	Caltag	1/200
CD117	APC	Stem Cells	α-mouse	BD	1/100
Gr-1	PE	Granulocytes	α-mouse	BD	1/400
pan NK	PE	NK Cells	α-mouse	Caltag	1/100
sav	APC	secondary reagent	α-biotin	BD	1/200
Sca-1	FITC	Stem cells	α-mouse	BD	1/100
TER119	PE	Erythrocytes	α-mouse	BD	1/200

Abbreviations: APC, allophycocyanin; BD, Becton and Dickson company; CD, cluster of differentiation; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyante; NA, NK, natural killer cells; PE, phycoerythrin; sav, streptavidin; T_H, Helper T cells; T_K, Killer T cells

2.1.16 Antibodies used for Western blotting

Table 5: Antibodies used for Western blotting

Antibody number	Antigen	Background	Company	Catalogue number	Working dilution
4	p38 rabbit	α-rabbit	Santa Cruz	Sc535	1/200-1/1000
7	α-Tet-repressor polyclonal rabbit	α-rabbit	MoBiTec	TET01	1/200-1/1000
16	α-mouse	IgG HRP	Dakocytomation	P0260	1/5000
59	α-HA rabbit polyclonal	α-rabbit	eBioSciences	14-6756	1/200- 1/1000
62	α-HA mouse monoclonal IgG	α- mouse	Santa Cruz	Sc7392	1/200- 1/1000
64	donkey α-rabbit	α-rabbit	Santa Cruz	Sc2313	1/5000

Abbreviations: HA, influenza A virus heamagglutinin; p38, mitogen activated protein kinase; Tet, tetracycline

2.2 Methods

2.2.1 Molecular biology

2.2.2 Manipulation of bacteria

Plasmid transformed bacteria were selected on LB plates with the appropriate antibiotic for 16-24 h.

2.2.2.1 Growth of bacteria

For overnight cultures, single colonies were picked and inoculated in LB medium with antibiotic and shaken overnight at 37°C.

2.2.2.2 Cryo-conservation of bacteria

This culture was then used for preparing frozen glycerine cultures, plasmid DNA. For storage of bacteria, a glycerol stock culture was prepared by growing bacteria to an OD of 0.8 at a wavelength of 600 nm in culture medium. 500 µl bacterial culture was taken and added to 500 µl 80% glycerine and then mixed thoroughly. This stock solution was subsequently frozen at -80°C. For the inoculation of an overnight culture, the bacteria was held at room temperature (RT) until the surface was thawed. A small amount of cells was picked and mixed with 2-5 ml culture medium and left to grow for several hours at 37°C in a bacterial culture shaker. The rest of the frozen stock was immediately returned to the -80^{o}C for freezing.

2.2.2.3 Preparation of electro-competent cells

First, an overnight culture was grown from a single colony in 2 ml LB or 2 x TY medium, incubated at 37°C while shaking at 150 rpm for approximately 16h. Using 4 ml of the culture in 400 ml of fresh media, the culture was grown at 37°C until the OD at a wavelength of 600 nm reached between 0.5 and 1.0. The culture was then put on ice for at least 15 min. All of the following procedures were carried out at 4°C. Subsequently, the cells were harvested by centrifuging in a 50 ml falcon tube for 10 min at 4500 rpm and then the supernatant was discarded. The bacterial pellets were thoroughly resuspended in 10 ml of ice-cold HEPES buffer per 50 ml falcon tube. The suspension was then diluted with HEPES buffer to a final volume of 30-40 ml vol. The cells were then spun down as before and the supernatant discarded carefully the pellets were resuspended in 5 ml HEPES-glycerol buffer. The suspension was

aliquoted in 50 to 100 µl portions and stored at -70°C. The transformation efficiency of the bacteria prepared by this method was about 10^6-10^8 transformants per µg of DNA supercoiled plasmid.

2.2.2.4 Electrotransformation of DNA into E. coli

A 50 µl aliquot of electro-competent cells was thawed at room temperature and put on ice immediately. 1 µl of plasmid DNA or ligation mix was added to the suspension of electro-competent cells, mixed thoroughly and transferred to a cooled 0.2 cm electroporation cuvette. Subsequently, the transformation mix was exposed to an electric field in the cuvette. The pulse controller and gene pulser were set at 2.5 kV, 25 µF and 200Ω. The time constant was measured between 4 to 5 ms. Immediately after the electo-pulse, 150 µl LB-medium was added to the cuvette. The transformed cells were shaken for 30 min at 37°C and plated out on agar plates containing the appropriate antibiotic for selection.

2.3 DNA Methods

2.3.1 Electrophoresis of DNA

According to their molecular mass on agarose gels, double-stranded DNA fragments with lengths between 0.5 kb and 10 kb can be separated accordingly. 1 x TBE buffer was added to obtain a 2-0.7% agarose gel respectively, containing 0.5 µg/ml ethidium bromide (EtBr). The DNA was loaded in gel loading buffer. The DNA fragments were separated in an electric field by using 1x TBE buffer at 80 V, 400 mA. The DNA was visualized using a UV-transluminator and documented with the TF video system.

2.3.2 Isolation of plasmid DNA from agarose (QIAEX II agarose gel extraction)

This protocol is designed for the extraction of 40-bp to 50-kbp DNA fragments from 0.3-2% agarose gels in TBE buffer. DNA molecules were absorbed to QIAEX II silica particles in the presence of high salt. All non-nucleic acid impurities such as agarose, proteins, salts and ethidium bromide were removed during washing steps.

The desired DNA band was excised from the agarose gel under the UV transluminator. The weight of the individual slice were determined and three volumes of buffer QG (w/v) per gel weight for DNA fragment sizes of 100 bp-4 kbp were used. For DNA fragments larger than 4 kb, 2 volumes of QG buffer plus 2 volumes of H_2O were used. Prior to use of the QIAEX II glass beads the solution was vortexed for 30 sec. Then 10 µl of QIAEX II was added to the sample, which contained

between 2-10 µg of DNA followed by incubation of 50°C for 10 min to solubilise the agarose and bind the DNA. To ensure optimal binding to the glass beads the mix was vortexed every 2 min. Subsequently, the extraction mix was centrifuged for 30 sec and the supernatant removed. The pellet was washed with 500 µl of buffer QG, followed by 2 washes with buffer PE. Then the pellet was air dried and the DNA eluted in 10-50 µl H_2O. For DNA fragments bigger than 4 kbp an elution step of 50°C was performed.

2.3.3 Purification of plasmid DNA (QIAquick PCR purification kit)

This protocol is designed to purify single- or double-stranded PCR products or DNA plasmids ranging from 100 bp to 10 kbp. DNA absorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. The impurities were washed away and pure DNA was eluted with low salt TRIS buffer or H_2O.

Five volumes of buffer PB was added to one volume of 1 volume of the DNA containing sample. The mixed sample was applied to a QIAquick column and centrifuged for 30-60 sec. The flow-through was discarded and the QIAquick column was placed into the same collection tube at 14,000 rpm. 0.75 ml of washing buffer PE was added to the column and centrifuged for 30-60 sec at 14,000 rpm. The flow-through was discarded and the column was centrifuged for an additional 1 min at 14,000 rpm. The DNA was eluted by adding 50 µl of elution buffer to the centre of the QIAquick column and centrifuged for 1 min at 14,000 rpm.

2.3.4 5’ Dephosphorylation with calf-intestinal-phosphatase (CIP) reaction

Alkaline phosphatase catalyses the removal of 5' phosphate groups from DNA. In order to prevent the re-ligation of vectors, the 5’ phosphate group of the vector was removed using CIP prior to ligation. 2.5 µg of previously digested vector DNA was dephosphorylated at 37°C for 40 min in a 50 µl reaction consisting of 1x CIP buffer and 1 µl of CIP. To inactivate the enzyme, 5 mM EDTA was added to the reaction followed by an incubation at -70°C for 10 min.

2.3.5 TOPO TA cloning (Invitrogen)

TOPO TA cloning utilises both a restrictive and a ligative capacity of an enzyme DNA topoisomerase I during DNA replication to integrate the 3´phosphate group from a PCR product. The *Taq* enzyme amplified PCR 3´A-overhang is covalently bonded into the TOPO vector containing a 3´ T-overhang. This enables the PCR product to be readily subcloned into the final vector.

The PCR product for cloning was amplified according to section 2.3.14 with varying $MgCl_2$ concentrations depending on the required conditions of the PCR. Then 2 µl of PCR product was used in a reaction of 1 µl TOPO 4 vector mix, 1 µl salt solution and 2 µl H_2O and incubated at RT for 1 h. Subsequently, 2 µl of TOPO reaction was used for electroporation with 100 µl of Top 10 *E.coli* as described in section 2.2.2.4.

2.3.6 Ligation

After the to be ligated fragments were cut with suitable restriction enzymes, a 1:3 molar of vector to insert ratio was incubated in 1 x ligation buffer containing 5 units of T4 ligase in a total volume of 10 µl. This ligation mix was then incubated either for 4 h at RT or overnight at 16°C. To inactivate the enzyme the ligation was heated at 70°C for 10 min. The ligation mix was then transferred into competant E.coli as described under section 2.2.2.3.

2.3.7 Mini-preparation of plasmid DNA (Qiagen kit)

A 3 ml bacterial culture was grown O/N in LB or 2x TY medium with 100 µg/ml ampicillin at 37°C. The bacteria were pelleted at 14,000 rpm for 1 min. The supernatant was removed and the pellet resuspended in 100 µl buffer P1. Subsequently, 200 µl buffer P2 (lysis buffer) was added and incubated at RT for 5 min. 150 µl of ice-cold 3 M potassium acetate buffer pH 4.8 was added followed by inversion of the tube for 6-7 times. It then was incubated on ice for 5 min and centrifuged at 14,000 rpm for 3 min. The supernatant was transferred to a fresh Eppendorf tube and 900 µl of pre-cooled 100% ethanol was added. The DNA was precipitated at -70°C for 10 min by centrifuging at 14,000 rpm for 10 min. The DNA was obtained as a precipitated pellet. Traces of salt were removed by 2 washes with 200 µl of 70% ethanol. Finally, the pellet was air-dried and resuspended in 30-50 µl H_2O.

2.3.8 Maxi-preparation of plasmid DNA (Qiagen kit)

The Qiagen plasmid maxi-kit method uses an alkaline lysis principle in the following way. A 50 ml culture of bacteria was grown overnight in a 37°C incubator with shaking at 150 rpm. The bacterial suspension was centrifuged at 4000 rpm for 10 min and the bacterial pellet was resuspended in 10 ml of buffer P1. To this suspension 10 ml of buffer P2 was added, mixed gently and incubated at RT for 5 min. Then 10 ml of prechilled buffer P3 was added and incubated on ice for 15 min to neutralise the reaction. To remove unwanted bacterial debris the mix was passed through glass wool

using a 100 ml plastic syringe. The precleared DNA solution was then applied to an equilibrated QIAGEN-tip 500 with 30 ml of QBT buffer. At this point the DNA was bound to the resin by gravitational pull. The QIAGEN-tip resin was cleaned of any remaining debris by washing twice with buffer QC. Plasmid DNA was eluted with 15 ml buffer QF. The flow through contained the DNA in solution, to allow for precipitation of the DNA 0.7 volumes of isopropanol (10.5 ml) was added and the solution centrifuged for a further 10,000 rpm for 1hr at 4°C. The DNA pellet was washed twice with 70% ethanol and followed by air-drying at RT and resuspended in ddH_2O.

2.3.9 Preparation of genomic DNA from mouse tails

For the genotyping of transgenic mice genomic DNA was obtained from 1 cm of mouse tail snip. The biopsies were incubated with 750 µl lysis buffer containing 1mg/ml Proteinase K at 55°C overnight until the tissue was completely digested. To pellet the unwanted debris the lysate was spun down at 14,000 rpm for 10 minutes. The supernatant was then transferred to a new tube and the genomic DNA was put in the supernatant 250 µl of 5 M NaCl and mixed for a further 10 min. Then 220 µl of isopropanol was added to the supernatant for precipitation of the DNA. The DNA pellet was obtained after centrifugation at 13,000 rpm for 10 min. The pellet was then washed with 500 µl 70% ethanol, dried and resuspended in ddH_2O

2.3.10 Boiling prep method

This method was used for DNA isolation from *E. coli* of individual clones. The major advantages of this method are the quick preparation and the high quality of the isolated DNA.

Bacteria were grown as described in section 2.2.2.1. Each culture was transferred to 2 ml eppendorf tubes, and they were centrifuged for 1 min at 5000 rpm. The obtained pellets were resuspended in 250 µl STET buffer and incubated for 3 min at RT. The probes were heated for 45 sec in an Eppendorf thermomixer at 95°C followed by centrifugation at 14,000 rpm for 5 min. Then a non-soluble pellet was formed, which could then be removed with a toothpick, and 220 µl isopropanol was added to the remaining clear supernatant. After brief vortexing the DNA was spun down by centrifugation for 5 min at 14000 rpm. Finally, the supernatant was removed and the pellet was eluted in 30 µl ddH_20 treated with RNase A buffer.

2.3.11 Determination of DNA concentrations

The DNA concentration was determined by using a UV-Vis GeneSys 5 spectrophotometer with the absorption of 1 at 260 nm corresponds to a concentration of 50 µg/ml double stranded DNA in solution. For photometeric measurement an amount of 5 µl DNA solution was added to 495 µl of water (1:100 dilution). The formula for determining the DNA concentration [µg/µl] of the original undiluted solution was measured OD_{260nm} x Factor 5. In addition the identity, integrity and purity of the DNA was subsequently analysed on an agarose gel as described in section 2.3.1.

2.3.12 Preparation of DNA copy number standards for genotyping

Copy standard controls can be used in conjunction with southern blotting for the analysis of the copy number of the transgenic DNA. Non-transgenic DNA was spiked with a known amount of transgenic DNA to produce transgenic copy standards. This sample was run at the same time as tail DNA samples from potential transgenic mice. To calculate the copy number standards, the following formula was used:

$$\frac{\text{Mass of transgenic DNA}}{\text{5 µg genomic DNA}} = \frac{\text{N bp transgenic DNA}}{3 \times 10^9 \text{ bp genomic DNA}} \quad \text{or}$$

$$\frac{\text{mass of transgenic DNA}}{} = \frac{\text{N bp transgenic DNA (plasmid) X 5 µg genomic DNA}}{3 \times 10^9 \text{ bp genomic DNA}}$$

Where N is the size of the plasmid used for injection. This calculation gives 1 copy number. A standard copy dilution range was prepared for each subsequent experiment between 1-100 copies of the transgenic DNA.

2.3.13 Screening of individual bacterial clones by PCR

First, 5 µl of the dH_20 was added to each well of a 96 well plate. Using a sterile yellow tip, a single colony was picked from the bacterial plate and transferred to one well of the 96-well plate making sure that the colony is completely resuspended. 2 µl of the bacterial suspension was used for a PCR reaction as described in section 2.3.14. Then PCR product was analysed by gel electrophoresis as described in section 2.3.1 and 1 µl of the resultant positive clone was electroporated as described in section 2.2.2.4 and stored at 4°C.

2.3.14 PCR (Polymerase Chain Reaction)

PCR is the method of choice for *in vitro* amplification of DNA (Mullis, 1990). Which is the utilisation of oligonucleotides (primers), allowing a choice of particular DNA sequences to be amplified. The PCR is basically a cyclic reaction chain consisting of three steps: 1. melting of dsDNA (denaturation), 2. primer annealing, 3. synthesis of new DNA strands by DNA polymerase (primer extension)

The standard PCR protocol for a final volume of 50µl was:
Reaction mix included:
5 µl 10x PCR buffer (-$MgCl_2$), 2 µl dNTP mix (25 mM each dNTP), 1 µl template DNA, 2 µl sense primer (10 pM), 2 µl anti-sense primer (10 pM), 1 µl Taq polymerase (5 U), 6-10 µl $MgCl_2$ (25 mM), made up to 50 µl with ddH_20.
The temperature protocol was:
step 1 (initial denaturation): 4 min, 94°C
step 2 (denaturation): 1 min, 94°C
step 3 (primer annealing):1 min, 60°C
step 4 (primer extension): 1 min, 72°C and steps 2 to 4 repeated for 30-35 times
step 5 (final elongation): 10 min at 72°C
The PCR product was then visualised using agarose gel electrophoresis as described in section 2.3.1.

2.3.15 Selection of siRNA sequences for targeting specific mRNAs

To design siRNA duplexes against a specific target mRNA it is necessary to know the correct sequence of at least 20 nucleotides of the targeted mRNA. Since recent studies which show that the RNAi silencing occurs within the cytoplasm intron sequences 19nt target sequences of mature mRNA were taken from EST and mRNA databases (e.g. www.ncbi.nih.gov, www.ebi.ac.uk or www.allgenes.org). In this study the target regions were selected from the open reading frame of the SCL cDNA (Begley *et al.*, 1994) within the first hundred nucleotides downstream of the start codon. Search criteria were determined with a sequence 5´-AA(N19)UU motif where N is any nucleotide. Each candidate sequences required a G/C-rich content of 40 to 60%. SiRNA oligonucleotides were synthesised as 5´-(N19)TT for sense and 5´-(N´19)TT for anti-sense orientation (N and N´ indicate any ribonucleotide, T was a 2´-deoxythymidine). To ensure that only the SCL gene was targeted, the siRNA sequences were additionally subjected to a blast-search against mouse EST libraries

and known mRNA sequences. Only siRNA sequences exclusively matching the murine SCL mRNA were used.

2.3.16 Preparation of siRNA duplexes

The 114 bp single stranded sense and anti-sense siRNAs of which 19 nt sequence-specific for SCL were diluted to a final concentration of 100 µM. Then for each of the above oligonucleotides 1 µl of the 100 µM dilution was added to 48 µl of annealing buffer. After denaturation for 4 min at 95°C the oligonucleotides were incubated for 10 min at 70°C and the siRNA duplexes were then slowly cooled down to 4°C. The annealed oligonucleotides were run on an 2% agarose gel described in section 2.3.1 and extracted from the gel according to section 2.3.2. These annealed siRNA oligonucleotides were then used for cloning into the RNAi vector RNAi EGFP.

2.4 RNA METHODS

2.4.1 RNA handling

All solutions were DEPC treated before use. Reagents and plasticware were taken only from fresh packages set aside for RNA work; glassware, spatulas, stir bars, etc. were also decontaminated by baking O/N at 180°C.

2.4.2 RNA isolation from cells

Total cellular RNA was isolated using TRIzol reagent. The cells were trypsinised, washed with cold PBS and spun at 1000 rpm at 4°C. 1 ml TRIzol reagent was added to each cell pellet. The cells were mechanically lysed by passing the cell lysate several times through a 10 ml pipette. The cells were frozen at -80°C or the RNA extracted by the TRIzol protocol as described in section 2.4.4 below.

The second method used the RNeasy kit from Qiagen (Hilden, D). The cells were prepared as before and a pellet of 1x 10^6 cells were isolated in 350 µl RLT buffer-1% ß-ME by centrifuging at 14,000 rpm for 2 min in a QIA shedder column (kit). Then 350 µl 70% EtOH was added to the suspension. The solution was added to a RNeasy mini column (kit) and centrifuged for 15 sec at 14,000 rpm. Then an addition of 350 µl RW1 buffer centrifuging at 14,000 rpm for 15 sec. and 80 µl of DNAse-RDD buffer was added to the column and incubated at RT for 15 min. Subequently, 500 µl RPE buffer was added to the column and centrifuged at 14,000 rpm for 2 min. Finally, 60 µl of DEPC-H_2O was added to the column to elute the RNA and collected in a 1.5 ml eppi by centrifuging for 2 min at 14,000 rpm.

2.4.3 RNA isolation from mouse tissue

After dissection of the mouse organs with snap freezing in N_2 the tissues were homogenised in 1 ml TRIzol per 50-100 mg of tissue. After each homogenisation step the tip was washed three times- once with 1.5 ml 1%SDS, then once with 0.75 ml 4M NaOH and then finally with DEPC-H_2O. The samples were frozen at -80°C or the RNA extracted by the TRIzol protocol section 2.4.4 below.

2.4.4 RNA extraction with TRIzol reagent (Invitrogen)

0.2 ml chloroform per 1 ml of TRIzol reagent was added to each sample. After vigorously mixing and centrifugation at 13000 rpm for15 min at 4°C, three phases are separated: lower red phenol chloroform layer, interphase and colourless upper RNA containing aqueous phase. For RNA precipitation the aqueous phase was transferred to a new tube, 0.5 ml isopropanol was added, mixed and centrifuged at 13000 rpm, at 4°C for 15 min. RNA pellet was washed with 75% ethanol, air dried and redissolved in 15-30µl DEPC-H_2O.

2.4.5 Measuring RNA concentration

The concentration of the RNA was determined by measuring the OD at 260 nm. A solution whose A260 = 1 corresponds to approximately 40 µg of mRNA per ml. The ratio between the readings at 260 nm and 280 nm was used to detect the purity of the RNA. A 260/280 absorbance ratio in the range of 1.8 to 2.0 indicated a pure preparation of RNA.

2.4.6 RT-PCR

RNA was reverse transcribed using the SuperScript™ one step system for RT-PCR (Invitrogen) allowing a "hot start" for a recombinant Taq DNA polymerase complex with a proprietary antibody which inhibits polymerase activity and Superscript II H-Reverse Transcriptase.

The standard RT-PCR protocol for afinal volume 50µl was:

Reaction mix:

25 µl RT/Platinum *Taq* Mix, 2 x reaction mix, 1 µl template RNA (1-5 µg), 1 µl gene sense primer (10 pM), 1 µl gene anti-sense primer (10 pM), 1 µl GAPDH sense primer (10 pM), 1 µl GAPDH antisense primer (10 pM) and made up to 50µl with ddH_2O.

The temperature protocol was:

step 1 (inactivate DNA polymerase): 30 min, 55°C

step 2 (initial denaturation): 2 min, 95°C

step 3 (denaturation): 30 sec, 95°C

step 4 (primer annealing): 30 sec, 60°C

step 5 (primer extension): 30 sec, 72°C and steps 3 to 5 repeated for 40 times

step 6 (final elongation): 10 min at 72°C

The RT-PCR product was then visualised using agarose gel electrophoresis as described in section 2.3.1. A control experiment without reverse transcriptase was performed for each sample to verify that the amplification was not caused by any residual genomic DNA. The mRNA for GAPDH was examined as the reference cellular transcript in each PCR reaction.

2.5 Cell Culture Techniques

2.5.1 Cell maintenance

The human embryonic kidney cell line HEK 293 and HEK 293 TetR (T-REx) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U penicillin and streptomycin per ml, and 2 mM L-glutamine, at 37°C in humidified air with 6% CO2. The M1 cells were obtained from ATCC and grown in RPMI 1640 (Gibco) supplemented with 10% heat inactivated (45 min., 60°C) fetal calf serum, 100 U penicillin and streptomycin per ml, 2 mM L-glutamine grown at 37°C, 5% CO_2 and 90% humidity.

2.5.2 Transient transfection of 293 cells and M1 cells (Lipofectamine 2000)

The day before transfection, cells were counted and plated so that they were 50-90% confluent on the day of transfection. Antibiotics were avoided at the day of transfection and during plating to help cell growth thus increasing the transfection efficiency. DNA was precomplexed to lipofectamine reagent according to the manufactures protocol. To do this the DNA was diluted in serum free medium (OptMEM). The complete mixture was incubated at RT for 5 min. Then the lipofectamine reagent was diluted and mixed in OptMEM in a second tube. Pre-complexed DNA and diluted Lipofectamine reagent were mixed and incubated for 20 min at RT. During this incubation period, cells were rinsed with serum free medium. DNA-Lipofectamine reagent complexes were added to each well containing fresh medium. The complexes were gently mixed with the cell medium and incubated at

37°C at 5% CO_2 for 4 h. After 4 h of incubation medium volume was increased to normal volume by adding medium containing 10% fetal bovine serum. After 24-48 hr transfection, the cells were harvested for RT-PCR and Western blot analysis. Transfection efficiency was monitored by EGFP fluorescence of the pRNAi EGFP based expression vectors.

2.6 Protein Methods

2.6.1 Determination of protein concentration (Roti-Quant reagent)

The Roti-quant protein assay is based on the observation that when Coomassie Brilliant Blue G-250 binds to the protein. The absorbance maximum shifts from 450 nm to 595 nm (Bradford, 1976). Equal volumes of cell lysate containing 1-20 µg of protein is added to diluted dye Reagent and mixed well (1:5 dilution of dye reagent concentrate in ddH_2O). After a period of 5 min to 15 min, the absorption at wavelength 595 is measured versus reagent blank (which contains only the lysis buffer).

2.6.2 Protein gel electrophoresis (SDS PAGE)

Proteins can be easily separated on the basis of mass by electrophoresis in a polyacrylamide gel under denaturing conditions.

Cells were harvested at 1 x 10^6, washed with PBS and lysed with 2 x Laemmli buffer. The cell pellets protein was then denatured by heating it to 95°C for 5 min. The mouse tissue sample preparation was by homogenisation with pre-chilled pestle and mortor in liquid nitrogen. Then 1 ml of tissue lysis buffer was added and the supernatant cleared by centrifugation at 4000 rpm for 30 min at 4°C. The protein measurement was then determined by bradford assay as described in section 2.6.1. Using 100-150µg of protein for mouse tissue in 6 x leammli buffer, the protein was heated to 95°C for 5 min. The SDS is an anionic detergent that disrupts nearly all noncovalent interactions in native proteins and ß-Mercaptoethanol was also included in the sample buffer to reduce disulfide bonds. The SDS complexes with the denatured proteins were then electrophoresed on a polyacrylamide gel. The vertical gels were set in between two glass plates sealed with a rubber band giving a internal thickness of 1.5 mm between the two plates. The gels are composed of two layers: a 10-15% separating gel (pH 8.8) that separates the proteins according to size and a

lower percentage (5%) stacking gel (pH 6.8) that insures simultaneous entry of the proteins into the separating gel at the same height.

	Separating gel	**Stacking gel**
1.5 M TRIS pH 8.8	2.5 ml.	-
1.0 M TRIS pH 6.8	-	1.25 ml
Acrylamide (30%)	2.0-5.0 ml	1.7 ml
10% SDS	0.1 ml	0.1 ml
ddH2O	5.4-2.4 ml	6.8 ml
10% APS	0.1 ml	0.1 ml

The separating gel was poured in between two glass plates, leaving a space of about 1cm plus the length of the teeth of the comb, ethanol was then added to the surface of the gel to exclude air bubbles. After the separating gel was polymerised, the ethanol was removed. The stacking gel was then poured on top of the separating gel, the comb inserted and the gel allowed to polymerise. The samples were loaded into the wells of the gel and electrophoresis buffer was added last to the chamber and any air bubbles expelled. The SDS-PAGE eletropration was preformed using 400 mA and 25V through the stacking gel and 50V through the separating gel. The negatively charged SDS-proteins complexes migrate in the direction of the anode at the bottom of the gel.

2.6.3 Western blotting

The proteins were transferred by electroblotting to a nitrocellulose membrane. The proteins that were separated on an SDS-PAGE gels were electroblotted at 250 mA with 9V in blotting buffer for 2hr. Ponceau S fixative dye solution (containing Ponceau S, trichloroacetic acid, and sulfosalicylic acid) was used for control and loading accuracy, by staining for 15 sec and washing the membrane in de-ionised water. For Western blot analysis, the membranes were incubated in blocking buffer for 1 hr at RT. or O/N at 4°C on a shaker. The first antibody was diluted in BSA/5% milk PBS buffer, added to the membrane, and incubate O/N at 4°C. The membrane was then washed three times with PBST, each time for 15 min. The appropriate peroxidase-conjugated secondary antibody was diluted in PBS, added to the membrane and incubate at RT for 30 min, and washed as above. Finally the membranes were exposed to an enhanced chemiluminescence reaction (ECL-system): incubate the membrane in a 40:1 mix of ECL solutions 1 and 2 (kit). The membrane

was then covered in a plastic film and placed in a developing cassette. This reaction is based on a peroxidase catalysed oxidation of Luminol, which leads to the emission of light photons that can be detected on X-ray film. Specific bands were visualised by exposure to a x-ray BioMax MS film (Kodak) on developing the film between 1-30 min.

2.7 Transgenic mice

Transgenic DNA for microinjection for the generation of transgenic mice must first be purified to prevent toxicity to the oocyte of the mouse during the microinjection technique. The DNA insert was isolated by restriction digestion and purified by gel extraction using QIAEX II described in section 2.3.2, and spun through a spinX column. The plasmid was diluted in micro-injection buffer (10mM TRIS-HCl pH 7.4, 0.1mM EDTA). The purified fragment was checked for integrity and concentration confirmed by dilution of the DNA on an agarose gel (see section 2.3.1). The purified insert was injected at a concentration between 1 and 5 ng/µl into the pronucleus of fertilised eggs of FVB/N mice which were transferred into the oviducts of pseudo-pregnant with NMRI foster females. The DNA was injected by Leonid Eshkind and Swetlana Ohngemach, Institute of Toxicology, Johannes Gutenberg-Universität Mainz.

The genotyping of putative transgenic founder animals were identified by Southern blot analysis and PCR. Transgenic lines were established by breeding transgenic founders into wildtype FVB/N mice. F1 or F2 males were used for mating with non-transgenic females to establish from an individual line a breeding colony.

2.7.1 Treatment of the transgenic mouse lines with doxcycline (Dox)

A regime of doxycycline treatment was established to activate the expression of the tetracycline system. To process this activation a solution of 7.5 mg/ml Dox in 1% sucrose was given twice a week for a duration of 6-8 weeks in the animals drinking water.

2.8 Microscopy and tissue sampling

2.8.1 Fluorescent Microscopy

For fluorescent microscopy, phenol red-free medium (Opti-MEM) was used and images were recorded by fluorescent microscopy by inverse optics (Olympus 1x70) and an attached Photonics image CCD camera (TILL-Photonics).The EGFP cells were illuminated with a wavelengths of 509 nm, using a monochrometor (Photonics Polychrome IV, TILL Photonics) The whole system was controlled by TILLvisION software (TILL-Photonics). The pictures were then edited by Photoshop software (Adobe Photoshop, version 7.0).

2.8.2 EGFP analysis by Fluorescence Activated Cell Sorting (FACS)

This method was used to assess the EGFP expression by transfected cells which harbour the RNAi and EGFP cassette. The cells to be tested were harvested at 1x 10^6 cell/well (6-well plate) trypsinized and were washed with FACS buffer, centrifuged at 1000 rpm for 5 min and resuspended in 1000 µl FACS buffer. collected into individual in a 5 ml Falcon tubes, counted and stored on ice till use. Propidium iodide (PI) (1 µg/ml) was added to the FACS buffer just before analysis to exclude dead cells and debris. GFP fluorescence using a 488nm Augan-laser was quantitated on a FACS-Vantage SE (Becton Dickinson, San Jose, CA) Positive cells were sorted on a Vantage SE equipped with an Augan laser from the FACS and Array Core Facility (Facs Lab) Johannes Gutenberg-Universität Mainz.

Quantifying FACS Data:

FACS data was displayed using by plotting the intensity of green fluorescence versus PI staining in a forward-side scatter graph. The data analysis was performed with Cell Pro-Quest software.

2.8.3 Preparation of transgenic mouse organ for FACS analysis

Mice were dissected for several cell lineages of the haematopoietic system. Blood was collected through a piercing in the aorta into a syringe coated with Heparin to stabilise the blood, the sample was then centrifuged for 10 min and washed in 1 ml FACS buffer. The thymus and spleen were dissected and the cells are dispersed through a cell strainer containing FACS buffer. Extraction of the bone marrow was by dissection of the femur and tibiae of the leg and washing through the shaft of the bone

with FCS buffer using a syringe. All samples were collected into individual in a 5 ml Falcon tubes, counted and stored on ice till use.

2.8.4 Single colour standard method (indirect detection)

The cell suspensions containing 3x 10^6/well (96 well plate) were washed with FACS buffer to block the Fc receptors and non-specific Ig binding sites by centrifuging at 1200 rpm for 10 min and the supernatant gently tapped off and left on ice. The antibody dilutions were then prepared as aliquots of 50 µl per antibody in FACS buffer and aliquoted into the 96 well plate as required. The antibody was gently agitated and left to incubate for 15 min in the dark on ice. The cells were washed by filling the wells with 200 µl of FACS buffer, pelleted by centrifuging as before and carefully tapped to remove the excess supernatant. Add 50 µl of detecting conjugate directly to the cell pellet at appropriate dilution and suspend the pellet well. (for antibody dilutions see Table 4). Incubate for 15 min in the dark on ice, wash as before with 200µl of FCS buffer and resuspend the pellet in 1ml of FCS buffer for analysis.
Flow cytometric analysis was performed on a FACS 4-column Calibur ™ (Becton Dickinson, San Jose, CA). On analysis, scatter gating and staining with PI (1 µg/ml) was used to avoid collecting data from debris and any dead cells. The data was first analysed by forward-side scatter analysis for viable cell identification and then further analysed by contour plot analysis. The data analysis was performed by Cell Pro-Quest software (BD Biosciences).

3. RESULTS

3.1 Generation of an EGFP cassette for the H1-driven RNAi expression vector (pRNAi-EGFP)

The aim of this study was to conditionally knock down the basic helix-loop-helix (bHLH) transcription factor SCL (Stem cell leukaemia) by RNAi processing in tissue culture and transgenic mice. In order to be able to conditionally express RNAi, we made use of the a tet On/Off plasmid system (Gossen and Bujard, 1992). A vector, pTER, containing a *tet*O H1 promoter with *Bgl II-Hind III* cloning sites, previously published by van de Wetering *et al.*, 2003 was available to us. However, this construct did not allow for the simultaneous expression of RNAi together with a marker gene. The introduction of an additional marker gene, for example the enhanced green fluorescent protein (EGFP) into the pTER RNAi expression vector. This has major advantages over the original vector as it allows for quick and easy determination of transfection efficiencies in cell culture experiments by FACS analysis and might be also used for visually genotyping of RNAi-knockdown mice.

The original vector pTER was a gift from Marc van de Wetering (van de Wetering *et al.*, 2003) and is based on the pSUPER vector (Brummelkamp *et al.*, 2002). An additional feature of the pTER is a modified H1 promoter containing an integrated tetracycline operator (*tet*O) binding site (H1 *tet*O). To generate the RNAi-EGFP co-expression vector TOPO TA cloning was utilized. The cDNA encoding the EGFP cassette was amplified by PCR with primers #161 and #162 (Table 3), using the pEGFP-C3 (Clontech #U57607) vector as a template. The 1.4 kb *Spe I*/*Xba I* PCR product contained the cytomegalovirus immediate early (CMV-i.e.) promoter, the EGFP coding region for GFP (Prasher *et al.*, 1992; Chalfie *et al.*, 1994) and an additional stop codon generated by PCR. The oligonucleotide #161 contained the 5´end of the CMV-i.e. promoter introducing a *Spe I* site with a additional G overhang for cloning. The oligonucleotide #162 incorporated an in-frame stop codon (TTA) for the termination of EGFP transcription and also included an additional *Xba I* site with a GC overhang for cloning purposes.

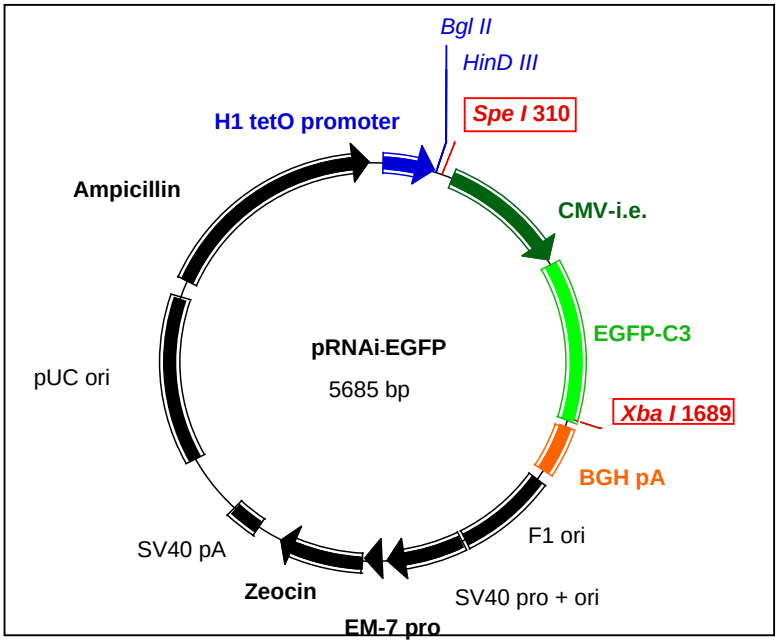


Figure 4: A Schematic diagram of the pRNAi-EGFP expression vector

The pRNAi-EGFP backbone shown in black is based on the pcDNA 3.1 expression vector (Clontech). The H1 *tet*O promoter and the *Bgl II* and *Hind III* cloning sites shown in blue originate from the pTER RNAi expression vector (van de Wetering *et al.*, 2003). The inserted CMV-i.e.-driven EGFP cassette is highlighted in green with the primary EGFP cloning sites shown in red. Numbers, show location of restriction enzymes sites used for cloning, the total size of the generated vector is 5685 bp.

H1 *tet*O promoter, histone 1 promoter containing a tetracycline operator sequence; CMV-i.e., human cytomegalovirus immediate early promoter; EGFP-C3, Enhanced green fluorescent protein gene–C3; BGH pA, bovine growth hormone polyadenylation signal; F1 ori, F1 single strand DNA origin; SV40 pro + ori, SV40 early promoter and origin of replication; EM-7 pro, synthetic prokaryotic promoter; Zeocin, Zeocin resistance (*Sh ble*) gene; SV40 pA, SV40 early polyadenylation signal; pUC ori, pUC plasmid replication origin; Ampicillin, Ampicillin (*bla*) resistance gene (ß-lactamase).

The PCR product containing the CMV promoter and EGFP cassette was digested with *Spe I* and *Xba I* resriction enzymes and inserted into the *Spe I/Xba I* sites downstream of the H1 *tet*O promoter of pTER. The resulting pRNAi-EGFP vector is shown in Figure 4. Picked clones of the pRNAi-EGFP ligation were analysed by restriction digest with *Puv II* and visualised by gel electrophoresis. Positive clones which were correctly ligated should give rise to a band at 3.6 kb and 1.8 kb. As shown in Figure 5A, clones 1, 4, 5, 7 and 10 did give this expected pattern of bands. To confirm candidate clones DNA picked from colonies 1, 4, 5, 6, and 10 were digested with *Spe I/Xba I*. The correctly ligated clones should give rise to two bands. A 1.4 kb containing the EGFP expression cassette and 3.9 kb backbone band (Figure 5B).

Clones 1, 5 and 10 were confirmed as positive clones with both *Pvu II* and *Spe I/Xba I* digests.

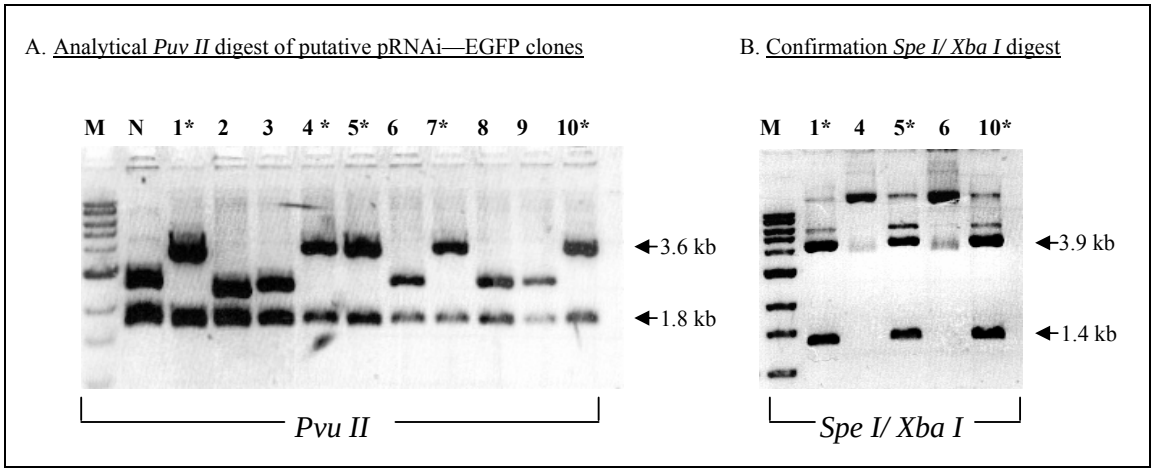


Figure 5: Diagnostic digest of putative pRNAi-EGFP clones

A. Picked clones of the pRNAi-EGFP ligation were analysed by restriction digest *Puv II* and visualised by gel electrophoresis. The *Pvu II* digest of clones # 1, 4, 5, 7 and 10 excised the expected bands at 3.6 kb and 1.8 kb. B. Confirmation of selected clones by *Spe I / Xba I* digest. Clones # 1, 5 and 10 gave the expected digestion pattern of 3.9 kb and 1.4 kb bands.M, 1 kb DNA marker (Fermentas); N, cloning vector pTER; 1-10 indicates clone numbers; (*), indictes positive clones; arrows, indicate expected band sizes.

Clone number 5 (designated plasmid #1551 pRNAi-EGFP, Table 1) was used for subsequent cloning of SCL-specific shRNA oligonucleotides.

3.2 ShRNA targeting, design and cloning for the RNAi-mediated knockdown of the SCL gene

Double stranded RNA (dsRNA) is degraded through a process known as RNAi (Sharp, 1999). However, long dsRNAs will induce cytotoxic effects in mammalian cells (Hunter *et al.*, 1975). This interferon response can be circumvented by using short synthetic (21-22nt) RNAi sequences (Elbashir *et al.*, 2001) which will result in specific identification and knockdown of the target mRNA without leading to a cytotoxic effect. To facilitate the knockdown of the murine SCL (mSCL) gene the RNAi system was utilised. For this purpose, four specific synthetic RNAi oligonucleotides against mSCL mRNA containing different RNAi target sequences were designed. These oligonucleotides were then cloned into the pRNAi-EGFP vector system. Possible targeting sequences were first tested in cell culture experiments for their knockdown efficiency prior to application in the mouse.

The mSCL gene has 8 exons and spans 16 kb. Transcription for this gene has been shown to be very complex producing several different splice variants, with all splice variants eventually leading into exon IV (Begley *et al.*, 1994). For this reason, sequences from exon V of the mSCL mRNA were used as RNAi-targets. In all cases the targeted sequences were located 100 nucleotide (nt) downstream of the splice accepter site of exon IV and 100 nt upstream of the mSCL translation stop codon of exon V. The 21 nt sequences of the four selected SCL targeted sites are listed in Table 2, designated SCL siRNA 1-4. To improve knockdown efficiency the following criteria were applied:

I. Target sequences were flanked 5´ with AA and 3´with TT/NN (alternative) nt.
II. Only sequences with a G/C content of between 30-70% were selected.
III. To prevent premature termination of the RNAi transcript, runs of As and Ts and G rich areas were avoided.
IV. To ensure unique targeting of the mSCL mRNA a BLAST search (www.ncbi.nlm.gov/BLAST) was preformed to prevent unspecific knockdown of unrelated mRNA sequences.

A. RNAi forward oligonucleotide:

sense orientation anti-sense orientation

5`-GATCCC**GACTCCAGTGGTAATCTAC**ttcaagaga**GTAGATTACCACTGGAGTC**TTTTTGGAAA-`3

B. RNAi reverse oligonucleotide:

sense orientation anti-sense orientation

3`-AGCTTTTCCAAAAA**GACTCCAGTGGTAATCTAC**tctcttgaa**GTAGATTACCACTGGAGTC**GG-5´

C. Cloning scheme for the SCL RNAi-EGFP knockdown vector:

Loop **Stop signal**

5`-GATCCC**GACTCCAGTGGTAATCTAC**ttcaagaga**GTAGATTACCACTGGAGTC**TTTTTGGAAA-`3

GG**CTGAGGTCACCATTAGATG**aagttctct**CATCTAATGGTGACCTCAG**AAAAACCTTTTCGA

Bgl II **Loop** ***Hind III***

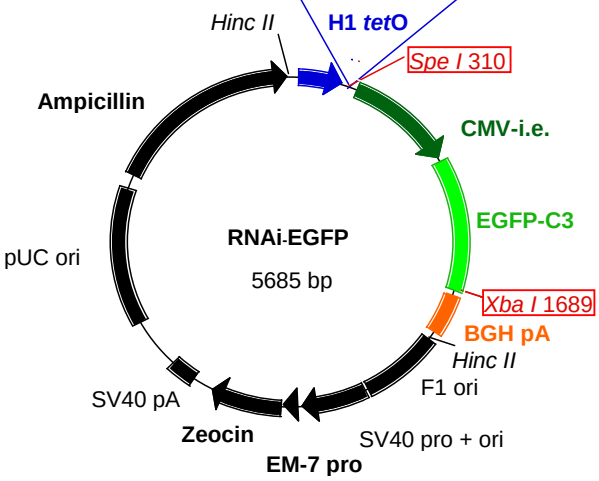
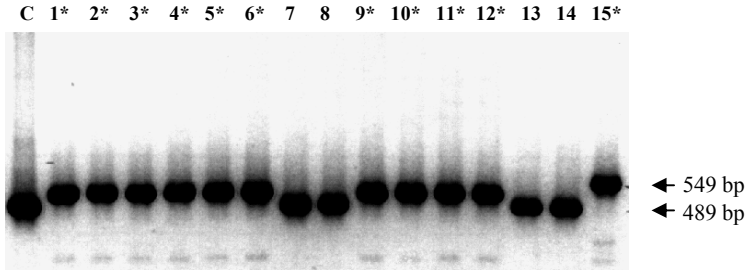


Figure 6: A schematic representation of the SCL specific pRNAi-EGFP knockdown vector

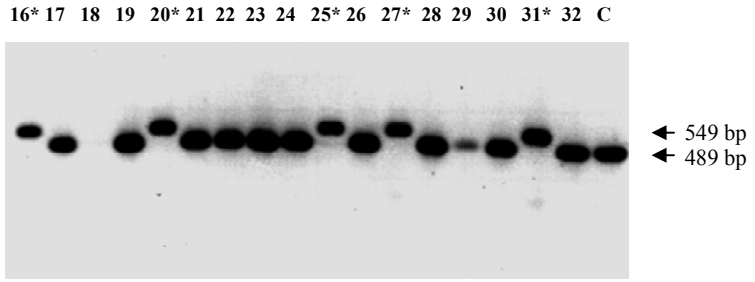
A. RNAi forward oligonucleotide: The left *Bgl II* cloning overhang (capitals, underlined) is followed by a 19 bp SCL specific knockdown sense sequence (bold), a 9 bp loop (lower case, underlined), the anti-sense SCL-specific knockdown sequence (bold), followed by the TTTTT stop signal and the *Hind III* specific overhang (capitals, underline). B. The RNAi reverse primer oligonucleotide contains the reverse complementary sequence for the forward oligonucleotide therefore producing an siRNA sequence which is then transcribed into a short hairpin loop (shRNA) by transcription with the *Pol III* enzyme. Cloning of the annealed A and B oligonucleotides into pRNAi-EGFP results in the SCL RNAi-EGFP knockdown vector. The pTER backbone is shown in black and the RNAi-oligonucleotide cloning region are indicated in blue. All other abbreviations are listed in Figure 4.

The RNAi targeting of mSCL sequences were then combined with an siRNA generating template (van de Wetering *et al.*, 2003) to be cloned into RNAi-EGFP (Figure 6).

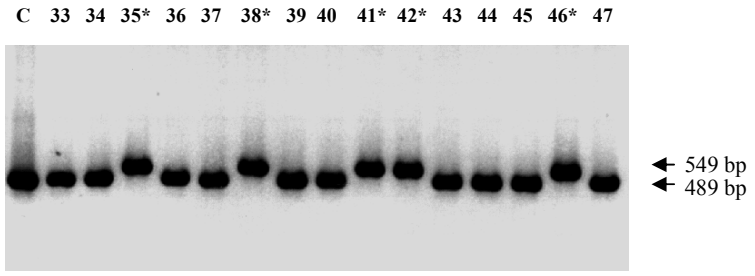
A. SCL siRNA 1



B. SCL siRNA 2



C. SCL siRNA 3



D. SCL siRNA 4

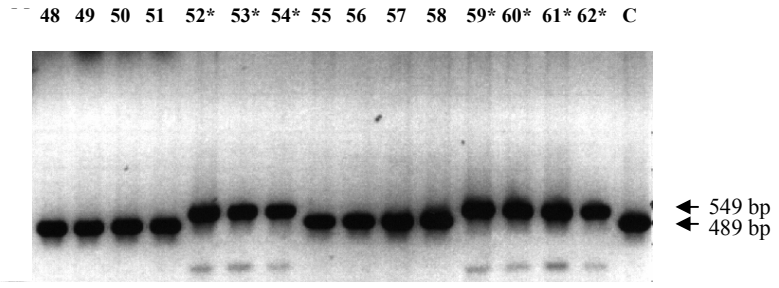


Figure 7: PCR screening of putative positive RNAi mSCL-specific pRNAi-EGFP vectors

Four different siRNA mSCL-specific pRNAi-EGFP vectors were generated using the pRNAi-EGFP vector as a recipient (Figure 6, for a detailed description of the cloning strategy). To identify correctly ligated plasmids, a PCR approach was established described in section 2.3.13. Resultant bands were run on a 2% agarose gel from

individual bacterial colonies with individual clone numbers indicated above the lanes. C, indicates the pRNAi-EGFP empty vector; 489 bp, indicates (arrow) the PCR fragment without the cloned insert. 549 bp, indicates (arrow) the presence of the ligated oligonucleotides in the vector; (*), an asterisks, indicates the positive clones.

The preparation and ligation of duplexes were preformed as described in section 2.3.18 and 2.3.6 respectively. The annealed DNA oligonucleotides (siRNA 1-4, Table 2) encoding the appropriate shRNAs were cloned into the *Bgl II* and *Hind III* sites of the pRNAi-EGFP vector and targeted different individual sequences within exon V of the mSCL mRNA. To confirm successful integration of the double stranded oligonucleotides into pRNAi-EGFP a PCR reaction was performed. The PCR reaction using oligonucleotides #185 and #186 (Table 3), flanking the RNAi cloning region of the pRNAi-EGFP H1 promoter, allowed for the identification of positive clones containing the insert having a 60 bp difference compared to the negative clone.

Figure 7, shows the result of these PCR experiments. DNA extracted from picked clones were amplified by PCR. A PCR band of 549 bp indicated successful ligation of the annealed oligonucleotide whereas negative clones, not containing the oligonucleotide amplified a PCR product of 489 bp. Eleven clones (1-6, 9-12 and 15) from fifteen candidates for SCL siRNA insert 1, five clones (16, 20, 25, 27 and 31) from seventeen candidates for SCL siRNA insert 2, five clones (35, 38, 41, 42 and 46) from fifteen candidates for SCL siRNA insert 3 and seven clones (52-54 and 59-62) from fifteen candidates for SCL siRNA insert 4 were shown to harbour the annealed oligonucleotide. DNA from positive clones was extracted as described in section 2.3.7 and integration of the insert was confirmed by nucleotide sequencing (Genterprise, Mainz, D). The result of the sequencing analysis demonstrated that all four resultant vectors analysed did harbour the selected anti-SCL-specific shRNA coding region (Table 2) in the correct orientation. The resultant vectors SCL siRNA 1-4, were further analysed for knockdown efficiency in cell culture (section 3.8). The most effective vector was then used to generate transgenic SCL-siRNA mice by pronuclear injection.

3.3 Generation of a CMV-i.e. ß-actin promoter driven tetracycline repressor (T-REx)

To be able to exogenously regulate the expression of specific shRNA molecules with the pSCL-siRNA vector system a tet On/Off repressor was required. Previous experiments have shown that the tet-dependent T-REx repressor (pcDNA6/TR, Invitrogen) can be utilised for regulating the expression of shRNAs with the pTER system (van de Wetering *et al.*, 2003). Since a *tet*O binding sequence is also integrated into the H1 promoter of pSCL-siRNA, controlled expression of RNAi under the regulation of the T-REx protein (TetR) should be possible. In the presence of tetracycline analog doxycxcline (Dox), the TetR protein is released and the expression of shRNA is resumed. Conversely, without Dox the T-REx repressor will tightly bind to the *tet*O consensus thus preventing the expression of shRNA. The advantage of using this tetracycline system is to allow for the inducible knockdown of mSCL mRNA. This conditional induction of the knockdown phenotype is critical as knockout experiments have shown that SCL loss-of-function results in an embryonic lethal phenotype at day E9.5 (Robb *et al.*, 1995; Shivdasani *et al.*, 1995; Visvader *et al.*, 1998; Sanchez *et al.*, 2001). However, conditional induction of the shRNA should allow for time-controlled loss-of-function thus circumventing the lethal phenotype. To generate a suitable expression system for the T-REx repressor, the T-REx coding region was cloned into a pcDNA 3.1 promoter vector (Figure 8). Expression of recombinant proteins in the pcDNA 3.1 promoter vector is governed by a CMV immediate early enhancer/ß-actin regulatory promoter known to results in high expression levels of the transgene both in tissue culture and transgenic mice (Niwa *et al.*, 1991; Okabe *et al.*, 1997; Hadjantonakis *et al.*, 2002).

The T-REx cDNA of vector pcDNA6/TR was used for cloning the T-REx coding region into the pcDNA 3.1 promoter vector, pCMB HDAC-4 (Table 1). The excised T-REx fragment contained the ATG site of the T-REx cDNA, a suitable 5´*Afl II* cloning site and a 3´ *Not I* cloning site after the SV40 pA. The 1.6 kb *Afl II* and *Not I* digestion product was sub-cloned into the pCMB-HDAC4 digested with *Alf II/Not I* which resulted in a 2.4kb TetR(B/E) HDAC-4 fragment dropping out. Ligating the 1.6kb *Alf II/Not I* T-REx fragment into the pcDNA 3.1 promoter *Alf II/Not I* receiving plasmid resulted in the T-REx expression vector seen in Figure 8.

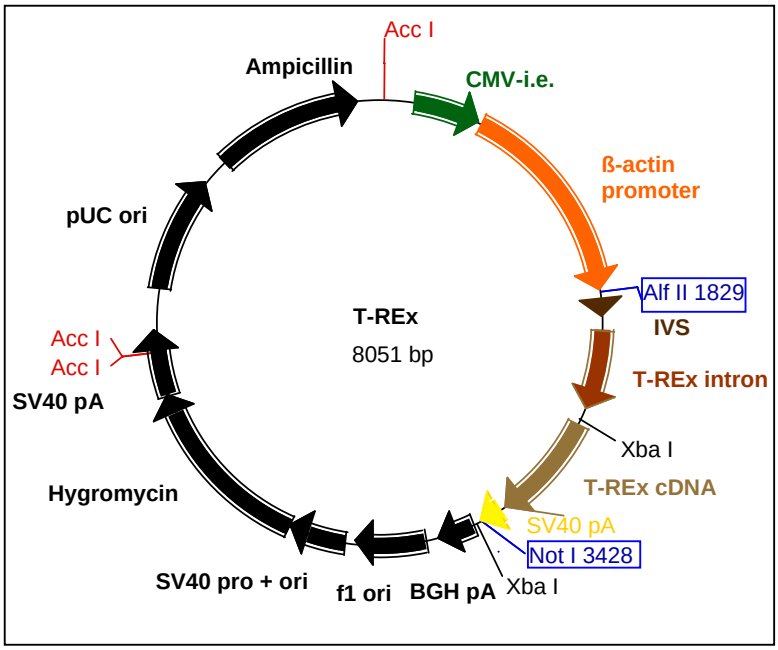


Figure 8: Schematic representation of the T-REx vector

The backbone of the T-REx is based on the pcDNA 3.1, indicated in black. The CMV-i.e./ ß-actin promoter of T-REx plasmid is shown in green (CMV-i.e.) and orange (ß-actin) respectively and originated from the pCAGGs vector (Nima H *et al*,.1991).The T-REx coding region shown in various shades of brown (IVS, T-REx intron and T-REx cDNA) followed by a SV40 pA shown in yellow, is based on the pcDNA6/TR vector (Invitrogen). Cloning sites are shown in blue boxes. *Acc I* digestion, was used before pronuclear injection. The T-REx vector size is 8051 bp. *Xba I* digestion sites were used for an analytical digest of putative T-REx clones.
All abbreviations are listed in Figure 4 with exception to T-REx intron IVS, Tetracycline regulated expression rabbit ß-globin intron II; T-Rex cDNA, cDNA of the TetR gene; Hygromycin, hygromycin B (*hph*) resistance gene.

To evaluate if the T-REx ligated clones contained the T-REx SV40 pA fragment a digest with *Xba I* was preformed. Figure 9, shows the resultant *Xba I* analytical digest of 19 picked clones as deduced from the electronic sequence for T-REx DNA, correct clones should drop out a 821 bp fragment. From nineteen T-REx clones tested six clones (1, 3, 7, 8, 13 and 16) dropped out a band at 821 bp signifying the presence of the T-REx and SV40pA coding region (Figure 9A). The correct *Xba I* digested clones were further confirmed by a second digest with *Not I/ Alf II*. All positive clones dropped a band at 1.5 kb in the correct orientation and all negative clones dropped a band at 2.4 kb. It was assumed that clones 1, 3, 7, 8 and 16 with a *Not I/ Alf II* digest

(Figure 9B) generating a 1.5 kb band contained the T-REx intron together with the T-REx cDNA and the SV40 pA.

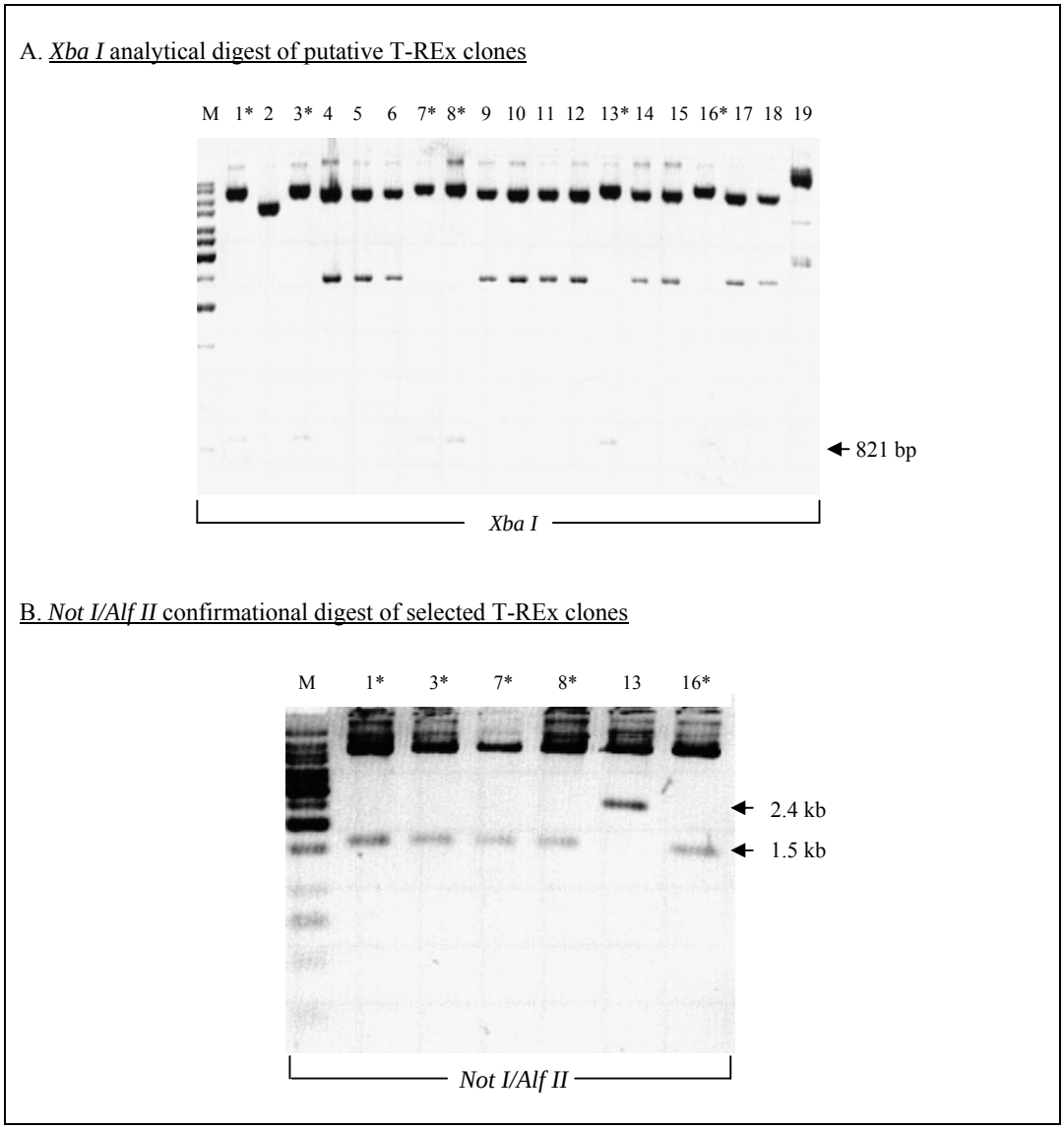


Figure 9: Analysis of putative T-REx expression vectors

A. Analytical digest of 19 clones from the T-REx ligation. Positive clones, in lanes 1, 3, 7, 8, 13 and 16 (*) releasing the expected 821 bp fragment (T-REx cDNA and SV40pA) after *Xba I* digest on a 1% agarose gel are indicated by an arrow. B. A conformational digest with *Not I/ Alf II*. Positive clones, indicated by an asteroid released the expected 2.4 kb and 1.5 kb fragments (indicated by an arrow). M, indicates 1kb DNA marker (Fermentas); numbers, indicate the gel lanes.

Clone 1 was then amplified, the T-REx coding region was then digested out with *Acc I* restriction enzyme (seen in Figure 8 in red) and the resulting 5822 bp fragment used for the generation of transgenic mice.

3.4 Generation of a copy controlled tetracycline repressor (T-REx) harbouring additional FRT and LoxP sites

To be able to induce shRNA expression in mice in a tissue-specific manner the *Cre*-LoxP system (Bockamp *et al.*, 2002) was utilised. For this purpose, a pair of LoxP binding sites (34 bp long sequences) for *Cre-recombinase* recognition, were strategically placed flanking the T-REx cDNA. This allows for insertion of the LoxP sites for T-REx trangenes to be excised from the genome of T-REx transgenic mice when crossed with a *Cre* deleter mouse (Bockamp *et al.,* 2002). Expression of *Cre* under the control of a tissue-specific promoter would allow for the removal of TetR expression in specific target organs. Therefore, TetR expression can be shut off leading to tissue specific expression of shRNA in the mouse (conditional *Cre*-mediated knockdown). Normally in transgenic mice the transgene is integrated in multiple tandem repeats into the genome. In order to be able to generate single copy transgenic mice containing only one copy of the T-REx expression cassette, a single *Flp recombinase* target (FRT) recognition site (Bockamp *et al.*, 2002), was also strategically placed into the T-REx FRT LoxP expression vector. Therefore in the case of multiple copies of T-REx in the mouse, a single copy transgene can be obtained when crossing the transgenic T-REx mouse with a general *Flp* deleter strain (Dymecki, 1996). Thus, removing all intervening T-REx transgenes by *Flp-recombinase* will leave only one T-REx transgenic expression cassette in the mouse genome. For the introduction of the FRT and LoxP sites into the T-REx expression vector, a 140 bp double stranded oligonucleotide containing annealed oligonucleotides # 203 and # 204 (seen in Table 3) was used. These annealed oligonucleotides were directly cloned into the *Not I/Afl II* digested T-REx vector. The annealed DNA strands contained in a 5´-3´ orientation a *Not I* site for cloning, a LoxP site, a *Nhe I* site for linearization, a FRT site, a second LoxP site and a final *Afl III* cloning site. The resultant T-REx FRT LoxP vector is seen in Figure 10. This cloning strategy also included two unique diagnostic sites (*Xho I* and *Sac I)* for analytical digests allowing for quick verification of correctly ligated T-REx LoxP FRT clones. To evaluate whether the 140 bp fragment was correctly inserted into the T-REx backbone, six independent clones were digested with *Sac I* and *Kpn I* restriction enzymes.

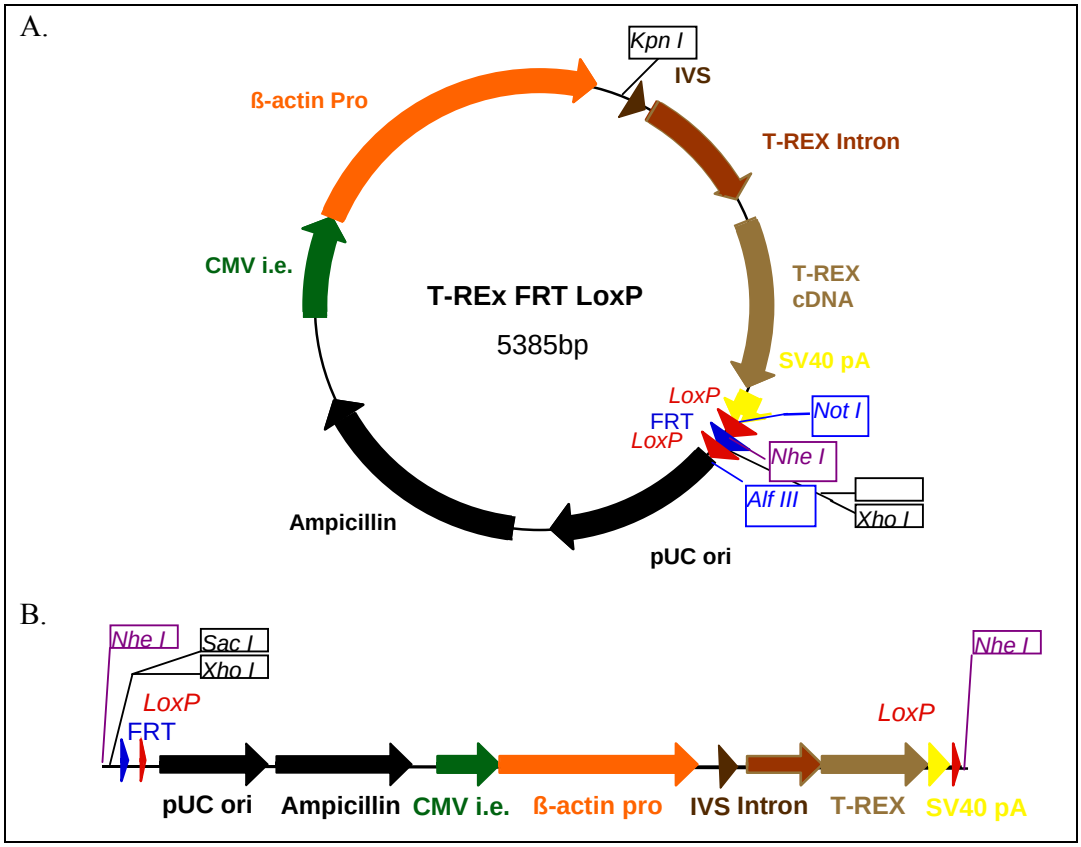


Figure 10: Schematic representation of the T-REx FRT LoxP vector

A. The ß-actin element of the promoter constitutively drives the expression of the T-REx transgene (T-REx) LoxP sites, shown in red; FRT site, shown in blue.
B. The linearised 5.4 kb *NheI* fragment, harbouring a single FRT site and two LoxP sites was used for pronuclear injection. LoxP, *Cre-recombinase* recognition site; FRT, *flp recombinase* recognition site. All other abbreviations are listed in Figure 9.

Clones number 2 and 5, dropped out a 1.6 kb band suggesting proper insertion of the insert (Figure 11A). The subsequent analytical digest of clone number 5 with different enzymes revealed the expected patterns for the T-REx FRT LoxP vector. Clone 5, was then amplified, the T-REx coding region was then digested out with a *Nhe I* restriction enzyme (seen in Figure 10, in purple) and used for the generation of transgenic mice.

Since all digests with different restriction enzymes resulted in the expected pattern clone 5 was used for the generation of transgenic mice.

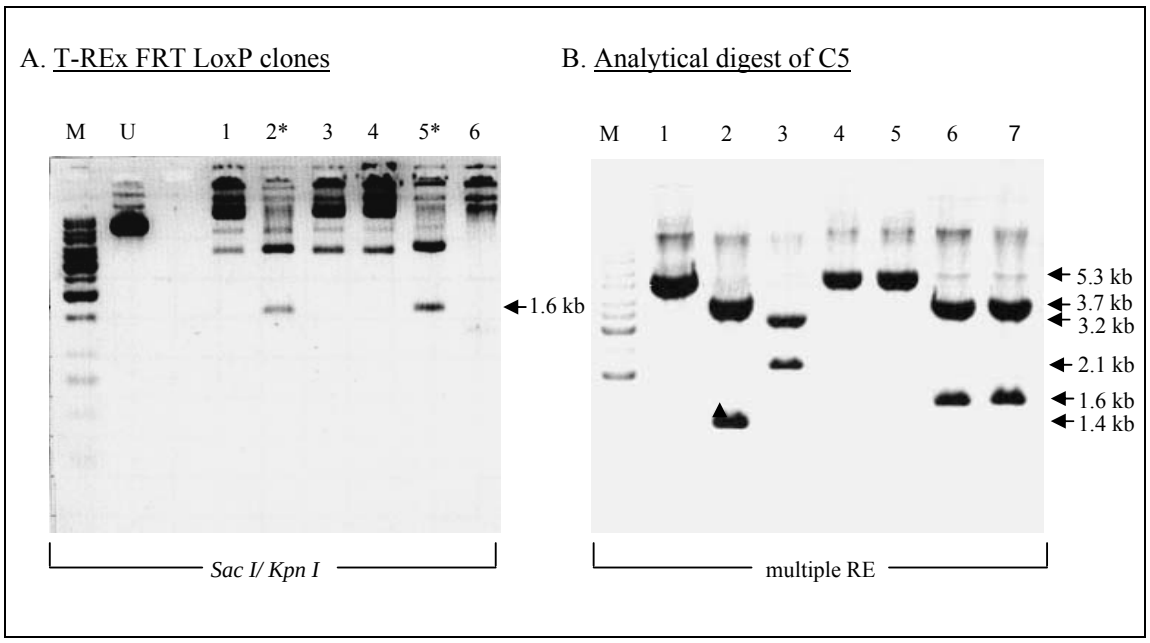


Figure 11: Analytical digest of putative T-REx FRT LoxP clones

A. A diagnostic digest with *Sac I* enzyme and *Kpn I*, with positive clones dropping out a 1.6 kb fragment, indicated by (*). B. Analytical digest of clone 5 with selected enzymes revealing the expected pattern for the T-REx FRT LoxP vector: 1 uncut vector 2 *Bam H1* (5.3 kb + 1.4 kb) 3 *Bgl II* (3.2 kb + 2.1 kb) 4 *Sac I* (5.3 kb) 5 *Xho I* (5.3 kb) 6 *Pme I* + *Not I* (3.7 kb + 1.6 kb) 7 *Sac I* + *kpn I* (3.7 kb + 1.6 kb)

M, indicates 1kb DNA marker (Fermentas); numbers, indicate individual digests; arrows indicate the expected molecular weight; RE, restriction enzyme digests.

3.5 Analysis of SCL mRNA expression and transfection efficiency of pRNAi-EGFP in M1 cells

The SCL gene product is a member of the basic helix-loop-helix (bHLH) class of transcription factors that are known to be involved in development and differentiation events in a wide variety of species and tissues (Begley and Green, review 1999). The M1 Mus *musculus* myleoid leukaemia cells (M1 cells) was shown to express SCL (Begley *et al.*, 1994; Bockamp *et al.*, 1994). For this reason it was assumed that this line was a suitable model for testing knockdown efficiency of the four different siRNA vectors generated under section 3.2. The M1 cell line was purchased from ATCC (TIB-192) and was maintained in RPMI medium as described in section 2.1.9.2. To determine expression of endogenous SCL in the M1 cell line RT-PCR was used. A, shows the result of the RT-PCR experiment. RNA was first extracted from the M1 cell line as described in sections 2.4.2 and 2.4.6 and the RT-PCR product was run on a 2% agarose gel. Oligonucleotides #195 and #196 (Table 3) were used amplify a 150 bp mSCL specific band. This band appeared only in the presence of reverse transcriptase (lane P, Figure 12A) and was absent in a parallel reaction carried out without the reverse transcriptase enzyme, lane N, confirming endogenous mSCL expression in the M1 cell line. GAPDH indicated by a 300 bp band in Figure 12A lane P, was amplified with oligonucleotides #63 and #64 (Table 3) and was used as an internal control for the integrity of the RNA.

The M1 cells were then transfected with 2.0 µg of the siRNA SCL vectors (1-4) according to the Lipofectamine 2000 (Invitrogen) protocol described in section 2.5.2 and analysed for EGFP expression. The transfection efficiency was determined by fluorescent microscopy seen in Figure 12B. Inspection in the EGFP channel (509 nm) revealed that only a very small fraction of the transfected M1 cells expressed EGFP. The EGFP positive cells were sorted by FACS analysis as described in section 2.8.1 and the forward-side scatter plots can be seen in Figure 12C. Each dot represents a cell and the EGFP positive cells can be seen in green. The transfection efficiencies of the M1 cell line with the pRNAi-EGFP vector were in all cases, below 3% efficiency (2.57%). This was also true for different transfection methods (data not shown). Therefore, it was concluded that the M1 cell line was not an adequate cell line for studying anti-mSCL different knockdown efficiencies described under section 3.2.

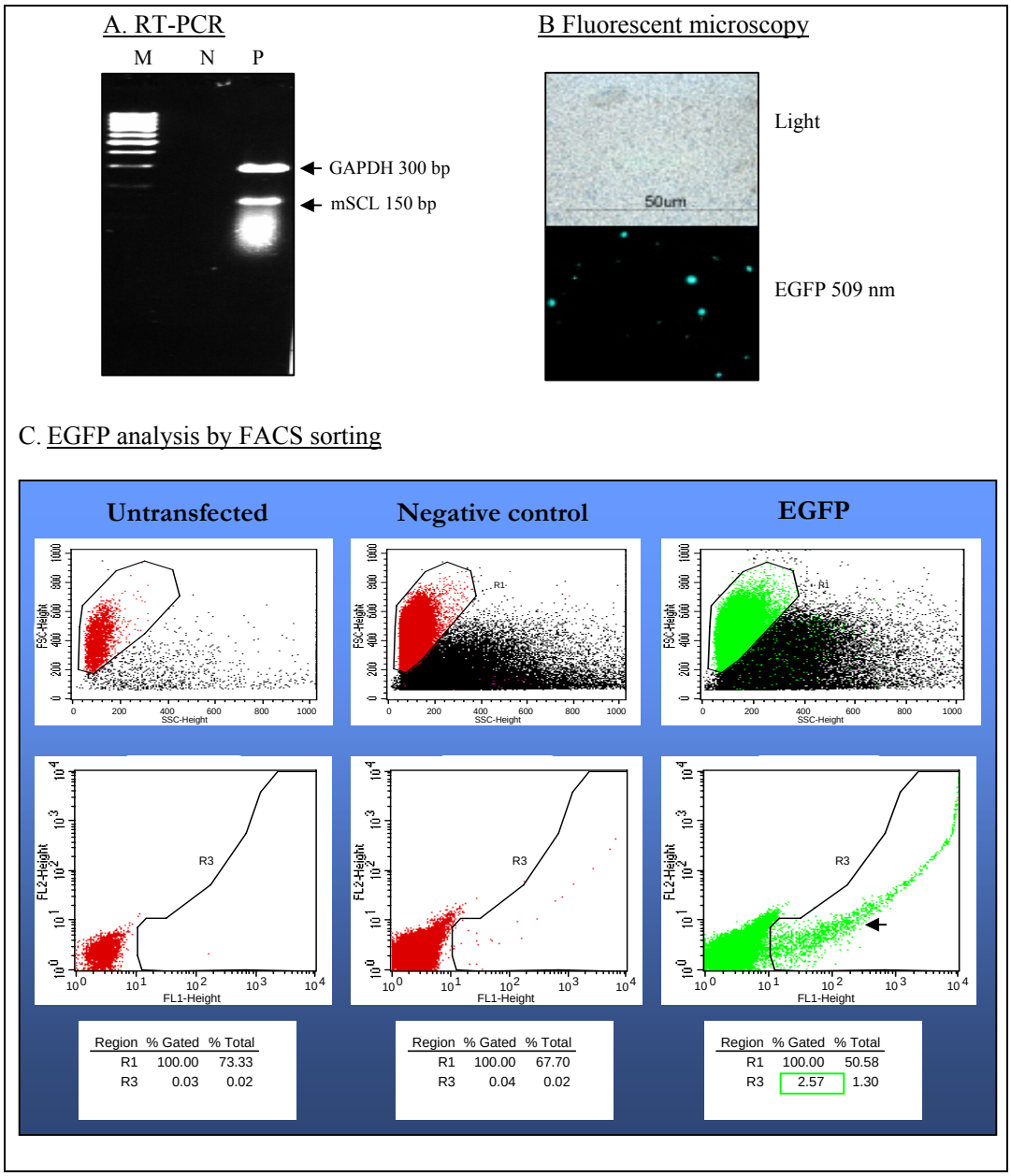


Figure 12: Analysis of mSCL in RNA from M1 cells and evaluation of M1 transfection efficiency

A. RT-PCR with total RNA extraction from M1 cells. As expected a 150 bp fragment was detected in M1 cells only in the presence of reverse transcriptase (RT-PCR, lane P). GAPDH internal control confirming the RNA integrity is seen by a 300 bp band (indicated by an arrow). B. As seen in under light microscope the M1 cells were small suspension cells (above panel). pRNAi-EGFP transfection efficiency in M1 cells is seen by EGFP at a wave length of 509 nm (Lower panel) (C). The EGFP marker was used for evaluating the transfection efficiency as analysed by FACS The EGFP population in C is indicated by an arrow. M, marker; N, reaction without reverse transcriptase; P, reaction in the presence of reverse transcriptase; Light, light microscopy; EGFP, enhanced green fluorescence protein seen under 509 nm wavelength.

3.6 Generation of a SCL expression HA-tag vector

As shown in section 3.5, M1 cells expressing mSCL were difficult to transfect with EGFP transfection efficiency less than 2% (Figure 12C). Therefore a SCL expression vector was generated coupling the mSCL coding region with a HA-tag epitope. The mSCL cDNA was therefore cloned in frame with the Y-P-Y-D-V-P-D-Y-A-S HA-tag peptide sequence. The mSCL HA-tagged protein can be detected by commercial HA tag antibodies in cell culture transfection experiments. It was planned to use the pSCL-HA tag expression vector for co-transfection experiments with the siRNA SCL vector. Therefore, a vector expressing a recombinant SCL protein with a HA-tag was needed to allow reliable and high transient transfection efficiencies. Another advantage of using a SCL HA-tag vector was that analysis by Western blotting could be approached using a commercially available HA-tag antibody against the recombinant mSCL protein over expression.

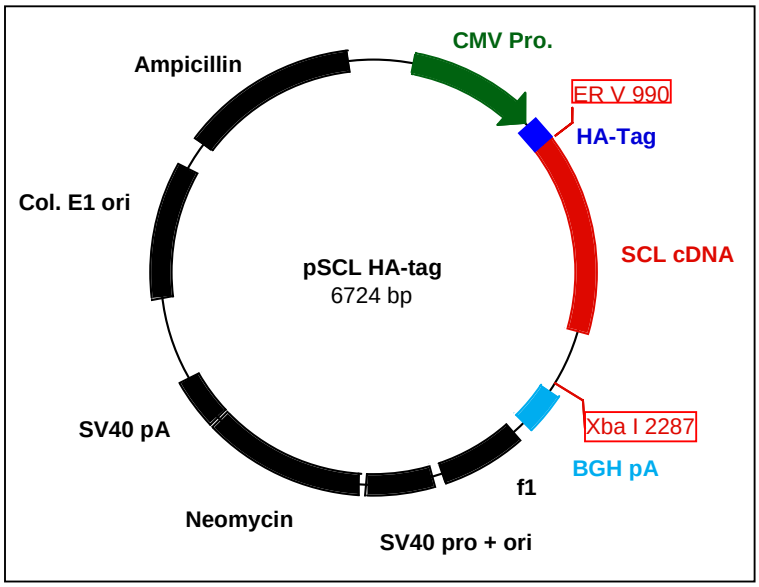


Figure 13: Schematic representation of the pSCL HA-tag expression vector

The pSCL HA-tag backbone is shown in black and is based on the pcDNA 3.1 expression vector (Clontech). The SCL cDNA and the *Xba I* and *ER V* cloning sites shown in red originate from the pBi SCL-EGFP expression vector (generated by Dr. Marko Maringer Institute of Toxicology, Johannes Gutenberg-Universität Mainz). The CMV promoter and BGH pA originate from pcDNA 3.1 are highlighted in green and light blue respectively.
The HA tag expression cassette is highlighted in dark blue originating from the pcDNA3.1 HA-tag vector (a gift from Dr. Carsten Weiss, Institute of Toxicology, Johannes Gutenberg -Universität Mainz) and encodes a (Y-P-Y-D-V-P-D-Y-A-S) HA-tag protein. Numbers, indicate the location of restriction enzymes sites used for cloning; SCL cDNA, Stem cell leukaemia gene coding region; HA-Tag, influenza A virus haemagglutinin tag; Neomycin,

neomycin resistance gene. The total size of the generated vector is 6724 bp. All other abbreviations are listed in Figure 4.

The SCL cDNA was excised from the pBi SCL EGFP (Table 1) expression vector with a *Nco I* restriction enzyme, filled in with *Klenow* enzyme, to generate a blunt end cloning site. This was followed by a second digest with *Xba I* restriction enzyme. The resultant 1.4 kb fragment was then cloned into *ER V/Xba I* sites of the pcDNA 3.1 HA-tag vector (Table 1) to produce the pSCL HA-tag expression vector seen in Figure 13. To confirm the correct ligation of the SCL coding region into the HA-tag pcDNA3.1 vector, eight individual putative SCL-HA tag clones were picked and digested with *Pst I.* As shown in Figure 14, clones 2, 4, 6 and 7 displayed the expected 4.0 kb and 2.3 kb fragments for a correctly integrated SCL insert as compared to the negative pcDNA3.1 HA tag clones releasing two bands at 4.0 and 1.3 kb.

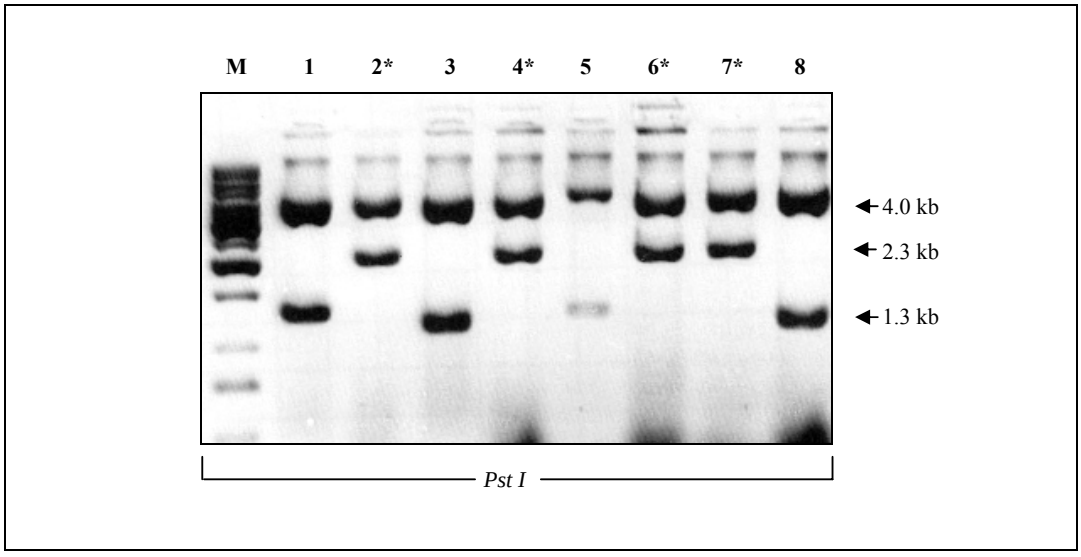


Figure 14: Analytical *Pst I* digest of putative SCL HA tag clones

Analytical digest of 8 clones from the pSCL HA-tag ligation. Positive clones (lane 2, 4, 6, and 7) are indicated by an asterisk (*) and show the two expected 4.0 kb and 2.3 kb bands (arrow) with a *Pst I* digest. Empty pcDNA 3.1 HA-tag clones released two 4.0 kb and 1.3 kb bands (arrow) visualised by gel electrophoresis. M, indicates 1kb DNA marker (Fermantas); numbers 1-8 indicate individual clone lanes.

Positive clone number two was used in the following RNAi cell culture experiments.

3.7 Evaluation of the pRNAi-EGFP vector in cell culture

To test the ability of the parental pRNAi-EGFP vector to express EGFP in eukaryotic cells a transfection assay was performed. Since the EGFP expression cassette of pRNAi-EGFP was generated by PCR (section 3.1) it was necessary to confirm the ability of the pRNAi-EGFP expression vector to express functional EGFP. Also, to establish the transfection conditions for planned experiments varying concentrations of the pRNAi-EGFP vector (1.0, 1.5 , 2.0 and 2.5 μg DNA) were co-transfected with 2 μg of vector pSCL HA-tag described under section 3.6 using a lipofectamine 2000 transfection assays into a HEK 293 cell line (ATCC) as described in section 2.5.2.

The result of the experiment is shown in Figure 15. The transfection was visually analysed under a fluorecsent microscope for the expression of EGFP. As expected the mock transfected 293 cells and cells transfected with pSCL HA-tag alone (Figure 15, A and B) did not show any EGFP expression and were used as negative controls for EGFP expression (right panel). By contrast co-transfection of increasing amounts of pRNAi-EGFP between 1.0- 2.5 μg and keeping the SCL HA-tag expressions vector at a constant concentration of 2.0 μg for each transfection. Which resulted in a proportionally augmenting number of EGFP-positive green cells after a period of 48 hr (Figure 15, C-F, left panel). These results demonstrated that the pRNAi-EGFP vector contained a fully functional EGFP-expression cassette. In addition, the titration of different DNA concentrations againist a fixed concentration of the SCL-expression vector established an efficient methodology for testing the knockdown efficiencies of the SCL shRNA expression vectors (SCL-siRNA 1-4) in HEK 293 cells described under section 3.2. In conclusion, these experiments demonstrated a fully functional EGFP-expression cassette contained in the pRNAi-EGFP. In addition, the established transfection protocol was used for further determination of RNAi knockdown of SCL protein in Western blot analysis seen in section 3.8 and 3.9.

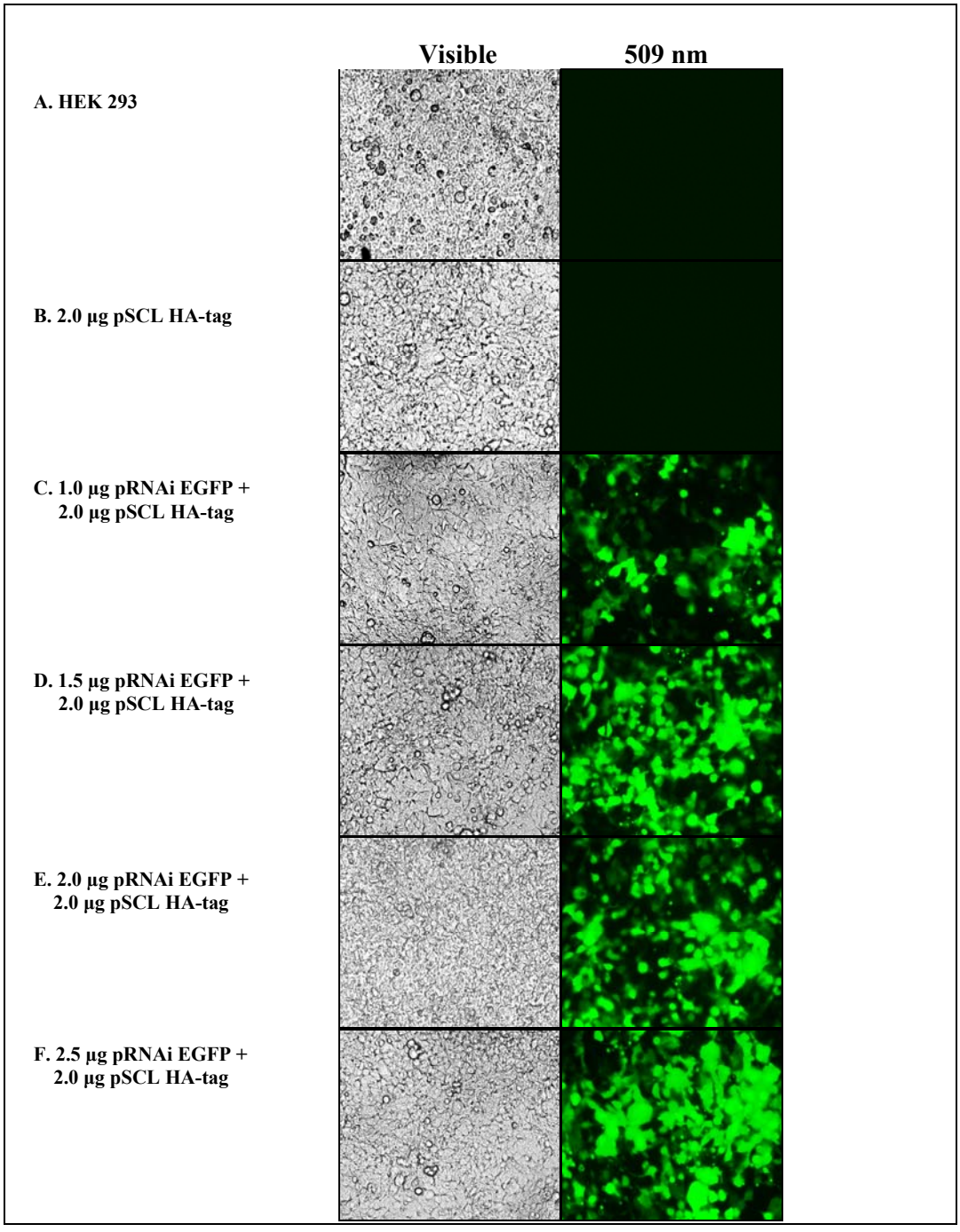


Figure 15: Fluorescent microscopy of a co-transfection of increasing concentrations of pRNAi-EGFP and pSCL HA-tag into HEK 293 cells

Transfected cells seen shown under normal light (left panel) and 509 nm to visualise EGFP fluorescence (right panel). A .Mock control (HEK 293), mock transfection of HEK 293 cells. B Negative control (pSCL HA-tag), transfection with pSCL HA tag vector. B. pSCL HA-tag vector alone. C-F, indicates different concentrations of the pRNAi-EGFP vector (1.0 µg -2.5 µg) used for co-transfection with 2.0 µg SCL-HA tag expression vector for each experiment.

3.8 Determination of the knockdown efficiency for different SCL-siRNA constructs

As shown previously in section 3.2, four different anti-SCL knockdown constructs were designed based on certain criteria including distinct 21-nt coding regions, relative position to SCL exon V including ATG and stop codon, GC-content, and unique targeting of the SCL gene. These theoretical consideration were tested to show their potential knockdown efficiency of SCL mRNA by the four individual shRNAs generated from SCL-siRNA vectors 1-4 seen in section 3.2. Although the M1 cell line expressing SCL gave poor transfection efficiency with the parent vector without siRNA insert, pRNAi-EGFP, this poor readout was circumvented by generating a SCL HA-tag vector described in section 3.6. A suitable transfection procedure for both parent vector pRNAi-EGFP vectors and pSCL HA-tag vector was established in section 3.7, therefore allowing for the analysis of knockdown efficiency of the four different SCL-siRNA vectors in transient co-transfection assays by Western blot analysis. In many recent studies RNAi knockdown assays have shown various efficiencies resulting from target homology, concentration or time dependent knockdown of the target gene of choice (Harborth *et al.*, 2003; Hong *et al.*, 2005).

It was assumed that titrating increasing amounts of anti-SCL shRNA and keeping a fixed amount of SCL HA-tag vector in co-transfection assays would possibly result in varying knockdown efficiencies of the SCL gene. This should result in the varying degradation of SCL HA tag mRNA and consequently a reduction in protein content in Western blotting assays, reflecting the SCL mRNA and protein knockdown efficiency of the different individual RNAi constructs (SCL-siRNA1-4).

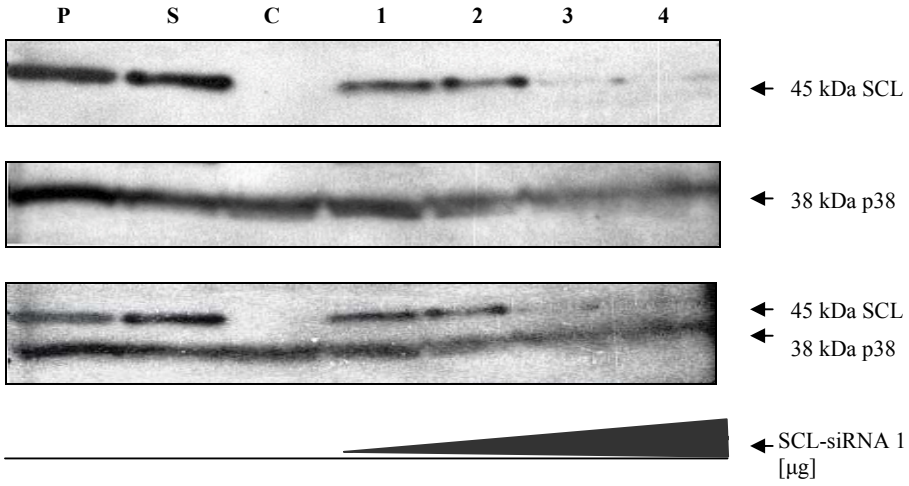
For this experiment the concentration of the siRNA SCL DNA is critical. Therefore, the concentrations of each individual anti-SCL knockdown constructs were precisely determined by an absorbance at 260 nm and further confirmed by serial dilutions on an agarose gel as described in section 2.3.11. A SCL-HA-tag DNA and a scrambled siRNA Dsg-2 knockdown construct targeting the unrelated protein desmoglein 2 were used in equal concentration as positive and negative controls, respectively. SCL siRNAs vectors (1-4) were co-transfected into 293 cells at a range of concentrations between 1.0- 2.5 µg with a fixed amount of 2.0 µg of pSCL HA tag (Figure 16, A-D)

as described in section 3.7. SCL HA tag protein (45 kDa) was detected by Western blotting at a 1:500 dilution with a α-HA tag antibody (Santa Cruz) for 1 hr at RT and secondary α-mouse antibody (Dakocytomation) at a 1:5000 dilution for 30 min at RT described in section 2.6.3, p38 was used as a loading control for each sample detected using the α-p38 antibody (Santa Cruz) at a 1:1000 dilution for 1 hr at RT and donkey α-rabbit secondary antibody (Santa Cruz) 1:5000 dilution for 30 min at RT as described in section 2.6.3.

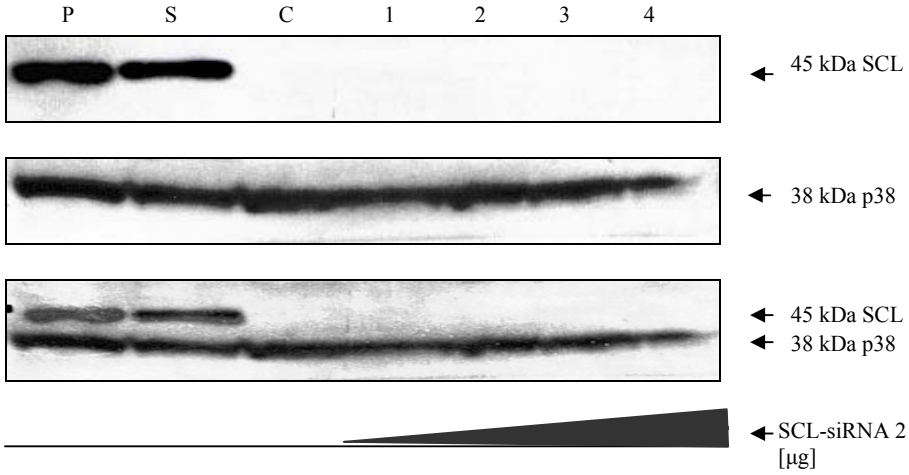
The SCL and p38 protein were analyzed by Western blot 48 hr after transfection. The resulting Western blot analysis is shown in Figure 16. Controls included cells that were either SCL-HA tag transfected (P, positive control), cells alone (C, cell line HEK 293 untransfected). Also transfected with scrambled vector (S, siRNA Dsg-2 targeting an unrelated protein desmoglein 2 co-transfected with pSCL HA tag) or siRNA Dsg-2 alone (N, negative control) in Figure 16C. Whole cell lysates from 2 x 10^6cells/ml for SCL HA-tag (P) transfected cells and siRNA Dsg-2, pSCL HA-tag (S)/ siRNA Dsg-2 (N) and cells transfected with SCL-siRNA 1 (A, lane 1-4), SCL-siRNA 2 (B, lane 1-4) SCL-siRNA 3 (C, lane 1-4) and SCL-siRNA 4 (D, lane 1-4) or cells only (N). Relative to SCL HA-tag transfection alone (P) and Dsg-2 scrambled siRNA transfection (S), all four SCL siRNAs reduced SCL protein content, each with different efficiencies (A-D, lanes 1-4 Figure 16).

SCL-siRNA 2 and SCL-siRNA 4 appeared to be the most effective with approximately 80-100% knockdown (Figure 16B and D). Whereas SCL siRNA 1 and 3 reduced protein levels by about 25-50 % respectively in comparison to the SCL HA-tag positive control compared to lane P and lanes 1-4 of Figure 16B and D. The negative control siRNA Dsg 2 showed no reduction in the SCL protein, lane S (scrambled control) in Figure 16 A, B and D. Expression of p38 in all Western blot experiment was used as a control and showed equal loading of the protein onto the SDS-PAGE gels. Also, for any given sequence, variations in the level of silencing in different experiments may be due to different transfection efficiencies. Therefore the transfections were analysed for EGFP expression from vectors SCL-siRNA (1-4) and siRNA Dsg 2 and was also repeated in two other independent experiments.

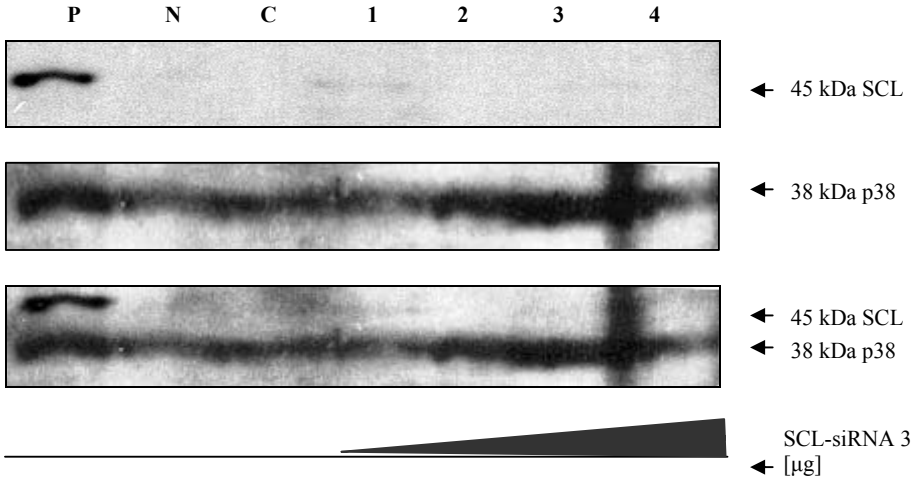
A. SCL-siRNA 1 Western blot



B. SCL-siRNA 2 Western blot



C. SCL-siRNA 3 Western blot



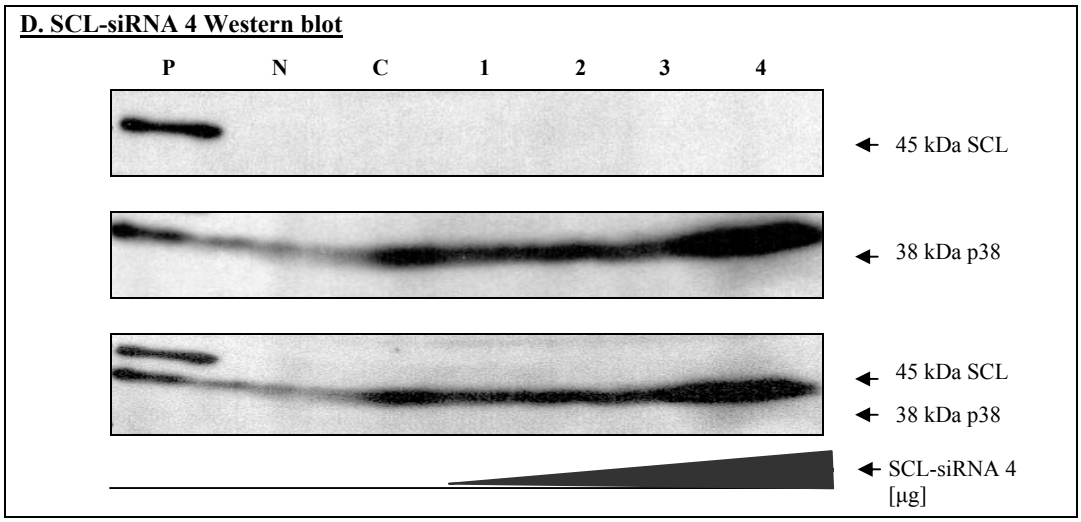


Figure 16: Western blot analysis of four SCL-siRNA dose-dependent knockdown efficiencies

SCL siRNA Western blot for RNAi expression vectors SCL-siRNA 1-4 (A-D respectively). Upper panel indicates the SCL-HA tag protein at 45 kDa, the middle panel indicates p38 protein at 38 kDa and the lower panel is an overlay of both the upper and middle panel with separate antibody incubation times. Arrows, indicate the size of the protein. Numbers 1-4, indicate the sample lanes. P, 2.0 µg pSCL HA-tag; S, 2.0 µg siRNA Dsg-2 + 2.0 µg pSCL HA-tag; N, 2.0 µg siRNA Dsg-2; C, 293 untransfected; 1, 1.0 µg SCL-siRNA + 2.0 µg pSCL HA-tag; 2,1.5 µg SCL-siRNA + 2.0 µg pSCL HA-tag; 3, 2.0 µg SCL-siRNA + 2.0 µg pSCL HA-tag; 4, 2.5 µg SCL-siRNA + 2.0 µg pSCL HA-tag.

This experiment demonstrated that SCL-siRNA 2 and 4 were the best candidates for SCL silencing and were used for the generation of SCL knockdown mice.

3.9 Switchability of the RNAi directed knockdown of SCL by tetracycline induction

To confirm the tetracycline-dependent switchability of RNAi *in vitro* with our modified SCL siRNA vector transfection assay of the RNAi vector into tetracycline repressor cell line HEK 293TR (T-REx) were performed to prove tetracycline switchability for later application in the mouse. The 293 TR cell line was a gift from Marc van de Wetering (van de Wetering *et al.*, 2003). To re-confirm the results published by van de Wetering *et al*., 2003 the same protocol was used. The TetR expressing stable cell line was co-transfected 2 μg SCL-siRNA vector and 2 μg of the HA tagged SCL expression vector (SCL HA-tag) using the lipofectamine 2000 protocol described in section 2.5.2. The cells were treated for 48 hr with a Dox concentration of 2 μg/ml medium and harvested at 2 x 10^6 cells/ml for protein extraction. The RNAi knockdown of SCL was determined by Western blotting. SCL HA tag protein (45 kDa) was detected by Western blotting at a 1:500 dilution with a α-HA tag antibody (Santa Cruz) for 1 hr at RT and secondary α-mouse antibody (Dakocytomation) at a 1:5000 dilution for 30 min at RT described in section 2.6.3.

In the regulatory T-REx system expression of shRNA (using either the pSCL-siRNA or pRNAi EGFP system) is repressed by the TetR protein in the absence of Dox (TetR On) and induced in the presence of Dox (TetR Off) as seen in Figure 17. As a condition for the tet On/Off regulation the 293 TR cell line constitutively expresses the TetR protein (van de Wetering *et al.*, 2003). The HEK 293 TR cells were co-transfected with SCL HA-tag and the SCL-siRNA 4 treated with Dox (TetR Off) or grown without Dox (TetR On). This leads to subsequent repression and de-repression of the TetR protein with the *Tet*O integrated into the promoter of the SCL RNAi vector. Therefore, suppressing (lane TetR On, Figure 17) or inducing (lane TetR Off, Figure 17) RNAi-specific knockdown against mSCL. To show that there was no interference with the Dox treatment and the TetR protein, the untransfected 293 TR was treated with Dox and was used as a negative control in Figure 17. HEK 293 cells stably transfected with T-REx (Invitrogen) were mock transfected and used as a negative control (293 TR, right lane).

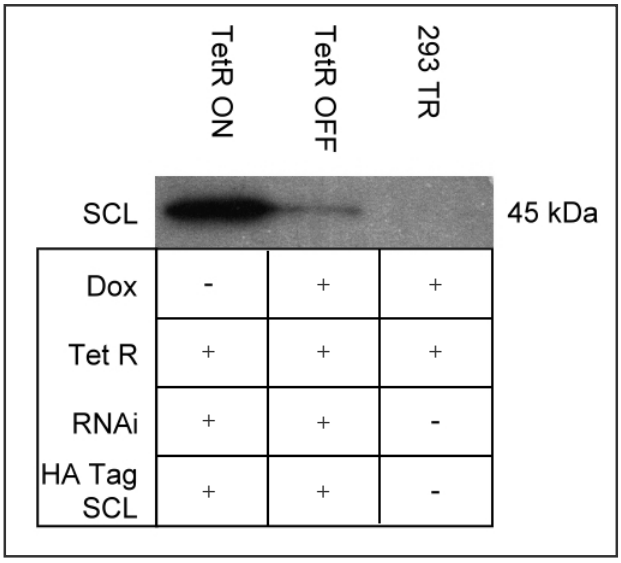


Figure 17: Western blot analysis demonstrating the switchability of shRNA expression

Expression of SCL-HA tagged protein was determined by Western blot after co-transfecting 239 TR cells with equal amounts of HA-tagged SCL expression vector pSCL HA-tag (2µg) and SCL-siRNA clone 4 (2µg) in the presence (lane TetR OFF) and absence of Dox (TetR ON). The 45 kDa SCL specific protein band. Lane 293TR shows mock transfected HEK 293TR cells which express the TetR protein only.
A table below the Western blot indicates the presence and absence Dox and the recombinant proteins expressed. TetR ON, T-REx repressor bound to the *tet* O binding site in the absence of Dox. TetR OFF, T-REx repressor unbound to the *tet* O binding site in the presence of Dox. Dox, doxycycline antibiotic; TetR, T-REx repressor; RNAi, SCL siRNA clone 4; HA-tag SCL, pSCL HA-tag (SCL expression plasmid); 293 TR, HEK 293 cell line stably transfected with T-REx plasmid (Invitrogen).

These results showed that specific RNAi knockdown of SCL can be regulated by Dox *in vitro* and suggests also that using the same combination of transgenes in the mouse should equally allow for conditionally knocking down SCL function *in vivo.* Importantly, as knockout experiments showed embryonic lethality of SCL null mice at day E9.5 (Robb *et al.*, 1995; Shivdasani *et al.*, 1995), conditionally expressing anti-SCL shRNA expression is critical.

3.10 Genotyping of tetracycline repressor mice

3.10.1 Genotyping of the tetracycline repressor T-REx mice

To generate T-REx transgenic mice, the plasmid T-REx was digested with *Acc I*. The restriction enzyme *Acc I,* cuts three times in the T-REx construct as shown in Figure 8, and releases a 5822 bp fragment, containing the ß-actin/enhancer promoter, the T-REx coding region and the SV40 polyA tail. The DNA was prepared as described in section 2.3.3 with gel extraction (Figure 18A) of the expected 5.8 kb band and subsequent purification using the Qiagen QIAquick column (Figure 18B). As an additional step of column purification prior to pronuclear injection, the 5822 bp fragment was passaged through with a Spin X costar 8160 column. This additional step dispenses of any unwanted debris which maybe toxic to the oocyte during pronuclear injection.

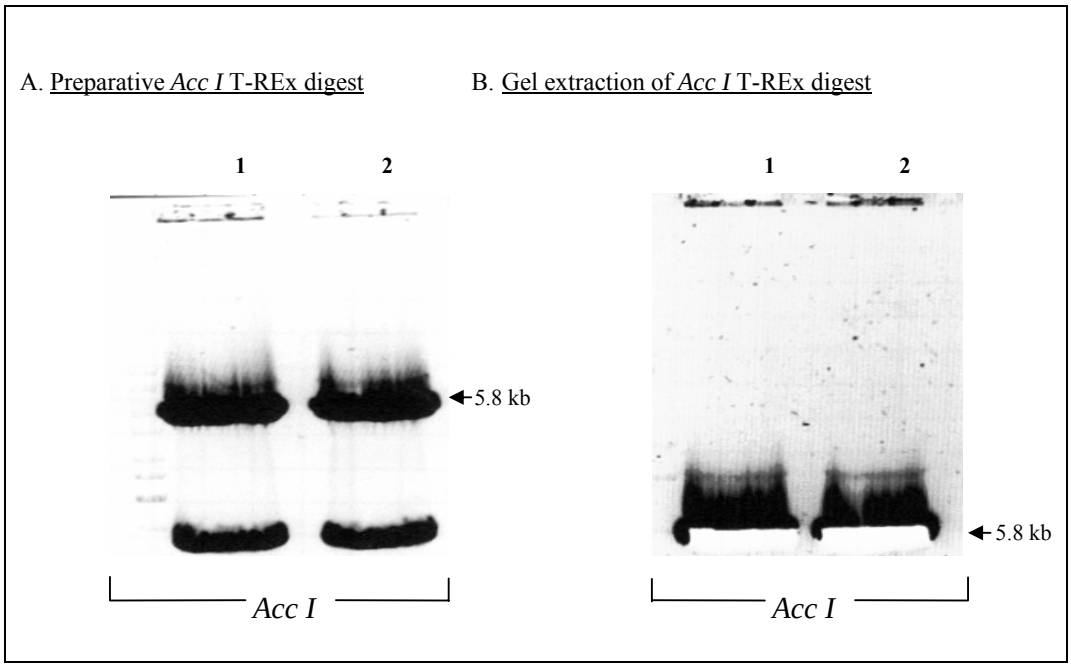


Figure 18:Preparative digest of T-REx with *Acc I*

A. The total eluted *Acc I* band at 5.8 kb is indicated by an arrow. B. *Acc I* T-REx digest ran longer for gel extraction purposes as described in section 2.3.3. Lanes 1-2, indicate *Acc I* DNA digests;. an arrow, indicates the gel extracted 5.8 kb digest band which was used for pronuclear injection.

To exactly determine the DNA concentration of the 5.6 kb fragment a serial dilution were performed, whereby the last diluted, still visible DNA band on the agarose gel

was assumed to equal an amount of 10 ng DNA. A calculation for the DNA concentration was determined by multiplying the dilution factor of the last visible band by 10 ng and subsequent division by the dilution volume used. For pronuclear injection a final DNA concentration of 2.5 ng/µl was prepared. This concentration is assumed to give 10-20% transgenic offspring without being toxic for the oocyte. The pronuclear injection process was undertaken by Leonid Eshkind and Swetlana Ohngemach, Institute of Toxicology, Johannes Gutenberg-Universität Mainz as described in section 2.7.

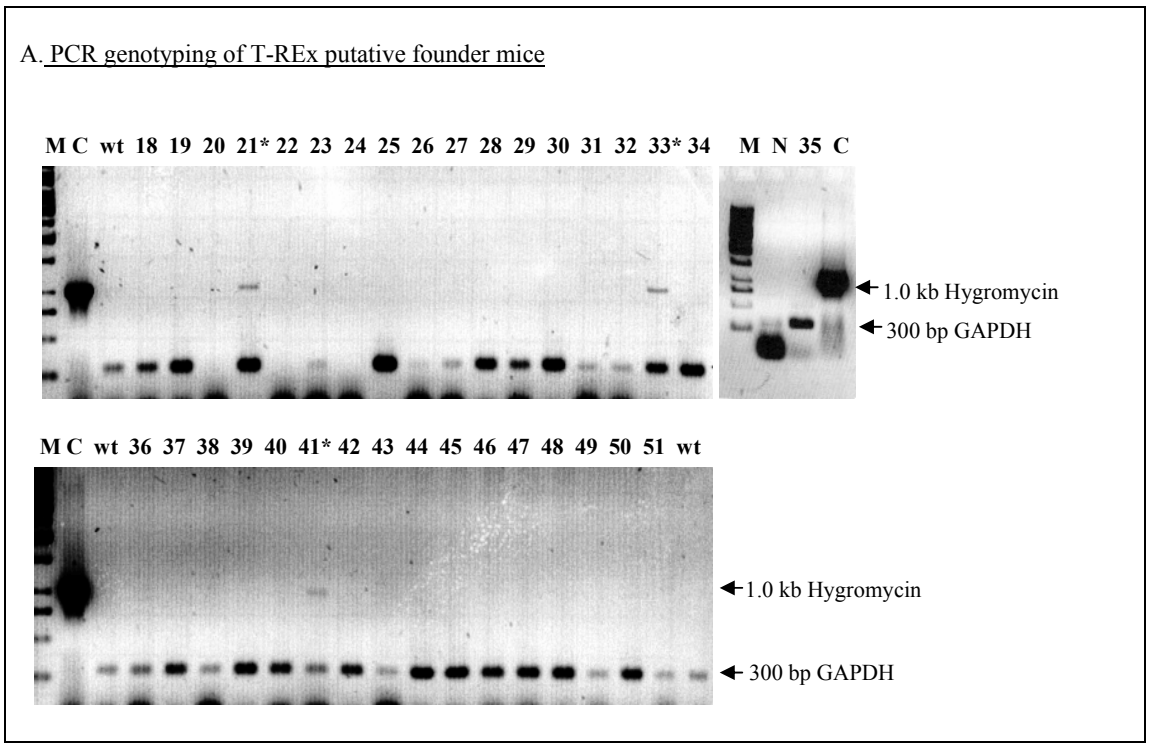


Figure 19: PCR genotyping to identify T-REx founder mice

A. A band at 1.0 kb represents an amplified hygromycin resistance gene fragment, indicating the presence of the transgene. GAP, GAPDH was used as an internal control and is indicated by a band at 300 bp. Hygromycin, hygromycin resistance gene specific for the T-REx construct and is indicated by a band at 1.0 kb; GADPH, GAPDH gene; numbers 18-51 represent individual mice DNA samples; (*), indicates positive T-REx founder mice; M, 1 Kb DNA marker (Fermentas); N, negative water control C, control plasmid T-REx; wt, wildtype mouse.

Resultant putative transgenic founder mice were screened by PCR using the hygromycin specific oligonucleotides # 125 and #126 (Table 3) to generate a specific 1.0 kb PCR band. GAPDH was used as an internal control to confirm the integrity of the genomic DNA and PCR reaction efficiency using oligonucleotides #63 and #64

(Table 3) which amplified a 300 bp band. For the T-REx construct, three positive transgenic founder mice (numbers 21, 33 and 41) were detected from a total of 33 putative founder mice as seen in Figure 19A. To establish a transgenic colony these three individual T-REx founder lines were crossed to the wildtype FVB strain.

3.10.2 Genotyping of the tetracycline repressor T-REX FRT LoxP mice

As a next step to facilitate tissue specific T-REx repression T-REx FRT LoxP mice were generated. These mice had the added advantage of having a unique FRT site allowing for the production of single copy transgenic mice when crossed with a *flp* deleter mouse. In addition, as these deleter mice have two LoxP sites flanking the T-REx expression cassette , deletion of the transgene repressor and initiation of the knockdown can be spatio-temporally controlled through *Cre* recombinase. Both systems were integrated into the T-REx FRT LoxP construct as described in under section 3.4, and shown Figure 8. To generate T-REx FRT LoxP transgenic mice, the construct T-REx FRT LoxP was digested with *Nhe I*. The unique *Nhe I* site was used to linearise the construct as shown in Figure 8, releasing a 5385 bp fragment, containing the ß-actin/enhancer promoter, the T-REx coding and the SV40 polyA tail together with the FRT and LoxP recognition sites (Figure 8B). The DNA was prepared as described in section 2.3.3 with gel extraction (Figure 20) of the required 5.3 kb band and subsequent purification as under section 3.11.

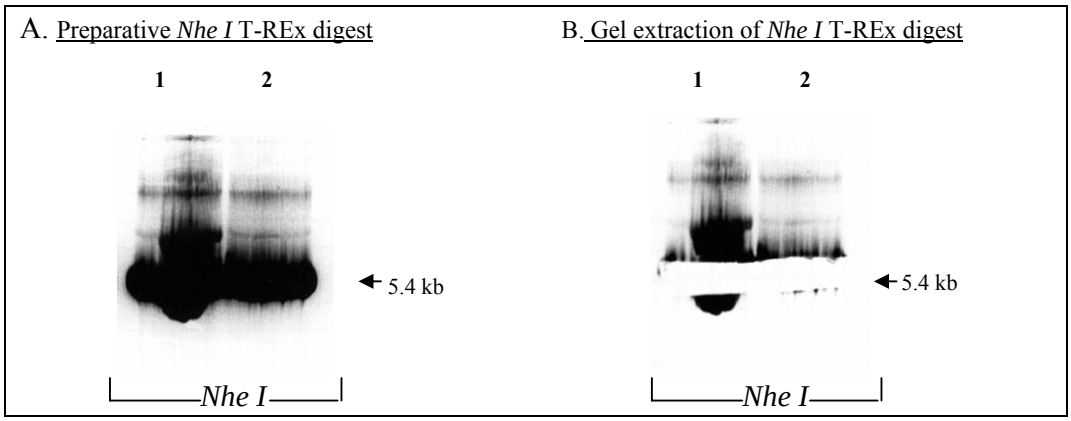


Figure 20: Preparative digest of *Nhe I* linearised T-REx FRT LoxP

A. The total eluted *Nhe I* band at 5.3 kb is indicated by an arrow. B. *Nhe I* T-REx FRT LoxP digest ran longer for gel extraction purposes as described in section 2.3.3. Lanes 1-2, indicate *Nhe I* DNA digests;. an arrow, indicates the gel extracted 5.8 kb digest band which was used for pronuclear injection.

Putative founder mice were screened by PCR using TetR specific oligonucleotides # 188 and #224 (Table 3) to generate a specific 500 bp PCR band. Positive founders were obtained in lanes 11, 23 and 31. As an internal control a PD specific PCR was used with oligonucleotides #121 and #122 (Table 3) which produced a 372 bp PCR band. For the T-REx FRT LoxP construct, three positive transgenic founder mice (numbers 8653, 8665 and 8687) were detected from 31 pronuclear injection mice as seen in Figure 21. To establish a transgenic colony for these three T-REx FRT LoxP lines, the founders were crossed to the wildtype FVB mice.

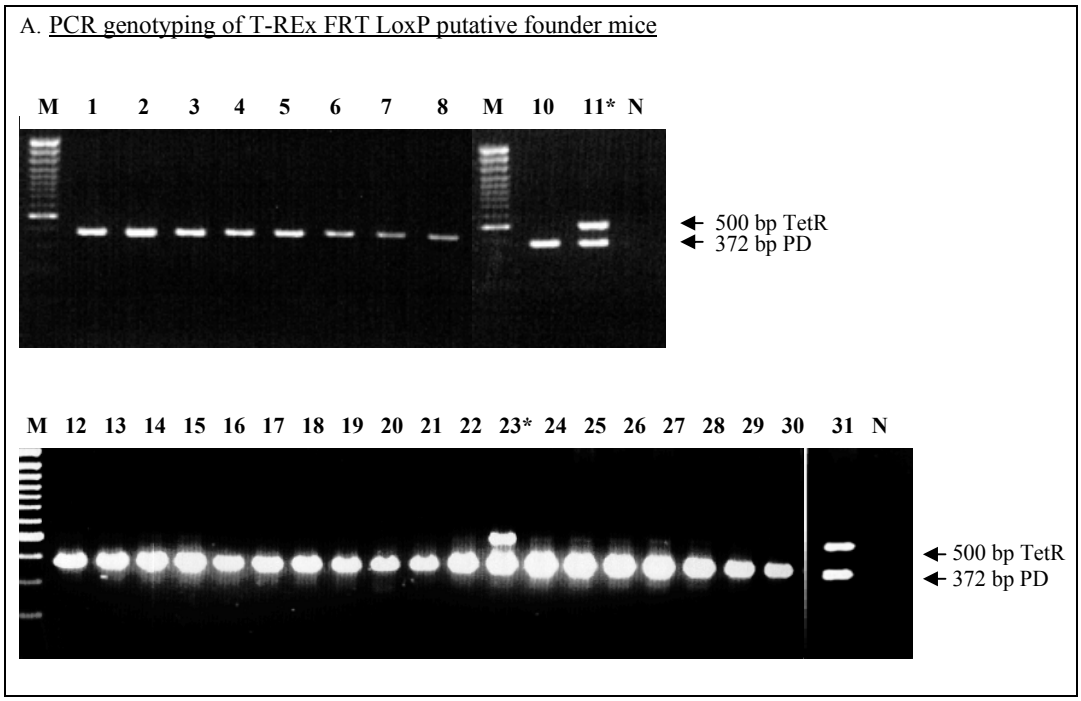


Figure 21: PCR genotyping gel to identify T-REx FRT LoxP founder mice

A. A band at 500 bp represents an amplified TetR gene as compared to a wildtype mouse thus indicating the presence of the transgene. The internal control PD is indicated by a band at 372 bp. TetR, tetracycline repressor gene specific for the pCMB T-REx FRT LoxP construct; PD, porphobilinogen deaminase gene; numbers 1-31 represent individual mice [8644-8666, 8681-8687] DNA samples; (*), indicates positive T-REx FRT LoxP mice; M ,100 bp DNA marker (Fermentas); N, negative water control

3.10.3 Genotyping of tetracycline repressor tTS KRAB mice

A second repressor strain was used as an alternative to the T-REx system. This repressor is a fusion of the KRAB domain of the Kox 1 gene with the TetR sequence. The transgenic mice tTS KRAB line were generously given from Moises Mallo (Mallo *et al.*, 2003). The tTS-KRAB mice were genotyped by PCR using oligonucleotides number #241 and #242 (Table 3) which amplified over the TetR sequence to produce a 300 bp PCR band as seen on a 2% agarose gel (Figure 22 A) as compared to the wild type DNA. A PD internal control was used to assess the integrity of the mouse genomic tail DNA which was prepared as described in section 2.3.9. A 372 bp PCR product was amplified with individual mouse genomic DNA to detect a 2 copy PD transgene confirming good PCR detection efficiency with oligonucleotides #121 and #122.

As shown in Figure 22, seven mice given to us from the Mallo group, six mice [#7412-7417] were positive for the tTS-KRAB transgene by PCR with a tTS-KRAB specific band being amplified at 300 bp. One mouse number 7411 was negative for the transgene and was discontinued.

A. PCR genotyping for confirmation of the tTS-KRAB transgene

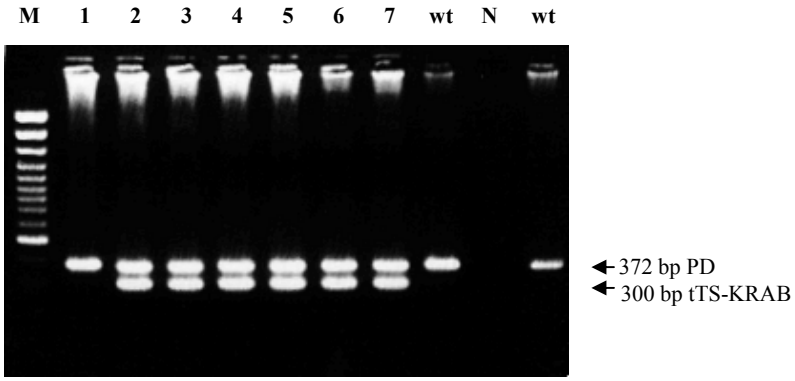


Figure 22: PCR genotyping gel of tTS-KRAB founder lines

A. Genotyping gel of founder lines tTS-KRAB (Mallo *et al*., 2003) to confirm tTS-KRAB transgene. Numbers 1-13 indicate individual mice [# 7411-7417 respectively]. The PD internal control band is seen at 372 bp, which was used to control the integrity of the genomic DNA, indicated by an arrow. PD, porphobilinogen deaminase gene; M,1 Kb DNA marker (Fermentas);N, negative control; wt, wild type mouse DNA.

All other mice were crossing with the FVB strain to keep the line. The tTS-KRAB transgenic progeny were used as egg donors for expression the tTS KRAB oocyte for pronuclear injection of the SCL-siRNA 4 construct. This allowed for the SCL-siRNA transgene to be repressed at all times and therefore it was hypothesised that there would be a higher efficiency of double transgenic mice which would survive into adulthood and not be embryonic lethal as single transgenic SCL-siRNA mice.

3.11 Genotyping of SCL-siRNA mice generated by co-injection with Tet-repressor vector

Aim of this work was to understand the *in vivo* function of SCL using SCL-RNAi knockdown mice as a tool to study the consequences of SCL deletion. In the first part of the work presented here, SCL-RNAi transgenic mice were created and characterized. The second part deals with the effects of SCL protein absence in RNAi-SCL mice on the molecular level. For this purpose red blood cells were sorted by the FACS analysis using the Lodish blot technique (Socolovsky *et al.*, 2001).

In previous work it was shown that *ab initio* ablation of SCL leads to embryonic lethality at around E9 (Begley *et al.*, 1994). For this reason it has to be assumed that *ab initio* SCL knockdown might equally lead to lethality during embryogenesis. Conditional strategies have been developed to bypass the problem of a lethal phenotype, which are mediated by *Cre* recombinase system (Nagy and Mar, 2001) In this study the tetracycline system (Gossen and Bujard, 1992; Freundlieb *et al.*, 1999), was developed which has the advantage of being able to endogenously switch the expression of RNAi. This On/Off system contrasts with the *Cre* recombinase mediated approaches which do not permit reversing the induced loss-of-function. For this purpose a RNAi targeting vector was constructed as described in section 3.1, see Figure 6 for details. This vector carries the *tetO* binding consensus in its promoter. In the absence of tetracycline analogues, bound TetR can repress transcription of RNAi when co-expressed in the same cell. By contrast, if Dox is added, TetR can not bind to the *tetO* sequence and expression of the shRNAi starts.

Double transgenic mice carrying both the *tetO* and TetR constructs were generated by co-injection of both constructs at a 1:3 ratio (SCL-siRNA clone 2 : T-REx FRT LoxP). This ratio was verbally communicated by Marc van de Wetering to be effective for repression of shRNA expression in the pTER RNAi vector (van de Wetering *et al.*, 2003). The DNA was injected by Leonid Eshkind and Swetlana Ohngemach, Institute of Toxicology, Johannes Gutenberg-Universität Mainz as described in section 2.7.

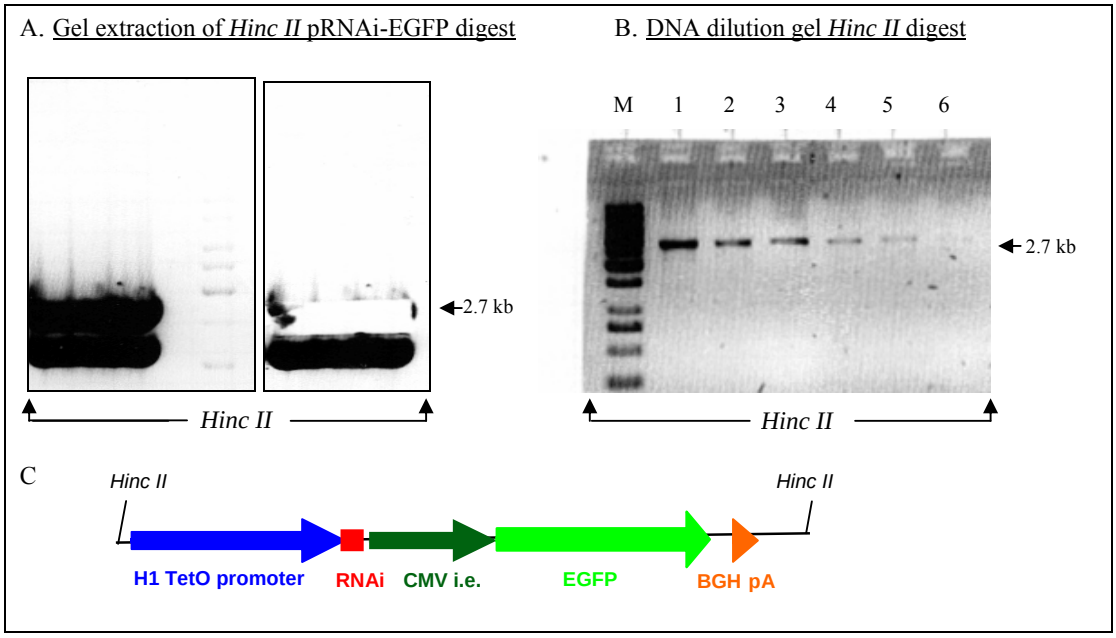


Figure 23: Preparative gel of pSCL-siRNA 2 fragment for pronuclear injection

A. Gel extraction of *Hinc II* pSCL-siRNA 2 digest releasing a 2.7 kb fragment. B. DNA dilution gel of the *Hinc II* digest was used to evaluate DNA integrity and total quantification. C. A diagrammatic representation of the 2.7 kb *Hinc II* fragment used for the generation of SCL-siRNA mice. Arrows, indicate the DNA size; M, indicates a 1 kb DNA marker (Fermantas); numbers 1-6, indicate the individual diluted DNA samples. All abbreviations are given in Figure 4.

Resultant putative founder mice from the co-injection were first screened by PCR using EGFP-specific oligonucleotides #161 and #162 (Table 3) to generate a 1.5 kb band. As an internal control GAPDH was used to confirm the integrity of the genomic DNA and PCR reaction efficiency (oligonucloetides #63 and #64 (Table 3)). The GAPDH specific PCR amplified a 300 bp band. Three positive transgenic mice (numbers 73, 74 and 77) for the pSCL-siRNA 2 construct were detected from 134 putative mice, as seen in Figure 24 A. A second PCR screening was then preformed to

detect the T-REx transgene. From a 134 mice only one mouse was double transgenic for both SCL-siRNA and T-REx FRT LoxP which was mouse (number 77) seen in Figure 24 A and B.

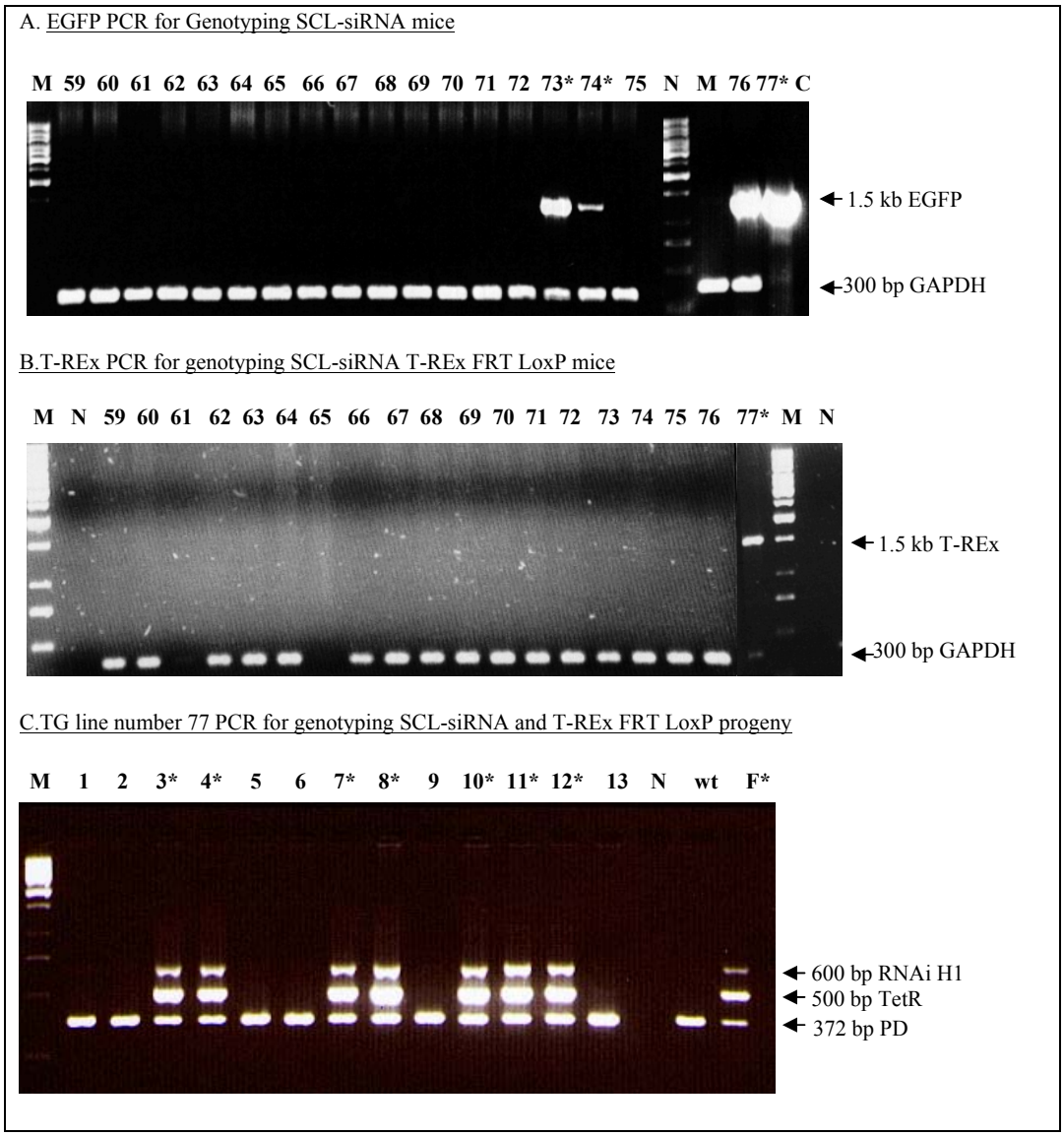


Figure 24: Representative PCR genotyping gels of putative RNAi-EGFP T-REx FRT LoxP mice

A. Analytical gel for RNAi-EGFP genotyping by PCR. A band at 1.5 kb indicated by an arrow represents an amplified EGFP gene (EGFP). Numbers 59-77, indicate individual mice.

B. A T-REx PCR gel for genotyping putative T-REx mice. A 1.5 kb fragment is specific the T-REx construct. Numbers 59-77, indicate individual mice. C. Genotyping of progeny of the TG 77. Numbers 1-13, indicate individual mice [# 7448-7460 respectively]. The internal control either GAPDH band (300 bp) or PD band (372 bp), are indicated by an arrow. EGFP, enhanced green fluorescent protein gene; GAPDH, GAPDH gene; PD, porphobilinogen deaminase gene; M ,1 Kb DNA marker (Fermentas); N, negative water control C, control plasmid RNAi EGFP; TG, transgenic DNA; wt, wild type mouse, F, Founder mouse.

PCR analysis of the progeny is seen in Figure 24C. This PCR analysis showed that both transgenes (SCL-siRNA and T-REx FRT LoxP) were successfully transmitted to the next generation.

3.12 Generation of SCL-siRNA TetR mice by injecting SCL-siRNA construct into repressor positive oocytes

As seen in section 3.11, there was only one resultant double transgenic mouse generated after screening by PCR (Figure 24). Thus, it was postulated that the low number of double transgenic mice was probable due to the single integration of the RNAi construct without the presence of the Tet-repressor. Whereby, single RNAi transgenic mice would knockdown the SCL gene during the post-transcriptional processing in the embryo at day E9.5 resulting in a lethal phenotype. Therefore an alternative strategy was implemented whereby the RNAi construct would be injected into the pronuclear of oocytes carrying the Tet-repressor transgene. Both the T-REx (section 3.10.1) and the tTS KRAB (section 3.10.3) mice line expressing the Tet-repressor by western blotting (3.13.1) and RT-PCR (Mallo *et al.*, 2003) respectively, were amplified for injection of the RNAi construct. Due to the insufficient knockdown of the SCL gene by construct SCL-siRNA 2 (designated #1556) *in vivo* by FACS analysis in bone marrow and blood. Therefore it was proposed that the SCL-siRNA 4 (designated #1558) construct showing effective knockdown in vitro by Western blotting (Figure 16) would be utilised.

Prior to nuclear injection the EGFP expressions cassette was removed from the RNAi construct due to possible methylation attracted to the promoter of the CMV-i.e. EGFP region of SCL-RNAi construct . Therefore, the SCL-siRNA 4 construct was digest seen in Figure 25 with a *Spe I* and *Xba I* digest and prepared for nuclear injection as described in 2.3.3. Resulting in a 4.3 kb band for pronuclear injection purposes after dropping out the 1.3 kb CMV-i.e. EGFP cassette.

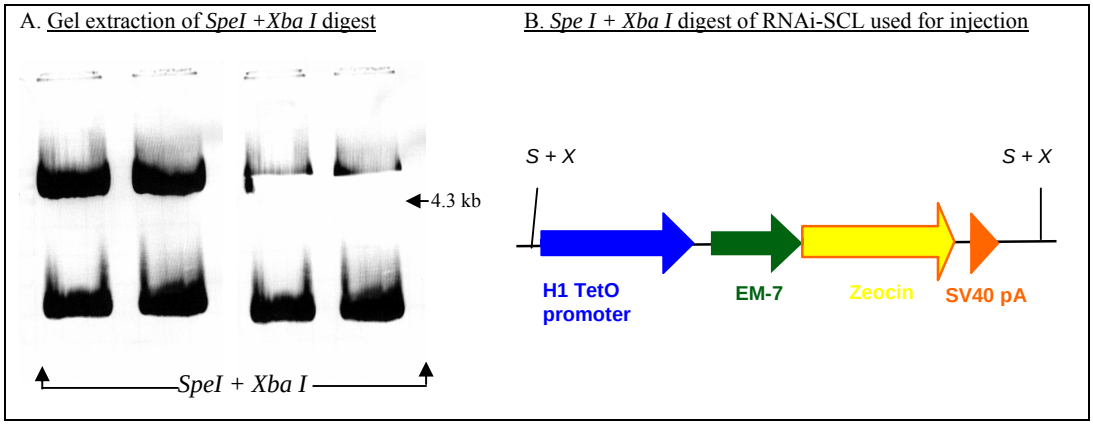


Figure 25: *Spe I* + *Xba I* used for the generation of SCL-siRNA Tet-repressor mice

A. Gel extraction of *Spe I* + *Xba I* pSCL-siRNA digest releasing a 4.3 kb fragment. B. A diagrammatic representation of the 4.3 kb *Spe I* + *Xba I* fragment used for the generation of SCL-siRNA mice. Arrows, indicate the DNA size; S + X, indicates the *Spe I* and *Xba I* restriction enzyme digest. All abbreviations are given in Figure 4.

A total of 257 mice were generated from the pronuclear injection. These mice were first screened for the tet repressor transgenes T-REx and tTS KRAB seen in Figure 26. Figure 26A, represents the genotyping of 112 putative SCL-siRNA T-REx double transgenic mice with the T-REx background. All positive T-REx mice amplified a band at 500 bp by PCR with oligonucleotides #188 and #224 (Table 3) as compared to the wildtype mouse. The amplification of the PD gene with a 372 bp PCR band using oligonucleotides #221 and #222 (Table 3), indicate DNA integrity and PCR efficiency. From 112 putative mice, 38 mice were positive for the T-REx transgene by PCR.

Also from 72 putative SCL-siRNA tTS KRAB double transgenic mice with a tTS KRAB background 35 mice were tTS KRAB transgenic by PCR represented in Figure 26B. All positive tTS KRAB mice amplified a 300 bp PCR band for tTS coding region with oligonucleotides #241 and #242 (Table 3), with the same PD internal control as in Figure 26A as compared to the wildtype mouse.

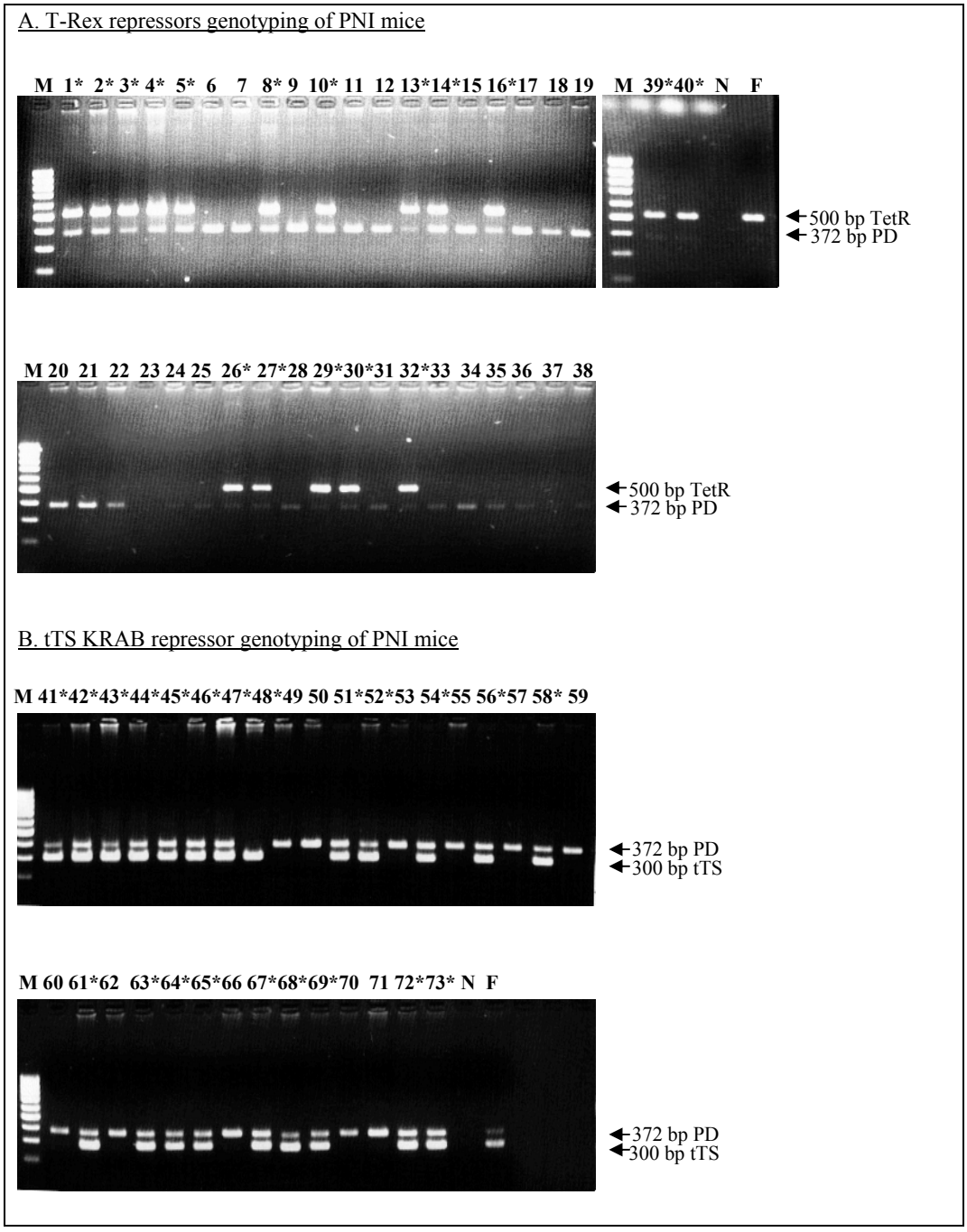


Figure 26: Genotyping of PNI mice for the Tet-repressor transgene

A T-REx PCR gel for genotyping putative T-REx mice, amplifying a band at 500 bp containing the T-REx coding region as compared to the wild type mouse DNA. Numbers 1-19 [#9735-53], 20, 21 [#9773, 9774] and 23-40 [#9754-9772], indicate individual mice. B. tTS KRAB PCR gel for genotyping putative tTS KRAB mice, amplifying a band at300 bp containing the tTS coding region compared to wild type mice. Numbers 41-73, indicate individual mice [# 9348-9381, respectively]. A PD band is used as an internal control at 372 bp. PNI, pronuclear injection mice; TetR, tetracycline repressor; tTS, tetracycline silencer; PD, porphobilinogen deaminase gene; M ,1 Kb DNA marker (Fermentas);N, negative control F, founder mouse DNA; wt, wildtype mouse; (*), indicates all positive transgenic mice by PCR.

All Tet-repressor positive mice were then screened by PCR for the pSCL-siRNA construct seen in Figure 26. A SCL-siRNA specific PCR band amplifying the zeocin within the coding region at 448 bp indicates was detected by PCR with oligonucleotides #269 and #272 (Table 3), compared to wild type mice. From a possible 38 repressor positive T-REx mice analysed by PCR only one was found to be double transgenic for the pSCL-siRNA construct and tet repressor, Figure 27A. As compared to no tTS KRAB repressor positive double transgenic mice detected by PCR (Figure 27B).

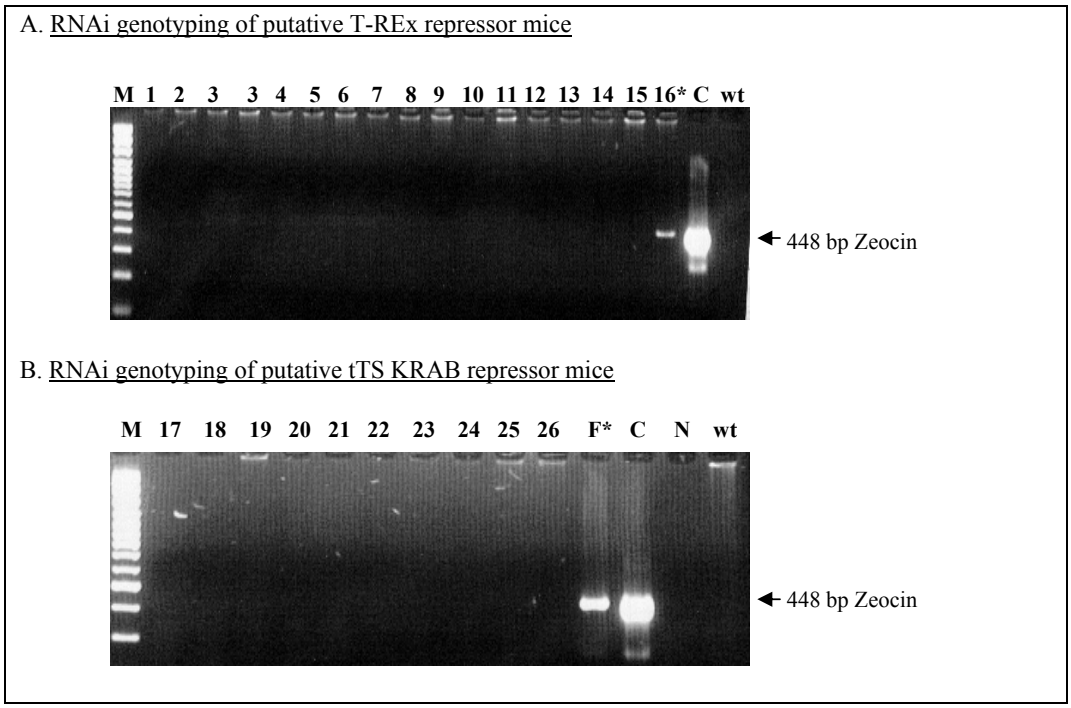


Figure 27: RNAi genotyping of putative double transgenic PNI mice

A. RNAi PCR gel for genotyping putative repressor T-REx and SCL-siRNA transgenic mice. A pSCL-siRNA specific PCR band amplifies zeocin at 448 bp. Numbers 1-16, indicate individual mice [# 9735-9774] respectively. B. RNAi PCR gel for genotyping putative repressor tTS-KRAB and SCL-siRNA transgenic mice. A pSCL-siRNA specific PCR band amplifies zeocin at 448 bp. Numbers 17-26, indicate individual mice [# 9141,9144, 9146, 9151-52, 9154-56 and 9159-60, respectively]. PNI, pronuclear injection mice; T-REx, tetracycline repressor expression ; tTS-KRAB, tetracycline silencer fused to the KRAB domain of the Kox 1 gene; PD, porphobilinogen deaminase gene; M ,1 Kb DNA marker (Fermentas);N, negative control F, founder mouse DNA; wt, wildtype mouse; (*), indicates all positive transgenic mice by PCR.C, control plasmid pSCL-siRNA.

This SCL-siRNA T-REx founder line number #9774 was found to be double transgenic for the RNAi SCL siRNA 4 and T-REx repressor constructs. Line #9774 was then crossed into the FVB strain for analysis of future progeny.

3.13 Tetracycline Repressor expression in the transgenic mice

3.13.1 Determination of T-REx expression in mouse tissue using Western blotting analysis

As shown by the SCL-siRNA T-REx genotyping the co-injection of the T-REx construct with the SCL-siRNA 2 construct together described in section 3.11 gave a low efficiency of transgenic mice probably due to embryonic lethality of the RNAi-EGFP single transgenic embryos. Therefore, a second strategy was established which makes use of pronuclear injection of the RNAi construct into TetR expressing oocytes. This strategy should circumvent the probability of a high number of embryonic lethal single transgenics because 50% of the injected oocytes should express the T-REx repressor which is expected to block , thus circumventing possible embryonic shRNA induced lethality.

A prerequisite of this strategy is of course a robust expression of the T-REx transgene. To evaluate the expression of the TetR protein in the mouse line number 33 (Figure 19), a Western blot was performed. Protein was extracted as described in section 2.6.3 from 10 different organs and 150 µg/µl protein from each sample was run on a SDS-PAGE gel. The TetR gene encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa. A TetR specific antibody (Tet 01 polyclonal rabbit, MobiTec) was used at a 1:200 dilution for 1 hr at RT with an α-rabbit secondary antibody (Santa Cruz) at a 1:5000 dilution for 30 min at RT and exposed to the ECL detection kit (Amersham) for the detection of the TetR specific band. In the Western blot the HEK 293 TR cell line expressing the T-REx construct (van de Wetering *et al*., 2003) is used as a positive control gave a clear signal at the expected size for the T-REx protein (23 kDa). As a loading control for each sample, α-p38 antibody (Santa Cruz) at a 1:1000 dilution for 1 hr at RT and donkey α-rabbit secondary antibody (Santa Cruz) 1:5000 dilution for 30 min at RT described in section 2.6.3 was used.

A clear T-REx specific signal was seen for the HEK 293 TR cell line stably expressing the T-REx protein at the expected size of 23 kDa. From ten analysed organs eight expressed the TetR protein, including the lung, heart, thymus, tongue, kidney, spleen and colon. The strongest TetR signal was detected in the heart, tongue and colon compared to the wildtype protein in the corresponding organs. The

intermediate to weak TetR signals were detected in the lung, brain, thymus and kidney compared to the wild type mouse organs. Only a very weak signal was detected in the brain. However, TetR expression was missing in the liver and bone marrow which maybe due to protein degradation during preparation or under loading of the protein onto the SDS-PAGE gel.

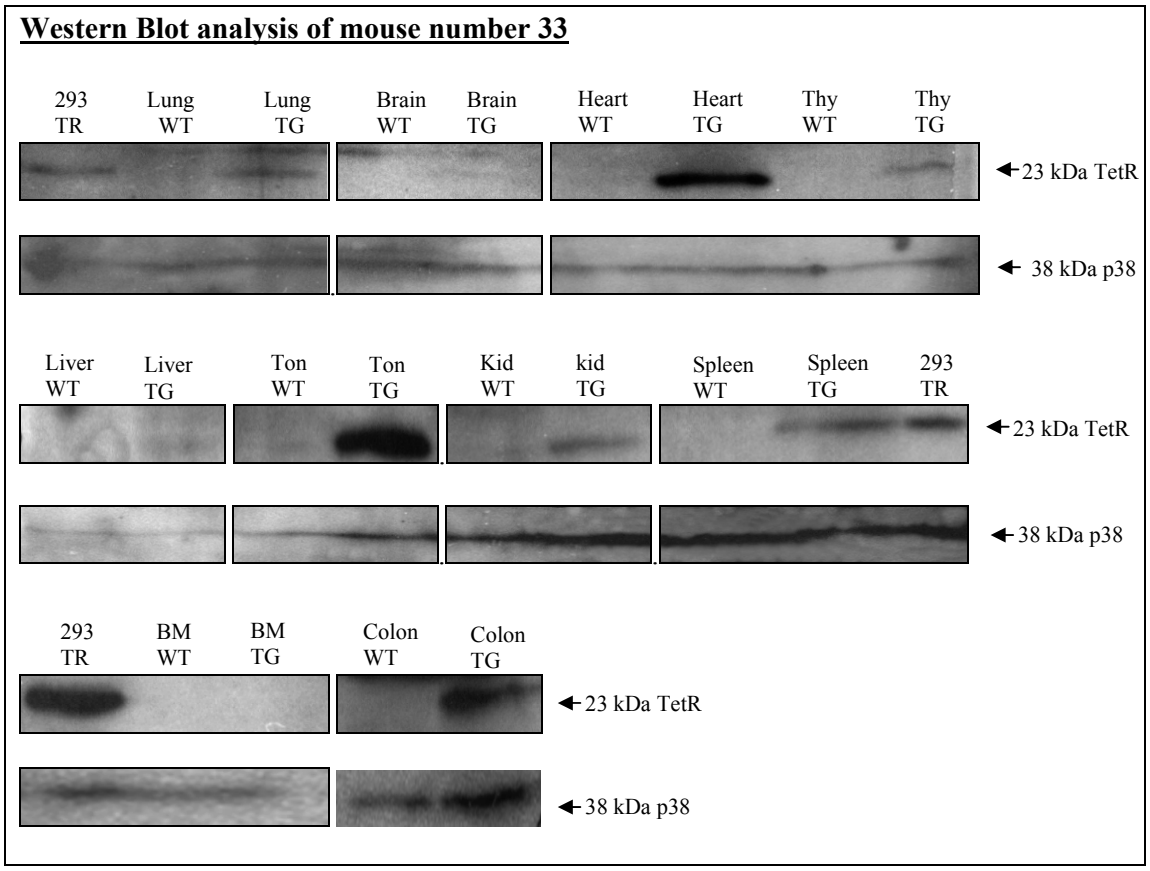


Figure 28: Expression of T-REx protein in different mouse tissues

Western blot analysis of ten organs from transgenic line # 33. T-REx expression protein analysed in the transgenic mouse (TG) in each case was compared to the corresponding tissue of a wildtype mouse (WT) (Upper panel). 150 µg/µl of protein was loaded for each sample and in addition analysed for p38 expression as a loading control (lower panel). T-REx protein gave a signal at 23 kDa and the p38 protein gave a signal at 38 kDa. Ton, indicates tongue; kid, indicates kidney; Thy, indicates thymus; BM, indicates bone marrow; 293 TR, HEK 293 TR (T-REx).

It was shown that transgenic line number 33 expressed the TetR protein in all analysed tissues but the liver and bone marrow. Since both thymus and spleen gave a positive signal, line number 33 was used as a donor for TetR expressing oocytes for injection of the SCL-siRNA4 construct.

3.13.2 Determination of T-Rex FRT LoxP expression in mouse tissue using Western blotting analysis

As described under section 3.10.2, the genotyping of 31 possible founder mice led to the identification of three transgenic T-REx FRT LoxP lines. In these lines T-REx expression was determined by Western blotting of selected tissues from adult mice as described under section 3.4. As shown in Figure 29, the first founder (mouse #8687) expressed substantial amounts of the T-REx repressor in the spleen, tongue, kidney, heart, liver, lung and thymus. Therefore seven out of eight organs analysed expressed the TetR protein with exception to the brain. Very high TetR signal was detected in the heart, liver, lung and thymus. Moderately high expression was detected in the tongue and kidney and weak expression in the spleen and no expression in the brain. As expected in the non-transgenic littermates not caring the transgene (WT) did not give any T-REx specific signal.

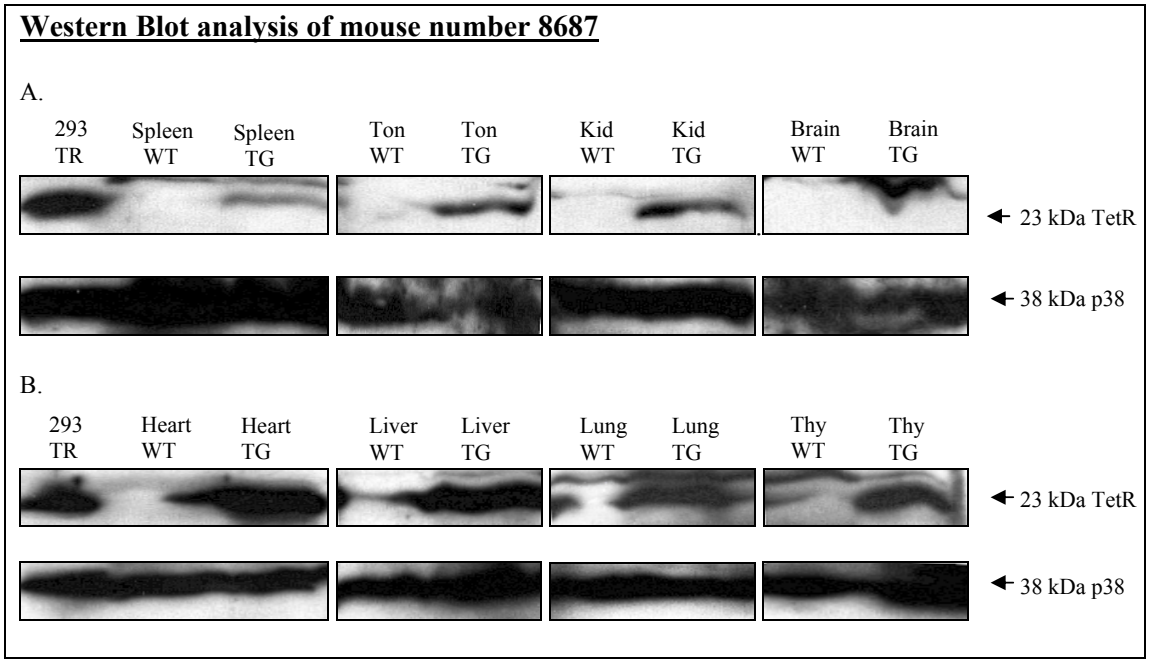


Figure 29: Expression of the T-REx protein in different mouse tissue for line T-REx FRT LoxP

Western blot analysis of eight organ from transgenic line #8687. A Shows TetR expression in spleen, tongue and kidney. B. Shows TetR expression in the heart, liver, lung and thymus. The transgenic protein denoted by TG was compared to wildtype mouse organ denoted by WT for the TetR signal (upper panel). 150 µg/µl of protein was loaded for each sample and analysed for p38 expression (lower panel). T-REx protein gave a signal at 23 kDa and the p38 protein gave a signal at 38 kDa. Ton, indicates tongue; kid, indicated kidney; Thy, indicates thymus; 293 TR, HEK 293 TR (T-REx)

T-REx expression was also demonstrated for a second founder line (mouse number 8665) as seen in Figure 30. Also, from eight organs TetR expression was detected in the spleen, tongue, brain, kidney, heart, liver, lung and thymus of T-REx FRT LoxP line # 8665 compared to the wild type mouse organs. High TetR expression was detected in the tongue, heart and lung with weaker expression detected in the spleen, brain, kidney, liver and thymus.

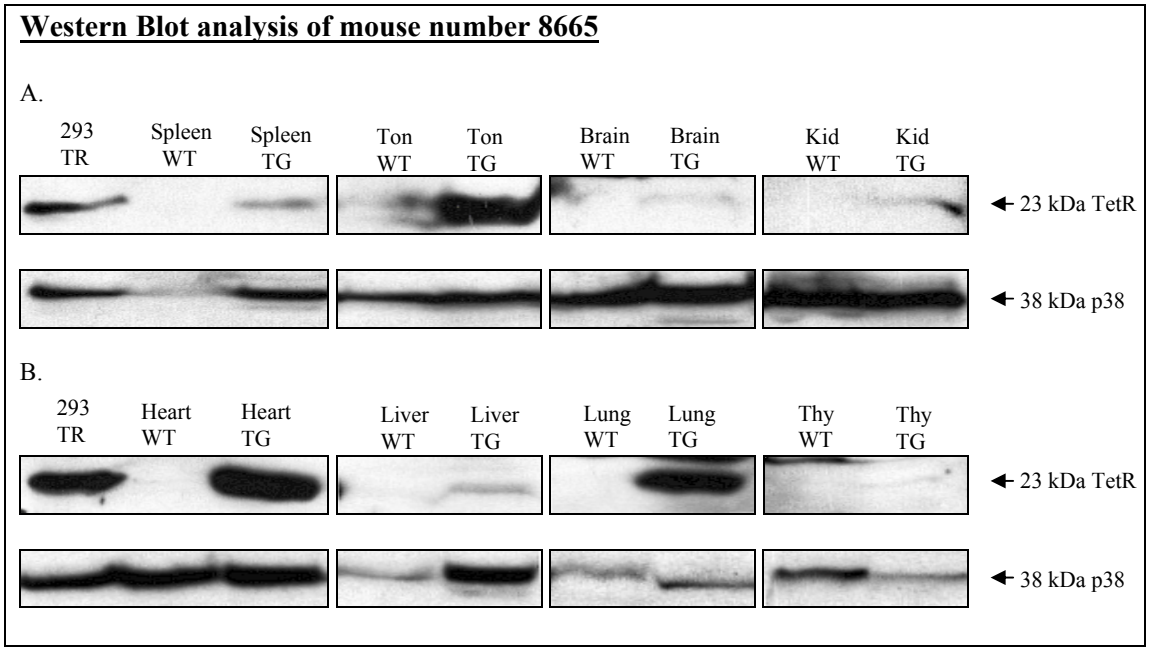


Figure 30: Expression of the T-REx protein in different mouse tissue for line T-REx FRT LoxP

Western blot analysis of eight organ from transgenic line #8665. A Shows TetR expression in spleen, tongue and kidney. B. Shows TetR expression in the heart, liver, lung and thymus. The transgenic protein denoted by TG was compared to wildtype mouse organ denoted by WT for the TetR signal (upper panel). 150 µg/µl of protein was loaded for each sample and analysed for p38 expression (lower panel). T-REx protein gave a signal at 23 kDa and the p38 protein gave a signal at 38 kDa. Ton, indicates tongue; kid, indicated kidney; Thy, indicates thymus; 293 TR, HEK 293 TR (T-REx)

The third mouse line #8653 did not express enough TetR protein in the eight organs as shown by western blotting, only the heart and liver expressed the TetR protein, the spleen, tongue, kidney, brain, liver, lung and thymus had no expression as seen in Figure 31. Therefore, it was concluded that the T-REx FRT LoxP line #8653 did have only substantial protein levels in some organs which were the heart and liver. Thus this mouse was not used in further experiments.

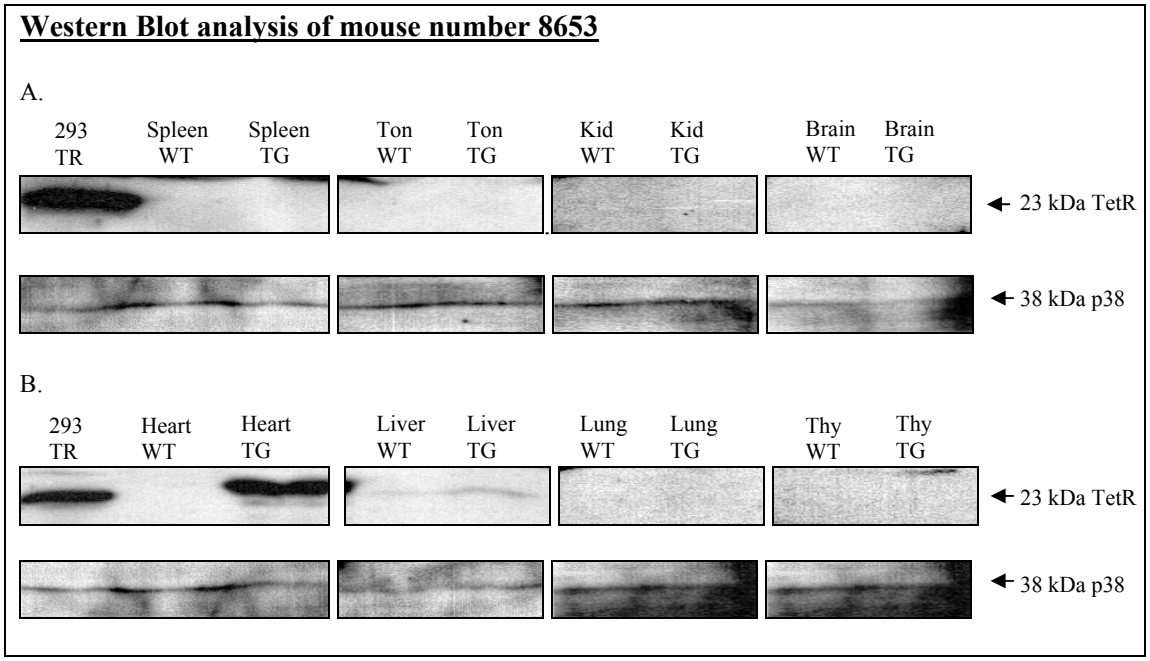


Figure 31: Expression of the T-REx protein in different mouse tissue for line T-REx FRT LoxP

Western blot analysis of eight organ from transgenic line #8653. A Shows TetR expression in spleen, tongue and kidney. B. Shows TetR expression in the heart, liver, lung and thymus. The transgenic protein denoted by TG was compared to wildtype mouse organ denoted by WT for the TetR signal (upper panel). 150 µg/µl of protein was loaded for each sample and analysed for p38 expression (lower panel). T-REx protein gave a signal at 23 kDa and the p38 protein gave a signal at 38 kDa. Ton, indicates tongue; kid, indicated kidney; Thy, indicates thymus; 293 TR, HEK 293 TR (T-REx)

As the first two transgenic mouse lines (#8665 and #8687) showed good expression in most organs we amplified both strains by crossing back into a FVB background.

3.14 Determinating the expression of adult SCL-siRNA Tet-repressor mice by FACS analysis

3.14.1 Lodish plot analysis in adult bone marrow and peripheral bood samples

Two founder mouse lines harbouring the doxycycline-dependent repressor T-REx and the tetracycline inducible RNA polymerase III H1 promoter governing the expression of specific shRNAs against the haematopoietic transcription factor SCL (SCL-siRNA) were analysed.

To determine whether the tetracycline system was functioning and whether there was a biological effect of the RNAi expression against the SCL protein after Dox induction, we utilized the "Lodish plot" technique (Figure 3). As we had no functional antibody against SCL the Lodish plot technique allowed us to have a quick and quantitative readout of the erythrocyte population in different haematopoietic compartments in the mouse. To test both available double transgenic lines for SCL knockdown efficiency, bone marrow and peripheral blood were analysed using the Lodish blot technique. A flow cytometry assay was developed in the Lodish group that allows quantitative evaluation of erythroid differentiation in neonatal and adult haematopoietic tissues (Socolovsky *et al.*, 2001). On the basis of the expression of the erythroid-specific TER119 and red blood cell PAN marker CD71 (transferrin receptor), erythroid cells are classified into four populations that correlate well with their maturation stages from pro-erythroblasts to mature erythrocytes. Then bone marrow and blood cells were double labelled for erythroid-specific TER119 and red blood cell marker (CD71) and analyzed by flow cytometry.

Flow cytometric analysis was performed on a FACS 4-column Calibur ^{TM} (Becton Dickinson, San Jose, CA). On analysis, scatter gating and staining with PI (1 μg/ml) was used to avoid collecting data from debris and any dead cells. The data was first analysed by forward-side scatter analysis for viable cell identification and then further analysed by contour plot analysis. Forward and side scatter are used for preliminary identification of cells. In a peripheral blood sample, lymphocyte, monocyte and granulocyte populations can be defined on the basis of forward and side scatter.

Forward and side scatter are used to exclude debris and dead cells. Forward scatter is plotted on the x-axis (a measure of cell size) and side scatter plotted on the y-axis (a measure of cell granularity) characterization.

Each dot represents one cellular event. Contour plots are produced when the points of equal density on dot plots are joined together presenting areas of high density in much the same manner as hills on conventional maps.

3.14.2 Lodish plot analysis in bone marrow and peripheral blood of transgenic line SCL-siRNA T-REx FRT LoxP

To analyse doxycycline induced SCL knockdown by RNAi, we generated first a transgenic mouse line by co-injection of both tetracycline repressor and responder constructs into wildtype oocytes. The SCL-siRNA 2 construct (Figure 16B) was used for conditional RNAi expression as it effectively gave full knockdown *in vitro* of the SCL HA-tag protein. Also for switchable, tissue-specific Tet-repressor expression the T-REx FRT LoxP construct described in section 3.4 was simultaneously co-injected into wildtype oocytes. Mouse line number 77 was genotyped positive for both constructs in section 3.11.

As conditional expression of the shRNA is dependent on the administration of the inducer doxycycline (Dox), the F1 generation of this line was treated with Dox for six weeks and the F2 generation mice were treated for seven weeks for the induction of RNAi. Two independent assays were undertaken examining the F1 and F2 generation, and all mice that were tested had the same age, sex and strain. The transgenic line was tested with and without Dox in two individual mice and as a positive control, compared to a wild type mouse which was assumed to show a normal haematopoietic population of red blood cells and progenitors.

We used flow cytometry analysis to monitor erythropoiesis in the bone marrow in the F1 and F2 generation of mouse line #77 with/without Dox treatment. We analyzed total bone marrow cells for the erythroid precursors. First the viable bone marrow cells were sorted by forward-side scatter analysis in which every dot represented an individual cell, this step discards of unwanted dead cells or debris (Figure 32 left panel, region 1/R1). The bone marrow cells co-stained a R613 sav biotinated anti-

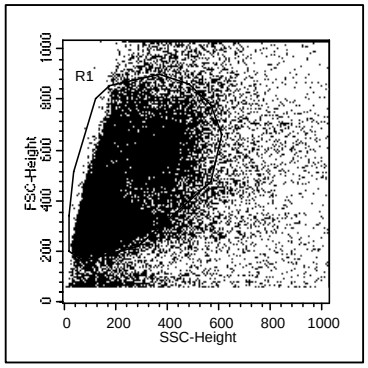
CD71 monoclonal antibody (mAb) and a PE-conjugated anti-TER119 mAb were analysed by relative logarithmic fluorescence units for PE (x-axis) and R613 sav (y-axis) seen in the contour plot (Figure 32AII right panel).

Our results showed that the adult bone marrow contained at least five distinct populations of cells, defined by their characteristic staining patterns: $CD71^{high}TER119^{med}$, $CD71^{high}TER119^{high}$, $CD71^{med}TER119^{high}$, $CD71^{low}TER119^{high}$, and $CD71^{low}TER119^{low}$ (Figure 32AII, contour plot, regions R2 to R6, respectively). In the normal adult a high number of mature erythrocytes was expected as opposed to a high number of pre-mature erythrocytes in a phenotype which was expected for SCL knockdown. The wildtype mouse which was the same age and background should showed a normal haematopoiesis phenotype. For the wildtype (WT) mouse the flow cytometry analysis of the bone marrow cells showed only a few basophilic erythroblast cells (R3) and almost no proerytroblast cells (R2) (4.96% and 0.07%, respectively; Figure 32A). For the double transgenic mouse off Dox basophilic erythroblast cells (R3) cells were less abundant, comprised of 0.02% and 2.57% for the proerytroblast cells (R2) and basophilic erythroblast cells (R3) cells respectively (Figure 32B). The double transgenic mouse on Dox showed, basophilic erythroblast cells (R3) cells become more abundant they comprised of 0.7% proerytroblast cells (R2) and 10.6% basophilic erythroblast cells (R3) total gated cells respectively (Figure 32C). The mouse treated with Dox showed a 50% increase in R3 basophilic erythroblast cells population compared with the wild type and transgenic mouse off Dox suggesting a Dox induced effect in bone marrow the transgenic line compared to the wildtype and transgenic mouse off Dox.

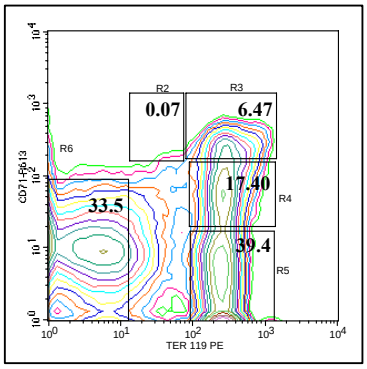
Lodish plot analysis of bone marrow cells of F1 generation SCL-siRNA T-REx FRT LoxP line

A Wildtype mouse without doxycycline treatment

I. Bone marrow forward-side scatter plot

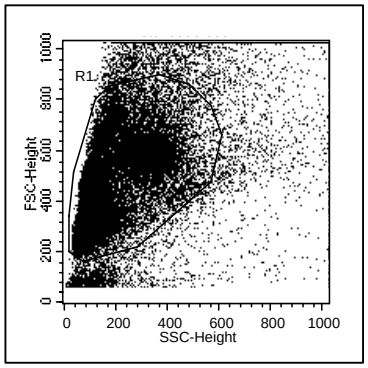


II. Bone marrow Lodish contour plot

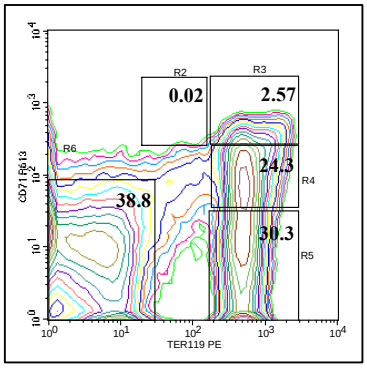


B. F1 generation of transgenic mouse without doxycycline treatment

I. Bone marrow forward-side scatter plot

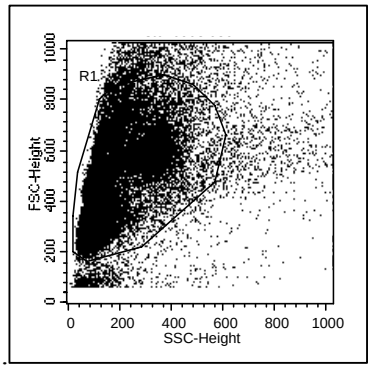


II. Bone marrow Lodish contour plot

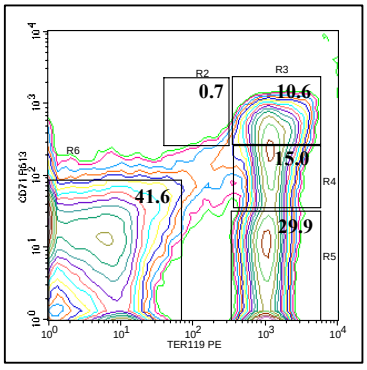


C. F1 generation of transgenic mouse with doxycycline treatment

I. Bone marrow Lodish forward-side scatter plot



II. Bone marrow Lodish contour plot



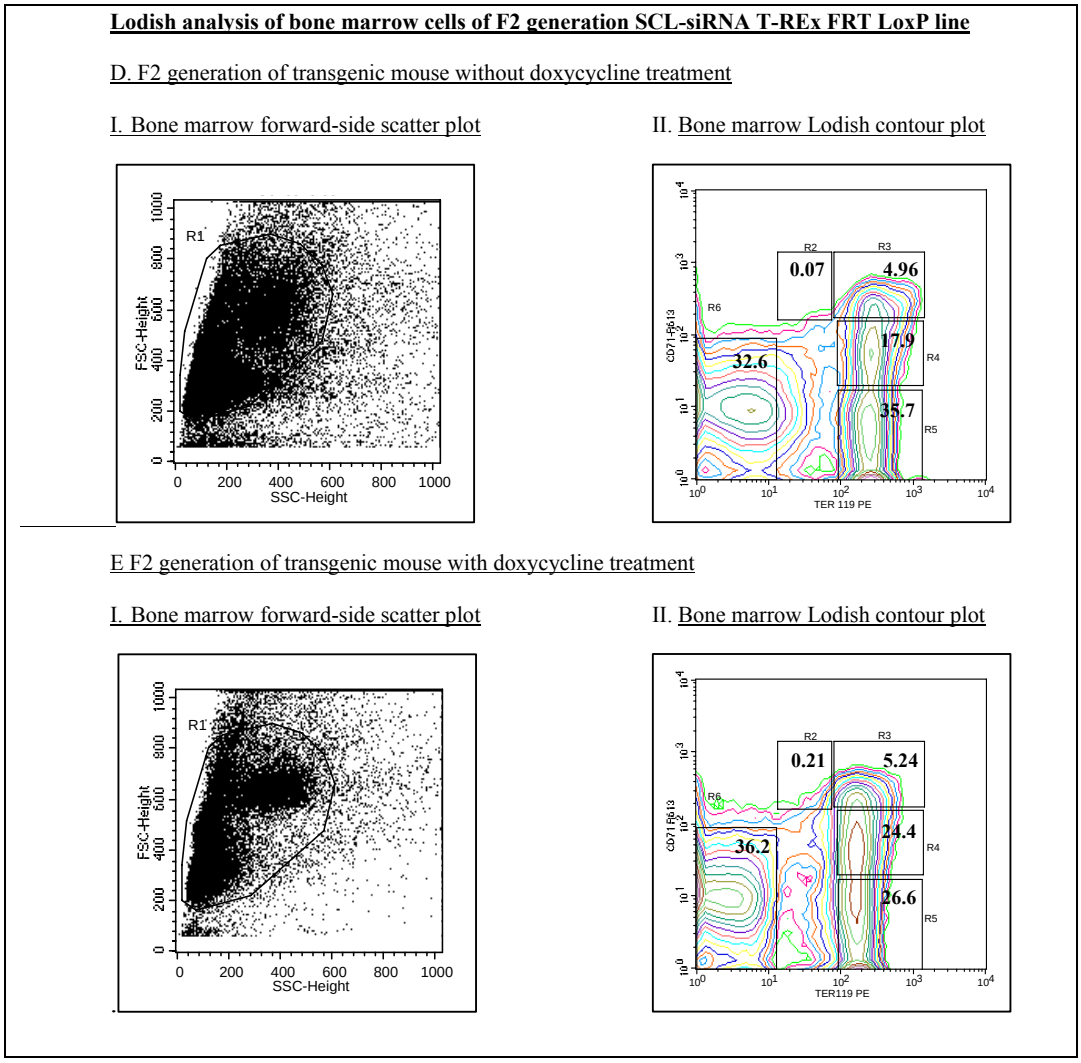


Figure 32: FACS analysis of adult mouse bone marrow of F1 and F2 generation SCL-siRNA T-REx FRT LoxP line

Wild type, F1 and F2 transgenic line (# 77) bone marrow cells for analysis of erythropoiesis, double labelled with a R613 sav biotinated anti-CD71 monoclonal antibody (mAb) and a PE-conjugated anti-TER119 mAb. Dead cells and debris (forward scatter left panel, R1) were excluded from the analysis. The right panel illustrates a contour plot of all viable cells in R1; axes indicate relative logarithmic fluorescence units for PE (x-axis) and R613 sav (y-axis). Regions R2 to R6 are defined by characteristic staining pattern of cells, including $CD71^{high}TER119^{med}$, $CD71^{high}TER119^{high}$, $CD71^{med}TER119^{high}$, $CD71^{low}TER119^{high}$, and $CD71^{low}TER119^{low}$, respectively. The percentages of $TER119^{-}$ cells (presented as total gated cells of R6) and $TER119^{+}$ cells (presented as total gated cells of R2 to R5 cells) are seen on the contour blot.

Lodish plot analysis of F2 generation revealed that the double transgenic mouse off Dox repeated the same pattern as the F1 generation. The F2 transgenic mouse off Dox had 0.07% and 4.96 % for the R2 and R3 regions respectively (Figure 32DII, contour plot) compared to 0.21% and 5.24% for the F2 transgenic mouse on Dox for

proerytroblast cells (R2) and basophilic erythroblast cells (R3) respectively (Figure 32EII, contour plot) showing a difference in the populations. One observation that was made was that a shift in the R4 cell population for the F2 transgenic mouse on Dox was higher compared to the F1 generation mouse off Dox which shows an effect in the later population of the erythrocyte differentiation pathway.

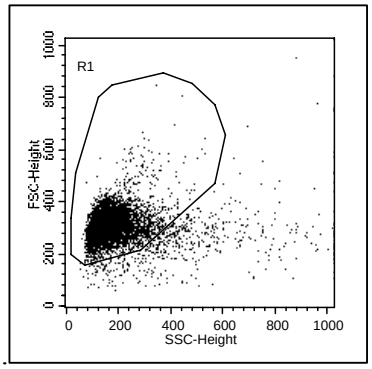
Next, the peripheral blood of transgenic line SCL siRNA T-REx FRT LoxP line was analysed by the Lodish plot technique using the same criteria as for the bone marrow. The F1 generation blood was analysed after Dox treatment for six weeks and co-stained with R613 sav biotinated anti-CD71 mAb and PE conjugated anti-TER119 mAb for Lodish plot analysis. The wildtype mouse showed a normal haematopoietic phenotype with 0.5% in the basophilic erythrocyte (R3) and 0.41% in the late basophilic erythrocyte (R4) population (Figure 33AII, contour plot). The transgenic mouse without Dox treatment showed a 0.44% in basophilic erythrocyte (R3) and 0.26% in late basophilic erythrocyte (R4) population (Figure 33BII, contour plot). In comparison, the transgenic mouse on Dox showed an increase with 0.62% in the basophilic erythrocyte (R3) and 0.23% in the late basophilic erythrocyte (R4) population (Figure 33BII, contour plot).

The F2 generation for mouse line SCL-siRNA T-REx FRT LoxP was analysed by Lodish plot under the same criteria as the F1 generation. In which, the F2 transgenic mice were treated with/without Dox for a seven week period. The transgenic mouse off Dox gave a 0.61% in the basophilic erythrocyte (R3) and 0.29% late in the basophilic erythrocyte (R4) population compared to the transgenic mouse on Dox which gave a 50% increase of 1.21% in the basophilic erythrocyte (R3) and 0.91% in the late basophilic erythrocyte (R4) population (Figure 33CII and DII, contour plots respectively).

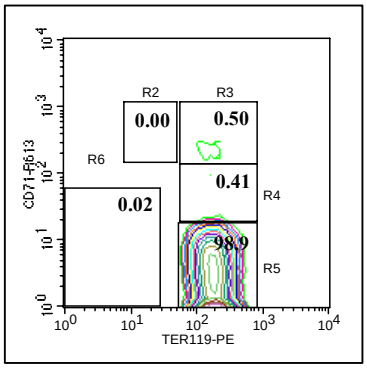
Lodish analysis of peripheral blood cells of F1 generation SCL-siRNA T-REx FRT LoxP line

A. Wildtype mouse without doxycycline treatment

I. Blood forward-side scatter plot

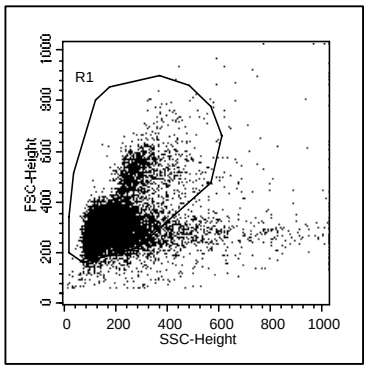


II. Blood Lodish contour plot

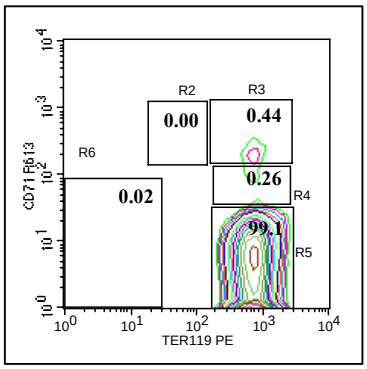


B. F1 generation of transgenic mouse without doxycycline treatment

I. Blood forward-side scatter plot

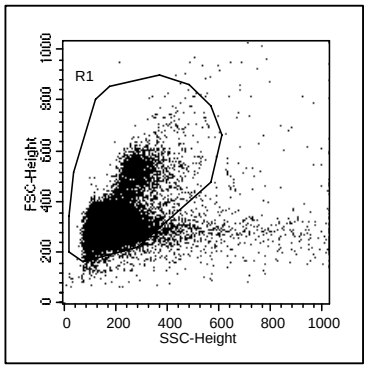


II. Blood Lodish contour plot

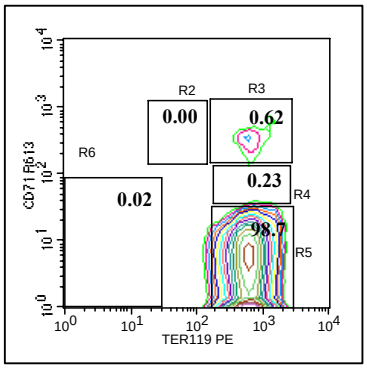


C. F1 generation of transgenic mouse with doxycycline treatment

I. Blood forward-side scatter plot



II. Blood Lodish contour plot



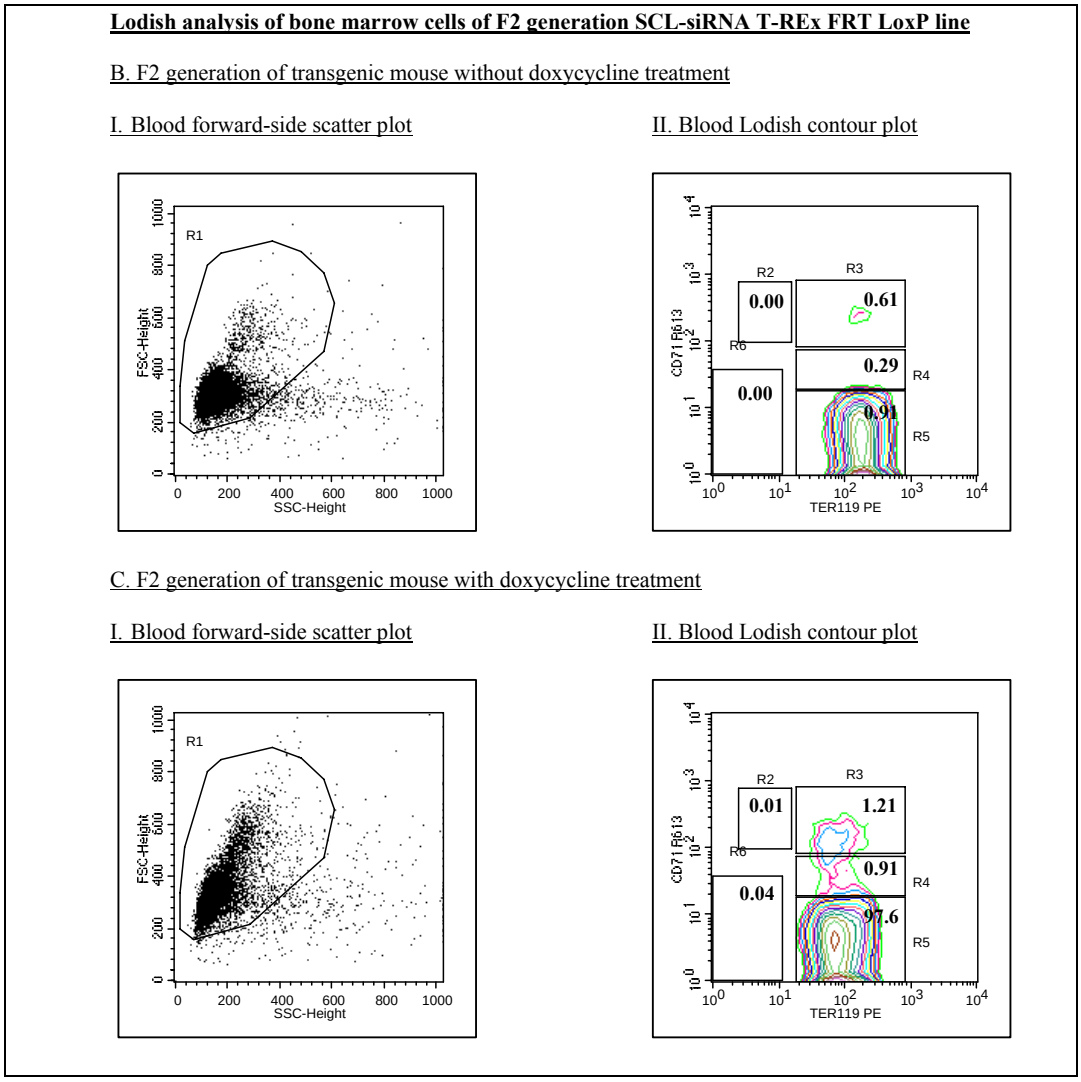


Figure 33: FACS analysis of adult peripheral blood of F1 and F2 generation SCL-siRNA T-REx FRT LoxP trangenic line

Wild type, F1 and F2 transgenic line (# 77) peripheral blood analysis of erythropoiesis, double labelled with a R613 sav biotinated anti-CD71 monoclonal antibody (mAb) and a PE-conjugated anti-TER119 mAb. Dead cells and debris (forward side scatter left panel, R1) were excluded from the analysis. The right panel illustrates a density plot of all viable cells in R1; axes indicate relative logarithmic fluorescence units for PE (x-axis) and R613 sav (y-axis). Regions R2 to R6 are defined by characteristic staining pattern of cells, including $CD71^{high}TER119^{med}$, $CD71^{high}TER119^{high}$, $CD71^{med}TER119^{high}$, $CD71^{low}TER119^{high}$, and $CD71^{low}TER119^{low}$, respectively. The percentages of $TER119^{-}$ cells (presented as total gated cells of R6) and $TER119^{+}$ cells (presented as total gated cells of R2 to R5 cells) are seen on the contour blot.

This suggests that the Dox induced state is inherent and that there is a dose-response seen in the F2 generation compared to the F1 generation after seven weeks of Dox induction.

3.14.3 Lodish plot analysis in bone marrow and peripheral blood samples of transgenic line SCL-siRNA T-REx

The transgenic line number 9774 was genotyped positively for the tetracycline repressor T-REx and tetracycline responsive RNAi construct SCL siRNA-4 as shown in section 3.11. This transgenic line was generated using co-injection of the RNAi vector into the Tet-repressor positive oocytes. The transgenic line was administered Dox for six weeks to induce an RNAi response. Subsequently, double transgenic mice for RNAi and T-REx were screened for erythroblast population maturity by Lodish blot analysis of the bone marrow and blood as described in section 3.14.

We analyzed total bone marrow cells for the erythroid precursors. First the viable bone marrow cells were sorted by forward-side scatter as described in section 3.14.1. As the first round of mice analysed by the Lodish plot technique showed a lower population size in average compared to the published values by the Lodish group (Socolovsky *et al.*, 2001), we decided to change the conjugated stain for CD71 from R613 to APC as a fluorescent dye. We were able to observe improved staining sensitivity using APC-CD71/PE-TER119 compared to R613-CD71/PE-TER119 during analysis. This alternative staining technique was used for the rest of the experiments. A wildtype mouse from the same age and background was expected to show no effect, revealing a normal haematopoiesis state compared to an increase in the pre-mature erythrocyte population expected for a SCL knockdown phenotype after Dox induction. Flow cytometry analysis of the bone marrow cells for the non transgenic littermate mouse on Dox showed that there was no effect in the basophilic erythroblasts (R3) cells or the proerythroblasts (R2) which comprised 0.29% and 19.75% respectively, when treated with Dox (Figure 34AII, contour plot). The double transgenic mouse on Dox showed, basophilic erythroblasts (R3) cells had no significant change. They comprised 0.28% and 19.75% of R2 and basophilic erythroblasts (R3) of the total gated cells respectively (Figure 34BII, contour plot). This suggests that there was no significant effect in the F0 generation SCL-siRNA T-REx transgenic mouse generated by pronuclear injection into repressor positive oocytes compared to the wildtype littermate.

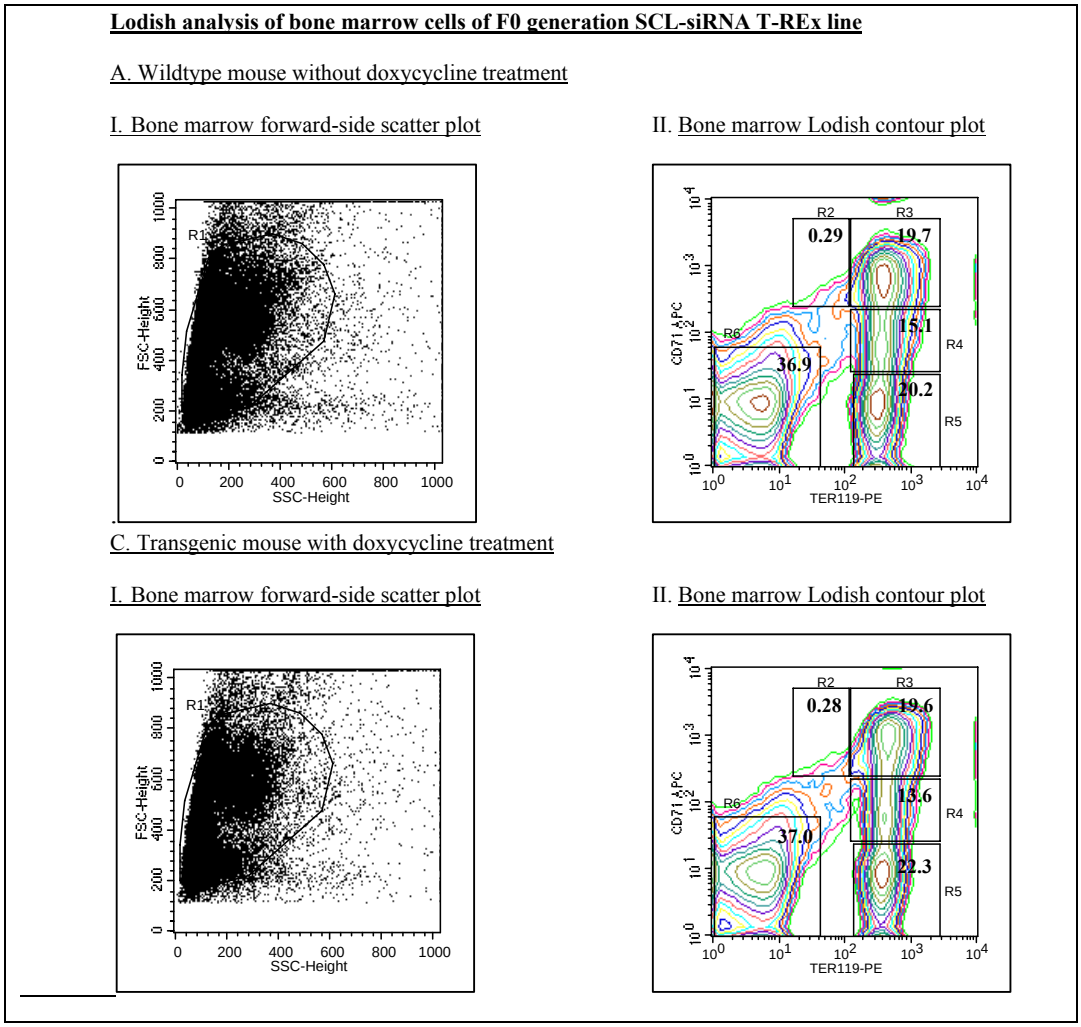


Figure 34:FACS analysis of adult mouse bone marrow in transgenic line SCL-siRNA T-REx

Wild type littermate and transgenic line (# 9774) bone marrow cells for analysis of erythropoiesis, double labelled with a APC-conjugated anti-CD71 monoclonal antibody (mAb) and a PE-conjugated anti-TER119 mAb. Dead cells and debris (forward scatter left panel, R1) were excluded from analysis. The right panel illustrates a contour plot of all viable cells; axes indicate relative logarithmic fluorescence units for PE (x-axis) and APC (y-axis). Regions R2 to R5 are defined by characteristic staining pattern of cells, including $CD71^{med}TER119^{low}$, $CD71^{high}TER119^{low}$, $CD71^{high}TER119^{high}$, $CD71^{med}TER119^{high}$, and $CD71^{low}TER119^{high}$, respectively. The percentages of $TER119^{-}$ cells (presented as total gated cells of R6) and $TER119^{+}$ cells (presented as total gated cells of R2 to R5 cells) are labelled at the bottom of each contour plot.

Next the peripheral blood of transgenic line SCL-siRNA T-REx FRT LoxP line (#9774) was analysed by the Lodish plot technique using the same criteria as for the bone marrow. The blood was co-stained with APC conjugate anti-CD71 mAb and PE conjugated anti-TER119 mAb for the wildtype mouse off Dox, non-transgenic littermate on Dox and the transgenic line SCL-siRNA T-REx on Dox for a period of

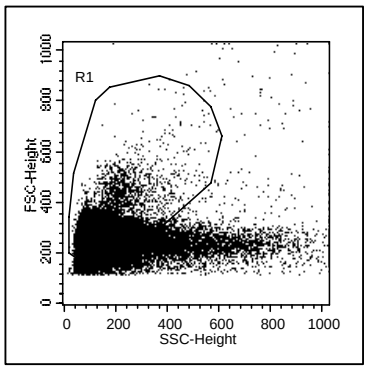
seven weeks. The wildtype mouse without Dox treatment demonstrated the normal haematopoietic phenotype expected with 0.65% in the basophilic erythroblasts (R3) and 0.38% in late basophilic erythroblasts (R4) population (Figure 35AII, contour plot). The non-transgenic littermate on Dox showed the same effect as the wildtype mouse off Dox with 0.79% in the basophilic erythroblasts (R3) and 0.39% in the late basophilic erythroblasts (R4) population therefore showing no unspecific effect of the Dox treatment (Figure 35BII, contour plot). No apparent difference was seen in the R3 (0.67%) and R4 (0.37%) population of the transgenic mouse on Dox (Figure 35CII, contour plot) compared to the wildtype and the non-transgenic off Dox.

Although an unexpected population was seen in the R6 region of the transgenic SCL-siRNA T-REx mouse on Dox. This population was CD71 negative and TER119 negative suggesting the effect was an increase in a non-erythrocyte population. After re-analysis of this population, we could see that the R6 population coloured in red for easy identification, were in the lower set of the forward scatter plot ruling out interference of dead cells and suggesting a larger type of cell, for example platelets (Figure 36BI, forward scatter plot). On further investigation and analysis of other mice this population was seen again in a wildtype mouse without Dox treatment (Figure 36AI, forward scatter plot). These results suggest that the effects in the R6 was not doxycycline induced and is not an apparent effect of the transgene on the adult peripheral blood population.

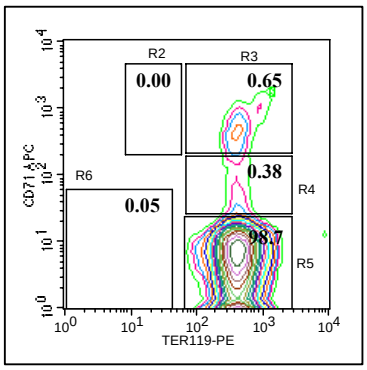
Lodish analysis of peripheral blood of F0 generation SCL-siRNA T-REx line

A. Wildtype mouse without doxycycline treatment

I. Blood forward-side scatter plot

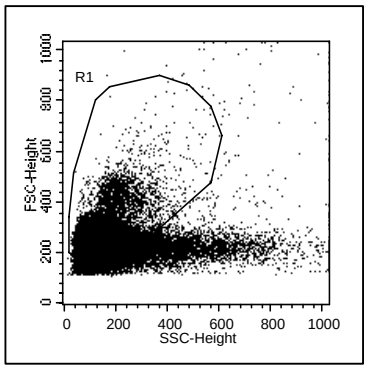


II. Blood Lodish contour plot

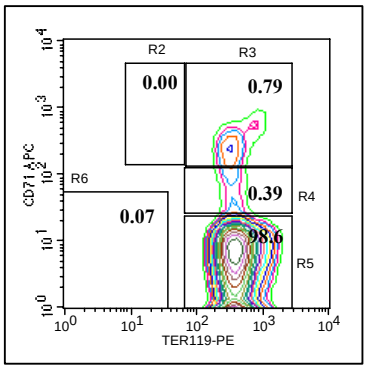


B. Wildtype mouse with doxycycline treatment

I. Blood forward-side scatter plot

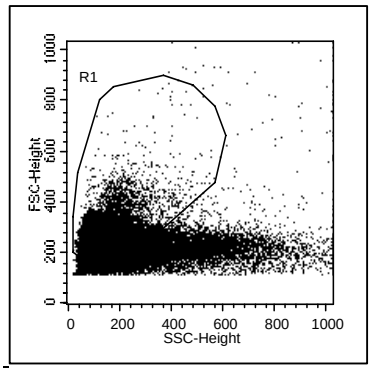


II. Blood Lodish contour plot



C. Transgenic mouse with doxycycline treatment

I. Blood forward-side scatter plot



II. Blood Lodish contour plot

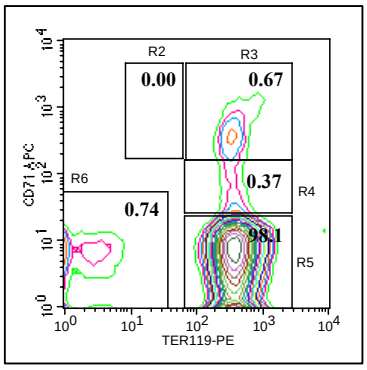


Figure 35:FACS analysis of adult peripheral blood

Wild type, littermate and transgenic line # 9774 peripheral blood analysis, double labelled with a APC-conjugated anti-CD71 monoclonal antibody (mAb) and a PE-conjugated anti-TER119 mAb. Dead cells and debris (forward scatter left panel, R1) were excluded from analysis. The right panel illustrates a density plot of all viable cells; axes

indicate relative logarithmic fluorescence units for PE (x-axis) and APC (y-axis). Regions R2 to R5 are defined by characteristic staining pattern of cells, including CD71^{med}TER119^{low}, CD71^{high}TER119^{low}, CD71^{high}TER119^{high}, CD71^{med}TER119^{high}, and CD71^{low}TER119^{high}, respectively. The percentages of TER119^{-} cells (presented as total gated cells of R6) and TER119^{+} cells (presented as total gated cells of R2 to R5 cells) are labelled at the bottom of each density plot.

Although the absolute number of cells in each of the R2 to R5 populations varied somewhat among mice, the flow cytometry profiles were consistent.

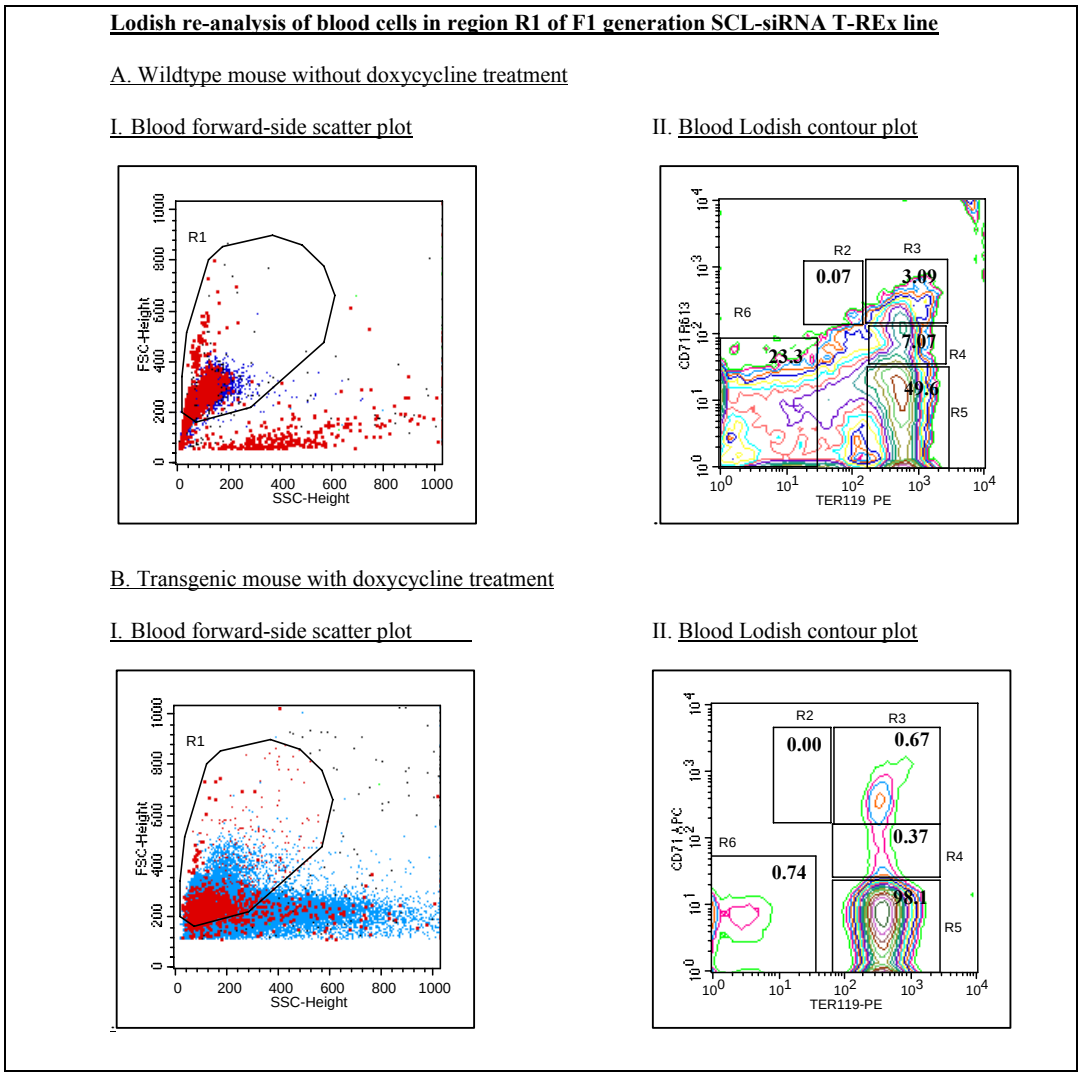


Figure 36: FACS re-analysis of mouse blood population for transgenic line #9774

Wild type and transgenic line (# 9774) bone marrow cells for analysis of erythropoiesis, double labelled with a APC-conjugated anti-CD71 monoclonal antibody (mAb) and a PE-conjugated anti-TER119 mAb. Dead cells and debris (forward scatter left panel, R1) were excluded from analysis. The right panel illustrates a density plot of all viable cells; axes indicate relative logarithmic fluorescence units for PE (x-axis) and R613 sav for the WT mouse and APC (y-axis) for transgenic mouse. Regions R2 to R5 are defined by characteristic staining pattern of cells,

including $CD71^{med}TER119^{low}$, $CD71^{high}TER119^{low}$, $CD71^{high}TER119^{high}$, $CD71^{med}TER119^{high}$, and $CD71^{low}TER119^{high}$, respectively. The percentages of $TER119^{-}$ cells (presented as total gated cells of R6) and $TER119^{+}$ cells (presented as total gated cells of R2 to R5 cells) are labelled at the bottom of each density plot.

Collectively, our results recapitulate the Lodish flow cytometry analysis (Socolovsky *et al.*, 2001) and can be used to monitor erythroid differentiation step by step.

With these results we were able to show a doxycycline induced effect in the of transgenic mouse line SCL-siRNA T-REx FRT LoxP in the bone marrow and peripheral blood after Dox induction. By contrast, the second analysed mouse line (SCL-siRNA T-REx) which showed no doxycycline induced effect in the bone marrow or peripheral blood compared to the wildtype or non-transgenic littermate on Dox.

In conclusion, the transgenic line #77 generated by co-injection SCL-siRNA T-REx FRT LoxP showed a phenotypic response as demonstrated by a significant increase in the immature red blood cell compartment when induced by Dox. This preliminary data therefore provides experimental evidence that the tet On/Off strategy used is suitable for controlling RNAi knockdown in mice.

4. DISCUSSION

RNA interference (RNAi) is a powerful tool to induce loss-of-function phenotypes by inhibiting gene expression post-transcriptionally. Synthetic short interfering RNAs (siRNAs) as well as vector-based siRNA expression systems have been used successfully to silence gene expression in a variety of biological systems. Here the development of an inducible siRNA expression system that is based on the tetracycline repressor and eukaryotic RNA polymerase III promoter H1 with a *tetO* sequence is described.

For proof of concept the expression of the murine stem cell leukaemia gene (mSCL) is selectively inhibited *in vitro* by using vector-derived short hairpin RNAs (shRNAs). Utilising a stable cell line expressing the tetracycline repressor (van de Wetering *et al.*, 2003), reduced levels of the mSCL protein were exhibited in a dose-dependent manner due to the expression of corresponding shRNAs in an inducible fashion *in vitro* as well as in an SCL RNAi mouse model. This inducible system for RNAi allows an unbiased and comparable analysis of loss-of-function phenotypes by comparing selected isogenic cell populations on the induced and non-induced level. In addition, conditional RNAi allows the study of essential and multifunctional genes involved in complex biological processes by preventing inhibitory and compensatory effects caused by constitutive knockdown.

4.1 EGFP expression for selection of shRNA vectors *in vitro* by fluorescent microscopy and flow cytometry

Green fluorescent protein (GFP) first derived from the jellyfish *Aequorea victoria* is a versatile reporter molecule which is used in many biological applications. The original molecule has been modified in order to enhance fluorescence intensity (EGFP, enhanced GFP) (Chalfie *et al.*, 1994). When utilized in a transgenic construct, cells expressing sufficient amounts of EGFP will fluoresce when exposed to a 488 nm light source.

To generate an inducible shRNA plasmid co-expressing EGFP we utilized the tetracycline responsive pTER vector (van de Wetering *et al.*, 2003), based on the pSUPER vector which harbours the RNA pol III dependent H1 promoter. We chose a

H1 driven shRNA vector as it was shown to function more efficiently than the U6 promoter (Boden *et al.*, 2003). In addition, the H1 promoter used was known to be expressed in human and mouse cells, thereby allowing us to broaden possible applications. As there was no available shRNA vector system co-expressing with EGFP at the start of this project, we developed a shRNA vector based on the pTER vector. The newly generated pTER-EGFP vector was developed to encode an independent expression cassette carrying EGFP. The additional constitutive EGFP expression cassette allows direct identification of transduced cells by fluorescent microscopy (Figure 15), and thereby provides a means of checking the efficiency of the transfection. Hans Clevers and colleagues developed an inducible siRNA system based on the tetracycline operator/repressor interaction (van de Wetering *et al.*, 2003). The idea behind their system was simple and easy to use, but first they needed to create cell lines that stably expressed the Tet repressor (by using blasticidin selection) and then stable integration of the siRNA constructs by zeocin selection. In other studies EGFP expression vectors have been co-transfected with the shRNA plasmid as a control for transfection efficiency and direct knockdown of the EGFP by siRNA-EGFP expression plasmids (Kim and Rossi, 2003; Cao *et al.*, 2004). This method was shown to be ineffective, as co-localization of the EGFP vector with the shRNA vector is not guaranteed, explained by the absence of a direct physical interaction between the co-transfected constructs *in vitro*.

Another EGFP inducible system utilises *Cre* recombinases by inserting a LoxP flanked EGFP cassette between the U6 promoter regulatory elements (the proximal site enhancer (PSE) and the distal site enhancer (DSE)), therefore impairing the promoter activity. In these studies, knockdown against EGFP was mosaic, suggesting only a partial activation of the shRNA or a non optimal shRNA structure (Coumoul *et al.*, 2004).

Until that time point the available systems were shown to be inadequate for co-expression of EGFP and shRNA. Our vector system allows for 100% transduced cell populations after FACS sorting of the RNAi-EGFP vector, without using viral means or antibody selection, which is often more expensive and time consuming. It offers highly efficient and consistent transient transfections for evaluating functional knockdown efficiencies. It also obsoletes the need for making and using stable cell lines for most applications. In addition to much faster assay set-up and cost reduction,

it also enables the functional assay of toxic genes due to its transient nature. Using this RNAi-EGFP system, we were able to quickly identify that the transfection efficiency of the M1 cell line which endogenously expressed our SCL target gene was difficult to transfect (Figure 12). This was also observed when several different transfection methods and reagents were used. We were able to circumvent time loss by working with an alternative system utilising the expression of an SCL HA-tag vector for co-transfection with the RNAi-EGFP vector into an inducible cell line for initial screening of our different shRNA vectors targeting four different sequences against the SCL mRNA *in vitro* (Figure 15).

4.2 Inducible RNAi knockdown of murine SCL *in vitro*

The Clevers group were the first to report a stable system for inducible expression of shRNAs. In their report, a tet-regulated (tet repressor (TetR)-responsive) variant of the RNA polymerase III-dependent H1 promoter was used for doxycycline (Dox)-induced shRNA expression and knockdown of β-catenin in stably transfected colorectal cancer cell clones (van de Wetering *et al.*, 2003). Inducible shRNA producing plasmids are considered to be 3rd generation siRNAs. Tetracycline based methods in the mouse (Chen *et al.*, 2003; Czauderna *et al.*, 2003; Dickins *et al.*, 2005) with an inducible siRNA expression system have also been based on the tetracycline repressor and the eukaryotic U6 and H1 RNA polymerase III promoters.

In the absence of its synthetic regulator, Dox, TetR binds to the operator and prevents expression of the shRNA transcripts by RNA polymerase III. In an initial step to assess inducible knockdown of mSCL RNA, a transient transfection was performed into a TetR expression cell line. Western blot analysis first revealed the RNAi dose-dependent knockdown (Figure 16) and then the Dox-dependent transcription of the mSCL-specific shRNA transcripts (Figure 17). In these experiments we observed knockdown efficiencies between 25 and 100%. Dox-induced shRNA transcription resulted in successful reductions of SCL-HA tag protein with all four different siRNA vectors tested by Western blotting (Figure 16, SCL siRNA 1-4). We employed a SCL HA tag-specific antibody alongside with the measurement of p38 to demonstrate functional knockdown of the target and equal loading of the protein used.
SCL protein expression was reduced by Dox-mediated de-repression of shRNA expression (Figure 17). As expected, mSCL transcription was not affected in samples

with an empty control plasmid lacking the mSCL sequence. Importantly, transient cell transfections in the absence of Dox did not result in SCL knockdown, demonstrating tight control of the system by TetR. With the pRNAi-EGFP Dsg 2 vector, a specific siRNA-based construct which was otherwise identical to the other SCL-specific vectors, no SCL knockdown was observed but EGFP expression was consistent with that of the SCL knockdown experiments (Figure 16A, lane S). We were able to show successful inhibition of mSCL protein expression correlated with induced shRNA expression, as shown in Figure 17 (TetR On/ TetR Off), comparing the induced knockdown to the non-induced control. Having demonstrated inducible protein knockdown of the mSCL in TetR cells (constitutively expressing the repressor), the addition of Dox itself showed no obvious effect on the knockdown of mSCL (Figure 16A, B and C, lane S). In conclusion, we have demonstrated that the generated plasmids are suitable for SCL mRNA knockdown and that the inducible shRNA knockdown was completely dependent on the Dox for induction of the tetracycline system. In addition, the constructed pRNAi-EGFP vector contained EGFP as a visual tag.

In relation to tetracycline kinetics, hairpin RNAs can be produced within 6 hours of the addition of Dox and increased thereafter in a time-dependent manner in RNAi clones (van de Wetering *et al.*, 2003). Another inducible RNA interference was shown in stable human colon cancer cell lines also using components of the tet system, in which the function of KILLER/DR5 is knocked down. Inducible silencing of KILLER/DR5 *in vivo* was demonstrated by injecting a stable cell line interperitoneally (IP) into mice. After exposure of mice to Dox, accelerated growth of bioluminescent tumour xenografts and conferred resistance to the chemotherapeutic agent 5-fluorouracil was seen (Wang and El-Deiry, 2004). In these experiments, the pSUPERIOR plasmid was used based on the pSUPER plasmid (Brummelkamp *et al.*, 2002). The KILLER/DR5 shRNA vector was co-transfected with a TetR-expressing vector pcDNA6/TR (Invitrogen) at a ratio of 1:2. KILLER/DR5 mRNA expression was dramatically decreased within 48 hours of Dox induction, and the increased expression of the hairpin RNAs correlated with the decreased expression of the KILLER/DR5 mRNA on Dox treatment. By contrast, withdrawal of Dox led to increased expression of mRNA, showing that the Dox-induced gene suppression was reversible.

Other inducible systems using a *Cre*-LoxP system are based on a modified U6 promoter with a LoxP site inserted into the TATA box (Coumoul *et al.*, 2004; Tiscornia *et al.*, 2004; Ventura *et al.*, 2004) and rely on a strategy where the LoxP-STOP-LoxP cassette is inserted into the hairpin loop (Fritsch *et al.*, 2004; Kasim *et al.*, 2004). All these approaches have been shown to result in inducible expression of shRNAs and subsequent knockdown of the target gene. However, these system has potential drawbacks: these systems do not allow for the reversal of the induced knockdown. Also, the *Cre* recombinase mediated recombination might be incomplete to an ineffective system (Lee and Saito, 1998). Therefore, choosing a tetracycline-mediated on/off strategy offers the advantage of complete exogenous and reversible control of the knockdown.

4.3 Generation of tetracycline repressor mice

Transgenic mice lacking specific genes have become very useful models for studying gene function *in vivo*. The classical approach is to create animal loss-of-function models for human diseases and gene function studies utilises constitutive gene knockouts involving the *Cre*-LoxP mediated systems (Bockamp *et al.*, 2002). The reason for this is to prevent any possibility of a lethality during embryogenesis, for example when the targeted gene is essential during development. We have chosen the tightly regulated tetracycline method for conditionally expressing shRNAs. This strategy was expected to prevent any abnormal or lethal phenotypes during uninduced time periods. It could for example prevent the embryo from reaching maturity resulting in an embryonic lethal phenotype.

4.4 Generation of tet repressor mice allowing for copy-number control and tissue specific expression

In order to study the knockdown of SCL in the mouse by a tetracycline responsive RNAi target vector, we needed to generate an *in vivo* tetracycline repressor (TetR) system. Knockout experiments had already shown that SCL loss-of-function results in an embryonic lethal phenotype at day E9.5 (Robb *et al.*, 1995; Shivdasani *et al.*, 1995; Visvader *et al.*, 1998; Sanchez *et al.*, 2001). Over the last decade, the tetracycline (tet) regulatory system has been extremely useful for generating reversible transgenic mouse models. This has led to a large collection of tet-controlled transgenic mice

suitable for studying gene function in many different cell types and also during selected time points (for electronically searchable databases see http://www.zmg.uni-mainz.de/tetmouse/ and http://www.tetsystems.com).

Important considerations for conditional transgene expression are that a substantial difference between the induced and uninduced state should be seen, with no constitutive activity during uninduced time points. Also, to have rapid switchability knockdown expression, the inducer (Dox or tamoxifen) should be non toxic and easy to administer. Lastly, the induced switch should be reversible so that defined developmental periods or critical stages in disease can be appropriately monitored (Bockamp *et al.*, 2002).

Switchable gene expression methods for mammalian cells allowing tight and specific regulation are rare. Using the regulatory elements of the Tn*10*-encoded tetracycline *(tetO*) resistance operon from *Escherichia coli* (Hillen and Berens, 1994), conditional systems can be developed for inducing shRNA expression. In the classic Bujard and Gossen tetracycline system, TetR is converted into a transcriptional activator by fusing it with the VP16 transcriptional activation domain which binds to *tetO* in the absence of Dox called tTA (Gossen and Bujard, 1992). Subsequently, a reversed TetR-VP16 transactivator (rtTA) was developed, which efficiently binds *tetO* only in the presence of Dox (Gossen *et al.*, 1995). To keep a gene switched off and to induce it rapidly at a given time, the rtTA system may be preferable. On the other hand, to keep a gene active and to turn it off occasionally, the tTA system may be better. In some cases however, applications of this system have initially been hampered by residual basal activity in the uninduced state (Furth *et al.*, 1994; Howe *et al.*, 1995).

We present two novel approaches in which activation by a tetracycline responsive RNAi is combined with repression in the uninduced state. For this purpose, both the RNAi responder and repressor (TetR) are expressed in the same cell and controlled by the inducer (Dox). This strategy holds all the criteria necessary for conditional expression of RNA interference (RNAi). RNAi has been extensively used for sequence-specific silencing of gene function in mammalian cells. Recently, several groups have demonstrated that RNA polymerase III expression constructs can be used for producing transgenic knockdown mice and rats (Prawitt *et al.*, 2004). Also, the

emergence of new molecular tools has allowed the design and generation of transgenic mice carrying subtle mutations whose expression can be targeted both spatially and temporally using cell type-specific (*Cre*-deleter) or inducible promoters (tetracycline system). However, until now it has not been possible to reversibly express shRNAs in transgenic animals. Our system incorporates three different Tet-repressor systems: Two were generated in our laboratory and the third one which will be discussed later, was carried out in collaboration with the Mallo group.

We first generated a novel TetR mouse expressing the TetR protein (T-REx) under the control of CMV-i.e. ß-actin regulatory promoter, based on the pCAGGs vector. This promoter contains the chicken ß-actin promoter and the first intron coupled to the CMV immediate early enhancer (Niwa *et al.*, 1991). This promoter/enhancer combination has previously been shown to drive expression in ES cells, embryos, mice and rats (Niwa *et al.*, 1991; Hadjantonakis *et al.*, 2002; Maruyama *et al.*, 2004) unlike the conventional CMV promoter contained in the original T-REx plasmid, which is very problematic and often leads to mosaic expression in mice (personal communication E. Bockamp).

The TetR expression in mouse tissue was determined by Western blot analysis using a TetR-specific antibody. The positive control a HEK 293 TR cell line stably expressing the T-REx protein has been published by Marc van de Wetering for RNAi induciblity *in vitro* (van de Wetering *et al.*, 2003). From transgenic mouse line #33 (T-REx), ten organs were analysed of which eight expressed the TetR protein. The strongest TetR signal compared to the wild type organs was from the heart, colon, tongue and spleen. Weaker TetR signals were detected in the lung, brain, thymus and kidney as compared to the wild type mouse organs. The bone marrow did not express the TetR protein. This may be explained by a insufficient detection limits of the assay or because of protein degradation. This male mouse expressing the TetR protein (T-REx mouse) was used for crosses with wildtype females to produce donor TetR-positive oocytes to give a mandelian inheritance of 50% positive transgenic mice. These TetR-expressing oocytes were used for pronuclear injection of the SCL siRNA vector to allow for tetracycline controlled repression of the RNAi embryonic lethal phenotype expected from the mSCL knockdown.

To allow for tissue specific and/or spatio-temporal knockdown of genes using the housekeeping H1 promoter, we have combined the *Cre* conditional regulation system and the tetracycline inducible system which established a second novel TetR approach, called T-REx FRT LoxP. We adopted the tetracycline regulation system and modified it one step further so that the TetR (T-REx, repressor described above) could be tissue-specifically removed from a selected cell type, thus resulting in the induction of shRNA expression.

In transgenic mice, the transgene is often integrated in multiple tandem repeats in the genome. In order to be able to generate single copy transgenic mice containing only one copy of the T-REx expression cassette, a single *Flp recombinase* target (FRT) recognition site was strategically integrated into the pCMB T-REx FRT LoxP expression vector seen in Figure 37A. Therefore, in the case of a multiple copy T-REx mouse, a single copy transgene can be obtained when crossing the transgenic T-REx mouse with a general *Flp* deleter strain (Figure 37B). Thus, removing all intervening T-REx sequences leaving only single T-REx transgenic sequence in the mouse genome.

In this regard, it was assumed that floxed single copy T-REx expression cassettes are more efficiently removed by *Cre*-recombinase than multiple copies. The TetR sequence in our mice was flanked by the two LoxP sites as shown in Figure 37A. When crossed with a tissue restricted *Cre*-deleter mouse, it is possible to induce a tissue-specific deletion of the TetR transgene (Figure 37C). The advantage of the single copy gene is that the LoxP sites can be saturated by the *Cre* recombinase enzyme allowing for efficient deletion of the gene, which has shown to be problematic in transgenic mice. *Cre* expression in general is driven by a promoter that is selected for a particular tissue-specific expression, therefore deleting the repressor in the organ of choice allowing for RNAi expression within that tissue.

To establish this system on a practical basis, we generated three transgenic lines harbouring the T-REx FRT LoxP construct. As described in section 3.13.2, determination of the TetR protein was performed by Western blot analysis.

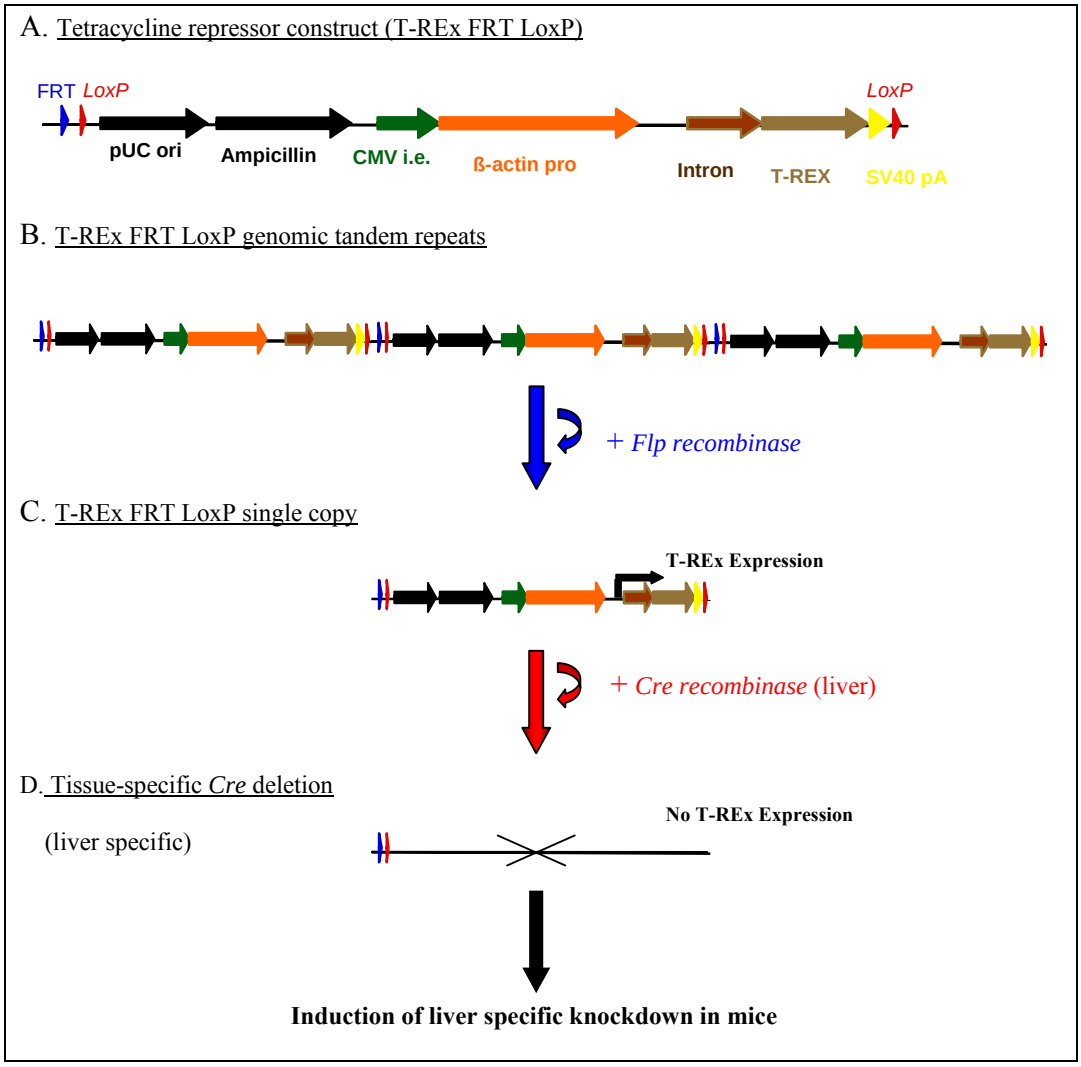


Figure 37: Conditional deletion of the tetracycline repressor results in tissue-specific knockdown

A. Schematic representation of the T-REx FRT LoxP repressor construct which harbours a FRT site and two LoxP sites for conditional expression. B. In the case of multiple tandem repeats in the genome, FLP recombinase recognizes a pair of FLP recombinase target (FRT) sequences flanking the TetR repeats. In the FLP-FRT system, FLP recombinase deletes the multicopy repeats down to a single copy of the transgene. C. P1 bacteriophage cyclization recombination (*Cre*) recombinase recognises a pair of LoxP binding sites flanking a genomic segment of interest. *Cre* enzyme deletes the intervening sequence which is flanked by complementary LoxP sequences. D. *Cre* expression is driven by a promoter that was selected for a particular tissue-specific expression pattern i.e. as shown here in the liver.

The first founder line (mouse #8687) expressed substantial amounts of the T-REx FRT LoxP repressor in the spleen, tongue, kidney, heart, liver, lung and thymus by detection with a TetR-specific antibody at 23kDa (Figure 29). Seven out of eight organs analysed expressed the TetR protein with exception of the brain. From the second line, T-REx FRT LoxP (mouse # 8665), of which eight organs were analysed,

TetR expression was detected in all of them as compared to the wild type mouse organs. High TetR expression was detected in the tongue, heart and lung while expression detected in the spleen, brain, kidney, liver and thymus was weaker (Figure 30). Analysis of the last T-REx FRT LoxP repressor line (mouse #8653) revealed that this mouse line, did not express high TetR protein levels in the eight organs analysed by Western blotting. Only the heart and liver expressed the TetR protein, whereas the spleen, tongue, kidney, brain, liver, lung and thymus did not show any detected expression (Figure 31). These results demonstrate that the TetR expression construct T-REx FRT LoxP expressed the TetR protein substantially in a variety of organs in mouse lines #8687 and #8665 under the control of the CMV-i.e. ß-actin regulatory promoter. Based on these results we have generated transgenic mouse lines expressing the doxycycline-dependent repressor and the inducible RNA polymerase III H1 promoter governing the expression of specific shRNAs. Conditional expression of the shRNA will depend on the administration of the inducer doxycycline to the drinking water of the animals. Therefore, using the repressor mouse line, in the future it should be possible to generate conditional mouse models with complete exogenous control and reversible induction of the desired knockdown phenotype for any given target gene.

The third strategy was devised in collaboration with the Mallo group (MPI, Freiburg). It involves a tetracycline repressor mouse, which utilises a transcriptional repressor domain. In this case, transcriptional repression is mediated by a tTS chimeric protein consisting of the N-terminus of the KRAB-repressor domain of the mammalian Kox1 protein fused to TetR. This transrepressor can bind to *tetO* in the absence of Dox to silence transcription of the gene of interest (Deuschle *et al.*, 1995). In the presence of Dox, the transrepressor dissociates from *tetO* binding consensus, resulting in activation of transcription of shRNA (Figure 2) by a human ß-actin promoter (Ng *et al.*, 1985). Since TetR forms dimers *in vivo*, heterodimerization could obscure these phenotypes. The tTS-KRAB expression has been seen as a TetR-KRAB fusion (TetR(B/E)-KRAB) in cells (Forster *et al.*, 1999). These have been shown not to dimerize with tTA and rtTA molecules. More recently, the TetR-KRAB was also shown to be expressed in tTS-KRAB mice (by communication of the Mallo group), which were used in this study (Mallo *et al.*, 2003).

In this study, the T-REx and tTS-KRAB repressor lines were used as oocyte donors for the injection of the tet-responsive RNAi construct. The reason for this was that the viability of the double transgenic SCL-siRNA TetR mice would be greatly enhanced by crossing the TetR mice with wildtype donor females to give a mandelian ratio of 50% transgenic offspring. It was obvious that by using TetR positive oocytes for pronuclear injection of the RNAi construct, the single transgenic lethal embryonic phenotype would be reduced and all double transgenic embryos carrying the TetR transgene and the RNAi transgene would be viable without Dox induction. As this tetracycline system was planned for the knockdown of SCL, which has an embryonic lethal knockout phenotype, this TetR mechanism, which constitutively represses RNAi expression from development on, was of prime importance for the knockdown strategy. For a better understanding of the pronuclear injection strategy involving the tetracycline repressor system, the planned experiment is depicted in Figure 38.

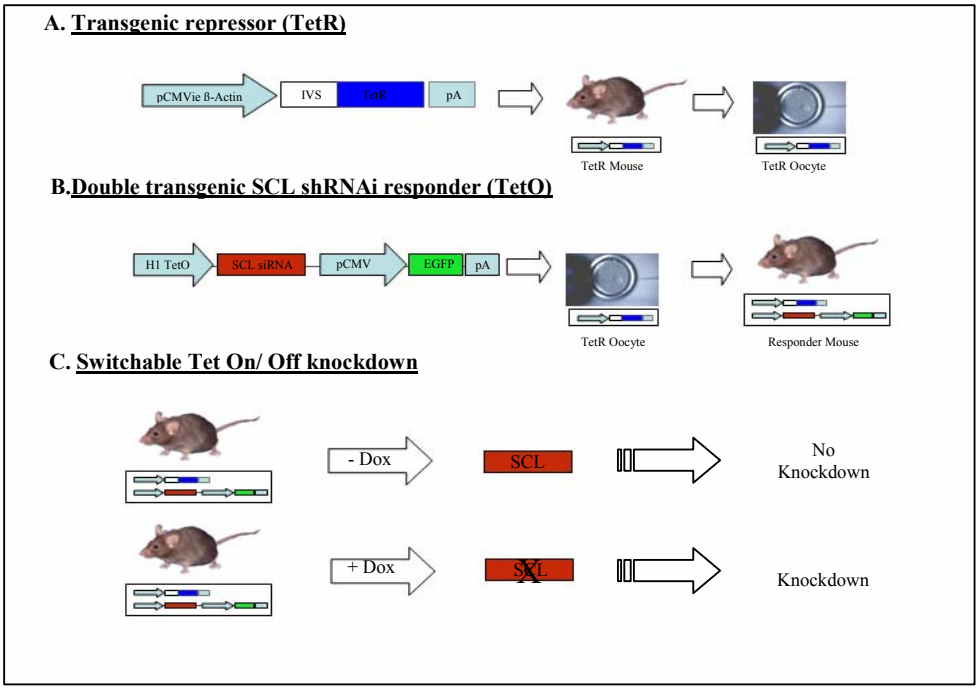


Figure 38: Conditional induction of RNAi using the tetracycline system

A conditionally induced RNAi expression system using a switchable T-REx (tetracycline-regulated expression) mouse model. Generation of a mouse expressing a ligand-dependent effector TetR (tetracycline-repressor) is used as a donor mouse for pronuclear injection of one-cell mouse embryos. Injection of the SCL shRNA responder should result in double transgenic mice, constitutively suppressing the shRNA expression and thus preventing a possible SCL lethal phenotype (TetR On,

without doxycycline (Dox)). As opposed to shRNA expression without TetR suppression (TetR Off, with Dox), in a double transgenic mice, gene knockdown can be regulated by Dox administration, making it a tightly regulated reversible system.

The three tetracycline repressor strategies applied were designed for inducible RNAi under the control of the tetracycline system, allowing reversible regulation of shRNA in the mouse with the additional possibility of tissue-specific silencing by RNAi.

4.5 Generation of tetracycline controlled RNAi mice

Inducible tetracycline-mediated shRNA expression allows for reversible, dose- and time-dependent knockdown in mice. Inducible, reversible and stable RNAi is feasible in mammalian tissue culture cells, for example by virtue of Dox-controlled suppression strategies (Matsukura *et al.*, 2003; van de Wetering *et al.*, 2003). To assess whether inducible RNAi-mediated shRNA shows a distinct knockdown of SCL-mRNA, resulting in a knockdown phenotype, we have generated double transgenic mice by pronuclear injection which harbour both tetracycline responder and repressor constructs (Figure 38 and section 3.12). Transgenic mice harbouring inducible shRNA expression constructs have been published using ectopic application (Lazrak *et al.*, 2004) or direct implantation (Lazrak *et al.*, 2004; Wang and El-Deiry, 2004), both of which do not allow for stable expression. In our study, two strategies were adopted to generate tetracycline regulated RNAi mice: the first strategy consisted in co-injection of the SCL-siRNA 2 vector with the T-REx construct driven by a constitutive promoter for high expression levels of T-REx. From 134 founder mice, two mice were found to be single transgenic for RNAi (1.49%) and one double transgenic (0.74%). Unexpectedly, all other mice were negative for both constructs. As the SCL knockout phenotype is embryonic lethal, it can be speculated that the two single transgenic mice harboured an inactive gene through DNA methylation or random integration into a silent chromosome or locus. The double transgenic mouse harbouring the two tetracycline components SCL-siRNA 2 and TetR seemed to be an adequate candidate for tetracycline induciblity as all progeny for this founder line (#77) were either wild type or double transgenic (see Figure 19C). There are two possible explanations to this observation: Either both transgenes had to be expressed to avoid shRNA induced embryonic lethality (negative selection) or both transgenes were co-injected into the same genomic locus. As mice were first screened for the presence of the siRNA vector at this stage both possibilities had to be considered.

As an alternative approach for generating conditional SCL knockdown mice, the RNAi construct was injected into tetracycline repressor positive oocytes as described in Figure 38. Table 6 summarizes the number of TetR positive mice (T-REx or tTS KRAB) and RNAi positive mice. From 112 putative T-REx mice from pronuclear injection (PNI) into T-REx oocytes, 38 (33.9%) were positive for the T-REx construct, of which only one was positive for the tetracycline responsive RNAi vector (2.6%). In contrast, from 72 putative founder mice from PNI into tTS KRAB oocytes, 35 (48.6%) were positive for the tTS KRAB construct and none were positive for the RNAi construct (0%).

Table 6: Statistical analysis of the RNAi and Tet-repressor positive mice generated from pronuclear injection (PNI)

Transgenic	PNI	Repressor positive	RNAi Bi-transgenic
T-REx	112	38	1
tTS KRAB	72	35	0
Total	**184**	**73**	**1**

A paper from the Erickson group recently described a large number of founders that had to be generated from constructs using Pol III promoters, which were employed to generate shRNA. The observed poor transmission to the next generation also suggested toxicity of the constructs, which may be explained by an interferon response (Cao *et al.*, 2005). Cao and colleagues targeted the *NAT1* and *NAT2* (N-acetyltransferases) genes which do not have a lethal phenotype in knockout studies. The percentage of live born mice that were positive for the transgene after pronuclear injection with shRNA constructs ranged from 5.9% for U6 promoter *NAT1* siRNA to 13.9% for H1 promoter *NAT1* siRNA. In the general range for other constructs, the transmission to subsequent generations was deficient, compared to 30% which are assumed to be germline mosaics (Wilkie *et al.*, 1986). This was unusual for a viable phenotype. The transmission ratio of offspring that survived until weaning was low (15%), compared to higher ratios among dead newborns (38%) and early foetuses

(30%). The fact that late foetuses (8%), also showed a low ratio indicates that the shRNA constructs were toxic to the developing embryos. As the same lack of transmission occurred with a non-endogenous target, it is unlikely that the toxicity was related to reaction with the endogenous target (*NAT1* or *2*). One possibility is that induction of an interferon response, expected for double-stranded RNAs of 30 bp or longer, but not for the shRNAs, was responsible for this fetal lethality. Induction of the mRNA of *Oas1*, an indicator for this response, ranged from 1.4 to 15.3 fold, comparable to the about 3 fold increases found using microarrays (Sledz *et al.*, 2003), but lower than the 100 fold responses found by the Bridge group (Bridge *et al.*, 2003). Thus, an interferon response to highly expressing shRNA constructs may contribute to apparent early fetal lethality of these constructs. This interferon response could be related to the amount of shRNA generated by the transgene and it is very likely that the transgenic founders which transmitted and allowed the establishment of lines had lower levels of shRNA than those which did not.

As our transgene is embryonic lethal at day 9.5, it is perfectly feasible to assume that the low number of RNAi transgenic mice is due to lethality at embryonic development and/or possible interferon toxicity as suggested (Cao *et al.*, 2005). The remaining two transgenics were probably low shRNA expressing lines, or low copy numbers, which are more likely to be transcriptionally controlled by the expression levels of the tetracycline repressor. As our internal controls (GAPDH and PD) are only 2 copies, our genotyping by PCR under sections 3.10 and 3.11 did not allow to determine copy numbers in a qualitative fashion.

More recently, lentiviral vectors have been shown to effectively deliver polymerase III promoter-shRNA constructs for knockdown transgenesis (Rubinson *et al.*, 2003; Tiscornia *et al.*, 2004; Dickins *et al.*, 2005). For CD8 silencing, copy numbers ranging from 2-6 did not correlate with percent decrease in expression (Rubinson *et al.*, 2003). Although numbers of offspring were not presented, it appears that there is a discrepancy between the percentage of positive mice (decreased) and embryos in the work of Tiscornia (Tiscornia *et al.*, 2004). Thus, the lentiviral approach to shRNA transgenics may suffer from some of the same limitations that we have found with standard pronuclear injection transgenics. After the successful generation of two

conditional SCL knockdown candidate mouse lines, it was very important to analyse a possible knockdown phenotype in these mice.

4.6 Haematopoietic expression in tetracycline regulated RNAi mice

Conditional ablation of SCL in the adult mice was recently shown to have an effect on red blood cell development by two independent groups (Mikkola *et al.*, 2003; Curtis *et al.*, 2004; Gothert *et al.*, 2005; Hall *et al.*, 2005). Since we wanted to test the two generated SCL knockdown lines for a possible phenotype, it was decided to initially focus on the known effect of SCL ablation in red blood cells. If the expression of shRNA results in the knockdown of SCL, we should be able to detect a reduction of the total number of mature red blood cells. This would indicate that conditional induction of shRNA indeed leads to a reduction of SCL protein, which is known to be required for red blood cell maturation.

In the erythroid lineage differentiation into the several morphologically defined stages, proerythroblasts (R2), basophilic erythroblasts (R3), polychromatophilic erythroblasts (R4), and orthochromatophilic erythroblasts (OEs) (R5) can be seen. The OEs extrude their nuclei (enucleation) and become reticulocytes, which further expel all organelles and detach from their microenvironment to form mature circulating erythrocytes. As erythroid differentiation proceeds, erythroblasts display a gradual decrease in cell size, increase in chromatin condensation and increase in haemoglobin concentration.

Socolovsky and collaborators have uncovered a new signal transduction pathway in which Epo receptor (EpoR) activation prevents apoptosis of red cell progenitors: direct activation by Stat5 of transcription of the anti-apoptotic protein BclX. This biochemical result is consistent, showing fetal anaemia and enhanced apoptosis in erythroid progenitors from Stat5 deficient mice (Socolovsky *et al.*, 2001). Stat5's role in adult erythropoiesis was not clear since many Stat5a$^{-/-}$5b$^{-/-}$ mice had near normal levels of red blood cells. However, in the study it was shown that adult Stat5a$^{-/-}$5b$^{-/-}$ mice with a near normal haematocrit are deficient in generating high erythropoietic rates in response to stress. Most adult Stat5a$^{-/-}$5b$^{-/-}$ mice had persistent anaemia in spite of a marked compensatory expansion in their erythropoietic tissue. Using a novel

FACS assay, the authors developed a technique showing erythroid progenitors at different stages of maturation: The Lodish plot.

The Lodish plot monitors erythroid differentiation stepwise and quantitatively by flow cytometry. In the Lodish plot experiment, CD71 (transferrin receptor) and TER119 (an erythroid-specific glycoprotein) double-stained erythroblasts are plotted against each other resulting in a distribution pattern which allows to determine the stages of erythroid differentiation (Figure 3). The settings used in our study for gating the different grades of red blood cell maturation were slightly different to the initially published gatings by Socolovsky and colleagues. Instead of determining four different populations, we were only distinguishing between very immature proerythroblasts/basophilic erythroblasts, polychromatophilic erythroblasts and mature orthochromatophilic erythroblasts/erythrocytes. In wildtype mice, these three different populations of very immature, maturated and mature cells were consistently detected. Interestingly, when the T-REx FRT LoxP expression founder line #77 (in section 3.4), generated from co-injection was analysed, an effect in the basophilic erythrocytes population was seen in the bone marrow after Dox induction (as seen in section 3.14.2). The effect was shown to be Dox dependent, compared to both the wildtype off Dox and the transgenic mouse off Dox (Figure 32). A shift in the peripheral blood population in the basophilic and late basophilic population (Figure 33) was also seen in this mouse in the F1 generation and extending to the F2 generation after a longer period of Dox administration. Therefore, implying an inheritable and dose-dependent response to the Dox-induced effect in the peripheral blood of transgenic line SCL-siRNA T-REx FRT LoxP.

The second line generated from the pronuclear injection (PNI) into the T-REx positive oocytes, SCL-siRNA T-REx (#9774), showed no obvious bone marrow or peripheral blood population change (seen in section 3.14.3). This result was compared to the positive control a wildtype mouse off Dox, expected to show a normal phenotype and a non-transgenic littermate on Dox, used to show that there was no side-effects of the Dox treatment. The lack of response on Dox of the transgenic founder suggests that there is no apparent expression of the RNAi in this mouse line for bone marrow or peripheral blood populations. This may be due to methylation in the genome of the transgene following random integration. However it is possible that there is knockdown of the SCL gene in other haematopoietic compartments in the mouse,

such as the spleen or thymus which are also affected by SCL expression, but this needs to be analysed further.

In conclusion, we have established tetracycline inducible RNAi lines harbouring two different shRNA constructs against the helix-loop-helix transcription factor SCL by new as yet undescribed techniques of co-injection and PNI into TetR oocytes. Lodish plot analysis of the doxycycline treated T-REx FRT LoxP founder line #77 showed a difference in the F1 and F2 progeny regarding the expected biological readout for SCL knockdown in bone marrow and blood. We have been able to establish a proof-of-principle technique for studying the SCL knockdown in an inducible and reversible manner. Unfortunately, due to time limitations we were unable to further analyse these mice. Furthermore, it has been possible to establish tetracycline repressor strains expressing the Tet-repressor in many tissues with the ability to be tissue-specific using both *Cre*-LoxP and *Flp*-FRT systems. These mice can be further used for analysis of any gene under tetracycline regulation and will be an added advantage to the functional analysis of gene expression in mice.

4.7 Future applications

Collectively, our data demonstrates that an inducible siRNA expression system can be applied to generate conditional loss-of-function phenotypes *in vitro* and *in vivo*. As shown in this study, the TetR-based system resulted in an extremely tight regulation of shRNA expression. This regulation allows for direct analysis of loss-of-function phenotypes in a controlled manner, phenotypic consequences for example can be studied in clonal cell populations expressing shRNAs, allowing the direct comparison between genetically identical cells where shRNA expression is repressed by the tetracycline system. It is to be expected that in future applications inducible shRNA strategies with viral vector systems for effective delivery will enhance the power of recombinant RNAi technology, both in functional genomics and for use as a potential therapeutic agent. RNAi is an alternative to the more laborious and cost intensive knockout techniques with comparative results in functional phenotypes. The interest in using RNAi in transgenic mice is also partly driven by the potency of RNAi compared to anti-sense oligodeoxynucleotides. Two studies have found shRNAs to be much more potent than similarly targeted anti-sense oligodeoxynucleotides (Miyagishi *et al.*, 2003) .

In chronic diseases, for example, long-term biological effects are desired. Thus, repeated delivery of synthetic siRNAs is needed, raising a range of possible problems (e.g. cost and route of delivery). To circumvent these problems, several research groups have shown that short hairpin siRNAs can be produced from expression plasmids that contain promoters that are dependent on either RNA polymerase (pol) II or pol III (Brummelkamp *et al.*, 2002; van de Wetering *et al.*, 2003). To date, pol III promoters are used most frequently because it is possible to express small RNAs that carry the structural feature of siRNAs (Figure 6). Mature miRNAs are single-stranded 21-nucleotide sequence. Notably, they have the potential to trigger either mRNA degradation or translational arrest (Figure 1) (Hutvagner and Zamore, 2002). Recently, RNA polymerase II driven conditional vectors have been shown to function in cell culture (Stegmeier *et al.*, 2005). This will facilitate the conditional expression of tissue specific promoters. SiRNA technology can be applied to a wide range of cancers and other proliferative disorders, in which aberrant gene expression occurs. Oncogenic and mutant tumour suppressor genes might represent potential targets for the RNAi approach. For example, p53 protein is mutated in 50% of human malignancies. In most cases, this abolishes the function of p53. Furthermore, mutant p53 transdominantly impairs the function of wild-type p53 (Bullock and Fersht, 2001) Specific elimination of p53 mutant protein by siRNAs has been shown to result in the restoration of wildtype function (Brummelkamp *et al.*, 2002; Martinez *et al.*, 2002)

While using gene-modified stem cells to correct inherited blood diseases or enhance immune response has been a very attractive idea, but it has been difficult to achieve this goal. A recent report indicates the ease with which siRNAs can be expressed in repopulating stem cells (Hemann *et al.*, 2003). In this respect, foetal liver stem cells that were infected ex vivo with a retrovirus expressing anti-p53 siRNAs were used to reconstitute the haematopoietic compartment of lethally irradiated mice. The treated animals exhibited various phenotypes, ranging from benign lymphoid hyperplasia to highly disseminated lymphomas (Hemann *et al.*, 2003). One could use this strategy to transfect, for example, stem cells from HIV-positive patients with anti-HIV siRNA constructs and re-infuse the genetically modified stem cells into the patients. The majority of patients with chronic myeloid leukaemia and a significant fraction of patients with acute lymphoblastic leukaemia have one of three aberrant fusion

transcripts encoding oncogenic proteins (Deininger *et al.*, 2000). Therefore, these patients might benefit from siRNA-modified haematopoietic stem cells.

Transfection of leukaemia cells with siRNAs targeting a BCR–ABL (proto-oncogene) fusion transcript induced apoptosis comparable to that triggered by the ABL kinase tyrosine inhibitor STI571 (signal transduction inhibitor 571) (Wohlbold *et al.*, 2003; Scherr *et al.*, 2005). Conceptually, RNAi technology can be used alone or in combination with other existing therapeutic tools. Taken together, these findings provide a basis for the development of siRNAs as therapies for cancer .

This approach of rational design could be used to create allosteric siRNAs. In certain circumstances, constitutive siRNA expression in cells or tissues is not desirable and should be controlled. In addition, such expression might provide either a growth advantage or a growth disadvantage to the cells expressing the siRNAs. Several approaches can be envisaged to overcome these problems. The tetracycline regulated expression system represents the most widely used example of regulated expression vectors (Gossen and Bujard, 1992). Our data on the regulation of siRNA expression in vivo, provides a solid first basis for further development of inducible and tissue-specific models.

5. APPENDIX

5.1 Appendix I– FACS analysis for transgenic mouse lines

5.1.1 FACS data 08.09.04: Lodish plot for adult bone marrow

File: st040908.016
Sample ID: BM Lodish
Patient ID: wt
Acquisition Date: 08-Sep-04
Gate: G1
Gated Events: 175985
Total Events: 200000

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	175985	100.00	87.99	762.90	65.37
R2	242	0.14	0.12	268.49	399.42
R3	11386	6.47	5.69	1424.72	439.95
R4	30613	17.40	15.31	1229.26	116.00
R5	69451	39.46	34.73	1094.34	12.77
R6	58929	33.49	29.46	8.79	16.26

File: st040908.068
Sample ID: BM Lodisch
Patient ID: -Dox
Acquisition Date: 08-Sep-04
Gate: G1
Gated Events: 171090
Total Events: 200000

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	171090	100.00	85.55	314.54	48.09
R2	37	0.02	0.02	83.07	307.22
R3	4399	2.57	2.20	708.44	351.23
R4	41714	24.38	20.86	548.89	108.35
R5	51873	30.32	25.94	496.83	9.87
R6	66521	38.88	33.26	5.57	14.59

File: st040908.056
Sample ID: BM Lodish
Patient ID: +Dox
Acquisition Date: 08-Sep-04
Gate: G1
Gated Events: 173898
Total Events: 200000

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	173898	100.00	86.95	647.45	85.65
R2	128	0.07	0.06	208.98	345.71
R3	18457	10.61	9.23	1342.12	471.58
R4	26191	15.06	13.10	1192.35	131.31
R5	52131	29.98	26.07	1022.26	8.72
R6	72349	41.60	36.17	7.20	18.72

5.1.2 FACS data 15.09.05: Lodish plot for adult bone marrow

File: LG040915.007
Sample ID: WT -Dox BM Lodish
Patient ID:
Acquisition Date: 15-Sep-04
Gate: G1
Gated Events: 161015
Total Events: 191888

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	161015	100.00	83.91	166.22	35.03
R2	111	0.07	0.06	43.48	209.69
R3	7991	4.96	4.16	349.93	263.40
R4	28954	17.98	15.09	281.10	66.30
R5	57617	35.78	30.03	249.78	4.84
R6	52545	32.63	27.38	4.67	13.66

File: LG040915.020
Sample ID: Tg +Dox BM Lodish
Patient ID:
Acquisition Date: 15-Sep-04
Gate: G1
Gated Events: 119728
Total Events: 139205

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	119728	100.00	86.01	105.78	40.43
R2	252	0.21	0.18	53.49	240.45
R3	6268	5.24	4.50	189.29	251.20
R4	29311	24.48	21.06	174.87	63.25
R5	31877	26.62	22.90	168.58	6.02
R6	43395	36.24	31.17	3.10	14.92

5.1.3 FACS data 08.09.05: Lodish plot for peripheral blood

File: st040908.084
Sample ID: Blood Lodish
Patient ID: - Dox
Acquisition Date: 08-Sep-04
Gate: G1
Gated Events: 195533
Total Events: 200000

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	195533	100.00	97.77	739.10	6.95
R2	0	0.00	0.00	***	***
R3	853	0.44	0.43	958.99	282.57
R4	517	0.26	0.26	779.18	77.03
R5	193890	99.16	96.95	736.61	5.21
R6	48	0.02	0.02	3.28	10.41

File: st040908.079
Sample ID: Blood Lodish
Patient ID: + Dox
Acquisition Date: 08-Sep-04
Gate: G1
Gated Events: 195646
Total Events: 200000

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	195646	100.00	97.82	635.80	7.81
R2	1	0.00	0.00	140.75	149.89
R3	1219	0.62	0.61	841.17	364.30
R4	446	0.23	0.22	742.24	74.38
R5	193209	98.75	96.60	635.06	5.29
R6	33	0.02	0.02	3.75	6.55

5.1.4 FACS data 15.09.05: Lodish plot for peripheral blood

File: LG040915.004
Sample ID: WT -Dox Blood Lodish
Patient ID:
Acquisition Date: 15-Sep-04
Gate: G1
Gated Events: 52520
Total Events: 55115

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	52520	100.00	95.29	187.69	5.30
R2	0	0.00	0.00	***	***
R3	265	0.50	0.48	238.13	290.55
R4	213	0.41	0.39	192.45	57.85
R5	51975	98.96	94.30	187.40	3.58
R6	9	0.02	0.02	3.63	8.16

File: LG040915.026
Sample ID: Tg -Dox Blood Lodish
Patient ID:
Acquisition Date: 15-Sep-04
Gate: G1
Gated Events: 53803
Total Events: 55895

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	53803	100.00	96.26	192.64	5.54
R2	1	0.00	0.00	13.58	152.61
R3	328	0.61	0.59	224.31	262.87
R4	157	0.29	0.28	222.13	35.67
R5	53231	98.94	95.23	192.20	3.69
R6	33	0.06	0.06	1.86	4.44

File: LG040915.015
Sample ID: Tg +Dox Blood Lodish
Patient ID:
Acquisition Date: 15-Sep-04
Gate: G1
Gated Events: 53496
Total Events: 55467

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	53496	100.00	96.45	90.68	6.26
R2	3	0.01	0.01	7.10	156.74
R3	648	1.21	1.17	94.67	161.35
R4	487	0.91	0.88	101.16	39.38
R5	52212	97.60	94.13	90.54	3.92
R6	24	0.04	0.04	1.25	6.00

5.1.5 FACS data 05.07.05: Lodish plot for adult bone marrow

File: LG050705.052
Sample ID: 9750 BM CD71 APC TER119
Patient ID:
Acquisition Date: 05-Jul-05
Gate: G1
Gated Events: 274561
Total Events: 302520

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	274561	100.00	90.76	238.08	167.48
R2	790	0.29	0.26	69.23	433.10
R3	54225	19.75	17.92	446.48	665.96
R4	41662	15.17	13.77	400.01	90.23
R5	55632	20.26	18.39	333.24	8.48
R6	101377	36.92	33.51	5.85	11.59

File: LG050705.033
Sample ID: 9774 BM CD71 APC TER119
Patient ID:
Acquisition Date: 05-Jul-05
Gate: G1
Gated Events: 200565
Total Events: 218955

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	200565	100.00	91.60	276.39	190.89
R2	552	0.28	0.25	67.17	455.02
R3	39373	19.63	17.98	518.21	815.98
R4	27435	13.68	12.53	438.30	89.59
R5	44774	22.32	20.45	387.90	8.51
R6	74276	37.03	33.92	6.22	12.08

5.1.6 FACS data 05.07.05: Lodish plot for peripheral blood

File: LG050705.003
Sample ID: WT Blood CD71 APC TER
Patient ID:
Acquisition Date: 05-Jul-05
Gate: G1
Gated Events: 467732
Total Events: 503100

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	467732	100.00	92.97	440.83	12.14
R2	3	0.00	0.00	23.52	1826.25
R3	3047	0.65	0.61	578.58	725.16
R4	1781	0.38	0.35	456.76	77.96
R5	461972	98.77	91.83	436.26	6.13
R6	244	0.05	0.05	4.84	7.84

File: LG050705.041
Sample ID: 9750 Blood CD71 APC TER119
Patient ID:
Acquisition Date: 05-Jul-05
Gate: G1
Gated Events: 558436
Total Events: 604528

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	558436	100.00	92.38	408.45	10.63
R2	3	0.00	0.00	39.31	1379.92
R3	4407	0.79	0.73	448.03	407.89
R4	2157	0.39	0.36	400.99	63.01
R5	550847	98.64	91.12	405.75	6.40
R6	386	0.07	0.06	7.98	8.75

File: LG050705.022
Sample ID: 9774 Blood CD71 APC TER119
Patient ID:
Acquisition Date: 05-Jul-05
Gate: G1
Gated Events: 584711
Total Events: 657285

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	584711	100.00	88.96	392.31	12.24
R2	5	0.00	0.00	23.66	1487.96
R3	3901	0.67	0.59	449.13	610.09
R4	2191	0.37	0.33	398.55	65.69
R5	573618	98.10	87.27	391.74	6.73
R6	4310	0.74	0.66	3.11	6.70

File: st040908.074
Sample ID: Blood Lodish
Patient ID: wt
Acquisition Date: 08-Sep-04
Gate: G1
Gated Events: 15839
Total Events: 20100

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	15839	100.00	78.80	351.67	34.27
R2	11	0.07	0.05	100.26	188.43
R3	489	3.09	2.43	603.45	226.54
R4	1120	7.07	5.57	491.60	68.99
R5	7859	49.62	39.10	524.64	10.47
R6	3704	23.39	18.43	9.45	7.42

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7. CURRICULUM VITAE

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Paper

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