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Conditional inactivation of *Emilin1* and *Col6a1* genes

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THESIS CONTENTS

ABBREVIATIONS

BMP: Bone Morphogenic Protein
BAC: Bacterial Artificial Chromosome
bp: base pair
BSA: Bovine Serum Albumin
Cre: Causes REcombination
DMEM: Dulbecco's Modified Eagle Medium
Dpc: Days post coitum
ECs: endothelial cells
ECM: Extracellular matrix
EDEN: Emi Domain Endowed Proteins
EDTA: Ethylenediaminetetraacetic acid
Emilins: Elastin Microfibril Interface Located Proteins
ER: Estrogen Receptor
ES cell: Embryonic Stem cell
FBS: Foetal bovine serum
gC1q: Globular C1q domain
Kb: kilobase
kDa: kilo Dalton
LAP: Latency Associated Peptide
LBD: Ligand Binding Domain
LIF: Leukemia Inhibitory Factor
LLC: Large Latent Complex
loxP: LOcus of X over in PI
LTR long-terminal repeats
LTBP: Latent TGF-β binding protein
MEF: Primary embryonic fibroblasts
MFS: Marfan syndrome
Neo: Neomycin
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PGK: Phospho-Glycerate Kinase
SLC: Small Latent Complex
SMC: Smooth Muscle Cells
SMMHC: Smooth Muscle Myosin Heavy Chain
TGF-β: Transforming Growth Factor beta
Tk: Thymidine kinase
WPRE: Woodchuck PostRegulatory Element

ABSTRACT

Conditional gene expression methods are important approaches for examining the function of particular genes in development and disease. In particular, a lentiviral vector system for RNAi-mediated in vivo silencing of *Col6a1* and a Cre-*loxP* based procedure for conditional-inducible knockout (KO) of *Emilin1* were used in this work.

The use of lentiviral vectors can accelerate the generation of animals with substantial suppression of gene expression in an inducible way. Some authors have observed limitations of the technique due to low efficiency of transgenesis and mosaicism in transgenic mice. We have generated several transgenic mouse lines expressing siRNAs that target the α1(VI) mRNA. Characterization of the different lines and comparison of the phenotypes with that of *Col6a1* knockout mice have allowed a systematic evaluation of the different factors affecting silencing of *Col6a1*, a gene of the extracellular matrix with a complex pattern of tissue-specific expression. The results, obtained with vectors pLVTHM and pLVPT-rtTRKRAB, point out three parameters as major determinants of the efficiency of interference: the choice of interfering sequence, the number of proviral copies integrated into the mouse genome and the site of integration of the provirus. A lentiviral vector (pLVPT-rtTRKRAB) with doxycycline inducible production of shRNA was also tested. Control of expression by the drug was stringent in many tissues; however, in some tissues turning off of shRNA synthesis was not complete. The data support the application of the lentiviral vectors used here in transgenesis (Frka, Facchinello, et al., 2009).

Emilin1 is a gene coding for a protein, Emilin-1, of the elastic extracellular matrix expressed in interstitial connective tissue and in the cardiovascular system starting form early stages of embryonic development to adulthood. *Emilin1* null mice display reduced diameter of blood vessels and arterial hypertension. The protein regulates the bioavailability of TGF-β, a cytokine with major effects on the cardiovascular system. Specifically, it has been shown that *Emilin1* inhibits proteolysis of the proTGF-β precursor to LAP/TGF-β, a complex from which the growth factor can be subsequently released for receptor binding. In the absence of *Emilin1*, the amount of active TGF-β is increased, reducing the proliferation rate of smooth muscle cells and the diameter of blood vessels. To establish whether the *Emilin1*$^{-/-}$ phenotype is the result of a developmental defect or the function of the protein is required for the regulation of blood pressure and arterial structure also in the adult, a conditional gene targeting procedure was used to inactivate *Emilin1* in a tissue and time-specific manner. The genetic set up of the transgenic mouse model included the use of floxed *Emilin1*, CreER^{T2} (a tamoxifen inducible Cre recombinase) tissue-specific drivers and Rosa26R-*lacZ*, an inducible reporter for histological visualization of gene rearrangement.

A targeting vector was synthesized in which exons 1-2 of the *Emilin1* gene were flanked by *loxP* sites (floxed allele). The correct integration of the targeting construct in embryonic stem (ES) cell clones was confirmed by Southern blot and PCR analyses. Four of the ES cell clones were subsequently injected into blastocysts resulting in chimeric mice with 10-100% chimerism.

The CreER^{T2} gene was driven by the *Emilin1* or the smooth muscle myosin heavy chain (*SMMHC*) promoters in order to be expressed in cells active in Emilin1 synthesis or in smooth muscle cells respectively. After tamoxifen administration, activity of both promoters was evident in vascular and visceral smooth muscle cells, where *SMMHC*-CreER^{T2} induced recombination more strongly than *Emilin1*-CreER^{T2}. PCR and RT-PCR analyses confirmed that the *SMMHC*-CreER^{T2} was expressed and efficiently excised the *loxP* flanked sequences in the *Emilin1*$^{flox/wt}$ locus sufficiently to reduce *Emilin1* mRNA expression.

Emilin1$^{flox/flox}$ mice appeared and bred normally and showed no difference in *Emilin1* mRNA expression and in blood pressure levels as compared to controls, confirming that introduction of the *loxP* sites did not interfere with regulation of the gene.

Moreover preliminary data showed that adult animals of the double transgenic lines *Emilin1*$^{flox/flox}$ and *SMMHC*-Cre-ER^{T2} treated with tamoxifen displayed increased blood pressure. This result suggests that hypertension in *Emilin1*$^{flox/flox}$ mice is not due to a developmental defect of blood vessels, but to the lack of a continuous effect of Emilin-1 on blood pressure regulation even in the adult.

ABSTRACT (Italiano)

Tecniche che prevedono il controllo dell'espressione genica tramite inattivazione condizionale sono di particolare utilità nello studio della funzione di geni durante lo sviluppo e nel determinare patologie. In questo lavoro sono stati utilizzati un sistema di silenziamento in vivo del gene *Col6a1* mediante RNAi utlizzando vettori lentivirali e un sistema "Cre/loxP" per generare un modello murino di *knockout* condizionale inducibile.
L'uso di vettori lentivirali può accelerare la generazione di animali transgenici con una consistente riduzione dell'espressione genica e in modo inducibile, anche se in letteratura alla tecnica sono state attribuite alcune limitazioni dovute alla bassa efficienza di transgenesi e alla presenza di mosaicismo nei topi così ottenuti. In questo lavoro abbiamo prodotto diverse linee di animali transgenici con diminuiti livelli di messaggero per il gene *Col6a1* grazie alla produzione di siRNA. La caratterizzazione delle varie linee e la comparazione del fenotipo con quello dei topi *knockout* per lo stesso gene ha permesso l'identificazione dei diversi fattori che sono in grado di influire sul processo di silenziamento di *Col6a1*, un gene codificante per una proteina della matrice extracellulare con un complesso pattern di espressione tessuto specifica. I risultati ottenuti con i vettori pLVTHM e pLVPT-rtTRKRAB, hanno permesso di definire tre importanti fattori come maggiori determinanti nell'efficienza dell'interferenza: la scelta della sequenza interferente, il numero di copie provirali integrate nel genoma e il sito di integrazione degli stessi. É stato inoltre utilizzato un vettore lentivirale (pLVPT-rtTRKRAB) per la produzione inducibile di shRNA mediante somministrazione di doxyciclina. Il controllo dell'espressione dopo doxycicilina è risultato essere stringente in molti tessuti, anche se in alcuni la inattivazione della produzione di shRNA non è stata completa. I risultati ottenuti dimostrano l'applicabilità di vettori lentivirali nella generazione di animali transgenici (Frka, Facchinello, et al., 2009).

Emilina1 è una proteina della matrice extracellulare presente nei tessuti connettivi interstiziali e nel sistema cardiovascolare a partire da stadi precoci di sviluppo embrionale e nell'adulto. I topi mancanti di *Emilina1* sono ipertesi e mostrano un diametro ridotto dei vasi. Emilina1 svolge la sua funzione regolando la biodisponibilità di TGF-β, una citochina che svolge importanti funzioni nel sistema cardiovascolare. In particolare, è stato dimostrato che Emilina1 inibisce la proteolisi del precursore pro-TGF-β a LAP/TGF-β, un complesso dal quale il fattore di crescita attivo deve essere successivamente rilasciato per permetterne il legame con il recettore. In assenza di Emilina1, la quantità di TGF-β attivo è aumentata, riducendo la proliferazione delle cellule muscolari lisce e quindi il diametro del vaso. Per stabilire se il fenotipo dei topi *Emilina1*$^{-/-}$ è il risultato di un'alterata morfogenesi dei vasi o se la funzione della proteina è richiesta nella regolazione della pressione e nella struttura del vaso anche nell'animale adulto, è stato necessario produrre un *knockout* condizionale del gene di *Emilin1* in modo da indurre l'assenza della proteina con un preciso controllo temporale e spaziale. Il modello murino prevede la presenza di siti *loxP* posti nel gene di *Emilin1* in modo da produrre l'inattivazione genica, una Cre-ER^{T2} (cioè una Cre ricombinasi inducibile tramite tamoxifen) e il locus Rosa26R-*lacZ* (un gene *reporter* inducibile per la rilevazione istologica delle cellule nelle quali si è avuto il riarrangiamento genetico).

É stato preparato un costrutto dove l'esone 1 e 2 del gene di *Emilina1* sono fiancheggiati da due siti *loxP*. Mediante *Southern Blot* e *PCR,* è stata verificata la corretta integrazione del costrutto nelle cellule embrionali staminali di topo. 4 cloni ricombinanti omologhi così identificati sono stati trasferiti in vivo mediante microiniezione in blastocisti ottenendo topi chimerici con un grado di chimerismo compreso tra il 10 e il 100%.
L'espressione del gene Cre-ER^{T2} è stato posto sotto il controllo delle sequenze promotoriali proprie di *Emilina1* o, alternativamente, del gene per la catena pesante della miosina di muscolo liscio, in modo da essere attivamente espressa dalle cellule che producono Emilina1 o da quelle muscolari lisce rispettivamente. Dopo la somministrazione di tamoxifen, l'attività di entrambi i promotori è evidente sia nelle cellule muscolare lisce vascolari e viscerali, dove *SMMHC*-CreER^{T2} induce una ricombinazione più efficiente rispetto al costrutto *Emilina1*-CreER^{T2}. Le analisi mediante *PCR* e *RT-PCR* confermano che la cre ricombinasi sotto il promotore della miosina di muscolo liscio induce efficientemente la ricombinazione dei siti *loxP* nel locus di *Emilina1,* permettendo in questo modo una riduzione consistente dei livelli di messaggero.
I topi *Emilina1*$^{flox/flox}$ generati sono fertili e presentano un fenotipo normale e inoltre comparati con degli animali di controllo non mostrano differenze per quanto riguarda l'espressione del messaggero di *Emilina1* e nei livelli di pressione sanguigna, confermando che l'introduzione dei siti *loxP* non interferisce con la regolazione dell'espressione del gene.
Risultati preliminari inoltre indicano un aumento della pressione sanguigna nelle doppie linee transgeniche *Emilina1*$^{flox/flox}$ e *SMMHC*-Cre-ER^{T2} in seguito a trattamento con tamoxifen. Questo risultato suggerisce che l'ipertensione non è dovuta ad un'alterata morfogenesi dei vasi sanguigni, ma al ruolo continuativo di Emilina-1 nella regolazione della pressione sanguigna anche nell'adulto.

INTRODUCTION

Over the last few years, the complete genome sequences for several metazoan organisms have been determined. As a mammal, the mouse shares similarity to humans in development, behaviour, anatomy, physiology, and pathology. Almost 99% of mouse genes have orthologues in humans. Molecular and functional characterizations of mouse genes have provided valuable insights to potential functions of human orthologues. Transgenic and knockout technologies allow gene function studies in the mouse that are not possible in many other organisms (e. g. the ability to create large scale mutations in ES cells). In addition, transgenic or knockout animals have increasing importance in biotechnological applications including improvement of livestock, xenotransplantation and the production of biologically active pharmaceuticals. The goal of my project is the use of existing systems for conditional RNAi and for conditional-inducible knockout

RNAi has the potential to permit the downregulation of virtually any gene, but in many circumstances it is desirable that the knockdown be externally controllable. For instance, in tissue culture, external control of the downregulation permits parallel analysis of the 'off ' and 'on' states of a gene. Inducible knockdown also allows researchers to examine the function of genes essential during development in differentiated tissues in transgenic animals. Conditional knockdown is also useful for modeling human pathologies in vivo, to induce and revert at will the shutoff of a disease-influencing gene, for example an oncogene or a tumor suppressor gene in human cancer cells xenotransplanted into rodents. Controllable gene knockdown can thus impact a wide range of fields, from developmental biology to therapeutic drug screening. In particular, an essential distinction should be made between reversible and nonreversible conditional systems. Reversible gene knockdown will generally rely on the pharmacologically controlled. The most popular systems are doxycycline and ecdysone controlled units, which take advantage of regulating molecules that can easily be added to cells in culture or administered to animals. Irreversible gene knockdown systems, in contrast, make use of recombinases such as Cre or Flp, which allow the one-time excision of an inactivating sequence that otherwise prevents the expression of a gene or interfering RNA. Excision is completed, the system cannot be switched back to its original state, hence the knockdown is permanently established or abrogated. The main field of application of this technique will likely be the generation of conditional-knockdown animals.

For the purposes of transgenesis, the primary benefit in using the lentiviral vector system is its efficient ability to integrate into the host genome (Naldini et al., 1996, Park et al., 2000). The latter characteristic could be a distinct advantage for lentiviral transgenesis compared with pronuclear microinjection by allowing for more rapid integration into the genome and reducing the potential for genetic mosaicism. In my PhD I used a system for lentiviral-mediated RNAi in vivo silencing of *Col6a1* and for conditional-inducible knockout (KO) of *Emilin1* gene. The use of lentiviral vector can accelerate the generation of animals with substantial suppression of gene expression in an inducible way and I analyze how different factors influence RNAi mediated silencing of *Col6a1*. The second project is the generation of a inducible-conditional knockout in which Emilin1 inactivation is induced in adult mice, to understand the general relevance of *Emilin1*/TGF-β regulation of blood pressure in the pathogenesis of hypertension.

KNOCKDOWN OF *COL6A1* EXPRESSION USING LENTIVIRAL VECTOR

In the first part of my PhD I completed the work just started for in vivo silencing of the *Col6a1* gene via RNAi using lentiviral vectors. An alternative procedure for targeted gene inactivation has become available in the recent years based on RNA interference (RNAi) (Mello and Conte, 2004). The method is particularly efficient in cells cultured in vitro, where interfering sequences can be either administered directly as transfected small interfering RNAs (siRNAs) or expressed as small hairpin RNAs (shRNAs), that are processed to siRNAs in the cell, using retroviral vectors. Transfer of shRNA producing constructs into mammalian embryos has been realized through pronuclear injection and transfection into embryonic stem (Xia et al., 2006). These methods suffer several limitations and a more promising method for large-scale application entails the use of lentiviral vectors, a gene delivery system that does not suffer developmental repression and that efficiently transduces embryos from many different species (Pfeifer, 2004). The method has been applied for a limited number of genes with variable effects on their expression levels. Moreover some authors have observed limitations of the technique due to low efficiency of transgenesis and mosaicism in transgenic mice (Kirilov et al., 2007).

For example, it is not known whether silencing of genes with a complex pattern of expression is equally efficient in all tissues where such genes are activated; likewise, no information is available on silencing achievable for genes whose protein products have a slow turnover and persist for long time in the tissues. These two aspects are particularly relevant for genes coding for extracellular matrix components. In this pproject I analyze how different factors influence RNAi mediated silencing of *Col6a1*, a gene that codes for one subunit of collagen type VI. Collagen VI is an extracellular matrix protein composed of three different polypeptides, α1(VI), α2(VI) and α3(VI), encoded by separate genes (Lampe and Bushby, 2005). All three chains are necessary for the assembly of the triple helical conformation that characterizes the native collagen VI molecule. When one of the three chains is missing, the other two are synthesized, but cannot assemble into the triple helical conformation and are degraded inside the cell (Lamandé et al., 1999). As a consequence, no collagen VI molecules are deposited in the extracellular matrix (Figure 1). Deficiency of collagen VI in the mouse induces muscle alterations that mimic those found in two human heritable diseases of collagen VI, Bethlem myopathy and Ullrich congenital muscular dystrophy (Bonaldo et al., 1998, Angelin et al., 2007). Information on the regulation of expression of collagen VI comes from studies on the α1(VI) chain (Braghetta et al., 1996, Vitale et al., 2001, Braghetta et al., 2008). Regulation is mainly achieved through different enhancers, each of which controls transcription only in a limited set tissues. Available evidence indicates that enhancer activation is the consequence of inductive signals on collagen VI producing from nearby cells (e.g. myoblasts on connective tissue cells of skeletal muscle; neurons on peripheral glia precursors). This type of regulation produces variable levels of α1(VI) collagen mRNA in different tissues. Considering that siRNA expression may be variable due to the disparate site of insertion of the interfering transgene into the mouse genome, the ratio of siRNA/mRNA may be critical to achieve efficient knock- down. This potential source of diversification makes collagen VI a useful model to study the factors influencing silencing in different tissues. Were generated several transgenic mouse lines

expressing siRNAs that target the α1(VI) mRNA. Characterization of the different lines and comparison of the phenotypes with that of Col6a1 knockout mice have allowed a systematic evaluation of the different factors affecting silencing of the target gene. *Col6a1* gene was chosen since KO mice exhibit a well-characterized myopathic phenotype (Bonaldo et al., 1998). Therefore, the phenotype of KD mice generated through RNAi can be easily compared with those of KO animals. In addition, the applicability of an inducible lentiviral vector system in gene knockdown experiments has also been analyzed. This system took advantage of the promiscuous repression activity of tTRKRAB, a fusion protein between the Krüppel-associated box (KRAB) domain found in many vertebrate transcriptional regulators and the tetracycline repressor (tetR) of *Escherichia coli*. About one-third of all zinc-finger DNA binding proteins contains a KRAB domain. When tethered to DNA, KRAB recruits a multimolecular complex that leads to histone deacetylation and methylation and binding of heterochromatin protein 1, thus creating a local heterochromatin domain extending over a regions of 2–3 kilobases on either side. The tTRKRAB-mediated repression of cellular Pol II or Pol III promoters juxtaposed to tet operator (tetO) sequences can be reversibly controlled by doxycycline (DOX). By fusing the KRAB domain with a reverse tetR was created a Tet-off version of system. In this Tet-off system in the absence of DOX, rtTR-KRAB is sequestered away from tetO, thus allowing transgene expression and downregulation of the target gene by RNA interference (Szulc et al., 2006).

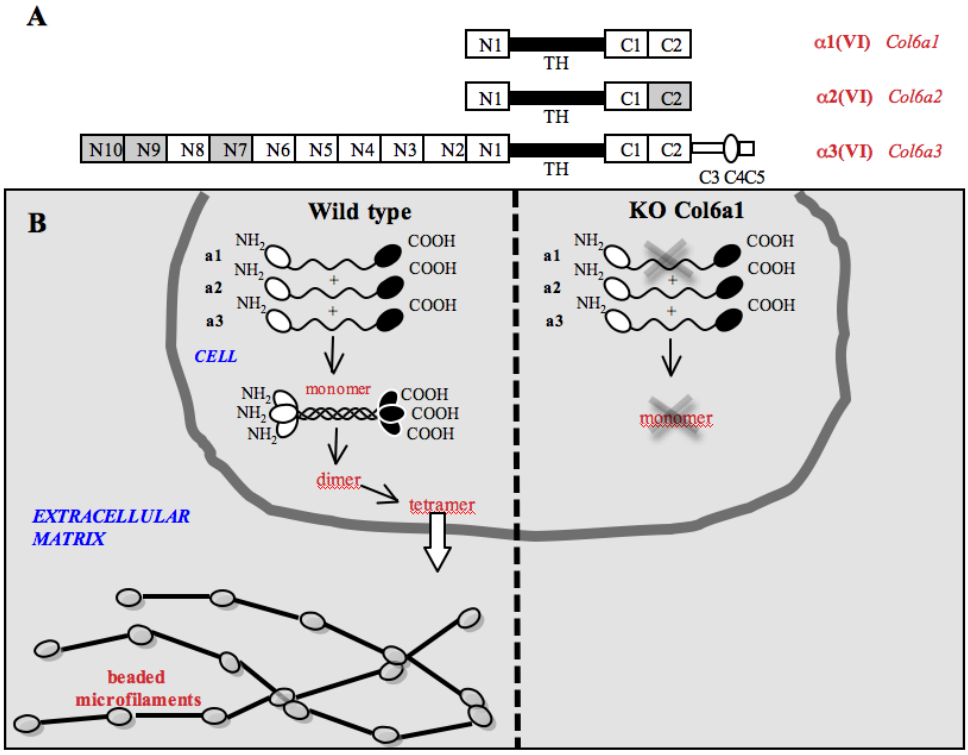

Figure 1. Schematic diagram of ColVI structure and assembly. (A) Protein domains of α1(VI), α2(VI), and α3(VI) chains. ColVI subunits contain a short triple helical (TH) region flanked by three vWF-A modules (N1, C1, C2). The α3(VI) chain contains nine additional vWF-A modules (N10-N2) at the N-terminal end, and three peculiar domains (C3-C5) at the C-terminal end. (B) Main steps of ColVI assembly and secretion.

DISSECTING THE ROLE OF EMILIN1 IN ARTERIAL HYPERTENSION THROUGH THE USE OF A CONDITIONAL AND INDUCIBLE GENE KNOCKOUT IN THE MOUSE

An adequate blood flow is essential for the function of different organs, and mammals have evolved complex mechanisms to adjust the levels of blood pressure of the systemic circulation necessary to force blood through the arterial tree. In humans, however, the blood pressure raises in an age-dependent fashion and, in more than 30% of people over 40 in most developed countries, results into a condition of high blood pressure, also called arterial or systemic hypertension, that is considered an important risk factor for heart, brain and kidney diseases. While in a proportion of cases hypertension is defined secondary, e.g. due to identifiable and curable causes, in the vast majority it is due to unidentified mechanisms that most likely entail a complex interaction between environmental factors and genetic predisposition and is dubbed essential hypertension. Genetic factors are major determinants of blood pressure, accounting for roughly 40% of blood pressure values variance (Corvol et al., 1992). However, as expected from the complexity of blood pressure regulation, different constellations of genes may contribute to the development of hypertension, so that, in most cases, it is not possible to trace back the alteration to the effect of a single gene. In this landscape, rare monogenic types of hypertension are of enormous value for the identification of genes that can influence the complex phenotypic manifestation of the disease and the disclosure of the cellular pathways involved.

A monogenic form of hypertension has been discovered in the mouse in a study on Emilin-1, a protein of elastic extracellular matrix, (Zacchigna et al., 2006). Emilin-1 controls blood pressure by regulating the activity of TGF-β1, a cytokine deeply involved in development, homeostasis and pathology of blood vessels (Bobik, 2006). In particular, Emilin-1, through its EMI domain, inhibits TGF-β1 signalling by binding specifically to the immature proTGF-β1 precursor and preventing its maturation by furin proprotein convertases thus limiting the bioavailability of active TGF-β1. Increased TGF-β1 signaling in *Emilin1* deficient mice decreased proliferation of smooth muscle cells and reduced the diameter and the thickness of the media. It was therefore hypothesized that hypotrophic remodeling of resistance arteries may be responsible for the increased peripheral resistance and elevated blood pressure of Emilin1-/- mice (Zacchigna et al., 2006). However, unpublished data suggest that this may not be the case. In fact, reversal of the hypertensive phenotype can be accomplished by reducing TGF-β activity by treating Emilin1-/- mice with antibodies to TGF-β (Lembo and Vecchione, unpublished results). This effect is specific and the normalization of blood pressure is rather quick, 5 days, a time insufficient to bring the reduced diameter of the arteries back to normal, indicating that the smaller lumen of blood vessels is not the direct cause of hypertension and that a continuous TGF-β activity is required for the maintenance of the hypertensive phenotype. Additional experiments have shown that the drop of contractile response to vasoconstrictors, such as phenylephrine, attained by inhibition of TGF-β in blood vessels is much higher in *Emilin1* null mice than control animals and that the administration of the α1-adrenergic receptor antagonist prazosin normalizes blood pressure in Emilin1 deficient mice (Lembo and Vecchione, unpublished results). Thus, our

present suggestion is that Emilin-1, through TGF-β regulation, sets the strength of the contractile response of smooth muscle cells to physiological vasoconstrictors and that hypertension of mutant mice is due to increased contractility induced by unrestrained TGF-β signaling.
In apparent contrast with this concept, however, is the observation that contraction of Emilin1-/- arteries is lower compared with controls. We think that the conflicting results can be reconciled by considering that Emilin1-/- arteries exhibit significant ultrastructural alterations, in addition to hypertension and hypotrophic remodeling. These consist in discontinuities and irregularities of elastic lamellae, reduced adhesion sites of smooth muscle cells to elastic fibers and the presence of enlarged membranous organelles (ER and mitochondria) within the cells (Zanetti et al., 2004). These ultrastructural changes are established during morphogenesis of the arterial wall and may be the outcome of either the absence of the adhesive function of Emilin-1 or the result of TGF-β dysregulation during development. Thus, it may be possible that these fine alterations of the arterial wall structure reduce the efficiency of contraction of Emilin1-/- arteries even if the actual contraction of cells is increased. To validate this hypothesis, a new transgenic mouse is needed in which the entire development is completed normally and then Emilin-1 expression is shut off in the adult, a task feasible through conditional and inducible gene targeting. The new mouse model not only should shed light on the role of Emilin-1 in the vascular system, but will also reveal whether Emilin-1 regulation of TGF-β may have a general role in the development of hypertension in the adult.
The inducible conditional *Emilin1* knockout model has been generated by using the Cre-ER^{T2} hybrid, in which the Cre recombinase is fused to a mutated ligand binding domain of estrogen receptor that does not recognize estrogens, but binds the synthetic estrogen analogue tamoxifen (Feil et al., 1997). As a consequence, the hybrid protein translocates into the nucleus and catalyzes recombination at *loxP* sites only upon administration of tamoxifen. As Cre driver, the *Emilin1* and the smooth muscle myosin heavy chain (*SMMHC*) promoters have been used (Fabbro et al., 2005, Wirth et al., 2008). The first guides Cre-ER^{T2} synthesis in cells that produce Emilin-1, while the second is active only in smooth muscle cells.

The next paragraph introduces some information necessary to understand the work carry out during my PhD.

EXTRACELLULAR MATRIX AND CELL SIGNALLING

The extracellular matrix (ECM) is a complex three-dimensional network of secreted macromolecules produced by different cell types. In addition to provide tissues with mechanical strength and stability, the ECM influences cell behaviour by the regulation of signals acting on the cell membrane. The best characterized of these functions is the adhesive one, that entails the assembly of signalling complexes at the cytoplasmic side of specialized sites of contact of the cells with the ECM called focal adhesions (Zamir and Geiger, 2001). The ECM can also affect cell behaviour in a way similar to growth factors, i.e. by binding surface receptors linked to specific signalling pathways (Discher et al., 2009). In addition, some ECM molecules modulate the activity of important growth factors like transforming growth factors β (TGF-βș and bone morphogenetic proteins (BMPs) (Bornstein, 2001, Rifkin, 2005). Moreover, growth factors can be stored as inactive complexes bound to the ECM and be released under particular conditions (Hynes, 2009) specific components of the ECM may directly influence cell proliferation and differentiation (Karnik et al., 2003). These functions of the ECM are particularly important in tissue morphogenesis during development and in pathological conditions, where they contribute to the refinement and/or the recovery of tissue structure (Hynes, 2009).

TGF-β REGULATION BY ELASTIC FIBER COMPONENTS

Elastic fibers are a component of the ECM responsible for elastic recoil and especially abundant in tissues such as lung, skin and blood vessels. Biochemical and ultrastructural analyses have demonstrated that elastic fibers are constituted by two morphologically distinct component (Greenlee et al., 1966): a central amorphous nucleus consisting of Elastin and responsible for the elastic properties of the fiber; and microfibrillar elements, composed primarily by Fibrillin-1 and –2, highly organized and arranged as a coating of the amorphous core (Sakai et al., 1986, Zhang et al., 1994). The structural complexity and the functional properties of this component are not fully understood and are now emerging thanks to genetic analysis in the mouse. For instance, Fibrillin-1 is involved in most cases of Marfan syndrome (MFS) (Collod-Béroud and Boileau, 2002). Marfan patients often exhibit emphysema; mice with mutations of the *Fibrillin1* gene also develop emphysema and it has been shown that this pathological alteration is due to dysregulation of TGF-β activation and signalling, resulting in apoptosis in the developing lung (Neptune et al., 2003). Another component of elastic fibers with regulatory activity is Elastin; null mice for this gene die soon after birth due to fibrocellular proliferation of the media layer that reduces considerably the lumen of blood vessels (Li et al., 1998). In fact, Elastin, a non-adhesive protein, inhibits cell proliferation via a non-integrin, heterotrimeric G-protein-coupled pathway and the observed alterations are due to lack of this inhibition (Karnik et al., 2003). Interestingly, Elastin has also a role in determining the structure of blood vessels: heterozygous

mutant mice have an increased number of elastic lamellae (Li et al., 1998), and loss of function of one *ELASTIN* allele in humans induces sopravalvular aortic stenosis, in which narrowing of the vessel's wall is the consequence of the increased thickness of the media determined by a higher number of elastic lamellae (Curran et al., 1993). Although the molecular details of how these modifications are brought about are not known, they indicate that Elastin has important effects on the behaviour of vascular cells. Interestingly, *Elastin* null heterozygous mice develop arterial systemic hypertension (Faury et al., 2003). This trait is also observed in mice deficient of *Fibulin5* (Yanagisawa et al., 2002) and *Emilin1* (Zacchigna et al., 2006), two other components of elastic fibers. All these data strongly suggest that elastic fibers are key modulators of vascular cells function and that, through action on the cells, they regulate structural remodelling of blood vessels and important physiological parameters such as arterial blood pressure.

Recent studies recognized that ECM proteins play a crucial role in the regulation of cytokine bioavailability in the vascular system, in particular, the release of latent TGF-β (ten Dijke and Arthur, 2007). Elastic fibers, the component responsible for elastic recoil of tissues like blood vessels lung and skin, are a good example of the role of extracellular matrix in cell signalling in addition to the mechanical one. Support to this concept comes from recent data on gene knockout experiments of elastic fiber constituents

The SLC can covalently attach to the large latent TGFβ-binding protein (LTBP) to form the large latent complex (LLC) (Saharinen et al., 1996). LTBPs are required for the secretion and correct folding of TGF-βs (Miyazono et al., 1991). The association with LTBPs results in the storage of latent TGF-β in ECM structures rapidly after secretion.

Four different LTPBs have been identified, of which LTBP1, LTBP3 and, to a lesser degree, LTBP4 covalently bind to LAPs of all three TGF-β isoforms (Saharinen and Keski-Oja, 2000). Localization of LTBP to the ECM is required for effective TGF-β activation (Nunes et al., 1997): LTPBs stabilize latent TGF-β complexes and regulate their activation at the cell surface (Rifkin, 2005). The significance of this interaction *in vivo* was examined by inactivation of these genes in mice. The *Ltbp1* disruption induces a reduced biological activity of TGF-β (Drews et al., 2008). The pathological changes of the *Ltbp-3* null mice are consistent with perturbed TGF-β signalling (Dabovic et al., 2002) and also the profound defects in the elastic fiber structure of *Ltbp-4* knockout mice are associated with reduced deposition of TGF-β in the extracellular space (Sterner-Kock et al., 2002).

ECM proteins play a crucial role in the regulation of TGF-β bioavailability in the vascular system. Defects in ECM, which were initially thought to affect the physical properties of vessels wall and thereby compromise the vasculature, are now linked to enhanced TGF-β-Smad signalling.

THE EDEN (EMILIN/MULTIMERIN) SUPERFAMILY

EMILINs are a family of proteins of the extracellular matrix that are characterized by a unique arrangement of structural domains, including a signal peptide and the EMI domain, a cysteine-rich sequence of about 80 amino acids, at the amino terminus; an alpha-helical domain with high probability for coiled-coil structure formation in the central part of the molecule; and a region homologous to the globular domain of C1q (gC1q domain) at the carboxyl-terminal end (Colombatti et al., 2000). In mammals the EDEN superfamily comprises seven members: *Emilin1, Emilin2, Multimerin1, Multimerin2, Emilin3, Emid1* and *Emid2* (Figure 1). The first four genes share extensive homology and represent the Emilin family proper within the superfamily (Colombatti et al., 2000, Braghetta et al., 2004). High sequence similarity between Emilins was found in EMI and gC1q domains. In particular, bioinformatics analysis of human and murine EMI domains revealed an average similarity of 60%. Moreover, the seven cysteines of EMI domains are located at highly conserved positions: although the total lengths of domains are variable between 71 to 79 aminoacids, the distances between cysteines are conserved. Only the second cysteine is shifted downstream of four residues in Multimerin-2, while it is absent in Multimerin-1 (Doliana et al., 2000a). Emilin-3 is the sole member of the truncated Emilin family and is characterized by the lack of the C-terminal gC1q domain. Emids are part of a separate family, being formed by the EMI domain followed by a collagenous domain with some triple helix interruptions (Leimeister et al., 2002). This thesis will deal principally with one member of the Emilin protein family, *Emilin1*. For this reason, information on this molecule is given below.

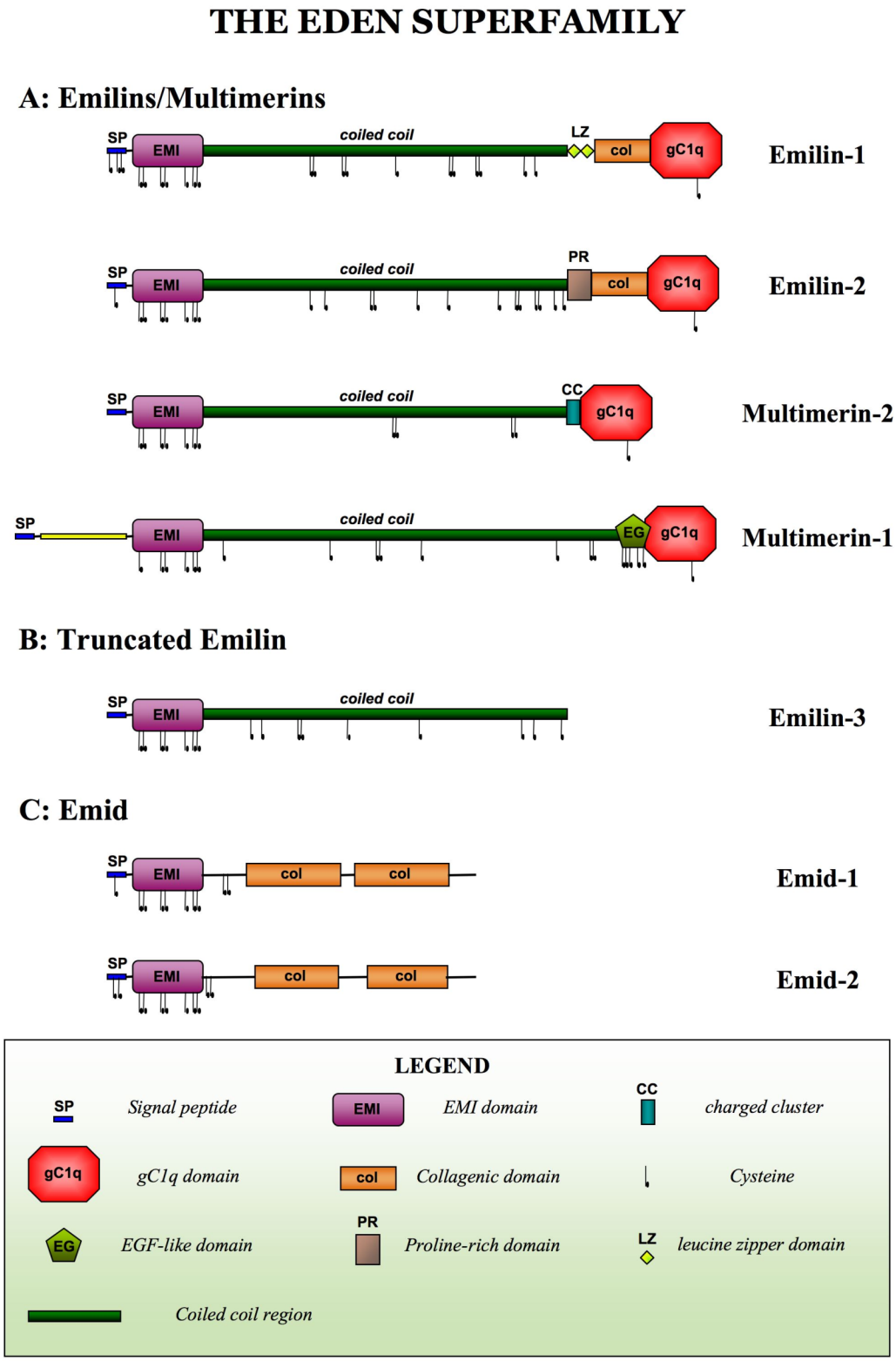

Figure 1: The EDEN (EMI domain endowed) gene superfamily

EMILIN-1

Emilin-1, initially named gp115, was isolated from chicken aorta and found to be particularly abundant in that tissue (Bressan et al., 1983). Immunofluorescence studies confirmed that this protein is strongly expressed in blood vessels and revealed its presence in the connective tissue of a wide variety of organs (Colombatti et al., 1985). At the ultrastructural level, the molecule was detected in elastic fibers, where it was located at the interface between the amorphous core and the coat of microfibrils (the name Emilin is an acronym assembled from Elastin Microfibrils Interface Located proteIN) (Bressan et al., 1993). Emilin-1 appears in early stage of aorta development in association with a network of maturing microfibrils. Moreover, the process of elastic fibers formation was altered by anti-Emilin antibodies suggesting that the protein may be involved in elastogenesis (Bressan et al., 1993).

A detailed analysis was performed on the expression pattern of Emilin-1 in the mouse during mouse development (Braghetta et al., 2002). In this study, RT-PCR analysis revealed the presence of Emilin-1 mRNA in morulae and blastocysts, corresponding to 2.5 and 4.5 days of embryonic development (days *post coitum*, dpc). The first, strong expression of Emilin-1 mRNA during development, as revealed by *in situ* hybridization, is found in the extraembryonic tissues, such as ectoplacental cone (6.5 dpc) and extraembryonic visceral endoderm (7.5 dpc). Staining of ectoplacental cone-derived secondary trophoblast giant cells and spongiotrophoblast is strong up to 11.5 days and then declines. In the embryo, high levels of mRNA are initially expressed in blood vessels, perineural mesenchyme and somites at 8.5 days. Later on, intense labeling is identified in the mesenchymal component of organs anlage (i.e. lung and liver) and different mesenchymal condensations (i.e. limb bud and branchial arches). At late gestation staining is widely distributed in interstitial connective tissue and smooth muscle cell-rich tissues. Once organogenesis is completed, Emilin-1 shows a decrease of expression that varies from organ to organ. RT-PCR studies in adult tissues revealed a high level of Emilin-1 expression in lungs, gut, kidney, bladder, uterus and spleen (Braghetta et al., 2004).The data suggest that EMILIN-1 may have a function in placenta formation and initial organogenesis and a later role in interstitial connective tissue

The role of the protein in elastic fibers assembly was confirmed with the disruption of the *Emilin1* gene in mice. *Emilin1* null mice have a normal development and are morphologically indistinguishable from wild-type littermates, but a closer examination shows alteration in elastic lamellae of elastic arteries and cellular defects in morphology and anchorage (Zanetti et al., 2004). Emilin-1 presents a multimodular structure including a C-terminal gC1q domain similar to those of type VIII and type X collagens endowed with cell adhesion-promoting functions (Colombatti et al., 2000); a short uninterrupted collagenous stalk and a long α-helical domain with high probability for coiled-coil structure formation in the central part; a unique cysteine-rich sequence of approximately 80 amino acids, the EMI domain, that follows the signal peptide and forms the N-teminal end of the mature molecule (Colombatti et al., 2000). Studies on Emilin-1 have demonstrated that the gC1q domain is necessary for the formation of non-covalent homotrimers and acts as a nucleation centre for triple helix and multimers formation (Mongiat et al., 2000). Moreover, it was demonstrated that Emilin-1, through its C1q domain, promotes

adhesion of smooth muscle cells (Doliana et al., 1999) by the interaction with integrin $\alpha_4\beta_1$ (Spessotto et al., 2003). Since Emilin-1 is expressed at the contact area of arterial smooth muscle cells and amorphous elastin, it is likely to contribute to their anchorage, and thus to regulate the formation of blood vessel wall. Considering that Emilin-1 contributes to cell adhesion (Spessotto et al., 2003), and that it interacts with both Elastin and Fibulin-5 (Zanetti et al., 2004), was demonstred that Emilin-1 can regulate elastogenesis and vascular cell maintenance by stabilizing interactions among different components of the elastic fiber and by endowing elastic fibers with specific cell adhesion properties (Zanetti et al., 2004)

After secretion, the protein is deposited into the ECM where it undergoes intermolecular cross-linking by disulfide bonds, finally leading to the formation of high molecular weight aggregates (Colombatti et al., 1988). Intracellularly, Emilin-1 is first produced as monomeric protein of ~90 kDa that is subsequently glycosylated on seven potential N-glycosylation sites, giving rise to a 105-115 kDa monomer. Once secreted into ECM, supermolecular formation proceeds with trimer formation and then with the quaternary assembly into higher order polymers, via intermolecular disulfide bonds (Mongiat et al., 2000).

The *Emilin1* gene is about 8 kb in size, with a coding sequence of 3.1 kb. It is a remarkably compact gene, organized into 8 exons interrupted by 7 small introns. The average size of exons is 500 bp and that of introns 550 bp. Thus, its coding capacity is around 50%, which is significantly higher compared to the average coding capacity of most vertebrate genes, which is around 10% (Doliana et al., 2000b). The first exon contains the 5'-untranslated region and the signal peptide sequence. The EMI domain is encoded by the second and third exons. A very large fourth exon (~2000 bp) codes for the long central region containing coiled-coil and leucine zipper sequences. Since coiled-coil and leucine zipper structures are important for the interchain association, it can be hypothesized that this fourth exon is a kind of functional unit. The fifth, sixth and a part of the seventh exons encode the short collagenous region, while C-terminal gC1q sequence is encoded by the remaining part of the seventh exon and by the eight exon (Doliana et al., 2000b). The human *EMILIN1* gene is located on chromosome 2 (2p23.3) between D2S305 and D2S165 markers, while the mouse homolog is present on the chromosome 5 (5B1), between D5Mit389 and Slc30a3 markers. Both human and murine genes are in the close proximity of the ketohexokinase (*KHK*) gene at their 3'-end. The two genes are in a head-to-tail orientation, and in the mouse the transcription start site of *Khk* is only 665 bp apart from the poly-adenylation site of *Emilin1* (Fabbro et al., 2005).

Studies on *Emilin1* null mice have revealed two striking phenotypes. One concerns the cardiovascular system as explained in the introduction (Zacchigna et al., 2006). More recent studies have shown that Emilin-1 is involved in the regulation of the growth and in the maintenance of the integrity of lymphatic vessels. Indeed, *Emilin1* deficiency results into hyperplasia and enlargement of lymphatic vessels and in a reduction of anchoring filaments (Danussi et al., 2008). The lymphatic vessels of *Emilin1* null mice are functionally altered, leading to a mild lymphedema associated with inefficient lymph drainage and increased leakage.
In a recent work was conducted a two-stage case-control study (Thomas et al., 2004) to investigate the associations of common variants of *Emilin1* gene with essential hypertension in

the northern Han Chinese population. Was designed a two-stage case-control study and selected three single nucleotide polymorphisms (SNPs), rs3754734, rs2011616 and rs2304682 from the HapMap database, which covered Emilin1 gene. Their work doesn't support positive association of *Emilin1* gene with essential hypertension, but the interaction of age and genotype variation of rs3754734 and rs2011616 might increase the risk to hypertension (Shen et al., 2009). Further replications in other populations and functional studies should be done.

THE CRE/*LOXP* SYSTEM

The field of gene targeting was revolutionized through the application of sequence specific recombinases as tools for genomic engineering. A number of bacterial and yeast recombinase enzymes are able to rearrange DNA at specific target sequences. Gene modification employing site specific recombinases was pioneered using the Cre/*loxP* recombination technology (Rajewsky et al., 1996). The site specific DNA recombinase Cre (causes recombination) of the bacteriophage P1 recognizes specifically 34 bp long sequences, called *loxP* (locus of X over in PI) (Sternberg, 1981, Sternberg and Hamilton, 1981). Cre-mediated catalysis results in a reciprocal recombination between the two similarly oriented loxP sites and the consequent excision of the DNA segment between the loxP sites leaving behind a single *loxP* site. (Sauer and Henderson, 1988). When the *loxP* sites are arranged in opposite directions, Cre catalyses inversion of the intervening DNA (Kano et al., 1998). In the conditional allele, an essential region (typically a critical exon) of a gene of interest is flanked by two directly repeated *loxP* sites (floxed) positioned in non-coding regions. The desired genetic modification including the *loxP* sites is introduced into the genome of ES cells via homologous recombination. Subsequently, the targeted allele is generated by site specific recombination of the *loxP* sites through expression of Cre by transient transfections of Cre-containing plasmid. Two types of clones have lost the positive selection marker (*neo*) by this strategy. In one, recombination between the two external *loxP* sites leads to complete deletion of the selection marker and the gene segment, whereas the other recombination between the *loxP* sites flanking the selection marker generates the *loxP*-flanked allele (floxed allele).The removal of positive selection markers is one important application of Cre-mediated recombination in ES cells, since the presence of the selection marker gene can interfere with expression of the targeted gene or adjacent genes (Pham *et al*., 1996). Therefore, the selection marker is flanked by two *loxP* sites in the same orientation, which allows its deletion by Cre-mediated recombination leaving a single *loxP* site in the genome. The floxed mice are generated by procedures similar to those used in the classical gene targeting approach, and require the construction of a targeting vector with *loxP* sites within the desired gene, electroporation of ES cells, selection for homologous recombinant clones and transfer of ES cell clones *in vivo* .

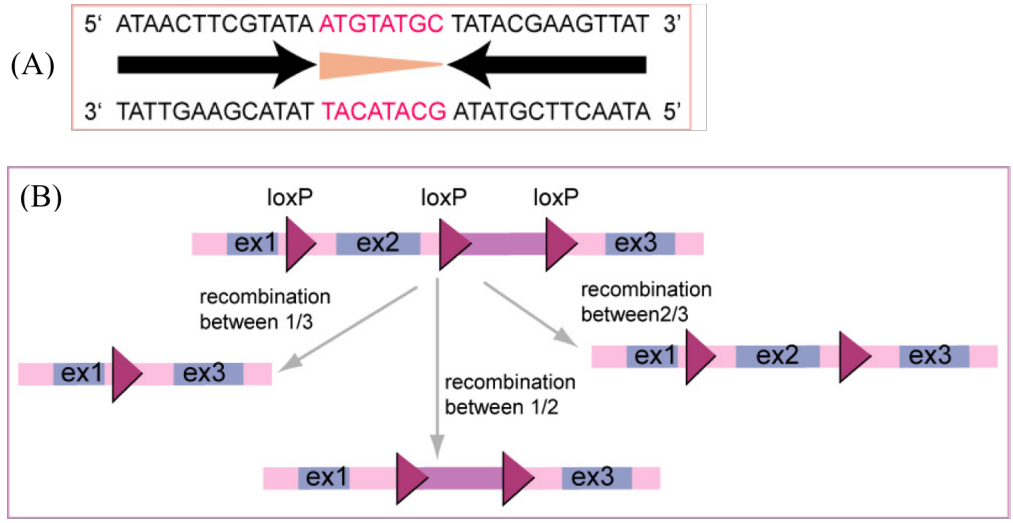

Figure 3: Tri-lox strategy (A) The 34bp *loxP* site consists of two 13-bp inverted repeats (black arrows) that flank an 8bp core sequence (arrow head). The core sequence imparts directionality to the *loxP* site. (B) In the tri-lox strategy, a *loxP* site is positioned in an intron and two *loxP* sites are placed flanking the positive selection cassette (purple) in another intron. This strategy was developed to conditionally remove the positive selection cassette (purple) from the targeted locus to make an essential gene floxed. This is achieved by a "partial" recombination event between *loxP2* and *loxP3* (right). The other two recombination products are also shown (left and middle). ex: exons.

CRE TRANSGENIC MICE AND INDICATORS FOR CRE ACTIVITY

The usage of the Cre/*loxP* system in conditional gene targeting requires both a mouse strain containing a *loxP*-flanked modification of the target gene and an additional mouse strain, which expresses the Cre recombinase in specific cell types. Crossing these two strains results in the generation of a conditional mouse mutant, which carries the mutation restricted to those cell types, in which the Cre recombinase is expressed.

A large variety of Cre-transgenic mouse lines has been developed, where the Cre recombinase is expressed under the control of different tissue-specific promoters. These mice allow the expression of the Cre recombinase in a highly restricted manner, and a database (http://www.mshri.on.ca/nagy/cre-pub.html) was established as a resource of Cre-transgenic mouse lines available worldwide. Such Cre expressing mouse strains can be generated either by using conventional random transgenesis, or by targeted insertion into a gene (knock-in), or by using a bacterial artificial chromosome (BAC) strategy. In any case, the expression pattern of the promoter used to express Cre determines onset and cell type specificity of the Cre mediated gene modification. The technique of conventional random transgenesis has several disadvantages: the limited understanding of promoter regions used to express the transgene, the potential unpredictable effects of enhancer/silencer elements present at the integration site on transgene expression and the necessity to analyze many different founder lines which may contain multiple copies, tandem repeats, broken and differently integrated transgenic constructs. Another

technique, which is currently used to generate Cre expressing mouse strains, is the insertion of the Cre gene into bacterial artifical chromosomes (BAC) and the subsequent generation of BAC transgenic mice. BACs carry large genomic fragments from mouse or human of up to 400 kb and can be obtained commercially. The Cre gene can be introduced into defined genes encoded by the BAC via homologous recombination in bacteria (Testa et al., 2003). The large size of the BAC renders it likely that most if not all of the transcriptional control elements of the gene, into which Cre has been introduced, are present. This should result in a Cre expression pattern closely related to the expression of the endogenous gene. This strategy was successfully used by S. Offermanns to express Cre under smooth muscle myosin heavy chain promoter by inserting the Cre gene in the SMMHC gene (Myh11) gene of a 180 kb BAC (Wirth et al., 2008).

As explained in the introduction *Emilin1*$^{-/-}$ phenotype is caused by disregulation of TGFβ1 maturation and *Emilin1* expressed in the endothelial cells or in the vascular smooth muscle cells has different contribution to this signalling. In my laboratory have been generated transgenic mouse lines to understand contribution of endothelial cells and vascular smooth muscle cells in cardiovascular phenotype development of *Emilin1* knockout mice. Was observed that Emilin1 is necessary and sufficient only in the smooth muscle cells to rescue Emilin1$^{-/-}$ vascular phenotype. For this reason we choose to use a *SMMHC* promotor, in this way Cre recombinase is expressed exclusively in smooth muscle cells. In my laboratory were also generated transgenic mice (*Emilin1*-Emilin1) in which Emilin1 cDNA is expressed by Emilin1 promoter, which consists of all regions to control mRNA transcription (Fabbro et al., 2005). The expression of *Emilin1*-Emilin1 transgene rescue knockout phenotype of *Emilin1* nulle mice. This suggest the generation of a transgenic mouse line that express CreER^{T2} under *Emilin1* promoter to understand *Emilin1*/TGF-β regulation of blood pressure in the pathogenesis of hypertension.

The success of Cre-mediated conditional gene targeting critically depends on stringent regulation of Cre expressing mouse strains, which have to be intercrossed with mice containing *loxP*-flanked target genes.

A convenient strategy for the generation of a Cre reporter strain would be to target a single reporter construct into a gene which is expressed ubiquitously throughout all developmental stages of the mouse. An excellent candidate for an appropriate locus is the Rosa26 locus, which was originally identified by gene trap (Zambrowicz et al., 1997). (Soriano, 1999) subsequently engineered the Rosa26 locus in ES cells by introducing a two *loxP* sites containing cassette, in which the two *loxP* sites flanked the selection marker to interrupt the expression of the lacZ gene. This mouse strain expressed *lacZ* only after Cre-mediated excision of the *loxP*-flanked region. Crossing Rosa26R mice with Cre expressing strains resulted in different or more restricted *lacZ* expression patterns. The Rosa26R mouse strain should be of wide use for monitoring Cre expression, as well as for analysing cell lineages during development, and is available from the Induced Mutant Resource of the Jackson Laboratory.

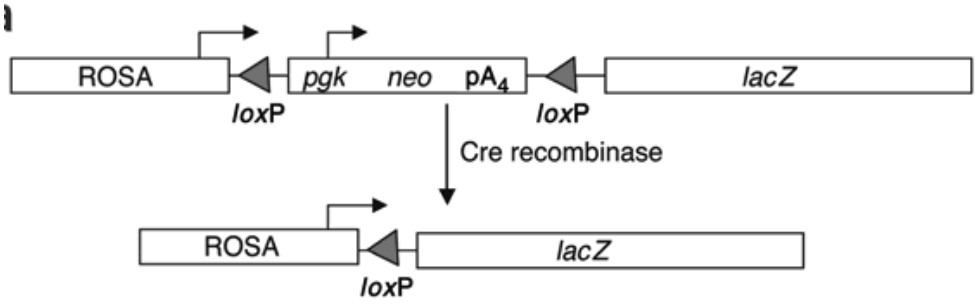

Figure 3. The ROSA26R transgene. It comprises the ubiquitous ROSA promoter followed by *loxP* (triangles), flanked by a transcriptional stop fragment and *lacZ* (Soriano, 1999). The stop fragment contains the phosphoglycerate kinase (*pgk*) promoter−driven neomycin phosphotransferase (*neo*) expression cassette and the four tandem polyadenylation (pA_4) sequences, which blocks transcription of the downstream *lacZ*. Transient Cre recombinase expression leads to irreversible deletion of the *loxP*-flanked neo cassette and permanent expression of β-gal. Adapted from (Garg et al., 2003)

TAMOXIFEN INDUCIBLE CRE RECOMBINASE ACTIVITY

Steroid hormones such as estrogen, progesterone, androgen and glucocorticoid regulate gene expression directly through classical intracellular steroid hormone receptors and possibly indirectly through novel unconventional membrane bound receptors belonging to the G protein coupled receptor superfamily (Wehling, 1997). The intracellular steroid hormone receptors possess a well characterized ligand binding domain (LBD), which can be used for posttranslational control of Cre activity. A fusion protein between Cre and the LBD can be expressed from any given cell type specific promoter. In order to generate a posttranslational system of Cre fused to the LBD of the estrogen receptor (ER), single amino acid substitutions have to be introduced in the ER-LBD to prevent its activation by the endogenous hormone 17 β−estradiol (E2) but, coincidently, to maintain its binding capacities for synthetic steroids (Figure 4).

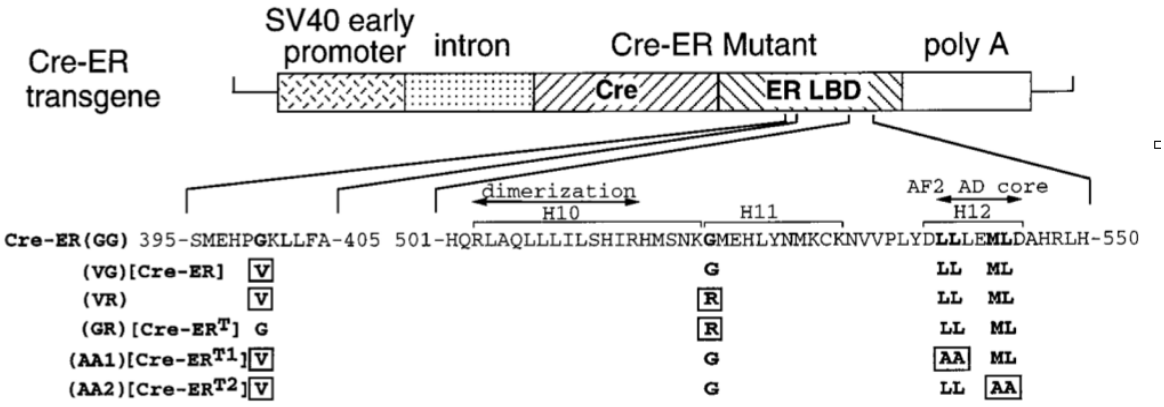

Figure 4: Modular structure of steroid hormone receptors. Numbers indicate the position of amino acids in the human estrogen receptor (ER). (Feil et al., 1997)

In the absence of steroid hormone, the LBDs are bound by heat shock proteins, which inactivate recombinase activity presumably by sterical hindrance. Only after addition of the steroid hormones as inducers, the fusion protein becomes activated and can mediate recombination of *loxP* sites flanked sequences in the nucleus (Figure 5). In particular in the absence of hormone, complexes of heatshock proteins retain the receptors in the cytoplasm (Passinen et al., 1999). Binding of hsp90 and other factors like the immunophilins hsp56 and hsp70 to the LBD maintain the receptors in an inactive but ligand-friendly conformation. Hormone binding causes dissociation of this large multiprotein complex and subsequent import of the receptors into the nucleus. The nuclear import is achieved by specific amino acid sequences termed NLS (nuclear localisation signal) which are masked by the binding of the hsp chaperones in the cytoplasm (Misrahi et al., 1987, Tyagi et al., 1998). Once in the nucleus, the steroid receptors dimerize, bind as homodimers to specific HRE (hormone responsive element) in the promoter of their target genes and change thereby expression levels of the latter by either activating or repressing transcription.

During the last years, numerous CreER^{T2} transgenic mouse strains were generated, which express the fusion protein ubiquitously, or restricted to muscle, to brain, and to several other tissues. In most of these strains, highly efficient deletion of *loxP*-flanked targets can be induced by injection of tamoxifen in the range of 1-3 mg daily for 5 days. The reduced doses of tamoxifen, which have to be injected and subsequently block endogenous estrogen receptor mediated signalling, do not interfere with most experimental applications. Coincidently, the background activity of the CreER^{T2} fusion protein in the absence of inducer is ranging at tolerable levels between 3-10% in mice.

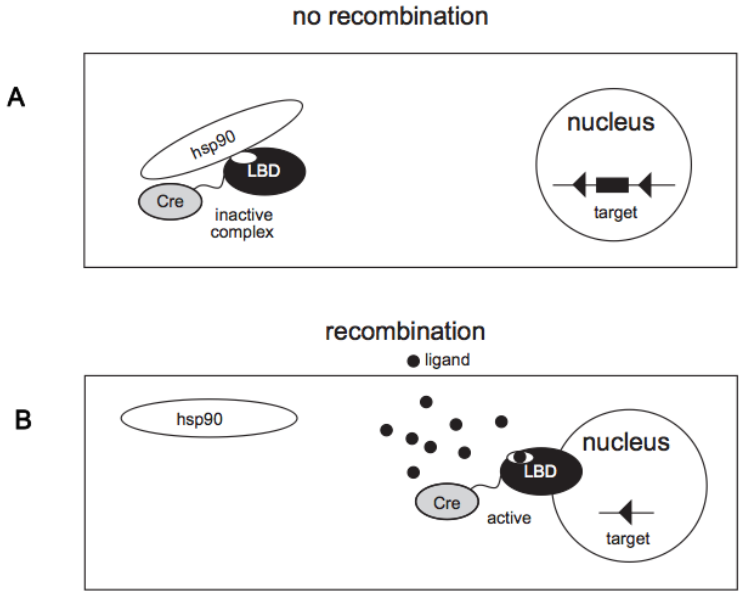

Figure 5: Cre-ER^{T2} Recombinase activity is inducible by Tamoxifen (A) Cre is fused to the ligand binding domain (LBD) of a steroid hormone receptor and kept as an inactive complex in the cytoplasm by bound heat shock proteins (hsp90). (B) The complex dissociates upon ligand binding and CreLBD translocates to the nucleus and mediates recombination.

COMPARISON OF PRONUCLEAR MICROINJECTION VS. LENTIVIRAL VECTORS FOR ANIMAL TRANSGENESIS

Here I present a comparison of the factors involved in the plasmid DNA microinjection approach compared with the lentiviral vector system.

The pronuclear microinjection is the classic method to generate transgenic animals, and few changes have been made in the pronuclear injection method using DNA as the vector since the pioneering studies by Gordon et al. (Gordon et al., 1980). The details of this approach have been extensively reviewed elsewhere (Chan, 1999, Rülicke and Hübscher, 2000, Wall, 2001). This procedure involves the transferring of genetic material by microinjecting DNA into the pronucleus of fertilized one-cell eggs, which are subsequently implanted into the oviduct of pseudopregnant surrogate females following mating with a vasectomized male. Even with the successes described for this method, it has been fraught with a low transgenic efficiency. The integration of the constructs is often poor, and the generation of transgenic founder mice can be generated at efficiencies ranging from 5 to 20% (Brinster and Avarbock, 1994, Wall, 2001). It is important to note that the production of founder transgenic mice has been shown to be strain dependent(Tesson et al., 2005). In the classic method of pronuclear microinjection the number of copies of plasmid DNA that can be integrated varies from one to several hundred copies, which can be found as concatemers with a predominant head-to-tail orientation (Bishop and Smith, 1989). Inhibitory effects from nearby genomic areas can alter the expression of the transgene (al-Shawi et al., 1990), which is why larger genomic fragments are often used that include insulator sequences to maximize the production of transgene-expressing transgenic animals (Rülicke and Hübscher, 2000). The unrestricted size limitation is the single most important advantage to creating transgenic animals through direct incorporation of plasmid DNA. Even extremely large genomic DNA pieces have been cloned into yeast artificial chromosomes (YAC; 1- to 2-Mb insert size) or bacterial/P1-derived artificial chromosomes (BAC/PAC; <300-kb insert size) before transgenic applications. However, there can be problems with the use of YAC/BAC/PAC because of rearrangements, shearing, and the technical nature of handling large constructs.

For the purposes of transgenesis, the primary benefit in using the lentiviral vector system is its efficient ability to integrate into the host genome (Naldini et al., 1996, Park et al., 2000). The latter characteristic could be a distinct advantage for lentiviral transgenesis compared with pronuclear microinjection by allowing for more rapid integration into the genome. The packaging size is an important factor in the use of lentiviral vector. Empirically, early generation lentiviral vectors were able to produce functional vector particles even with a genome size of 16 kb (Kumar et al., 2001). In reality, however, lentiviral vectors of this size may not be useful for biological applications, since the viral titers are dramatically reduced by up to 1,000-fold compared with lentiviral constructs of 5–7 kb. To calculate the cloning capacity, it is important to include the backbone of the lentiviral vector that is essential for the production of functional vector, which is generally 1.6 kb (without WPRE) or 2.2 kb (with the WPRE). From the previous studies by Kumar (Kumar et al., 2001)the maximal size of the lentiviral vectors was estimated to be 13.5 kb. Expression cassettes (promoter-transgene) markedly larger than 4 kb would likely

have reduced transgenesis rates using lentiviral vectors because of effects on vector integration, so studies are needed to determine the upper limit of the lentiviral vector packaging capacity. At the present time, however, the issue of packaging size may not be a major limitation, since promoter-transgene constructs <10 kb within the scientific community are fairly prevalent, so this approach should become a routine method in animal transgene.

Even with the disadvantage of a low transgenic efficiency using pronuclear microinjection, this approach still remains the most simple and least labor-intensive routine method to produce transgenic animals, particularly in mice. A comparison between the lentiviral vector system and the pronuclear microinjection method can be found in Table 1.

Table 1: Comparison of pronuclear microinjection vs. lentiviral vectors for animal transgenesis

	Pronuclear microinjection of DNA	**Lentiviral Vector**
Plasmid/vector preparation	Minimal	Labor intensive
Packaging capacity	<50 kb (plasmid)	Most effective <10 kb
	<1 Mb (BAC/PAC)	
	<1–2 Mb (YAC)	
Site-specific gene targeting	Possible	Unlikely
Sites of integration	Random	Fairly random, but prefers active transcription units
Embryo manipulation skill	High	Low-to-moderate
Embryo survival	Low-to-high	High (>70%)
%Born transgenic/implanted embryo	Very low (1–2%)	Low-to-moderate (8–50%)
Proportion born transgenic	Low (5–20%)	High (> 63%)
Integrated transgene copies	Low (1–5 for large transgenes)	Single-to-multiple
	High (>5–10 for small transgenes)	
Individual or multiple copies following integration	Concatemers	Individual
Expressing founders	50%	High (at least 60%, but generally >90%)
Expressing F_1 progeny	Moderate (50%)	Moderate (>50%)
Efficiency of cell types modified		
• **Zygote**	Moderate	Extremely high
• **Sperm**	Extremely low	Moderate-to-high
• **Germline stem cells**	Low	Moderate-to-high
• **Somatic cells**	Extremely low	Low-to-moderate

Table adapted from (Park, 2007)

LENTIVIRAL VECTORS FOR ANIMAL TRANSGENESIS

There are a plethora of viral vector systems that have been developed for gene therapy applications, but the vast majority of these vectors are nonintegrating, which precludes their usefulness for transgenesis. The best characterized integrating viral vectors originate from the *retroviridae* family, the members of which are ideal for the genetic manipulation of mammalian cells because of their intrinsic ability to integrate into genomic DNA. At present, the use of simple retroviruses based on murine Moloney leukemia virus (MLV) (Naviaux and Verma, 1992) is becoming overshadowed by more sophisticated lentiviral vector systems that have superior efficiency in modifying cells in vitro and in vivo compared with MLV-based vectors. For this reason, the main topic of this paragraph will focus on the lentiviral vector system and its potential role in transgenesis. The genetic composition and the applications for transgenesis of complex lentiviral or retroviral vectors are illustrated in Figure 6. The lentivirus genome derived from immunodeficiency viruses, such as human immunodeficiency virus-1 (HIV-1), has been split into multiple fragments to minimize the potential formation of replication-competent viruses. These components are broken into the following categories: *1*) transfer or integrating vector, *2*) structural and packaging, and *3*) envelope plasmids. Since the transfer (integrating) vector plasmid is the only component that is transferred to the transgenic animal, the molecular composition of this plasmid will be described in this section, while the details regarding the packaging/structural and pseudotyping plasmids and detailed protocols used to create the infectious lentiviral vector particles can be found elsewhere (Trono, 2000, Cockrell and Kafri, 2003, Pfeifer, 2004). There are two main regions within the transfer plasmid that express the viral RNA genome following transfection during vector production: *1*) a multiple cloning site for the insertion of various expression cassettes and *2*) flanking long-terminal repeats (LTR) that have several distinct functions. First, the 5'-LTR can act like an RNA pol II promoter. Second, the 3'-LTR acts to terminate transcription and promote polyadenylation. Third, the LTR has recognition sequences necessary for integration into the genome. The RNA vector genome is ultimately reverse transcribed and integrated into the genomic DNA as a provirus using viral proteins obtained from the structural and packaging plasmids in trans during vector production. Two important *cis*-acting DNA elements, the central polypurine tract sequence (cppt) and the woodchuck postregulatory element (WPRE), are also included in the transfer (integrating) vector to enhance the transduction efficiency and transcript stability, respectively (Follenzi et al., 2000). The cppt is a small DNA fragment found in the *pol* gene of HIV that is usually cloned 5' to the internal promoter region, whereas the WPRE is cloned 3' to the inserted transgene so that it is in close proximity to the poly(A) signal in the 3'-LTR. Another important feature in the lentiviral transfer plasmid is a 400-bp deletion in the U3 region of the 3'-LTR, which debilitates the 5'-LTR RNA pol II promoter activity following integration (Park and Kay, 2001).

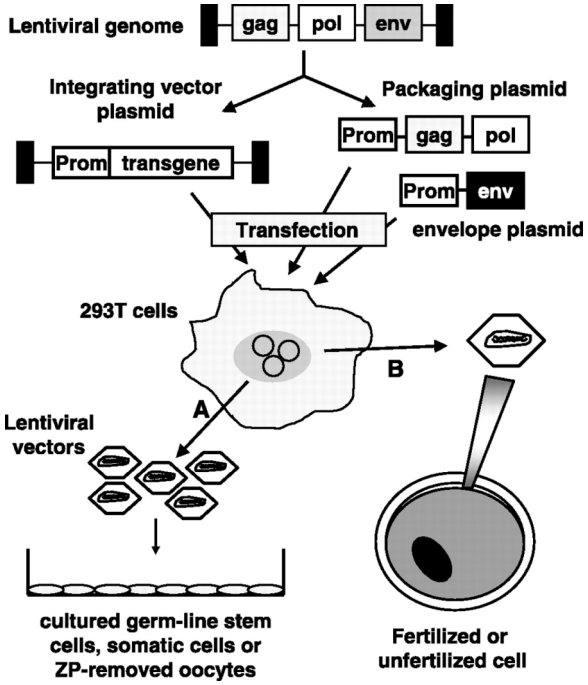

Figure 6: Lentiviral vector production. The wild-type lentiviral genome is made up of 3 main genes (*gag*, *pol*, and *env*) and it is surrounded by long-terminal repeats (black color). The vector system is a split-genome format whereby the packaging and structural genes have been separated from the portion of the virus that promotes integration into the host genome. The *env* gene can be replaced with a heterologous glycoprotein from various viruses, including the prominently used VSV-G. The 3 plasmids (or more) are transfected into a cell line (293T cells) to produce the viral vectors, which are collected in the supernatant for in vitro transgenic applications either by infection (*A*) or by direct injection (*B*). Prom, viral or cellular promoter; ZP, zona pellucida. (adapted from (Park, 2007).

MATERIALS AND METHODS

MATERIALS

SOLUTIONS

DNA Sample buffer 10x: 0.05% bromophenol blue (w/v); 1 mM EDTA, pH 8; 55% glycerol (v/v).

LB broth and LB-agar plates were used for *Escherichia coli* DH5α strain.

LB broth (for 1 liter): 10 g bacto-tryptone; 5 g bacto-yeast extract; 10 g NaCl; pH 7.

LB-agar plates: LB broth supplemented with 10 g/l bacto-agar.

HEPES buffer: 1 M pH 7.4.

SSC solution 20x: 3 M NaCl; 0.3M Na citrate; final pH: 7.

TAE buffer 50x: 0.04 M Tris base; 57.1 ml glacial acetic acid; 2 mM EDTA pH 8.

TE buffer: 10 mM Tris-HCl, pH 7.5 or 8; 1 mM EDTA pH 8.

TE buffer low EDTA: 10 mM Tris-HCl, pH 7.5 or 8; 0.1 mM EDTA pH 8.

Avertin 40x: 10 *g* 2,2,2- tribromoethyl alcohol, 10 ml tert-amyl alcohol

Tail buffer: 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 0.3 M Na acetate, 1% SDS, Proteinase K

ES complete medium: DMEM (4.5 g/l glucose, Gibco); 20% ES qualified fetal bovine serum (FBS, Gibco); 0.1 mM β-mercaptoethanol; non-essential amino acids (10 mM each, Gibco); 1 mM sodium pyruvate (Gibco); 2 mM L-glutamine (Gibco); $2x10^3$ units/ml recombinant LIF (Leukemia Inhibitory Factor, Chemicon).

Freezing medium: ES complete medium supplemented with 10% FCS (final 30% FBS) and 10% DMSO.

PBS 10x : (for 1 liter) 80 g NaCl; 2 g KCl; 11.5 g $Na_2HPO_4x7H_2O$; 2 g KH_2PO_4 pH 7.2.

Trypsin/EDTA: 0.05% Trypsin; 0.53 mM EDTA pH 7.2

M2 medium: 0.25 mg/ml $CaCl_2{\cdot}2H_2O$; 0.14 mg/ml $MgSO_4$; 0.36 mg/ml KCl; 0.16 mg/ml K_2HPO_4; 5.53 mg/ml NaCl; 4 mg/ml albumin, bovine fraction V; 1 mg/ml D-glucose; 11 μg/ml phenol Red; 40 μg/ml sodium pyruvate; 4.97 mg/ml HEPES free acid.

M16 medium: 0.25 mg/ml $CaCl_2{\cdot}2H_2O$; 0.16 mg/ml $MgSO_4$; 0.36 mg/ml KCl; 0.16 mg/ml K_2HPO_4; 5.53 mg/ml NaCl; 4 mg/ml albumin, bovine fraction V; 1 mg/ml D-glucose; 11 μg/ml phenol Red; 40 μg/ml sodium pyruvate; 3.32 mg/ml sodium lactate.

PLASMIDS

pBluescriptII-KS^+ and pMC1-TK were obtained from Stratagene. pCreERT2 was a gift of P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Strasbourg, France), pIC-Cre of V. Broccoli (Stem Cell Research Department, San Raffaele Scientific Institute, Milan, Italy), ploxP and ploxP-neoloxP of E. Hirsch (Molecular Biotechnology Center, Department of Genetics, Biology and Biochemistry, University of Turin, Turin, Italy).

BACTERIAL STRAINS

E.coli DH5α: F-(phi) 80lacZ DM15 D(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk^-, mk+) phoA sipE44 thi-1 gyrA96 relA1 λ-

Epicurian coli XL-10 GOLD: Tet^r D(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F'proAB $lacI^q$ ZDM15 Tn10 Tet^r Amy Cam^r]^a

MOUSE STRAINS

All the procedures involving animals have been performed according to institutional guidelines in compliance with national laws. *Emilin1*$^{flox/flox}$ mice and *Emilin1*-CreERT2 were generated as previously described (Bonaldo *et al.*, 1998; Zanetti *et al.*, 2004) using standard procedures (Nagy *et al.*, 2003). The homologous recombination experiment with embryonic stem cells and the microinjection of these cells into blastocysts were carried out by P. Bonaldo and P. Braghetta (Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Padova, Italy). Mice carrying Cre recombinase under SMMHC promoter were kindly provided by S. Offermmans (Institute of Pharmacology, University of Heidelberg, Germany). Mice Rosa26R were obtained from S. Piccolo (Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Padova, Italy). All animals analysed in the experiments here described were between 2 and 4 months of age.
The following mouse strains have been used: C57BL/6NCrl and B6D2F1 (Charles River) as embryo donors and for maintaining the mouse colonies; CD1 (Charles River), as recipient females and vasectomized males.

EXPERIMENTAL PROCEDURES

PREPARATION OF THE *EMILIN1* CONDITIONAL KNOCKOUT CONSTRUCT

A 16.8 kb *Hind*III fragment, containing the entire Emilin1 gene and 5'- and 3'-flanking sequences was subcloned from a 135-kb BAC clone (Zanetti et al., 2004) into pBluescriptII KS+ (pKS). This construct will be referred hereafter as pK16. Using *Cla*I and *Eag*I restriction enzymes a DNA fragments of 10,1 kb was isolated from pK16 and subcloned into pKS. The obtained construct (pKS-10 kb) was digested with *Cla*I and *BamH*I in order to isolate the 5' region (1,8 kb) including the promoter region of mouse Emilin1 ("left arm" (LA) of the final homologous recombination construct). After treatment with Klenow enzyme, this fragment was subcloned into the pKS-loxP plasmid digested with SmaI, dephosphorylated with alkaline phosphatase producing pKS-LA-loxP. After this blunt-end ligation, in order to find clones that contained correctly orientated fragment diagnostic restriction digestions were performed. Exons 1 and 2 (1,7 kb) were isolated from the vector pKS-10 kb by *BamH*I and recloned into pKS-loxP-neoloxP in a SmaI site to generate pKS-Ex1,2-loxpneoloxp. The 3' region of the construct ("right arm" RA) (6,6 Kb) was isolated from pKS-10kb by BamHI and EagI restriction digestion and cloned into pMC1-TK (Mansour et al., 1988) in a XhoI site producing the construct pMC1-

RA-TK. Afterwards the "left arm" and loxP site were isolated from pKS-LA-loxP after *BamH*I and *Hind*III digestion and then cloned into pKS-Ex1,2-loxpneoloxp using a *BamH*I site. (pKS-LA-loxp-Ex1,2-loxpneoloxp). The last component that was added in order to complete the targeting construct, was the Herpes simplex virus thymidine kinase (TK) gene driven by a its own promoter. The negative selectable marker (1.8 kb) and the right arm (6,6 kb) were derived from the pMC1-TK-RA plasmid by cleavage with *Cla*I and *Sal*I enzymes. Subsequently the right arm and TK cassette were introduced into a *Cla*I site of pKS-LA-loxp-Ex1,2-loxpneoloxp, located at the 3'-end of the homology locus with *Emilin1* gene. The final gene targeting construct thus obtained (pKS-LA-loxp-Ex1,2-loxpneoloxp-RA-TK) is shown in Figure 2.
Given the known difficulties in the ligation with such large inserts, some modifications of the usual cloning strategies were applied. The Epicurian XL-10 Gold bacterial strain was used instead of the usual E. coli DH5α strain because it is more suitable for the transformation of large DNA molecules.

ES CELLS AND ELECTROPORATION

CULTURE, PROPAGATION AND MAINTENANCE OF ES CELLS

ES cells were grown on plastic culture dishes (Falcon) and maintained at 37°C in an incubator (Forma Scientific) at 5% CO_2 and 95% humidity. Before addition of ES cells, plastic dishes were coated with gelatine (0.1% in PBS) for 30 min or longer at 37°C. The gelatin solution was then removed and mitomycin-inactivated murine embryonic fibroblasts (IEF) were seeded at fixed concentrations. After the IEF were uniformly attached to the Petri dish, the ES cells were plated in ES complete medium.

FBS was heat inactivated at 56°C for 30 min, LIF (10^7 units in 1 ml PBS) was diluted in DMEM at a final 100x concentration, sterile filtered with 0.2 μm filters, and kept at 4°C in 2 ml aliquots. All the media and trypsin were stored at 4°C and prewarmed to 37°C in a water bath prior to use. In order to prevent the spontaneous differentiation, which can lead to the loss of their totipotency ES cells need to be cultured with particular care, avoiding an excessive growth of the colonies.

At the time of splitting, the dishes were washed twice with PBS, treated with 0.5-2 ml of prewarmed trypsin/EDTA solution and incubated for 5 min at 37°C. A suspension of single cells was obtained through extensive pipetting with a sterile Pasteur pipette. A dilution of the single cell suspension (i.e., 1:3) was then plated onto a gelatin-treated culture dish, previously covered with the feeder layer of IEF.

In the case of freezing, the ES cells were trypsinized as above and resuspended in an appropriate volume of freezing medium. Cells were aliquoted in small tubes (Cryovials, Falcon), incubated for 2 hours at -20°C, transferred overnight to -80°C, and finally stored in liquid nitrogen. When necessary, ES cells were quickly thawed in a water bath at 37°C, washed in ES medium and finally plated onto gelatin-treated culture dish, previously covered with IEF. R1 ES cell line was used for all the experiments (Nagy et al., 1993).

ELECTROPORATION OF ES CELLS AND POSITIVE-NEGATIVE SELECTION OF ES CELL CLONES

The plasmid DNA was linearized with *Not*I restriction enzyme, and the digestion was performed for 2-3 hours at 37°C in a total volume of 500-700 µl. The linearized vector was purified by phenol/chloroform extraction and precipitated with ethanol. Cleaned DNA was dissolved in sterile PBS buffer at the concentration of 1 mg/ml.

ES cells were cultured on a layer of IEF feeder cells until sub-confluent, trypsinized to gain a single cell suspension, washed with warm PBS, counted and resuspended in PBS at the concentration of $2x10^7$ cells/ml. The linearized targeting DNA (25-40 µg) was added to 0.8 ml of ES cell suspension and the mixture was gently mixed with a pipette. After 5 min incubation at room temperature, cells were transferred to the electroporation cuvettes (BioRad) and electroporated with a BioRad Gene Pulser unit, by applying a pulse at 500 µF and 250 V. The electroporated ES cells from each cuvette were plated onto 15-cm petri dishes, containing G418-resistant IEF, and cultured overnight in ES complete medium to allow attachment and recovery.

About 24 hours after electroporation, the culture medium was replaced with fresh ES complete medium supplemented with 350 µg/ml of G418 (Gibco), for the positive selection of the *neo* gene. At day 2 after the electroporation and for 4 days, the negative selection against the *TK* gene was also applied, supplementing the ES complete medium with 2 µM gancyclovir (GANC; Syntex). Double resistant ($G418^R$, $GANC^R$) ES colonies were visible macroscopically after 6-7 days of culture. When the colonies reached an appropriate size, they were picked with a sterile Gilson micropipette under a stereo-microscope (Olympus SZX12), transferred as single clones in 96-well plates containing 50 µl/well of prewarmed trypsin/EDTA solution and incubated for 10-15 min at 37°C. After addition of 50 µl/well of ES complete medium, a suspension of single cells was obtained by thourough pipetting with a sterile multi-channel micropipette (Eppendorf). The isolated clones were then transferred to 96-well culture dishes (Falcon) containing a layer of G418-resistant IEF feeder cells and cultured in the presence of ES complete medium supplemented with 250 µg/ml G418. After 4-5 days each 96-well plate was trypsinized and split in two 96-well plates: one plate was further cultured for clones expansion and DNA isolation , while the other was processed for freezing after adding an equal volume of 2x freezing medium and storage at -80°C.

DNA ISOLATION FROM ES CELLS

Confluent ES clones grown in 24-well plate were washed twice with PBS and incubated overnight at 37°C with 0.5 ml/well of a lysis solution containing 0.3 M Na-acetate, 10 mM Tris-HCl pH 7.8, 1 mM EDTA pH 8.0, 1% SDS and 200 µg/ml Proteinase K (Sigma). Each lysed clone was then transferred to a clean sterile Eppendorf tube and frozen at -80°C for 20 min or overnight at -20°C. The samples, still frozen, were centrifuged at 13500 rpm for 15 min at 4°C to remove SDS. Genomic DNA was then subsequently extracted with phenol-chloroform (1:1) and

chloroform solutions. Finally, the DNA was precipitated by adding 1/30 volume of 3 M Na-acetate, (pH 5.2) and 1 volume of isopropanol, followed by incubation for 10 min at room temperature. The DNA pellet was collected by centrifugation for 5 min at 13500 rpm, washed with 70% ethanol and finally resuspended in appropriate volume of TE buffer-low EDTA, in order to obtain a final concentration of about 2 mg/ml.

IDENTIFICATION OF CORRECTLY TARGETED ES CLONES

PREPARATION OF cDNA PROBES BY POLYMERASE CHAIN REACTION (PCR)

All PCR reactions were performed in a total volume of 50-100 µl, in the presence of 1x PCR Mg-free buffer (Finnzymes), 1.5 mM $MgCl_2$, 200 µM dNTPs (Invitrogen), 1-5 pmol of forward and reverse oligonucleotide primers, and 0.25-1 unit Taq DNA polimerase (Finnzymes). Oligonucleotides were syntesized by MWG DNA oligonucleotide service (www.mwg-biotech.com) or Invitrogen (www.invitrogen.com). The reactions were carried out in a PCR thermocycler (PT-100 Thermal Controler, MJ Research), using different cycling conditions depending on the primer pairs (Table 1). Analysis of the PCR reaction products was performed by agarose gel electrophoresis.

Probe I	Probe5'for	AAAGGATCCCCCATCTGTTTTGGCATGA	890 pb
	Probe5'rev	TTTGGATCCGTGAAAGGGAACGGGAGAA	
Probe II	Probe3'for	AAGGATCCGCTAGGCCAGAACCACTGC	400 pb
	Probe3'rev	TTTGGATCCGAGGACCGTGTCCCAACTT	

Table 1: List of primers and PCR conditions used to prepare cDNA probes for the detection of correctly targeted ES cell clones by Southern blot.

PREPARATION OF RADIOACTIVE LABELED PROBES

Radiolabeled probes were prepared by random priming, in the presence of random sequence hexamers to prime DNA synthesis on a denaturated DNA template. 50 ng of appropriate cDNA fragment and random nanomers primer mix (3 mg/ml) were resuspended in ddH_2O to a final volume of 11 µl, denaturated by heating to 95°C for 10 min and then rapidly cooled on ice. Radiolabeling was performed by addition of a 1x buffer containing all dNTPs except dCTP (10 mM each), 5 units of Klenow enzyme (2 U/µl, Promega), 0.5 µg BSA and 5 µl [α^{32}P]dCTP (10 mCi/ml, Amersham Biosciences). After incubation for 2 hours at 37°C, the reaction was stopped by addition of 100 µl TE buffer. Unincorporated nucleotides were removed from the labeled DNA fragment by mini Quick Spin Columns (Roche). The eluted radiolabeled DNA was denaturated at 95°C for 10 min immediately before use.

SOUTHERN BLOT

Approximately 7 µg of genomic DNA cut with the appropriate restriction enzyme was loaded onto an 1% agarose gel and run in 1x TAE buffer until the DNA had reached a proper migration, as determined by visualisation under UV-light. The gel was then removed and incubated for 30 min in a solution of 0.4 M NaOH at room temperature. During this incubation, a blot chamber was set up. A Tupperware box was filled with transfer buffer (0.4 M NaOH) and a platform was then prepared by covering it with Whatman 3MM filter paper soaked in 0.4 M NaOH. The gel with wells facing up was carefully laid down and covered with Gene Screen Plus nylon membrane (PerkinElmer Life Sciences), cut to gel size and previously equilibrated for 20 min in 0.4 M NaOH solution. The transfer was allowed to proceed overnight by capillary forces. The nylon membrane was then removed, washed in 0.4 M NaOH for 5 min, dried at room temperature and DNA fixed by a UV cross- linker (Bio Rad). Pre-hybridization was carried out for 30' at 42°C in ULTRAhyb-oligo (Ambion). After addition of the appropriate radiolabeled probe (final concentration 3.3 ng/ml, $5.1x10^6$ cpm/ml), hybridization was performed overnight at 42°C in a hybridization oven (Bachofer). The membrane was then washed three times as follows: *i)* 10 min at room temperature in 2x SSC and 1% SDS ; *ii)* 10 min at room temperature in 1x SSC and 0.5% SDS; *iii)* 5 min at 65°C in 0.2x SSC and 0.5% SDS. The membrane was finally rinsed in 2x SSC and subjected to autoradiography with X-Omat (Kodak) films.

CRE MEDIATED REMOVAL OF *NEO* CASSETTE

For Cre recombinase-mediated deletion at loxP sites in ES cells, four cuvettes with $1•10^7$ ES cells from two different homologous recombinant clones (ES-102 and ES-155) were electroporated with 30–40 µg of supercoiled pIC-Cre vector in 800 µL PBS. The electroporated cells were diluited with complete ES medium and plated at about $3\text{-}5•10^6$ cells/10-cm dish on IEF. After 48 hr they were trypsinized and replated on three plates at 1000, 2500 and 10000 cells/10-cm dish. After 5-6 days the colonies were picked under a stereomicroscope and split into two 96-well plate for further culture: one plate was treated ES cell medium containing G418 at a concentration of about 400 µg/ml (i.e., 25% higher concentration of that used for the initial selection of homologous recombinants), the second plate was let grow without any selection.. G418-sensitive colonies that have undergone Cre-mediated deletion were clearly visible after 3 days of selection. These clones were expanded from the other 96 well plate, frozen and DNA was prepared for analysis by PCR (list of primer used in Table 2) and Southern blot.

P1for	GCCCATGGTCACTCTTGTG	loxP1: 361 pb wt: 236 pb
P2rev	CTCAGTTCCTTCCGCTCTCC	
P3for	TTGAGGATGGAGTGGAGACC	Δneo: 261 pb Wt: 171 pb
P4rev	CTTTGCACAGACCCCCTAGA	

Table 2: List of primer used to screening the Cre-mediated deletion

CYTOGENETICAL ANALYSIS OF ES CELL CLONES

ES cell clones were cultured in complete ES medium, in 10 cm petri dish. After reaching the 90% of confluency, cells were incubated 4 hours with 140 μl of fresh colchicine (final concentration 10^{-6} M). The cells were then removed and centrifuged. About 500 μl of an hypotonic solution (Na-citrate 1%) was added and incubated for 10 minutes at 37°C. After removing the hypotonic solution, ES cells were treated with 1 ml of fixative (ethanol/acetic acid, 3:1) for 10 minutes at room temperature. The fixative was then removed and microscope slides were prepared and stained with Giemsa staining (Sigma).
For each clones, an average of 50 metaphases were examined. Metaphases were considered to be abnormal when their number of chromosomes was dissimilar to 40. Clones were considered aneuployd only when abnormal metaphases were found more than five times in the same ES cell clone.

FROM TARGETED ES CELL CLONES TO *EMILIN1* CONDITIONAL KNOCKOUT MICE

ISOLATION OF BLASTOCYSTS

C57BL/6NCrl mouse strain is widely used for the isolation of blastocysts and their subsequent injection with ES cells. The C57BL/6NCrl strain differs from the 129/Sv strain, from which R1 ES cells are derived, in coat color and at other genetic loci that are useful as markers (e.g., glucose phosphate isomerase). Blastocysts isolated from C57BL/6NCrl mice are good host for ES cells, thus allowing to obtain chimeric mice at relatively high frequencies and a good rate of germ-line transmission.
To set up matings, two C57BL/6NCrl females of 6 weeks of age were placed with one C57BL/6NCrl male in one cage. The following morning, the females were checked for vaginal plug. Plug-positive female were separated and sacrificed by cervical dislocation at 3.5 dpc (*days post coitum*). 6-10 blastocysts per mouse were isolated, harvested in M2 medium and transferred into a dish with M16 medium which was placed at 37°C for few hours, until ES cells were ready for injection (Nagy et al., 2003).

BLASTOCYST MICROINJECTION WITH ES CELLS

The ES cells from a selected knockout clone were prepared by standard procedure in order to obtain a single cell suspension in ES complete medium.

Approximately 10 single, well isolated ES cells were injected in the blastocoel cavity of a blastocyst following established procedures (Nagy et al., 2003), using a micromanipulator/microinjector device combined with an inverted microscope (Zeiss Axiovert 35M) and a mechanical suction-and-force syringe device, placed on a pneumatic anti-vibration

table. The injected blastocysts were transferred into a drop of M16 medium and incubated in a CO_2 incubator at 37°C until ready for implantation into foster mothers.

TRANSFER OF INJECTED BLASTOCYSTS INTO FOSTER MOTHERS

Transfer of injected blastocyst was performed by implantation into pseudo-pregnant 'foster' CD1 females of about 6 weeks of age or older, obtained by natural mating with vasectomized CD1 males. Copulation resulted in a hormonal cycle in the female, which made the foster mother pseudo-pregnant.

The injected blastocysts were transferred into the uterus of 2.5 dpc (*days post coitum*) foster mothers, following a standard procedure established in our lab. Eight to ten blastocysts were implanted into each uterine horn.

GENERATION OF *EMILIN1*-CREER^{T2} TRANSGENIC MOUSE LINES

A oligo adaptor was designed for the insertion of *Mlu*I as a unique restriction enzyme site that wasn't present in the vector or in the insert, necessary for the isolation of *Emilin1*-CreER^{T2} fragment and the subsequently pronuclear injection. The following complementary oligos (For TCGAGACGCGTAAGCTTACGCGTT and Rev CTAGAACGCGTAAGCTTACGCGTC) were annealed by mixing at equal molar ratios. The oligo adaptor contained two stiky ends *Xho*I and *Xba*I in order to be ligated into pKS *Xho*I-*Xba*I linearized plasmid. This product was called pKS-*Mlu*I-*Hind*III-*Mlu*I.

A SV40 polyadenylation signal was added at the Cre recombinase gene. The pCreER^{T2} plasmid (kindly given by P. Chambon) was digested with *Eco*RI, the insert containing the gene was subcloned in pCS2+ in a *Sna*BI site. A 16.8-kb HindIII fragment, containing the entire *Emilin1* gene a was isolated from pK16 using *Hind*III and subcloned into pBluescriptII KS+ (pKS) containing the oligo adaptor *Mlu*I-*Hind*III-*Mlu*I via a sticky-end ligation cloning. In the last step the fragment CreER^{T2} with SV40 was isolated, by *Eco*RI-*Not*I digestion and subcloned into a *Sal*I site of pKS-*Mlu*I-Emilin1-*Mlu*I in order to express the Cre recombinase under the *Emilin1* promoter. The identity of pKS-*Mlu*I-Emilin1-CreER^{T2}-*Mlu*I was confirmed by restriction enzyme mapping and DNA sequence analysis (data not shown), indicating that the *Emilin1* promoter and Cre recombinase were correctly inserted and in frame.

For pronuclear injection, pKS-*Mlu*I-Emilin1-CreER^{T2}-*Mlu*I was digested with SalI and HindIII to remove vector sequences. The insert was isolated by agarose gel electrophoresis and purified by electroelution, and then by a PCR purification kit (Biorad). The purified DNA was resuspended in a buffer containing 10 mM Tris, pH 7.9, 0.1 mM EDTA.

Transgenic mouse lines and embryos were produced from B6D2F1 females mated with B6D2F1 males (Charles River Italia) using standard procedures (Hogan et al., 1986). DNA was microinjected into pronuclei of one-cell embryos and the surviving embryos were implanted into CD1 pseudopregnant foster mothers.

In particular, recombinant Emilin1-CreER^{T2} fragment was microinjected into the male pronucleus of the 241 fertilized eggs of B6D2F1 mice, which were transferred into the oviducts of 14 pseudopregnant recipient mice. 10 out of 14 recipient mice became pregnant and gave birth

to 105 offspring mice. Potential founder animals were screened by polymerase chain reaction (PCR) and the number of integrated copies of transgene into the genome was determined by Dot Blot. For PCR, mouse tail DNA was amplified by 33 cycles on a thermal cycler using primers from Cre recombinase (List of primer, Table 3). The size of the amplified product is approximately 455 bp.

DOT BLOT HYBRIDIZATION

Dot blot analysis, originally described in 1979, permits the determination of the number of integrated copies of transgene into the genome. Basically, the denatured genomic DNA (5 ug) from the different transgenic mouse lines were applied by vacuum directly to a GeneScreen Plus Membrane (Perkin Elmer). Accurate determination of the DNA concentration is absolutely necessary to obtain reliable results. Standard curves of gene copy number were constructed using known amounts of a plasmid containing the $CreER^{T2}$. The strategy is to include increasing amounts equivalent to 1, 2, 4, 6, 8, 10, 15, 20, 25 or 30 gene copies of the injected transgene (previously mixed with nontransgenic DNA) in lanes parallel to the samples to be analyzed. The amounts of plasmid used for the standard curves were calculated considering that the plasmid size is 21,8 kb.

The membrane was then dried at room temperature for 30 min, and DNA fixed by heating at 80°C for 30 min. A 800 pb DNA probe (pCreER^{T2} digest with BamHI and HindIII), directed against the Cre recombinase was radioactively labeled. Procedures of labeling, hybridization, and detection were performed as illustrated previously.

CROSS-BREEDING OF MICE AND INDUCTION WITH TAMOXIFEN

Male Emilin1-CreERT2 and SMMHC-CreERT2 transgenics were crossed to females that carried the Rosa reporter allele, Rosa26R (Soriano, 1999). Offspring were genotyped by PCR for Cre recombinase (as above) and Rosa26R alleles (Soriano, 1999), and mice that carried both genetic modifications were used for administration of Tamoxifen. Tamoxifen (Sigma) was dissolved in Miglyol 812 (Sasol) at a concentration of 20 mg/ml. For the initial analysis of the transgenic founders of Emilin1-CreERT2 and Rosa26R, was treated with Miglyol alone as control or with Miglyol containing Tamoxifen at the dose of 1, 3, 6 mg of Tamoxifen/40 g of mouse body weight intraperitoneally for 5 consecutive days. For the cotransgenic progeny from SMMHC-$CreER^{T2}$ and ROSA26-LacZ crosses with the treatment with tamoxifen was performed as described in Wirth et al., 2008 that is 1 mg intraperitoneally per day for five consecutive days. Twenty days after the last injection, various organs from the mice were fixed and subjected to X-gal staining.

WHOLE MOUNT *LACZ* STAINING AND HISTOLOGICAL ANALYSIS

Mouse tissues were dissected, quickly washed in PBS, and processed for fixation (PFA 4°C for 15 minutes) and staining for β-galactosidase as described (Bonnerot and Nicolas, 1993). Briefly, the tissues were incubated overnight at 30°C in the staining solution containing X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) as substrate for enzyme activity, washed in PBS and subsequently dehydrated through a graded series of ethanol. Fixed dehydrated tissues were prepared for paraffin embedding by incubation in 100% xylene and then infiltrated by incubation through xylene:paraffin solutions and three final incubations in 100% paraffin before embedding in 100% paraffin. Serial sections (6 µm) were placed on glass slides and then dried. Sections were cleared in multiple washes of 100% xylene and rehydrated through a graded ethanol series to a final incubation in PBS. Pictures were acquired with a Zeiss Axiophot Microscope equipped with a Leica DC500 digital camera.

REVERSE TRANSCRIPTASE PCR (RT-PCR) ANALYSIS

RNA was extracted from normal and mutant tissues by using TRIzol reagent (Gibco-BRL) as recommended by the manufacturer. RT-PCR analysis was performed as follows. First strand cDNA synthesis using 1 µg total RNA and random hexanucleotides was synthetized by M-MLV reverse transcriptase (Invitrogen). Amplification was carried out in 50 µl reaction mixtures containing 0.1–0.3 µg cDNA, 10 mM Tris–HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM dNTPs, 25 pmol of each specific primer and 2U Taq DNA polymerase (Fynnzymes). The reaction products were separated in 1-2 % agarose gels and stained with ethidium bromide. The oligonucleotides used for these reactions are reported in Table 3. To correct for sample variations in RT-PCR efficiency, the amplification of Gapdh was used as internal control.

Emilin1 mRNA	Ex1for	TGCTGTCTGCTGACCATAGC	266 pb
	Ex2rev	CCAGCCACAGGGTTGATAGT	
Cre-ERT2 mRNA	Crefor	TCGATGCAACGAGTGATGA	455 pb
	Crerev	AAGCAATCCCCAGAAATGC	
Gapdh mRNA	Gapdhfor	CAACAGCAACTCCCACTCTTCC	170 pb
	Gapdhrev	GGTGGTCCAGGGTTTCTTACTC	

Table 3: List of primers and PCR amplificated

GENOTYPE ANALYSIS

Genomic DNA was isolated from tail biopsies as described in common applied methods for genotyping of transgenic mice (Laird et al., 1991). The oligonucleotides used for these reactions are reported in Table 4.

SMMHC-CreER^{T2}	SMWT1	TGACCCCATCTCTTCACTCC	Cre-negative: 225 pb Cre-positive: 225 pb and 287 pb
	SMWT1	AACTCCACGACCACCTCATC	
	phCREAS1	AGTCCCTCACATCCTCAGGTT	
Emilin1-CreER^{T2}	Crefor	TCGATGCAACGAGTGATGA	Cre-positive: 455 pb
	Crerev	AAGCAATCCCCAGAAATGC	
Rosa26R	LacZfor	CGGTGATGGTGCTGCGTTGGA	LacZ-positive: 400 pb
	LacZrev	ACCACCGCACGATAGAGATTC	
Emilin1$^{flox/flox}$	Floxfor	TGACCCCATCTCTTCACTCC	wt: 280 pb Emilin1$^{flox/+}$: 280 and 366 pb Emilin1$^{flox/flox}$: 366 pb Emilin1$^{flox/flox}$ + Tam: 589 pb
	Floxrev	AACTCCACGACCACCTCATC	
	FloxforB	CACTGGGCCTCCTATTCAGA	

Table 4: List of primers and PCR amplificated used to genotype the different transgenic mouse line and conditional Emilin1 mice

EVALUATION OF BLOOD PRESSURE

Measurements of cardiovascular parameters in mice were carried out in the laboratory of Prof. G. Lembo (Department of Angiocardioneurology, I.R.C.C.S. Neuromed Institute, Pozzilli (IS), Italy). Blood pressure was evaluated by non-invasive tail-cuff plethysmography.

SEQUENCING AND BIOINFORMATIC SEQUENCE ANALYSIS

RT-PCR products were cloned into pGEM-T Easy (Promega) according to the manufacturer's protocol. Sequencing was performed with the Sanger method by BMR Genomics (Padova-Italy) with SP6 and T7 promoter primers.
Sequence alignments were performed with Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

RESULTS

The results of my PhD thesis are divided in two parts. The first part concern the completion of a work just started for in vivo silencing of the *Col6a1* gene, via RNAi, using lentiviral vectors (Appendix). The second part concerns the generation of conditional null mice of *Emilin1*, and characterization of transgenic mice carrying Cre recombinase. The main steps of the experimental procedures carried out are summarized in Figure 1.

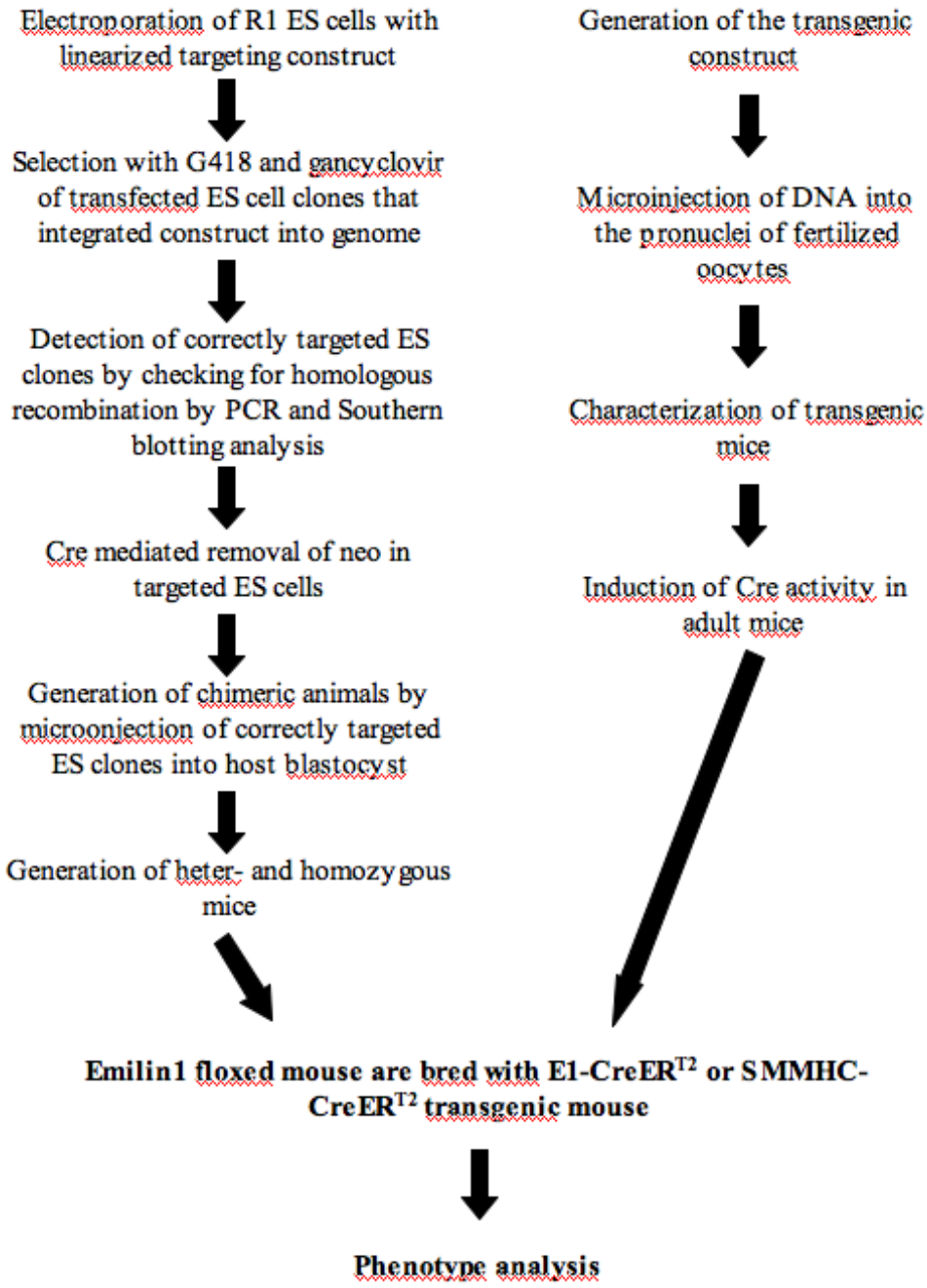

Figure 1: Main experimental steps in the generation of a floxed allele of *Emilin1* gene.

TARGETING STRATEGY FOR THE MURINE *EMILIN1* GENE

Based on the determined exon-intron structure and restriction site map of the genomic subclone, a Cre/*loxP* based gene targeting strategy specific for the murine *Emilin1* gene was designed. According to the literature, the minimal length of homology between the gene targeting construct and the endogenous gene of interest should be at least 3.0 kb in size (Hasty et al., 1991, Deng and Capecchi, 1992). To enable conditional inactivation of the *Emilin1* gene, a gene targeting construct was generated in which a *loxP* flanked *neo* cassette allowing positive selection in embryonic stem cells, was introduced downstream of exon 2. A third *loxP* site was introduced in the same orientation upstream of exon 1. A 1,7 kb genomic fragment was included in 5' end as the short arm of homology and another 6,6 kb genomic sequences were included at the 3' end as part of the long arm of homology in order to allow the endogenous *Emilin1* allele to be replaced with the targeting construct by homologous recombination in ES cells. The floxed allele, which differs from the endogenous *Emilin1* allele only in the two remaining *loxP* sites flanking exons 1-2, was expected to allow normal expression of the *Emilin1* gene product until Cre mediated deletion of the floxed gene segment. Based on the targeting strategy, Cre-mediated excision of the floxed exons 1 and 2 was predicted to result in deletion of the ATG start codon and of EMI domain in the *Emilin1* gene

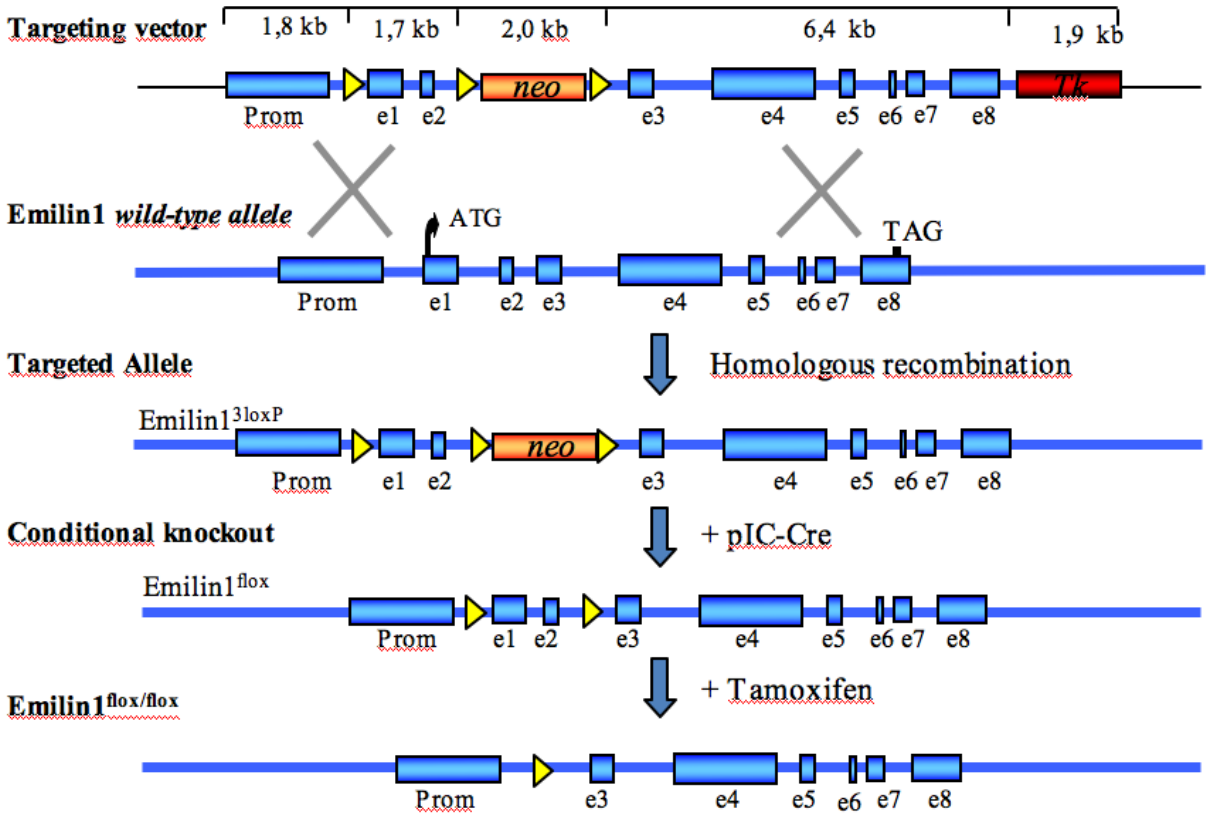

Figure 2: Schematic representation of *Emilin1* gene targeting vector. *Emilin1* gene contains eight exons (e1-e8) and seven introns (i1-i7). Initiation of transcription is indicated with an arrow, as well are the ATG start codon and the TAG stop codon. In the targeting construct, the position of the two selectable cassettes (*neo* and *TK*), as well as the loxP sites, are indicated.

ISOLATION AND CHARACTERIZATION OF HOMOLOGOUS RECOMBINANT ES CELL CLONES OBTAINED WITH THE *EMILIN1* FLOXED TARGETING CONSTRUCT

In total, 170 resistant ES cell clones were picked and expanded for subsequent genomic DNA isolation and characterization. To detect ES clones with correctly integrated targeting construct, Southern blotwas performed on genomic DNA with external DNA probes, recognizing a region of murine *Emilin1* gene which lies outside the targeting construct sequences. Three different probes were used: probe I (890 pb, positioned at the 5'-end of *Emilin1* gene outside the left arm of homology of targeting construct; probe II (400 pb, positioned at 3'-end of *Emilin1* gene outside the right arm of homology of targeting construct) and probe neo (1900 pb) (Figure 3).

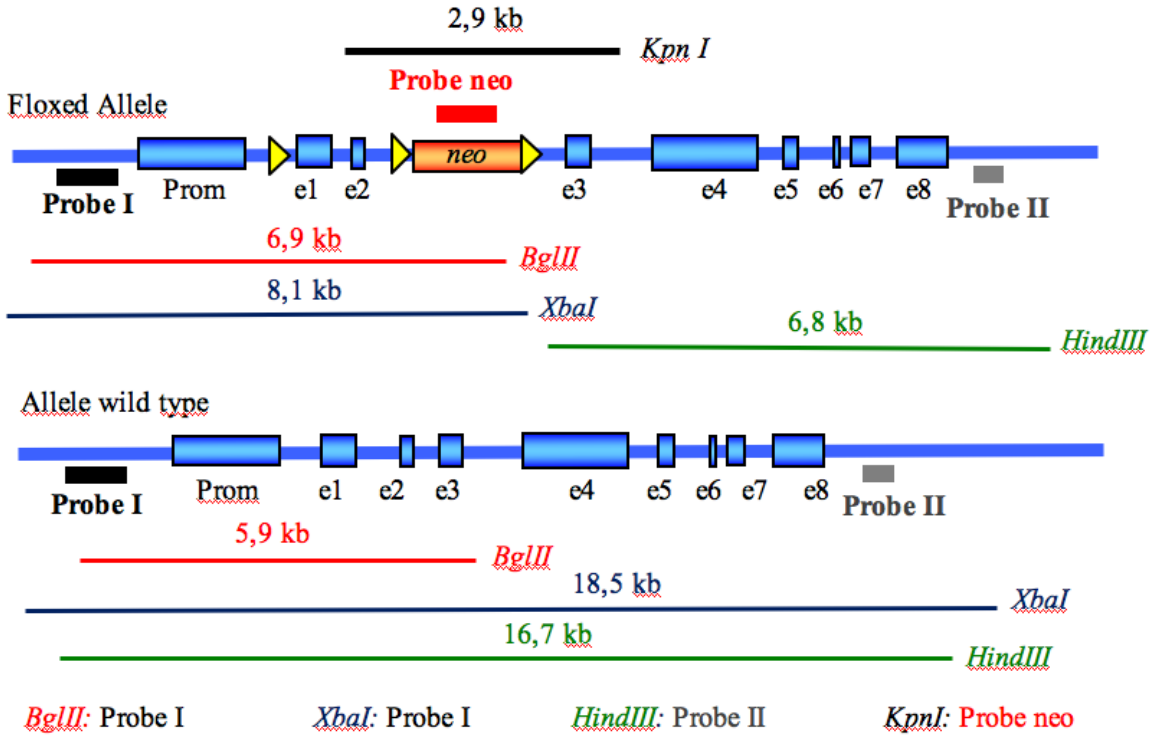

Figure 3. Strategy used for the identification of the correctly targeted ES cell clones by Southern blotting. The scheme represents the wild-type allele and the targeted allele, with the position of PCR generated external probes (probe I, probe II and neo probe) and the fragments generated by digestion with diagnostic restriction endonucleases *(Bgl*II, *Xba*I, *Hind*III and *Kpn*I).

Out of 170 ES clones analyzed by digestion with *Bgl*II restriction and Southern blotwith probe I, only six clones (assigned as E1-27, E1-37, E1-45, E1-102, E1-155 and E1-164) were found to contain two bands. Non-targeted clones should contain only one DNA band, which corresponds to the wild-type allele (5.9 kb in size). On the other hand, correctly targeted clones should have two bands, corresponding to a wild-type allele (5.9 kb) and to the targeted one (6.9 kb). Homologous recombination between the targeting construct and the endogenous gene should increase the size of the band to 6.9 kb in the targeted allele, as the consequence of the presence of *neo* cassette (Figure 4).

The six positive ES clones identified during the primary screening with *Bgl*II and probe I, were further characterized by *Xba*I and *Hind*III digestion and Southern blotwith either probe I and probe II. Hybridization of *Xba*I-digested DNA with external probe I revealed expected fragments for the wild-type allele (18.5 kb) and for the targeted one (8.1 kb) in the targeted ES clones, while the non-targeted ES clone contain only a fragment corresponding to the wild-type allele. Furthermore, hybridization of *Hind*III digested DNA with external probe II confirmed that the six clones contain correctly targeted *Emilin1* gene, without any evidence of rearrangements.

Hybridization of *Kpn*I-digested DNA with the *neo* probe showed the presence of one expected bands (2,9 kb) in the mentioned six clones, indicating a single insertion of *neo* cassette in *Emilin1* gene, but not in other genomic loci. No signal was detected in the six targeted clones with the amplification of *TK* by PCR, indicating that the *TK* cassette was lost during homologous recombination as expected for correctly targeted ES cells.

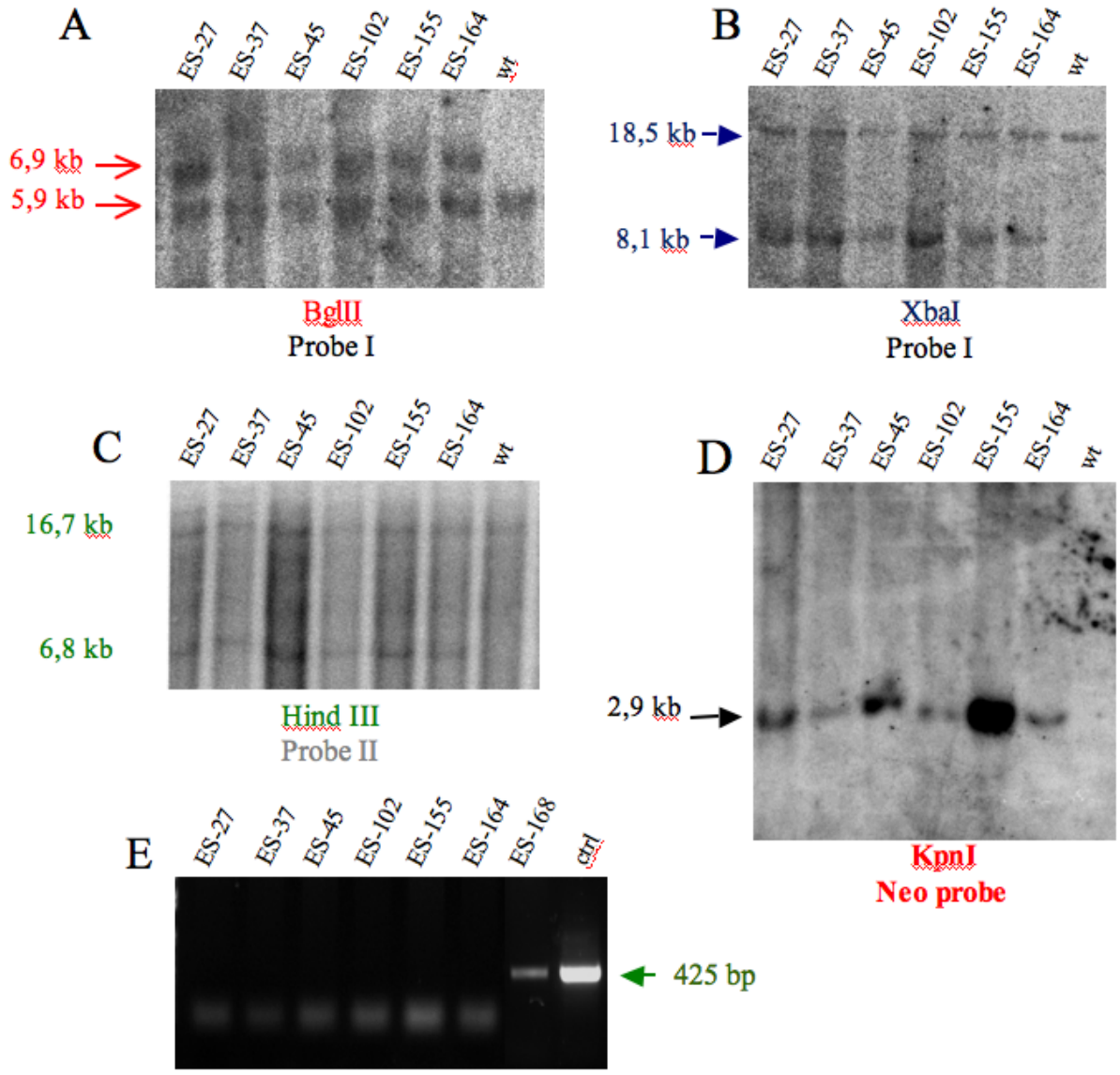

Figure 4: Characterization of targeted ES cell clones by Southern blot and PCR. *(A-D)* Southern blot analysis of targeted clones (lanes 1–6) and DNA from parental R1 (lane 7) using different diagnostic enzymes and different probes. A) Hybridization of *Bgl*II-digested DNA with external probe I reveals the expected fragments for the normal allele (5.9 Kb) and the mutated allele (6.9 kb). B) Hybridization of *Xba*I-digested DNA with an external probe (probe I) reveals the expected fragments for the normal allele (18.5 Kb) and the mutated allele (8.1 kb) C) Hybridization of *Hind*III-digested DNA with an external probe (probe II) reveals the expected fragments for the normal allele (16.7 Kb) and the mutated allele (6.8 kb) D) Hybridization with the *neo* probe show a single insertion of the cassette at the correct size. E) No signal is obtained with the amplification of *TK*, indicating that the *TK* cassette was lost during homologous recombination.

CRE MEDIATED REMOVAL OF NEO

As the *neo* selection cassette represents a strong transcriptional unit predicted to disrupt expression of the *Emilin1* gene product from the targeted allele even in its intronic position, it had to be removed from targeteted ES clones.

The *neo* resistance cassette was removed in ES cells by transient expression of Cre recombinase in targeted ES cells. The expression of Cre in targeted ES cell clones results in two types of clones which have lost the selection marker (*neo*). Recombination between the two external *loxP* sites leads to deletion of the selection marker and the gene segment (Type I deletion), whereas recombination between the *loxP* sites flanking the selection marker generates the *loxP*-flanked version of the gene (Type III deletion, Figure 5).

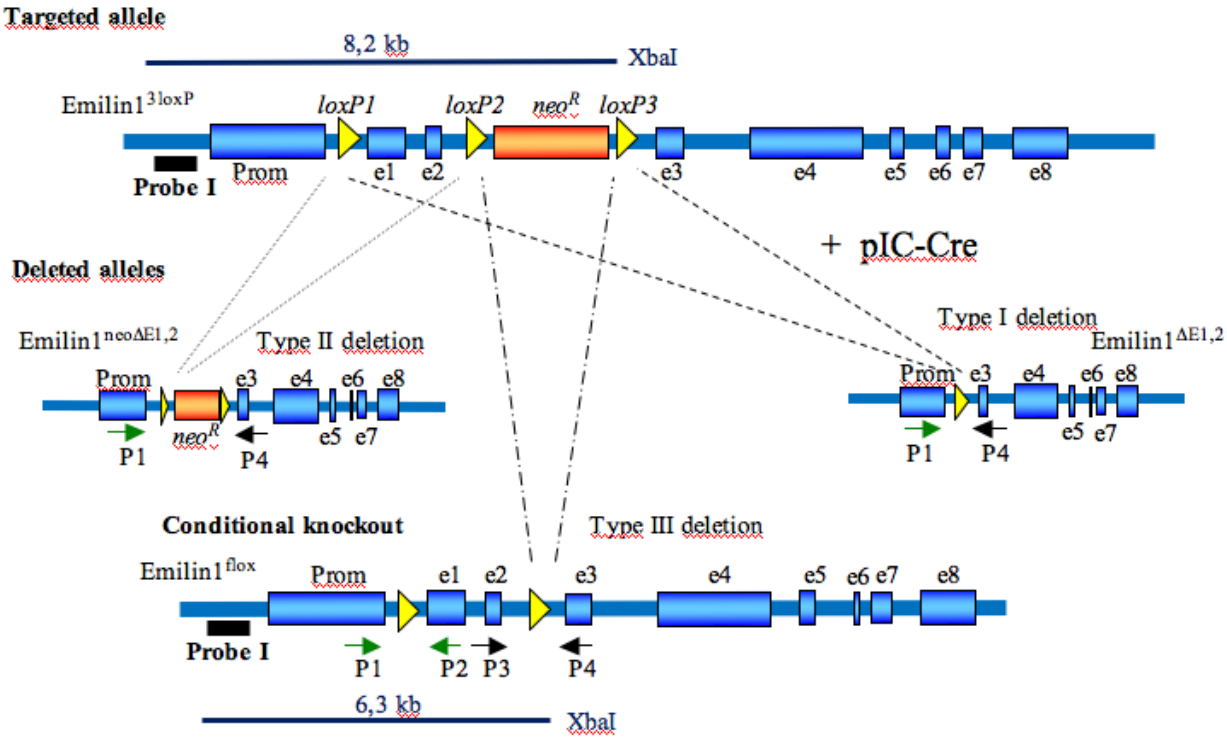

Figure 5: Cre/*loxP* strategy

Two targeted ES clones (ES-102 and ES-155) were transfected independently by electroporation with Cre expression vector pIC-Cre (Gu et al., 1993). For each of the two targeted parental clones, about 190 (for the clone ES-102) and 230 (for the clone ES-155), ES clones were isolated expanded and screened for G418 sensitivity. Cre mediated deletion was checked in G418 sensitive subclones and the type of deletion was determined by PCR and then confirmed by Southern blot analysis as is shown in Figure 5.

ES cells clone DNA was analyzed by PCR using several sets of primers. To demonstrate the presence of the floxed exon1-2, primer 1, located upstream of the first *loxP* site and primer 2 located between the first *loxP* site and exon 1 were used, amplifying a 236 bp wild-type band and a 361 bp mutant band (loxP 80 bp extra). To confirm the deletion of the *neo* cassette, the primer 3 located upstream of second *loxP* site and primer 4 located in the exon 3 were used, generating a 171 bp wild-type band and a 261 bp mutant band (Figure 5 and Figure 6).

About 23% of ES clones isolated had undergone the deletion of *neo* cassette through Cre mediated recombination of the first and third *loxP* sites and of the second and the third *loxP* sites (Type I and III deletions). About 85% of G418 sensitives ES clones (or about 19,5% of total clones isolated) had recombined the first and the third *loxP* sites (Type I deletions) and carried the deleted *Emilin1* allele and *neo* cassette (Table 1). Such clones could have been used to generate conventional Emilin1 knockout mice.

Only 15% of homologous recombined G418 sensitives ES clones (or 3,6% of total clones isolated) carried the *loxP* flanked Emilin1 allele shown in Table 1, in which, by excision of the *neo* selection cassette, exons 1 and 2 are flanked with *loxP* sites (Type III deletions).

Table 1: Summary of site-specific recombinations

ES Clone	No ES clones isolated	Neo present: no recombination or type II deletion	Neo lost: type I deletion (%)	Neo lost: type III deletion (%)
ES-102	190	144 (75,8%)	39 (20,5%)	7 (3,6%)
ES-155	220	171 (77,7%)	41 (18,6%)	8 (3,6%)

The 15 positive ES clones identified during the primary screening by PCR, were further characterized by Southern blotwith probe I. Hybridization of *Xba*I-digested DNA with external probe I revealed expected fragments. The Southern Blot confirmed that the 4 clones contain correctly targeted *Emilin1* gene, without any evidence of rearrangements (Figure 6).

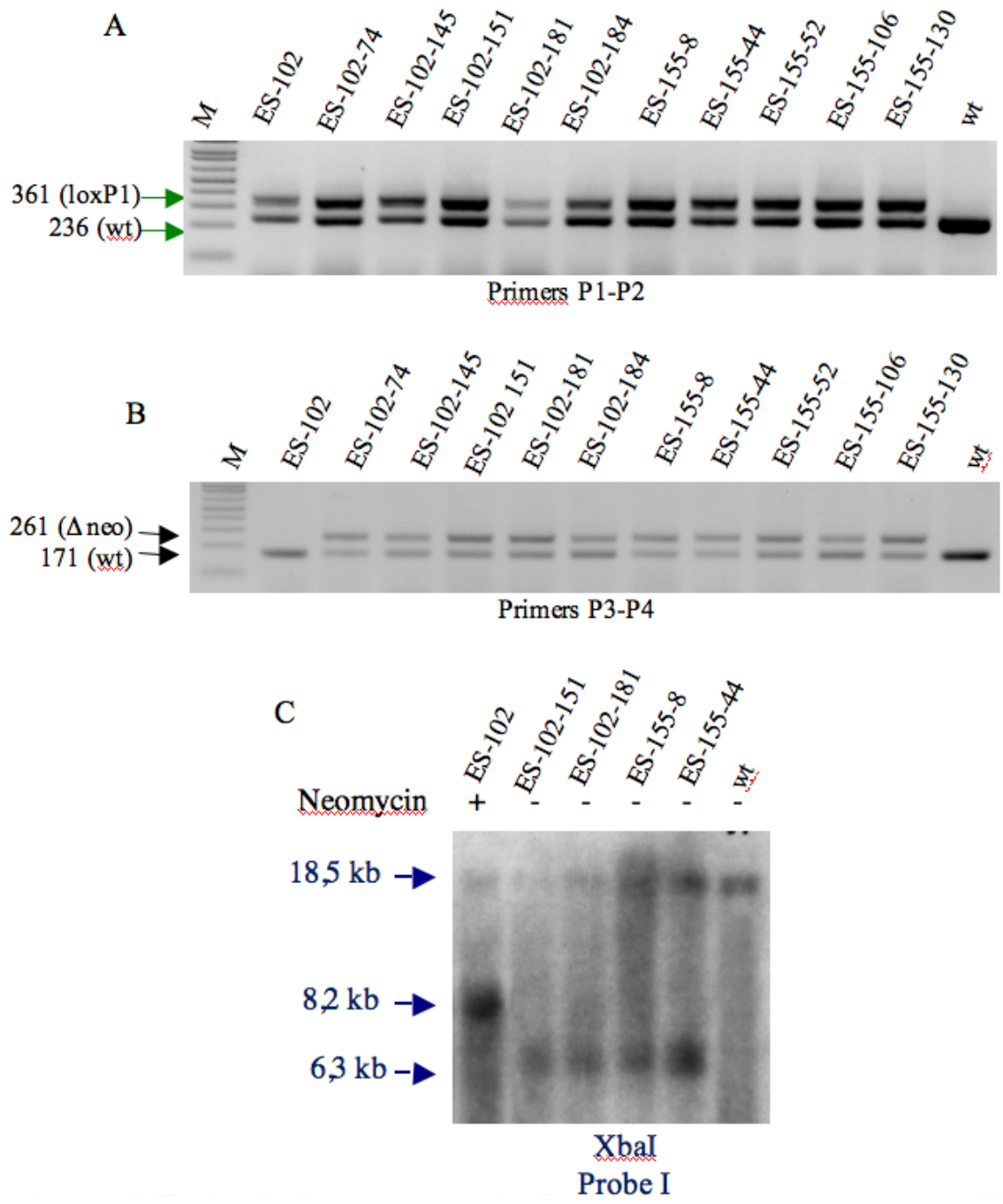

Figure 6: Cre mediated deletion of *neo* cassette in targeted ES cells. PCR analysis was used to screen ES cell clones. Arrowheads in Figure 6 indicate the location and direction of the primer sets (P1,P2 and P3,P4) used for identification of specific alleles. A-B) Expected lengths of the PCR products for detecting loxP1 (A) and neo deletion alleles (B): lane 1, 1 kb DNA ladder; lane 2, emilin1^{3loxP} ES clones; lane 3-12, Emilin1^{loxP} ES cell clones; lane 14, wt ES clones. C) Genomic DNA from ES cell clones were digested with *Xba*I and hybridized with probe I, which detects a targeted emilin1^{3loxP} 8.2 kb fragment (lane 1), a conditional knockout Emilin1^{loxP} with the deletion of the *neo* cassette through recombination of *loxP* sites 2 and 3 and this results in detection of 6,3 kb fragment (lane 2-5) and a wild type 18,5 kb fragment (lane 6).

CYTOGENETIC ANALYSIS OF TARGETED ES CELL CLONES AND THE YIELD OF CHIMERISM

A Possible cause for the inability of ES cells to give rise to high frequency chimeras and the lack of germ-line transmission of the targeted gene mutation is aneuploidy (Longo et al., 1997) (Liu et al., 1997). Mouse pluripotent embryonic stem (ES) cells, once reintroduced into a mouse blastocyst, can contribute to the formation of all tissues, including the germline, of an organism referred to as a chimeric. The steps of electroporation, selection with G418 and in particular the use of gancyclovir (a potential mutagen), can negatively affect the generation of germline chimeras. The chromosome make-up may be important in contributing both to somatic cell chimerism and to germ line transmission. The percentage of chimerism of ES cell-embryo chimeras, the absolute number of chimeras and the ratio of chimeras to total pups born all correlate closely with the percentage of euploid metaphases in the ES cell clones injected into the murine blastocyst (Longo et al., 1997). Thus, a karyotype analysis of ES cells was performed which included the original ES R1 cell line, from which all the clones used in these experiments were derived; and the correctly targeted *Emilin1* clones before and after the deletion of *neo* cassette. Metaphase chromosome spreads were prepared from colchicine-treated cells, stained with Giemsa and count the number of chromosomes (Figure 7). Since it has been reported that an extensive number of passages during *in vitro* culture affects negatively the potential of ES cells to contribute to host tissues as well as to the germ line, the number of passages of the different ES cell clones used in the karyotype analysis is also listed in Table 4 and Table 5.

Table 4: Karyotype analysis of ES cell clones

Clone no.	% euploid cells	Passages no.
ES R1	85	15
ES-27	67	20
ES-37	65	20
ES-45	12	20
ES-102	**80**	**20**
ES-155	**70**	**20**
ES-164	60	20

Karyotype analysis of different ES cell clones after electroporation and double drug selection. ES R1 corresponds to the original ES cell R1 line, used for electroporation. Numerical aberrations are found in clone ES-45. At least 50 metaphase figures for clone were evaluate.

Table 4 show that the original ES cell line R1 contained a normal number of chromosomes in some of metaphases analysed for karyotype. In all clones investigates between 60%-80% of the metaphases analysed are euploid (40 chromosomes) and only in one clone ES-45 about 12% the metaphases analysed were euploid. Two ES cell clones with the high proportion of euploid cell (ES-102 and ES-155), were used for the transfection with Cre-recombinase,

Table 5: Karyotype analysis of different ES cell clones

Clone no.	% euploid cells	Passage no.	Pup yield (%)	Chimeras among pups (%)	% Agouti color	Offspring (F1) no Agouti/total
ES-102-74	57	26	/	/	/	/
ES-102-145	60	26	/	/	/	/
ES-102-151	71	26	30	27	60-95	0/75
ES-102-181	78	26	64	48	20-100	53/80
ES-102-184	67	26	/	/	/	/
ES-155-8	70	26	28	16	70-100	0/38
ES-155-44	76	26	37	35	10-95	11/40
ES-155-52	56	26	/	/	/	/
ES-155-106	0	26	/	/	/	/
ES-155-130	52	26	/	/	/	/

Karyotype analysis of different ES cell clones after the transfection with Cre recombinase and their fate regarding chimeric mice contribution. Numerical aberrations are found in clone ES-155-106. At least 50 metaphase figures for clone were evaluated.
Pup Yield: Number of pups per number of blastocysts implanted. (/): experiments were not performed.

The ten ES cell clones tested after the deletion of neomycin cassette, which were obtained from two clones show between 57 to 78% euploid metaphases and only in one clone ES-155-106 no euploid metaphases were observed.

It has been demonstrated that the ability of recombinant clones to contribute to the germline is lost when the proportion of euploid cells dropS below 50% (Longo et al., 1997). The euploidy of ES cell clone does not guarantee the germline transmission, but it is an effective method to exclude the clones that will not produce germline chimeras. The clones with the highest percentage (at least 70%) of euploid cell were chosen for chimeras' production. Therefore, systematic screening of ES cell clones by cytogenetic analysis prior to injection into the murine blastocyst is extremely useful in predicting their ability to contribute to the germline.

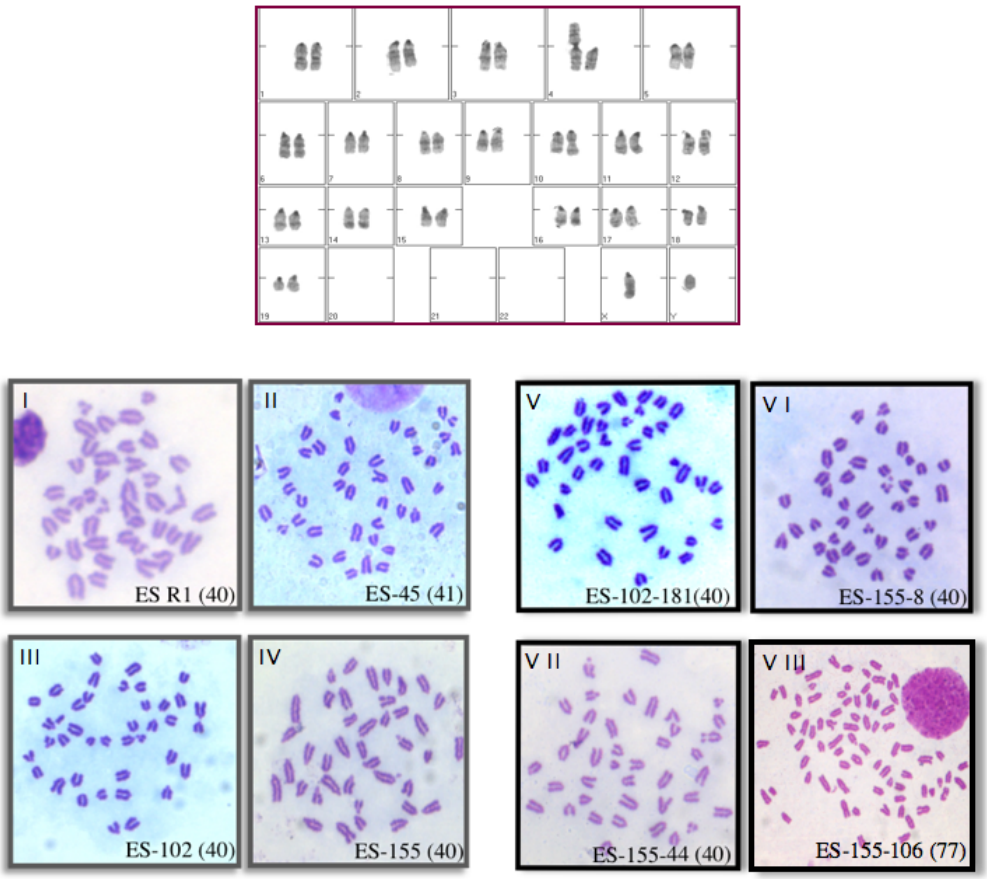

Figure 7: Metaphase analysis in targeted ES cell clones. The normal diploid karyotype of *Mus musculus* has 40 chromosomes. (I-IV) Clone ES-102 and ES-155 normal metaphase with 40 chromosomes; clone ES-45 abnormal metaphase with 41 chromosomes; ESR1 corresponds to the original ES cell R1 line, used for electroporation; (V-VIII) Clone ES-102-181, ES-155-8, ES-155-44 normal metaphase with 40 chromosomes; clone ES-155-106 abnormal metaphase.

GENERATION OF FLOXED *EMILIN1* CHIMERIC MICE

Four ES cell clones (ES-102-151, ES-102-181, ES-155-8, ES-155-44), with a correctly targeted recombination of *Emilin1* gene were microinjected into wild-type blastocysts which were subsequently implanted into CD1 foster mothers.

The ES cells that were used for the gene targeting are of Sv129/Ola origin and therefore carry an *agouti* allele. This allele is not present in the blastocyts of C57Bl/6xBDF-1 origin used for injection.

The *agouti* allele which results in brown coat colour allows to estimate the degree of chimerism of a particular animal since regions of brown coat colour are of injected ES cell origin and black coat colour is contributed by the recipient blastocyst. Figure 8 shows the mouse chimeras (G_0 generation) derived from the targeted *Emilin1* ES cell clones.

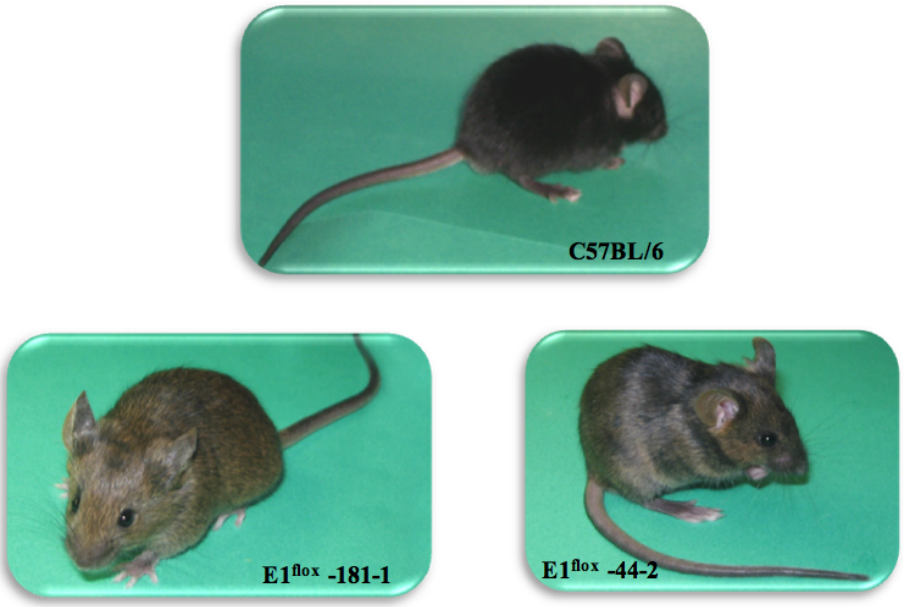

Figure 8: Chimeric animals obtained from clones ES-102-181, ES-155-44. Areas of lighter coat color (brown compared to black) reveal ES cell contribution to the host embryos, thus indicate the level of chimerism. Mouse 181-1 (male) has about 95% of ES cell contribution. Mouse 44-2 (male) has about 40% chimeric character.

Results obtained by the different ES cell microinjection experiments and the chimeric animals thus obtained are summarized in Table 6. As it can be noticed, all the targeted clones gave rise to mouse chimeras in which ES cells contributed between 10-100% to host embryonic tissues. For example, 42 blastocysts were microinjected with ES clone ES-102-181. Twenty-seven newborns were obtained in the G_0 generation and thirteen of them were chimeras ranging between 20% to 100% of ES cell contribution, respectively, as determined by their coat color.

Table 6: Results of the blastocyst injection of the ES cell clones.

Clone	Number of blastocyst injected	Number chimeras/born	Characteristic chimeras		
181	42	13 / 27	$E1^{flox}$ -181-1	95%	M
			$E1^{flox}$ -181-2	90%	M
			$E1^{flox}$ -181-3	70%	M
			$E1^{flox}$ -181-4	100%	M
			$E1^{flox}$ -181-5	100%	M
			$E1^{flox}$ -181-6	90%	M
			$E1^{flox}$ -181-7	80%	F
			$E1^{flox}$ -181-8	95%	M
			$E1^{flox}$ -181-9	95%	M
			$E1^{flox}$ -181-10	70%	M
			$E1^{flox}$ -181-11	30%	M
			$E1^{flox}$ -181-12	20%	M
			$E1^{flox}$ -181-13	60%	F
44	46	6/ 17	$E1^{flox}$ -44-1	95%	M
			$E1^{flox}$ -44-2	40%	M
			$E1^{flox}$ -44-3	40%	M
			$E1^{flox}$ -44-4	30%	M
			$E1^{flox}$ -44-5	30%	M
			$E1^{flox}$ -44-6	10%	F
8	67	3 / 19	$E1^{flox}$ -8-1	100%	M
			$E1^{flox}$ -8-2	50%	M
			$E1^{flox}$ -8-3	70%	M
151	37	3/11	$E1^{flox}$-151.1	60%	M
			$E1^{flox}$-151.2	95%	F
			$E1^{flox}$-151.3	95%	F

For all four ES cell clones chimeric animals could be obtained. The degree of chimaerism (given in %) was estimated based on the extent of agouti coat color of a particular animal. m:male; f:female. Chimeric animals for which germline transmission was tested are highlighted in gray dark.

EMILIN1 CHIMERAS TRANSMIT THE *EMILIN1* FLOXED ALLELE THROUGH THE GERMLINE

Highly chimeric animals are likely to have also germ cells of ES cell origin which is a prerequisite for germline transmission of the mutated locus. Concerning the chimeric animals listed in table 6 only the male animals with a high degree of chimerism where used to test them by breeding with C57Bl/6 females and genotyping the *agouti* coloured offspring. Female chimeras were generally not used because they are often sterile due to the contribution of the male ES cells to their germline. In chimeric females most of the cells of ES cell origin are expected to have a X0-genotype

Out of the total of the 9 chimaeras tested, 3 were shown to transmit the mutated *Emilin1* allele to the next generation (Table 7). The new mouse strains were called Emilin1$^{flox/wt}$. Emilin1$^{flox/wt}$ mice of both lines (44.2 and 181.1) were separately intercrossed to obtain Emilin1$^{flox/flox}$ mice and maintained on a C57BL/6 background.

Table 7: Results of testing chimeric animals for germline transmission of the *Emilin1* floxed locus.

Clone	Chimera		Birth	Litters	Offspring Agouti	Germline transmission
	no.	Sex (% Chimersim)				
ES-102-181	1	Male 95%	27/3/09	50	yes (35)	Yes (12)
ES-102-181	4	Male 100 %	27/3/10	-	-	-
ES-102-181	5	Male 100%	27/3/10	30	yes (18)	Yes (8)
ES-155-44	1	Male 95%	4/4/09	40	yes (11)	Yes (5)
ES-155-44	2	Male 40%	4/4/10	-	-	-
ES-155-8	1	Male 100%	11/4/09	14	no	no
ES-155-8	3	Male 70%	11/4/09	24	no	no
ES-102-151	1	Male 60%	19/4/09	31	no	no
ES-102-151	2	Female 95%	19/4/09	20	no	no
ES-102-151	3	Female 95%	19/4/09	24	no	no

Germline transmission of the *Emilin1* locus was assessed by PCR in *agouti* coloured offsprings since the *agouti* allele and the *Emilin1* locus are transmitted toghether. For some clones also females chimeras and animals with a low degree of chimerism were tested due to the lack of better candidates. Chimeric animals for which germline transmission could be demonstrated are highlighted in dark gray.

Continous crossing of heterozygous *Emilin1* mice to C57Bl/6 animals is referred to as backcrossing and is required to generate a so-called congenic mouse line. The genetic background can very often have a profound influence on the phenotype of a transgenic mouse line (Sanford et al., 2001) (Doetschman, 1999). With each backcross of heterozygous animals with animals of a defined inbred background the contribution of other genetic backgrounds is reduced. In a congenic mouse line all individual animals are genetically identical for all alleles of a particular inbred background.

Germline trasmission of Emilin1 locus was tested by Southern blot and PCR. Genomic DNA isolated from tail biopsies was digested using *Xba*I and probed with probe I (Figure 5). Southern blot confirmed the proper configuration of the *Emilin1* locus with a 18,5 kb band for the wt; 18,5 kb and 6,3 kb bands for the Emilin1$^{loxP/wt}$ and only a 6,3 kb band for the Emilin1$^{loxP/loxP}$. (Figure 9A). For future genotyping purposes a PCR based screening strategy was developed. PCR is performed using two primer: primer 5 (P5) anneals between exon 2 and upstream of second loxP, while primer 6 (P6) anneals downstream the second loxP before exon 3. Wild type animals are identified by the detection of a single band at 280 bp, for homozygous the PCR gives a single product of 366 pb size. Heterozygous *Emilin1* locus configuration allows amplification of both products as show for a representative PCR in the figure 9B.

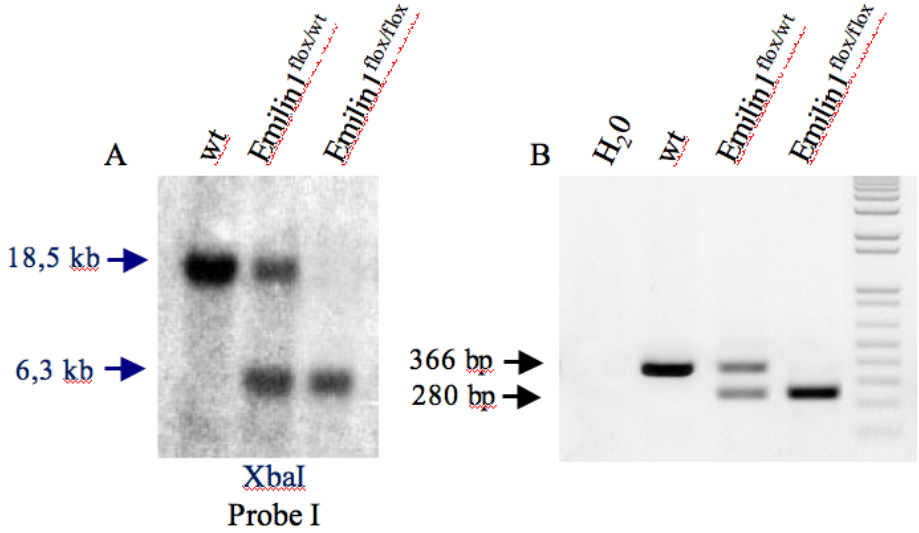

Figure 9: Genotyping of the *Emilin1* floxed allele by Southern blot and PCR. Southern blot analysis of genomic DNA of tail biopsies. Genomic DNAs were digested with *Xba*I and probed with the Probe I, resulting in a 18.5 kb band in case of the wild-type allele and a 6,3 kb band for the *Emilin1* floxed allele. B) Genotyping of *Emilin1* locus by PCR. WT, wild type animal; M, marker; H_20, no template added to the PCR as a control.

As expected, the homozygous mice are indistinguishable from their heterozygous and wild-type litter-mates. Normal amount of *Emilin1* expression in homozygous mutants was confirmed through study of mRNA. RT-PCR analysis with primers amplifying between exon 1 and exon 2 showed that levels of *Emilin1* mRNA was comparable to that detected in control mice, confirming that introduction of the *loxP* sites flanking exons 1-2 did not interfere with gene function (Figure 15).

USE OF *EMILIN1*-CREER^{T2} AND *SMMHC*-CREERT2 FOR CONDITIONAL TRANSGENESIS

We obtained a transgenic line called *SMMHC*-CreER^{T2} (Wirth et al., 2008) expressing a fusion protein of the Cre recombinase with the modified estrogen receptor binding domain (CreER^{T2}) under the control of the smooth muscle myosin heavy chain (*SMMHC*) promoter. Moreover, was generated a transgenic mouse lines expressing the Cre recombinase under the control of the *Emilin1* promoter, which allow inducible deletion of *Emilin1* floxed gene in cells that produce *Emilin1*.

Here is report the generation of Emilin1-CreER^{T2} and characterization of transgenic mice expressing Cre recombinase under the control of the *Emilin1* promoter and *SMMHC*-CreER^{T2}. These crosses enabled to determine the specificity of cre activity in adult mice, by assaying for expression of cre-dependent *LacZ* activity

CHARACTERIZATION OF *EMILIN1*-CREER^{T2} TRANSGENIC MOUSE LINES

Transgenic mice were generated with Cre recombinase under *Emilin1* promoter. For investigation of the pattern of promoter expression, a total of 3 independent expressing lines (with a efficiency of transgene of 2,8%) were generated using the constructs shown in Fig. 1 A. The founders were back crossed to set up F1 offsprings with other inbred C57BL/6 mice. All offsprings were screened by PCR (Figure 12B), indicating that the integrated *Emilin1*-CreER^{T2} gene was stably transmitted to subsequent generations. Both males and females of these mouse lines appear to have normal fertility. Dot blot analysis, permits the determination of the number of integrated copies of transgene into the genome. Basically, the genomic DNA from the different transgenic mouse lines were incubated with a radioactive-labeled probe. By employing known concentrations of the construct, the transgene copy number for each line can be estimated. The results show that the number of copy of transgene integrated into the genome was higher for mouse line 65 with 30 copies, than for mouse line 5 about 20 copies and line 20 only with 2 copies (Figure 10C)). However, the identification of transgenic mice carrying very low numbers of copies may be difficult, especially under high-hybridization background conditions. In addition, the method can be used only if there is no homology of the probe to endogenous sequences. A further and more important drawback is that the result is rather uninformative concerning important aspects such as construct integrity and the number of integration sites.

The three different transgenic mouse lines (No. 5, 20 and 65) were retained for detailed analysis of Cre mRNA expression.

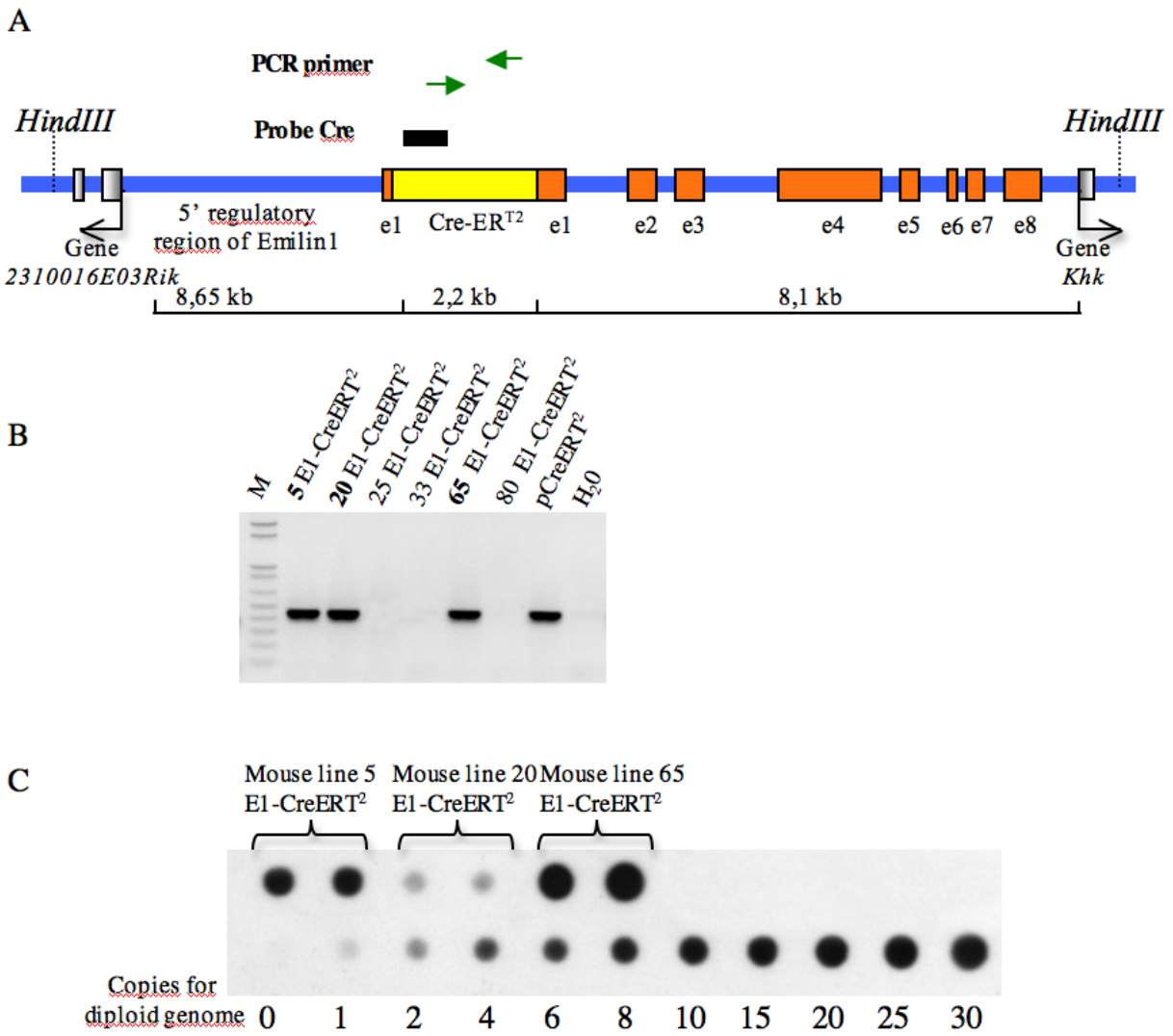

Figure 10: Generation and characterization of *Emilin1*-CreER^{T2} transgenic mouse line. A) Schematic diagram of the a *Emilin1*-CreER^{T2} transgenic construct. The Emilin1 promoter was fused to the Cre recombinase followed by a 3' end composed of 206 bp of SV40 splice and poly A. Primers for genotyping by PCR and the probe for Dot Blot are indicated. B) Genotyping of Emilin1-CreER^{T2} transgenic lines by PCR analysis of genomic tail DNA. C) Dot Blot gene copy number determination. The lane 1 containing genomic DNA of different mouse line, lane 2 containig increasing amounts of standard plasmid.

Subsequent screening of these transgenic founder lines was performed by RT-PCR using primer specific for the Cre recombinase to the identification of the Cre-mRMA expression in the tissues of the three different lines.

No Cre-mRNA expression was detectable by RT- PCR in mouse tissues derived from mice that were tested negative for the integration of the transgene by genomic PCR analysis.

We observed a similar expression of *Emilin1*-CreER^{T2} mouse line 5 and 65 in different tissues (heart, aorta, lung, kidney, skin and urinary bladder). In the mouse line number 20 there is a lower expression of Cre-mRNA in the different tissues (Figure 11).

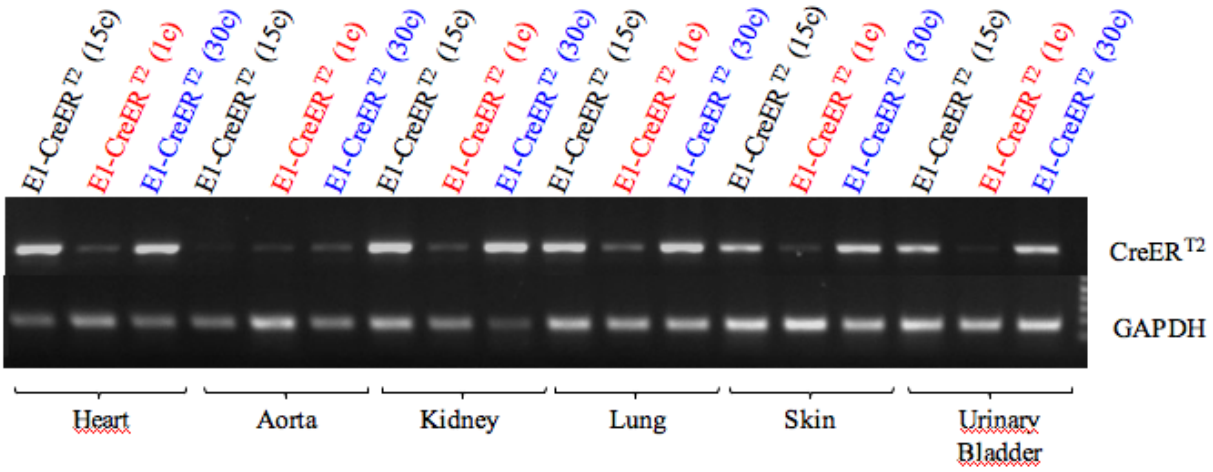

Figure 11: Expression of Cre-*mRNA* in tissues of 3-week-old mice. The expression of Cre RNA was evaluated by RT-PCR analysis using a specific set of primers in tissues taken from 3-week-old mice. The amplified fragments of 450 bp correspond to the products of the transgene, respectively. *Gapdh* was used as an internal control. M: DNA molecular size marker.

ANALYSIS OF CRE-ER^{T2} ACTIVITY IN ROSA26R REPORTER MICE

To further test function and specificity of these *Emilin1*-CreER^{T2} transgenic mouse lines in vivo, was used the mouse line number 65. *Emilin1*-CreER^{T2} (line number 65) and *SMMHC*-CreER^{T2} were crossed with Rosa26R mice that contain the bacterial *lacZ* gene preceded by a transcriptional stop sequence flanked by *loxP* sites (Soriano, 1999). Upon Cre mediated deletion of the transcriptional stop sequence this reporter line is able to mark the tissue and cells that express Cre recombinase.

To avoid any harmful effects of tamoxifen, we determined the least toxic concentration of tamoxifen that was still effective in inducing Cre recombination. Was assayed β-galactosidase activity in a variety of tissues 20 days after 5 consecutive days of intraperitoneal injection of 1, 3 and 6 mg of TM injection into an adult (40 g) mouse. The recombination was detected in different tissues with 3 mg. The lower dose is not sufficient to lead to a widespread modification of gene activity, while 6 mg leads sometimes to the death of the animals (data not shown). We selected 3 mg tamoxifen as the maximum dose that produced no toxcicity.

To test the potential use of Rosa26R/*Emilin1*-CreER^{T2} and Rosa26R/*SMMHC*-CreER^{T2} line in adult tissues, the recombination efficiency was compared following administration of Tamoxifen into 8-week-old animals. Mice were treated for 5 consecutive days with 1 mg (for 40 g mouse) of tamoxifen for Rosa26R/*SMMHC*-CreER^{T2} line and 3 mg (for 40 g mouse) for Rosa26R/*Emilin1*-CreER^{T2}. Multiple injections of these doses did not lead to any obvious change in the behavior, feeding, or appearance of the mice over the period of study.

After 20 days, a variety of tissues were analyzed for β-galactosidase expression. Cre mediated recombination was observed in different tissues examined, in particular in organs rich of smooth muscle cells (Figure 12). Background staining in some organs from control mice results from

endogenous β-galactosidase activity. C57BL6 mice (i.e., mice lacking the Rosa26R reporter) injected on five consecutive days with 1 mg/ml of Tamoxifen also displayed only background levels of *LacZ* expression (data not shown). Whole-mount examination of the thoracic aorta (Figure 12D) suggested that SMCs were labeled. When examined histologically, the aorta in *SMMHC*-CreER^{T2} mice as well as Emilin1-CreER^{T2} showed staining of the medial wall (Figure 13A). The X-gal staining indicated a stronger expression of the *LacZ* in tha aorta of Rosa26R reporter mice carrying the *SMMHC*-CreER^{T2} than the *Emilin1*-CreER^{T2} transgene.

Whole-mount staining of the heart revealed staining of the coronary arteries as well as discrete patches of staining in the myocardium (Figure 12A). The whole-mount staining of lung (data not shown) showed staining of arterioles in *SMMHC*-CreER^{T2} and *Emilin1*-CreER^{T2} mice. On histological examination of lung (Figure 13D), it is clear that vascular smooth muscle was stained for β-gal activity. The staining of very small arteries/arterioles in the mesenteric arcade and in the cerebral vessels was difficult to observe in *SMMHC*-CreER^{T2} and *Emilin1*-CreER^{T2} transgenic mice. This may reflect the following: there is a limitation in visualization of the β-gal reaction product in such a thin-walled vessel; small arteries/arterioles express lower levels of *SMMHC* and expression of the *SMMHC*-CreER^{T2} or *Emilin1*-CreER^{T2} transgene may be below the level necessary for detection.

In addition to the cardiovascular system, the *SMMHC*- CreER^{T2} and *Emilin1*-CreER^{T2} construct was active in all other smooth muscle tissues (skin, colon). Histologically analysis of colon, showed homogenous staining restricted to the circumferential and longitudinal smooth muscle layers (Figure 13B). Additional β-galactosidase activity was detected in skin of both transgenic lines (Figure 12B), while tissues from mice treated with vehicle only were negative (Figure 12A3,B3,C3,D3).

Moreover, the smooth muscle layer of the esophagus was also stained for β-gal activity in *SMMHC*-CreER^{T2}(data not shown). In the genitourinary tract, cre mediated *LacZ* expression was observed in the SMCs of the bladder (Figures 12C and 13C) of *SMMHC*-CreER^{T2} and *Emilin1*-CreER^{T2}.

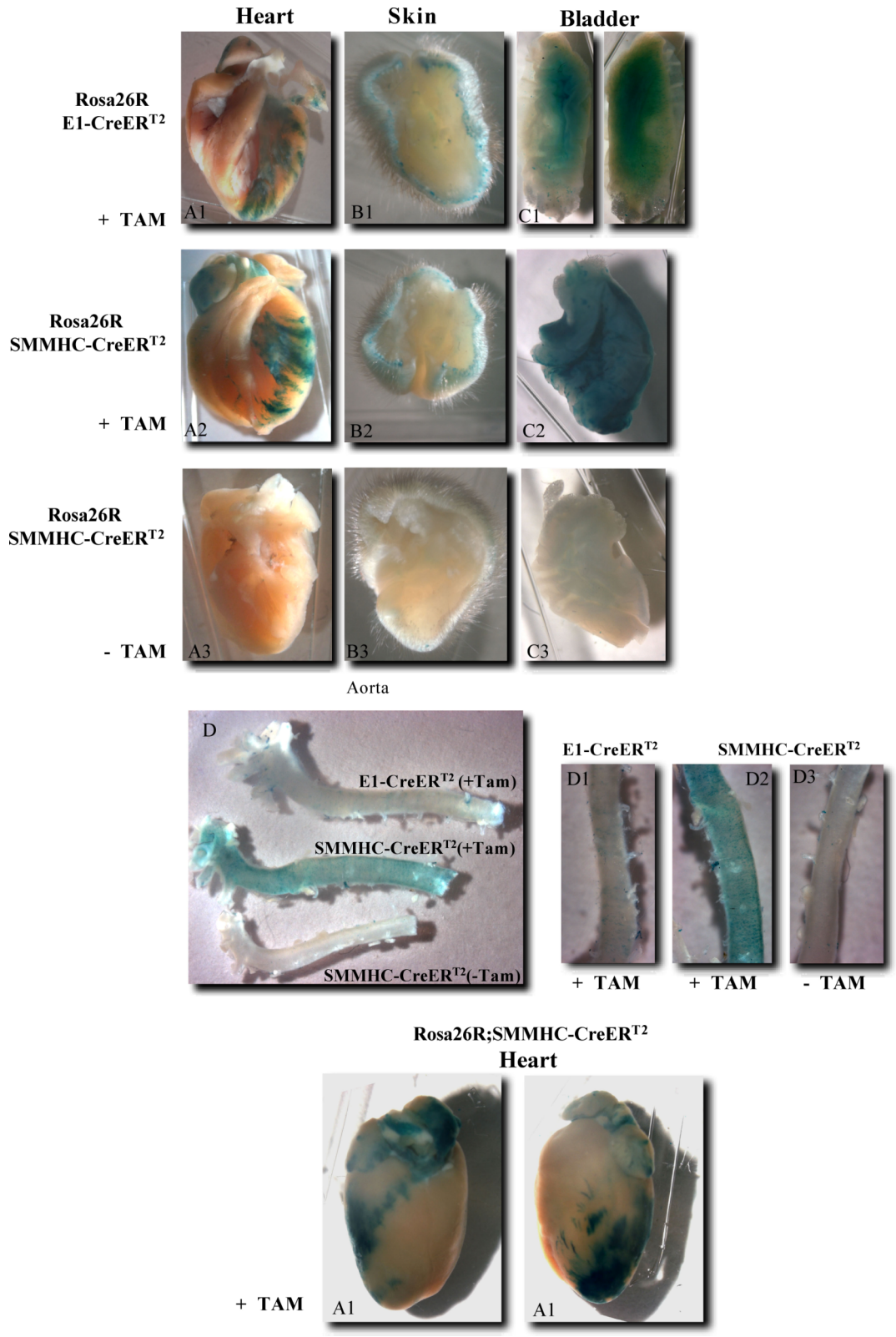

Figure 12: Whole-mount staining of various organs from adult mice that contain both the *SMMHC*-CreER^{T2} or *Emilin1*-CreER^{T2} transgene and the Rosa26R gene. Tissues were processed and stained as described in Materials and Methods. A) heart, B) skin, C) urinary bladder, D) aorta.

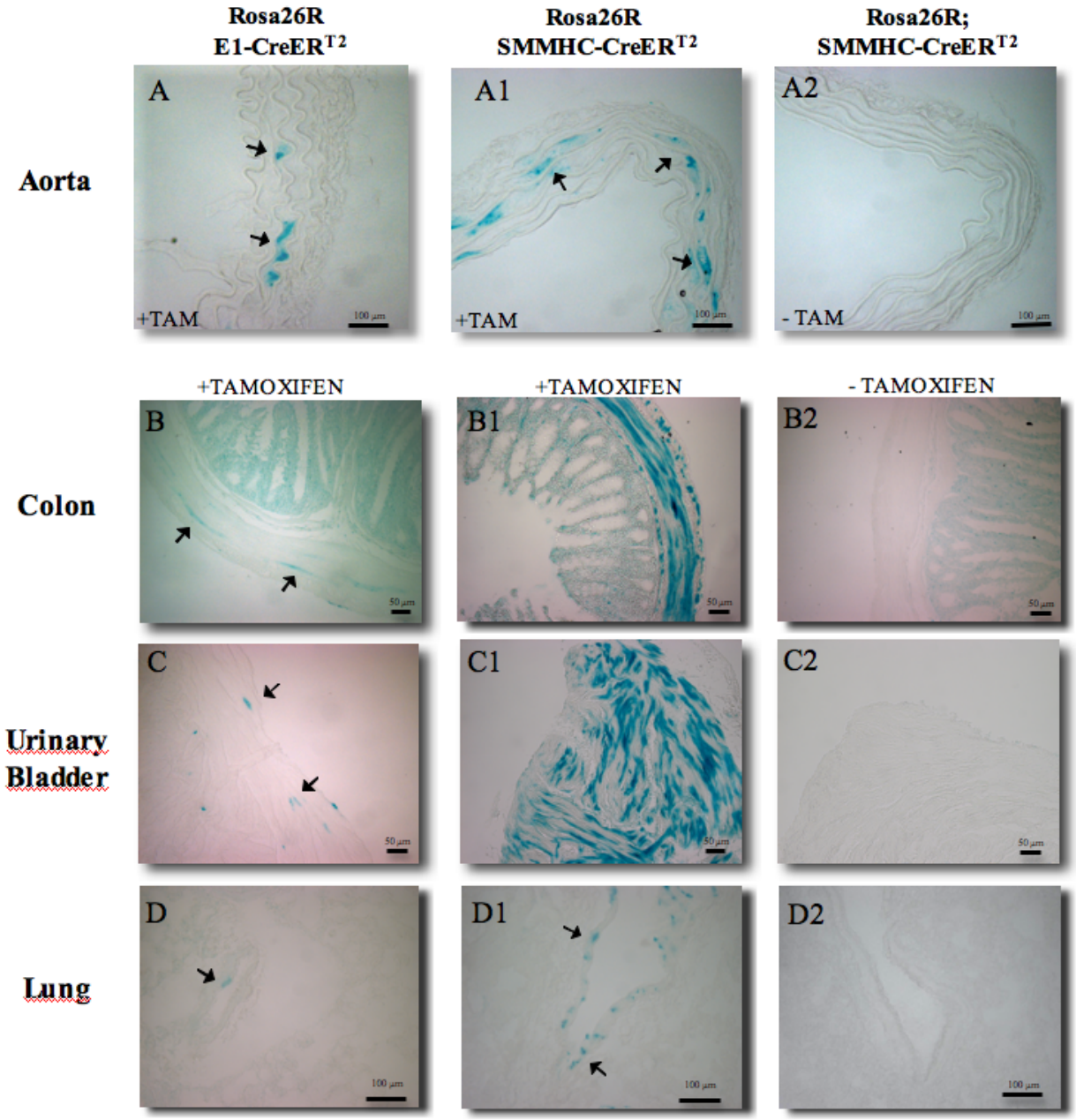

Figure 13: Histological examination of β-galactosidase expression. Twenty days after the last injection, the indicated organs were removed, stained with X-Gal, and sectioned at 12 µm. (A) X-Gal staining of lacZ expression in aorta, B) Colon, C) Urinary Bladder D) Lung. Scale bars, 100 µm (aorta and lung) and 50 µm for the other tissues.

ANALYSIS OF CONDITIONAL *EMILIN1* KO GENERATED WITH *SMMHC*-CreER^{T2}

Mice carrying Cre recombinase under the *SMMHC* promoter were mated with floxed *Emilin1* heterozigous mice in order to generate double transgenic mice. The efficiency of recombination was assessed in mice injected intraperitoneally with 1 µg/ml of tamoxifen daily for five days. The floxed allele generates a unique PCR product of 2380 bp that was measured at three, six and sixteen days from the administration of tamoxifen in aorta and urinary bladder. The percentage of cells undergoing recombination increased after 3, 6 and 16 days. Recombination was particularly strong in aorta after 16 days (figure 14). Moreover Emilin1 mRNA transcript abundance was used as a sensitive indicator of Cre/*loxP* dependent gene disruption in the aorta. We found that 5 injections of tamoxifen were sufficient for efficient gene disruption with maximal reduction of Emilin1 mRNA in aorta after 16 days from tamoxifen treatment (Figure 15).

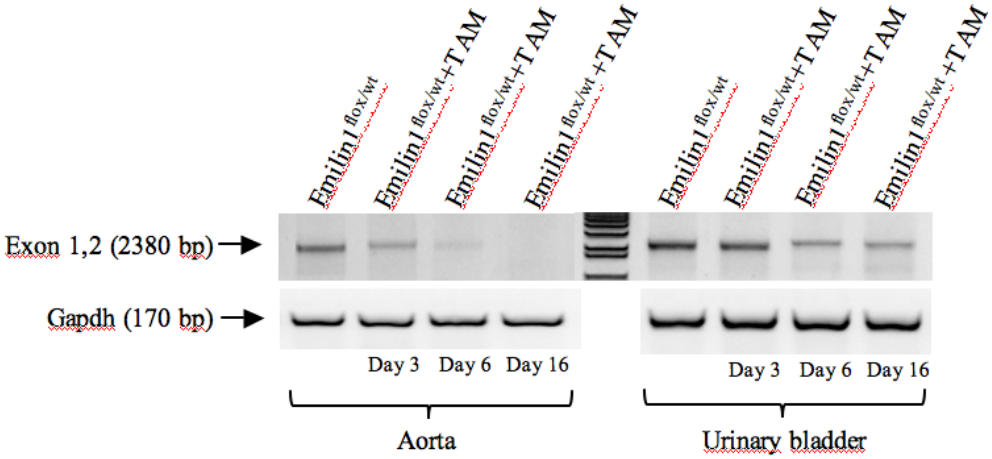

Figure 14: *In vivo* detection of Cre-mediated recombination. PCR product of floxed allele decreases due to recombination following the administration of tamoxifen.

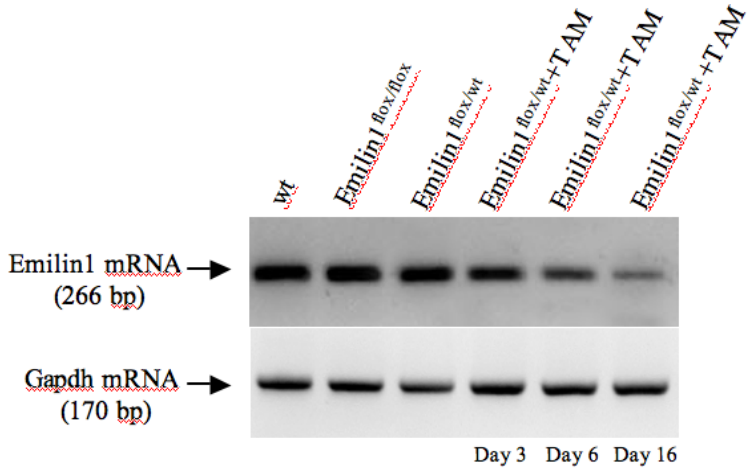

Figure 15 : RT-PCR analysis of Emilin1 mRNA expression. The primers used amplified between exon1 and exon 2. The amount of mRNA decreases after the administration of tamoxifen.

Blood pressure values were measured for 1 week before administration of tamoxifen (days –4 to -1), during the administration of tamoxifen (days –1 to 1) and during the second week after induction with tamoxifen (days 2 to 8). Systolic blood pressure, measured by non-invasive methods, increased in *Emilin1*$^{flox/flox}$ and *Emilin1*$^{flox/wt}$ compared to wild-type littermates(figure 16). Remarkably, even blood pressure of heterozygous animals increased, confirming haploinsufficiency of *Emilin1* gene for this phenotype.

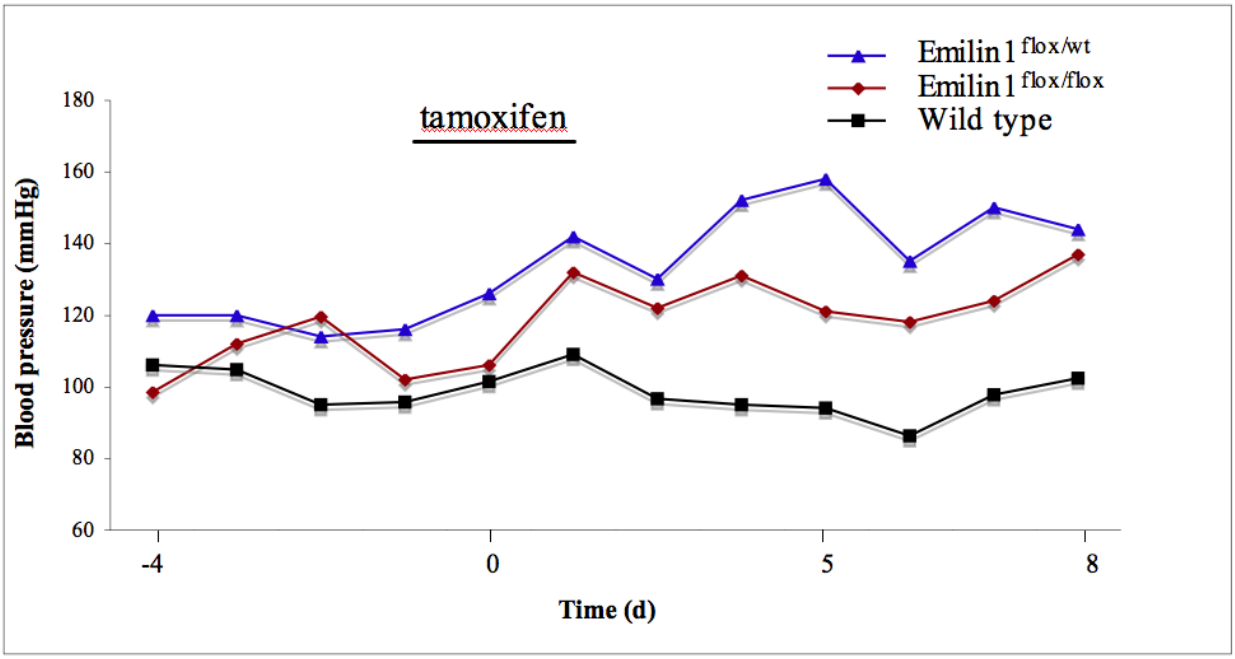

Figure 16: Preliminary recordings of systolic blood pressure. Was evaluated in wt (n=3), E1$^{flox/flox}$ (n=1) and E1$^{flox/wt}$ (n=1) mice non-invasively by tail-cuff plethysmography. Mice were induced by administration of tamoxifen on three consecutive days .

DISCUSSION

Aim of this work is to establish whether the hypertensive *Emilin1*$^{-/-}$ phenotype is the result of a defective embryonic development affecting arterial structure or the consequence of the lack of the continuous role of blood pressure regulation by Emilin-1. Although very preliminary and limited, the data obtained so far favor the latter explanation. In addition, the results concerning the characterization of the experimental design strongly suggest that the system used is adequate to the settle the issue.

Concerning the second point, it should be pointed out that the final results depend not only on the molecular device used for gene inactivation but also from the appropriate tissue targeting of the recombination event. The advent of conditional knockout technology has provided an important tool to manipulate gene expression in mice, allowing to specifically activate or inactivate genes in a cell type–specific manner, thereby significantly alleviating several limitations of conventional knockout approaches. The further development of inducible nuclear targeting of recombinases, such as the CreER^{T2} used here, has also introduce temporal flexibility to the procedure. The genetic set up of the mouse model used in this study includes floxed *Emilin1* as recombination target and *Emilin1*-CreER^{T2} and *SMMHC*-CreER^{T2} as recombinase drivers induced by the administration of tamoxifen. Considering that tamoxifen enhances TGF-β signaling (Buck and Knabbe, 2006) and that TGF-β is a regulator of blood pressure (Suthanthiran et al., 2000, Zacchigna et al., 2006) the use of the drug to stimulate a recombination event that is expected to induce hypertension may stir some concern. However, it should be noted that tamoxifen treatment in our protocol is very short (3-5 days) and insufficient to give rise to long-term increase of TGF-β activity. Moreover, precedent work has shown that the enhancement of arterial blood pressure during the tamoxifen treatment, if present, is very transient (2-3 days) and that tamoxifen-induced Cre/*loxP* recombination may be successfully applied to studies on blood pressure regulation (Wirth et al., 2008).

The first, crucial and usually the bottle-neck step of gene targeting is the construct preparation for the generation of *Emilin1* floxed mice. The DNA sequence of murine *Emilin1* gene was already isolated and characterized in my laboratory (Fabbro et al., 2005). The presence of a unique restriction site linearizing the targeting vector is important, since homologous recombination occurs more frequently when using linear foreign DNA (Hasty et al., 1991). Previous studies demonstrated that the frequency of homologous recombination is higher when the genomic DNA used for the preparation of targeting vector is derived from the same strain of mice from which the ES cells were originally isolated (te Riele et al., 1992). For this work, an ES cell line derived from mouse strain 129/Sv was used, and the source of the DNA used for the targeting vector was isolated from a murine 129/Sv. In this way, any potential decrease of targeting frequencies caused by sequence polymorphisms between targeting vector and ES cell DNA was avoided. The second important fact that needs to be considered when preparing targeting construct is the length of the region of homology between endogenous DNA sequence and foreign DNA construct. In fact, it has been demonstrated that longer regions of homology usually correspond to higher frequencies of homologous recombination, the lower limit of

homology length being in the range of about 3 kb (Deng and Capecchi, 1992). The homologous region in targeting vectors prepared for *Emilin1* gene inactivation was about 8.3 kb in total, of which 1.7 kb corresponded to the short arm and 6.6 kb to the long arm of homology.
In total, 170 ES cell clones were screened for the recombination event introducing *loxP* sites into the *Emilin1* gene and 6 correctly targeted clones were identified (3,5% frequency). Two ES cell clones with a high percentage of euploid cells were further expanded for excision of the *neo* cassette by the electroporation with Cre recombinase. The expression of Cre recombinase could generate three possible deletion alleles: recombination between *loxP1* and *loxP2*, deleting exons 1 and 2 only; between *loxP2* and *loxP3*, removing the *neo* cassette only; and between *loxP1* and *loxP3*, excising exons 1, 2, and the *neo* cassette. A substantial number of clones with neo-cassette excision were isolated (3,6% of total clones analyzed). However, the number of clones with high percentage of aneuploidy also increased; actually, while the original R1 ES cells contained about 85% of euploid cells, only 4 out of 10 clones analysed with the excised neo cassette showed a euploid cell percentage over 70%. Considering that ES cell clones having more than 50% of aneuploid metaphases are unable to colonize host tissues, and thus are unable to contribute to embryonic tissue formation and to the germ line (Longo et al., 1997), our results highlight the importance of the karyotype analysis for selection of the clones to be injected into blastocysts for generation of chimeras with efficient germline transmission. In fact, the four selected ES cell clones with the exon 1 and 2 floxed and the excision of *neo* cassette produced 25 chimeras with a high rate of chimerism (between 10% to 100%), without any obvious abnormalities, and were fertile. In addition, out of the total of the 9 chimaeras tested, 3 were shown to transmit the mutated *Emilin1* allele to the next generation, an outcome to be considered satisfactory. As expected, the homozygous mice are indistinguishable from their heterozygous and wild-type litter-mates. In addition, the blood pressure of Emilin1$^{flox/flox}$ mice carrying *SMMHC*-CreER^{T2} before tamoxifen treatment was normal confirming that the introduction of the *loxP* sites flanking exons 1-2 did not interfere with gene function.

Among the different inducible systems of Cre recombinase expression (Nagy, 2000, Kwan, 2002, Branda and Dymecki, 2004), a tamoxifen regulated one was chosen. This method is based on the use of Cre-ER, a fusion protein between a mutated ligand binding domain of the human estrogen receptor (ER) and Cre recombinase that translocates into the nucleus in the presence of tamoxifen, but not natural ER ligands. (Feil et al., 1996, Kellendonk et al., 1996, Indra et al., 1999). In particular, the CreER^{T2} version used here showed a tamoxifen sensitivity higher than other Cre-ER mutants (Indra et al., 1999). This high sensitivity reduces the amount of tamoxifen administered to the animals needed for efficient recombination, thus avoiding toxic effects of the drug.

As Cre drivers, the *Emilin1* and the smooth muscle myosin heavy chain (*SMMHC*) promoters were used to target gene inactivation. The *SMMHC* promoter is active almost exclusively in smooth muscle cells (Madsen et al., 1998, Regan et al., 2000, Wirth et al., 2008). On the other hand, the *Emilin1* promoter is active in a broader group of tissues, including, in addition to visceral smooth muscle, interstitial connective tissue and the entire blood vessel wall (endothelium vascular smooth muscle cells of the media adventitial fibroblasts) (Braghetta et al.,

2002). Despite these differences, recombination induced through both promoters is expected to affect blood pressure, the reason being that, for blood pressure control, Emilin-1 expression is required in vascular smooth muscle cells (Litteri et al., unpublished results).

Transgenic mice with a frequency of 2,8% were generated by microinjection of the *Emilin1*-CreER^{T2} transgene. A total of three lines were obtained, and dot blot indicated that each line conteined from two to thirty copies of the transgene.

The two strains of transgenic mouse expressing Cre-ER^{T2} under the control of two different promoter were assessed in vivo the inducibility and activity of Cre-ER^{T2} after administration of tamoxifen. The data show that the two promoters achieve different expression levels in tissues, the *SMMHC* one being more strongly expressed at most locations. This is suggested by the more intense histochemical staining for *lacZ* in the Rosa26R background. This is not unexpected, because high production of myosin heavy characterizes differentiation of smooth muscle cells, while Emilin1 is more abundant during development and its expression subsides in adulthood (Braghetta et al., 2002). It should be noted, however, that the *Emilin1*-CreER^{T2} transgene was express in the adult, as indicated by the detection of Cre mRNA by RT-PCR in all tissues analyzed in the three lines generated. Moreover the *SMMHC*-CreER^{T2} efficiently excised the *loxP* flanked sequences in the Emilin1$^{flox/wt}$ locus and the cre recombinase activity was sufficient to decrease the *Emilin1* mRNA expression.

Will be interesting to understand the meaning of the results showing a different levels of Cre expression from the two promoters in the context of this study. Should we expect more pronounced phenotypes concerning blood pressure control with the *SMMHC*-CreER^{T2} or *Emilin1*-CreER^{T2} promotors for *Emilin1* inactivation? This is really hard to say, mainly because Emilin-1 regulates blood pressure by modulating peripheral resistance, a parameter that depends on the contraction of small arteries, and neither promoter gave evidence of recombination in second branch mesenteric arteries or small vessels in any other district. This may reflect a limitation in visualization of the β-gal reaction product in such a thin-walled vessel. The expression of the *SMMHC*-CreER^{T2} or *Emilin1*-CreER^{T2} transgene may be below the level necessary for detection.

The scanty data obtained so far on the blood pressure following tamoxifen induction of recombination indicate that at least the *SMMHC*-CreER^{T2} construct was effective in altering this phenotype. The value of the *Emilin1*-CreER^{T2} setup will be appraised shortly.

A result raising some questions on the use of *SMMHC*-CreER^{T2} and *Emilin1*-CreER^{T2} as drivers for recombination is the staining of cardiac myocytes in a diffuse region of the left ventricle and atria in the Rosa26R background with both constructs. A similar finding was reported previously with a different *SMMHC*-Cre transgene (Regan et al., 2000). Although this targeting event should not influence blood pressure, the effect on cardiac function should be closely scrutinized. On the other hand, the result is intriguing, since the function of smooth muscle myosin heavy chain in cardiomyocytes is presently unknown.

The experiments recording the effect of *Emilin1* inactivation in adult animals is still ongoing. The scant data obtained so far are, however, very encouraging, although their statistical significance cannot be stated at the moment. Amazingly, while tamoxifen treatment did not

induce any variation of arterial blood pressure in *SMMHC*-CreER^{T2} mice with an *Emilin1* wild-type genotype, animals with the *Emilin1*$^{flox/flox}$ or *Emilin1*$^{flox/wt}$ context exhibited a stepwise increase of blood pressure peaking at the latest day of examination (8 days from the beginning of tamoxifen treatment) in the case of the homozygous floxed animal. Any speculation on these data is really premature; nevertheless, it should be noted that such behavior is the one expected on the hypothesis that hypertension in *Emilin1* deficient mice is not due to a developmental defect of blood vessels, but the lack of Emilin-1 function of keeping TGF-β signaling continuously at bay even in the adult.

Subsequently the analyses will focus on the characterization of the vascular phenotype induced by the conditional knockout and the effects on TGF-β1 signaling. *Emilin1*$^{flox/flox}$;*Emilin1*-CreER^{T2} and *Emilin1*$^{flox/flox}$;*SMMHC*-CreER^{T2} animals will be treated with tamoxifen and then different structural and functional parameters of blood vessels and TGF-β signaling will be investigated.

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APPENDIX

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Lentiviral-mediated RNAi *in vivo* silencing of *Col6a1*, a gene with complex tissue specific expression pattern

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ABSTRACT

RNA interference (RNAi) through the use of lentiviral vectors is a valuable technique to induce loss of function mutations in mammals. Although very promising, the method has found only limited application and its general applicability remains to be established. Here we analyze how different factors influence RNAi mediated silencing of *Col6a1*, a gene of the extracellular matrix with a complex pattern of tissue specific expression. Our results, obtained with vectors pLVTHM and pLVPT-rtTRKRAB, point out three parameters as major determinants of the efficiency of interference: the choice of interfering sequence, the number of proviral copies integrated into the mouse genome and the site of insertion of the provirus. Although low copy number may produce efficient interference with low frequency, the general trend is that the number of integrated proviral copies determines the level of silencing and the severity of phenotypic traits. The site of insertion not only determines the overall intensity of expression of the small interfering RNA (siRNA), but also introduces slight variability of silencing in different organs. A lentiviral vector (pLVPT-rtTRKRAB) with doxycycline-inducible production of siRNA was also tested. Control of expression by the drug was stringent in many tissues; however, in some tissues turning off of siRNA synthesis was not complete. The data support the application of lentiviral vectors used here in transgenesis.

1. Introduction

The development of transgenic animal technology represents an invaluable tool for understanding gene function and to dissect genetic interactions. In addition, transgenic animals have increasing importance in biotechnological applications including improvement of livestock, xenotransplantation and the production of biologically active pharmaceuticals.

One major advancement has been the establishment of procedures for targeted gene inactivation in the mouse using embryonic stem cells (Capecchi, 1989; Mansour et al., 1988). This method and its more recent improvements based on site-specific recombination allow a systematic study of gene function and are now an indispensable aid in the study of molecular mechanisms of human diseases (Branda and Dymecki, 2004). Despite the sophisticated genetic manipulations achievable, however, this method suffers major limitations. First of all, the preparation of constructs and the generation of transgenic animals are time consuming and costly. Secondly, the method can be carried out efficiently only in the mouse at the moment among mammals.

An alternative procedure has become available in the recent years for gene silencing based on RNA interference (RNAi) (Mello and Conte, 2004). The method is particularly efficient in cells cultured *in vitro*, where interfering sequences can be either administered directly as transfected small interfering RNAs (siRNAs) or expressed as small hairpin RNAs (shRNAs), that are processed to siRNAs in the cell, using retroviral vectors. Transfer of shRNA producing constructs into mammalian embryos has been realized through pronuclear injection and transfection into embryonic stem cells (Xia et al., 2006). These methods suffer several limitations and a more promising method for large-scale application entails the use of lentiviral vectors, a gene delivery system that does not suffer developmental repression and that efficiently transduces embryos from many different species (Dann, 2007; Pfeifer,

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2004). The method has been applied for a limited number of genes with variable effects on their expression levels. At the moment the general applicability and relevance of this method remains to be established. For example, it is not known whether silencing of genes with a complex pattern of expression is equally efficient in all tissues where such genes are activated; likewise, no information is available on silencing achievable for genes whose protein products have a slow turnover and persist for long time in the tissues. These two aspects are particularly relevant for genes coding for extracellular matrix components.

In this report we analyze how different factors influence RNAi mediated silencing of *Col6a1*, a gene that codes for one subunit of collagen type VI. Collagen VI is an extracellular matrix protein composed of three different polypeptides, α1(VI), α2(VI) and α3(VI), encoded by separate genes (Lampe and Bushby, 2005). All three chains are necessary for the assembly of the triple helical conformation that characterizes the native collagen VI molecule. When one of the three chains is missing, the other two are synthesized, but cannot assemble into the triple helical conformation and are degraded inside the cell (Lamande et al., 1999). As a consequence, no collagen VI molecules are deposited in the extracellular matrix. Deficiency of collagen VI in the mouse induces muscle alterations that mimic those found in two human heritable diseases of collagen VI, Bethlem myopathy and Ullrich congenital muscular dystrophy (Angelin et al., 2007; Bonaldo et al., 1998; Irwin et al., 2003).

Information on the regulation of expression of collagen VI comes from studies on the α1(VI) chain (Braghetta et al., 1996, 2008; Fabbro et al., 1999; Girotto et al., 2000; Vitale et al., 2001). Regulation is mainly achieved through different enhancers, each of which controls transcription only in a limited set tissues. Available evidence indicates that enhancer activation is the consequence of inductive signals on collagen VI producing from nearby cells (e.g. myoblasts on connective tissue cells of skeletal muscle; neurons on peripheral glia precursors). This type of regulation produces variable levels of α1(VI) collagen mRNA in different tissues. Considering that siRNA expression may be variable due to the disparate site of insertion of the interfering transgene into the mouse genome, the ratio of siRNA/mRNA may be critical to achieve efficient knockdown. This potential source of diversification makes collagen VI a useful model to study the factors influencing silencing in different tissues.

We have generated several transgenic mouse lines expressing siRNAs that target the α1(VI) mRNA. Characterization of the different lines and comparison of the phenotypes with that of *Col6a1* knockout mice have allowed a systematic evaluation of the different factors affecting silencing of the target gene. In addition, the applicability of an inducible lentiviral vector system in gene knockdown experiments has also been analyzed.

2. Materials and methods

2.1. Interfering sequences

Two interfering sequences corresponding to distinct regions of α1(VI) mRNA, as well as *Silencer*® negative control #1 with no homology for human or mouse genes, were obtained from Ambion. Sequences are: siRNA1, 5′-GGUGAAGUCCUUCACUAAG-3′; siRNA2, 5′-GCCACGUCUAAGUAUCAUU-3′ and siRNAC, 5′-TTCTCCGAACGTGTCACGT-3′.

2.2. Plasmid constructs

siRNA1, siRNA2 and siRNAC were adapted for expression of shRNAs from a gene transfer vector. A 9-nt loop (TTCAAGAGA) was inserted between sense and anti-sense sequences. According to described sequences, pairs of phosphorylated oligonucleotides (Invitrogen) were annealed and inserted into the non-inducible pLVTHM vector downstream from the H1 promoter (http://www.addgene.org/Didier_Trono). The resulting vector is indicated here for brevity as nLVsh1/2/C (n stands for non-inducible plasmid).

For RNAi silencing regulated by doxycycline, the MscI-FspI fragment containing the tetO-H1-shRNA1/C cassettes was excised from npLVsh1/C and cloned into the corresponding sites of pLVPT-rtTRKRAB-2SM2 (Szulc et al., 2006). We will refer to these constructs as iLVsh1/C (i stands for inducible plasmid). The plasmid constructs were sequence verified and used for virus stock production.

2.3. Cell lines and transfection

293FT (Invitrogen) and NIH3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. Transfections were carried out with Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer using 7.5×10^5 NIH3T3 or 3×10^6 293FT cells in 10 cm Petri dishes. Total RNA was extracted from NIH3T3 cells 48 h after transfection.

2.4. Lentivirus production and viral titer determination

All recombinant lentiviruses were produced by transient transfection of 293FT cells. Briefly, 3×10^6 293FT cells were seeded on 10 cm Petri dishes and, when subconfluent, they were cotransfected with 15 μg of the appropriate gene transfer vector, 4.5 μg of pMD2.G and 10 μg of psPAX2 by Lipofectamine 2000 reagent (Zufferey et al., 1997). Following one change after 24 h, the medium containing recombinant lentivirus was harvested at 48 h, centrifuged at 100,000 × *g*, 4 °C for 3–5 h, and re-suspended in PBS. Viral stocks were used for immediate transduction or stored at −80 °C.

To determine the viral titer 3×10^5 293FT cells were plated on 6 cm dishes and infected at 60% confluence with serial dilutions of viral stock and 8 μg ml^{-1} of polybrene (hexadimethrine bromide, Sigma). 48 h after the transduction cells were harvested, washed and analysed by FACS for green (GFP) fluorescence.

2.5. In vitro gene silencing

3×10^5 NIH3T3 cells were plated on the 6 cm dishes and medium containing recombinant lentiviruses (10^6 I.U. ml^{-1}) and polybrene was added after 16 h. At 48 h from transduction cells were split and, when confluent, analyzed by FACS and by Northern blotting to determine the percentage of infected cells and the corresponding gene silencing level, respectively.

2.6. Production, characterization and manipulation of transgenic mice and embryos

B6D2F1 females were superovulated with 5 I.U. of pregnant mare serum (Folligon, Intervet) and 5 I.U. of human chorionic gonadotropin (Chorulon, Intervet) (Nagy et al., 2003) and mated with B6D2F1 males. The concentrated viral suspension was microinjected into the perivitelline space of one cell embryo prepared using standard procedures (Nagy et al., 2003). The embryos were washed 20 times by successive transfer into drops of KSOM medium (Erbach et al., 1994) and implanted into the oviduct of CD1 pseudopregnant mice.

Transgenic mice were identified by PCR on DNA from tail biopsies using GFP primers and the number of copies of proviral integrations was determined by dot blot analysis using a GFP probe.

To control expression of siRNA in transgenic mice generated with the inducible vector pLVPT-rtTRKRAB-2SM2, doxycycline (2 mg ml^{-1}) was added to drinking water together with 5% sucrose for 15 days (Szulc et al., 2006).

Some embryos were cultured in KSOM medium at 37 °C in 5% CO_2. At different developmental stages the embryos were fixed for 5 min with 4% paraformaldehyde in PBS, stained with 5 mg ml^{-1} propidium iodide and observed in a confocal microscope (BioRad).

2.7. RNA isolation, Northern blotting and real time RT-PCR analysis

Total RNA was extracted from diaphragms with TRIzol® Reagent (Invitrogen) according to the supplier's protocol. Northern blot analysis was performed as described using 15 μg of total RNA (Piccolo et al., 1995).

For real time RT-PCR, total RNA (1–5 μg) was digested with DNase I (Epicentre) and then reverse-transcribed with oligo-$dT_{12–18}$ (Amersham Pharmacia) using Superscript III (Gibco-BRL) and treated with RNase H (Epicentre). cDNA was amplified with AmpliTaq Gold DNA polymerase (Applied Biosystem) and quantified using SYBR Green (Sybr Green PCR core reagents kit, Applied Biosystems) on a Rotor-Gene 3000 real-time analyzer (Corbett Robotics, Australia). The following primer sequences were used for the α1(VI) chain: forward primer: 5′-GATGAGGGTGAAGTGGGAGA-3′; reverse primer: 5′-CACTCACAGCAGGAGCACA-3′. In each sample, the mRNA expression of collagen VI was normalized to S16 expression (forward primer: GCAGTACAAGTTACTGGAGCC; reverse primer: CGGTAGGATTTCTGGTATCG) and the relative quantitation was calculated utilizing the mathematical model described by Pfaffl (Pfaffl, 2001). Data were expressed as the mean ± SEM and analyzed for significance using one-way ANOVA and Bonferroni's modified *t*-test at the 95% confidence interval.

2.8. Quantification of siRNA

For RT-PCR detection of mature siRNAs/small RNAs, we followed an established protocol (Shi and Chiang, 2005) with minor modifications. Briefly, 5 μg of total RNA were polyadenylated with *E. coli* poly(A) polymerase (Ambion). After phenol–chloroform extraction and ethanol precipitation, the RNAs were dissolved in water and reverse-transcribed with 200 U SuperScript™ II reverse transcriptase (Invitrogen) and 0.5 μg poly(T) adaptor 5′-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN-3′. PCR products corresponding to mature siRNA were amplified (30–36 cycles) using a forward primer specific for each mature siRNA and an universal reverse primer: siRNA1 (5′-GGTGAAGTCCTTCACTAAG-3′), siRNAC (5′-TTCTCCGAACGTGTCACGT-3′), mouse snRNA U2 (5′-ACGCATCGACCTGGTATTG-3′), reverse primer (5′-GCGAGCACAGAATTAATACGACTCAC-3′).

2.9. Preparation of tissue extracts

Frozen mouse tissues were pulverized by pestle and mortar and lysed with a solution containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 2% SDS, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 3 mM glycerol 2-phosphate, and protease inhibitors (Complete, Roche). Proteins were solubilized by heating at 70 °C for 10 min and the samples clarified by centrifugation at 4 °C.

2.10. Gel electrophoresis and immunoblot

Samples were reduced with 5% β-mercaptoethanol and subjected to SDS–PAGE on 4–12% (w/v) gradient polyacrylamide gels (NuPAGE, Invitrogen). Proteins were electrophoretically transferred to Immobilon-P transfer membrane (Millipore). The collagen VI α1 chain was detected using a rabbit polyclonal antibody (H-200, Santa Cruz Biotechnology) at 1:1000 dilution. For loading control, antibodies against glyceraldehyde-3-phosphate dehydrogenase (MAB374, Chemicon, 1:2000) or β-actin (Sigma, 1:4000) were used. Filters were treated with secondary antibody conjugated with horseradish peroxidase (GE Healthcare, 1:1000) and bands were detected by chemiluminescence (SuperSignal West Pico, Pierce).

2.11. Detection of apoptosis

Apoptosis was detected using the ApopTag *in situ* apoptosis detection kit (Chemicon) on paraffin-embedded mouse diaphragm muscle sections (7 μm). Samples were stained with peroxidase-diaminobenzidine to detect TUNEL-positive nuclei and with Hoechst 33258 (Sigma) to mark all nuclei. The number of total and TUNEL-positive nuclei was determined in randomly selected fields using a Zeiss Axioplan microscope equipped with a digital camera. Data were analyzed with the unpaired Student *t* test.

2.12. Mitochondrial membrane potential

Fibers from *flexor digitorum brevis* (FDB) muscle were isolated as described (Irwin et al., 2002). The tissue was incubated in Tyrode's solution (Sigma) supplemented with 0.1% collagenase type I (Sigma) and 10% FBS for 45 min at 4 °C. The temperature was raised

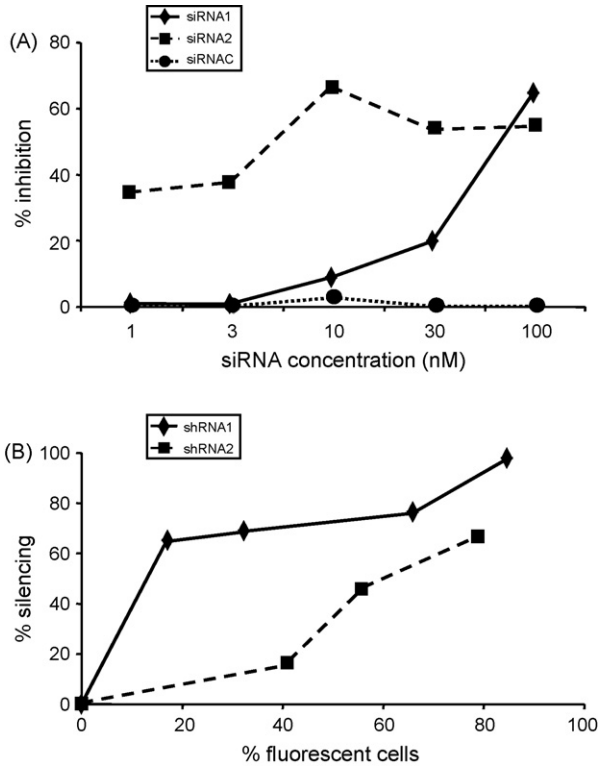

Fig. 1. Test of selected interfering sequences *in vitro*. (A) Dose-response analysis for sequence specific (siRNA1 and siRNA2) and control (siRNAC) siRNAs. NIH3T3 cells were transfected with the indicated concentrations in the culture medium of siRNAs using lipofectamine. Total RNA was extracted after 48 h and α1(VI) collagen expression analyzed by Northern blotting. (B) Silencing effect of shRNAs. Cells were transduced with different dilutions of the viral particles suspension and the infection efficiency and α1(VI) mRNA determined by FACS and Northern blotting analysis respectively. shRNA 1 and shRNA 2 are the short hairpin RNAs expressed by the viral vectors nLVsh1 and nLVsh2.

Table 1
Production of transgenic animals.

Viral construct	Average titer (U ml^{-1})	Zygote transfers	Number of born animals	Number of transgenic mice (%)	Range of proviral copy number (average)
nLVØ	5.1×10^8	400	123	34(28)	1–20(2)
nLVsh-ctrl	3.7×10^8	444	143	27(19)	1–6(2)
nLVsh-1	6.4×10^8	290	127	38(30)	1–≥20(4)
nLVsh-2	4.1×10^8	394	119	25(21)	1–7(2)
iLVsh-1	1.0×10^8	270	111	26(23)	1–4(2)
Total	4.1×10^8	1798	623	150(24)	1–12(2)

Table 2
Distribution of proviral copy number in transgenic animals.

Viral construct	No. of transgenic mice	No. of mice with the indicated copy number						
		1 (%)	2 (%)	3–4 (%)	5–10 (%)	11–15 (%)	16–20 (%)	20 (%)
nLVØ	34	22	6	4	1	–	–	1
nLVsh-ctrl	27	17	4	4	2	–	–	–
nLVsh-1	38	23	8	1	4	1	–	1
nLVsh-2	25	16	7	1	1	–	–	–
iLVsh-1	26	17	5	4				
Total	150	95(63.3)	30(20)	14(9.4)	8(5.3)	1(0.7)		2(1.3)

to 37 °C, and the incubation continued for additional 45 min. The muscle mass was removed and washed twice in DMEM and the fibers, suspended in Tyrode's solution with 10% FBS, were plated on glass coverslips (24 mm diameter) precoated with laminin (Roche) (3 μg cm^{-2}). The cells were cultured in DMEM containing 10% FBS for 1 day before starting the experiment. Only apparently healthy fibers (i.e. fibers without structural alterations) adhered to the coverslips. For measurement of mitochondrial membrane potential FDB myofibers were cultured in 1 ml Tyrode's solution and processed as described (Merlini et al., 2008).

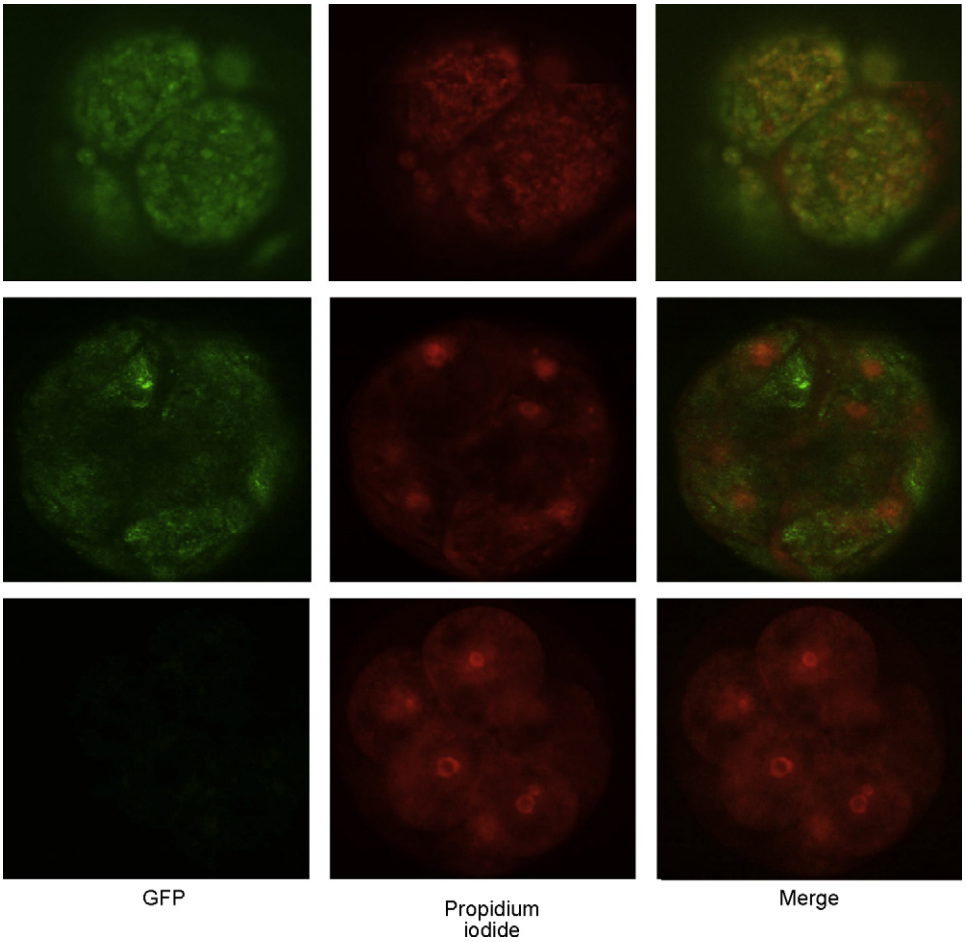

Fig. 2. Expression of green fluorescent protein (GFP) in mouse embryos transduced with lentiviral particles at the stage of one cell. Upper panels, transgenic 2-cell embryo; middle panels, transgenic 16-cell morula; lower panels, control (non-transgenic) 8-cell embryo. Embryos were fixed with paraformaldehyde, stained with propidium iodide and observed in a fluorescence microscope. All cells of transgenic embryos express the GFP marker.

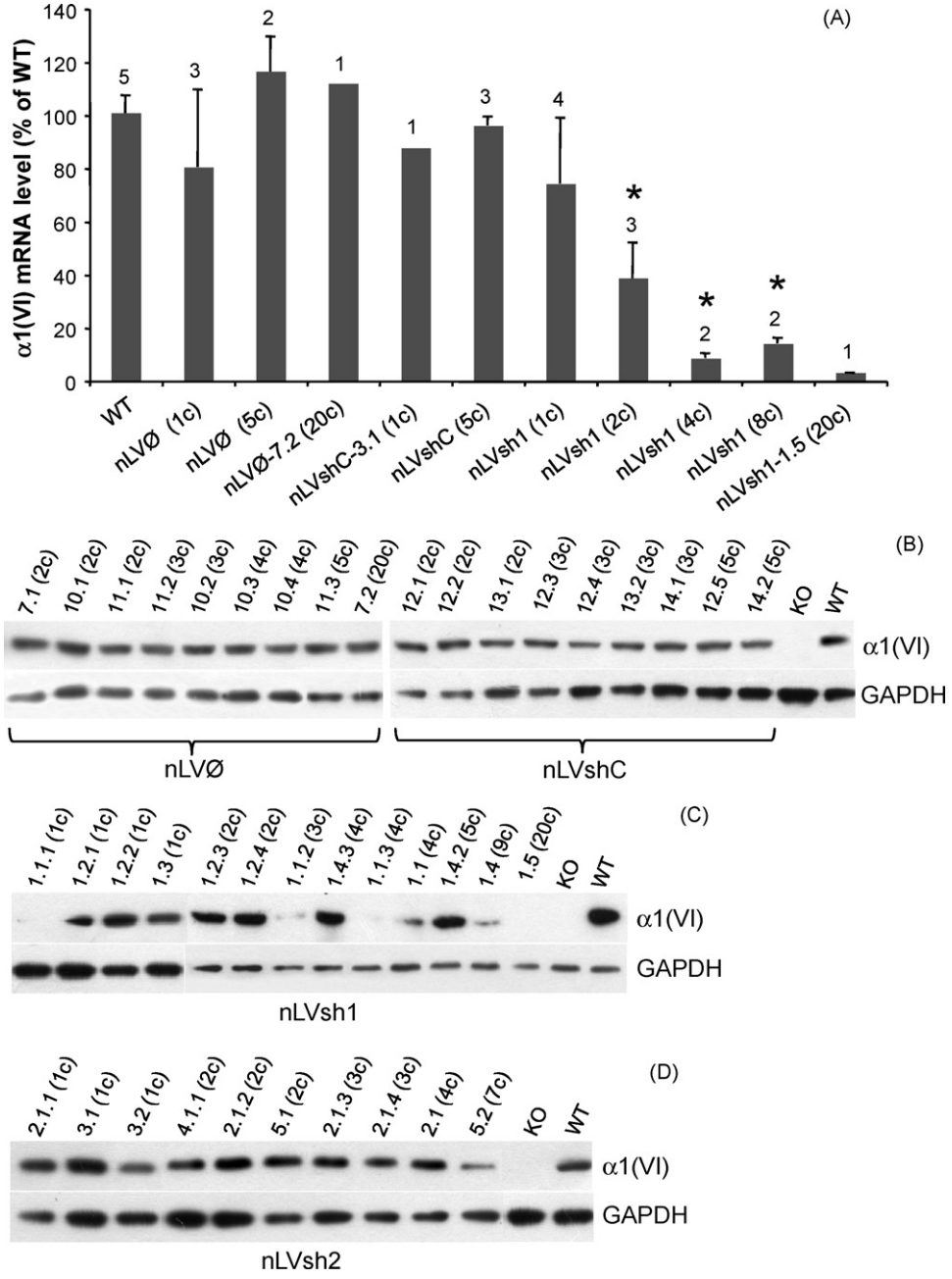

Fig. 3. Silencing of α1(VI) collagen expression *in vivo*. (A) Investigation of mRNA in diaphragm muscle by real time RT-PCR. The number of animals analyzed in each group is indicated above the bars. (B–D) Comparison of protein levels in diaphragm muscle of different mice by western blotting. None of control samples, derived from transgenic mice carrying either the empty lentiviral vector (nLVØ) or an unrelated sequence (nLVshC) (B), showed reduced synthesis of the protein. Silencing was frequently detected in mice expressing the shRNA1 sequence (C), whereas only a few animals with shRNA2 exhibited a decrease of the peptide chain (D). WT, wild-type animal; KO, knockout animal. The different relative intensity of α1(VI) and GAPDH bands in wild-type (WT) animals of panels C and D is due to the fact that the immunoblot analyses were not run in parallel.

3. Results

3.1. In vitro testing of interfering sequences

Among several siRNA tested, two (siRNA1 and siRNA2) were chosen that produced the highest levels of inhibition of α1(VI) collagen mRNA expression after transfection into NIH3T3 fibroblasts (Fig. 1A). Although the two siRNA were similarly effective at high concentrations, their dose-response properties were different, with siRNA2 being effective at lower concentrations (Fig. 1A). When expressed as shRNAs from lentiviral vectors, the two sequences reduced efficiently the expression of the α1(VI) mRNA; however, complete inhibition was achieved only with the shRNA1 sequence (Fig. 1B).

3.2. Inhibition of Col6a1 expression in vivo

Injection of lentiviral particles into the perivitelline space of one-cell embryos resulted into the generation of transgenic mice with high frequency (average 24%) (Table 1). The number of copies of the provirus integrated into the genome varied from 1 to more than 20. However, most transgenic mice (63%) contained only one copy of integrated provirus, while the percentage of animals with high copy number (≥5) was low (about 7%) (Table 2). A limited number of

embryos (8–10 for each viral construct) were allowed to develop in culture after microinjection and analyzed for GFP expression at different developmental stages (2-cell to blastocyst) (Fig. 2). All cells of fluorescent embryos were positive for GFP, indicating that integration of the proviral vector occurred before the first mitotic division.

The inhibition of *Col6a1* gene expression in transgenic mice was initially analyzed by real time RT-PCR. As shown in Fig. 3A, the levels of α1(VI) mRNA was inhibited by interfering, but not by control sequences. The efficiency of inhibition was strongly influenced by the number of integrated provirus copies: statistical significance was reached only with 2 or more copies and the decrease of mRNA was more pronounced by increasing the copy number. The effect was more intense for shRNA1 than for shRNA2 sequence (data not shown).

We next investigated the inhibition of *Col6a1* expression at the protein level by western blotting on extracts from individual diaphragms. While no inhibition of protein production was noted in the tissue from animals containing control constructs (vector alone without any interfering sequence or vector expressing a control sequence unrelated to the α1(VI) collagen mRNA) (Fig. 3B), a variable level of α1(VI) polypeptide was observed in diaphragms from transgenic mice expressing shRNA1 or shRNA2 (Fig. 3C and D). The frequency of animals with reduced levels of α1(VI) protein was higher for shRNA1 (6/13 animals tested) than for shRNA2 (2/13 animals tested). The overall efficiency of inhibition was dependent on the number of integrated copies of provirus. In addition, the site of integration was also an important factor determining silencing efficiency; this is suggested by the detection of efficient (although at low frequency) inhibition even in mice with only one copy of provirus (Fig. 3C, sample 1.1.1) and the variable effect on silencing of a few copies of integrated provirus (compare the different levels of α1(VI) protein in mice with 2–5 proviral copies in Fig. 3C).

3.3. Expression of interfering sequences

In RNA interference, inhibition of gene expression is brought about by degradation of mRNA by siRNA (Mello and Conte, 2004). In our transgenic mice a shRNA is produced and processed to siRNA that is predicted to induce degradation of the α1(VI) mRNA. Thus, efficiency of gene knockdown should be determined by the levels of siRNA present in the cells. To test this prediction, the levels of siRNA from different transgenic mice were compared. As shown in Fig. 4, the intensity of the band amplified from siRNA was stronger in the diaphragm from mice exhibiting the highest reduction of the α1(VI) protein (Fig. 4A, lanes 1, 5 and 9), it was weaker in mice where silencing was ineffective and showing normal amount of the protein (Fig. 4A, lanes 2, 3, 4 and 6) and had an intermediate intensity in samples where interference was only partially effective (Fig. 4A, lanes 7 and 8). In transgenic animals expressing control sequences, the siRNA species did not show any effect on the amount of the α1(VI) protein (Fig. 4B). These results indicate that the efficiency of silencing is dependent on the levels of shRNA expressed in different transgenic animals.

3.4. Variation of interference in different tissues

As most of the extracellular matrix components, collagen VI is endowed with exquisite systems of tissue specific transcriptional regulation (Braghetta et al., 1996). This condition adds to the complexity of inhibiting the expression of the *Col6a1* gene *in vivo* by RNAi. We therefore investigated how the levels of α1(VI) chain were reduced in the different tissues of individual mouse lines and whether this inhibition varied among different lines. We first evaluated the levels of the α1(VI) chain in various muscles

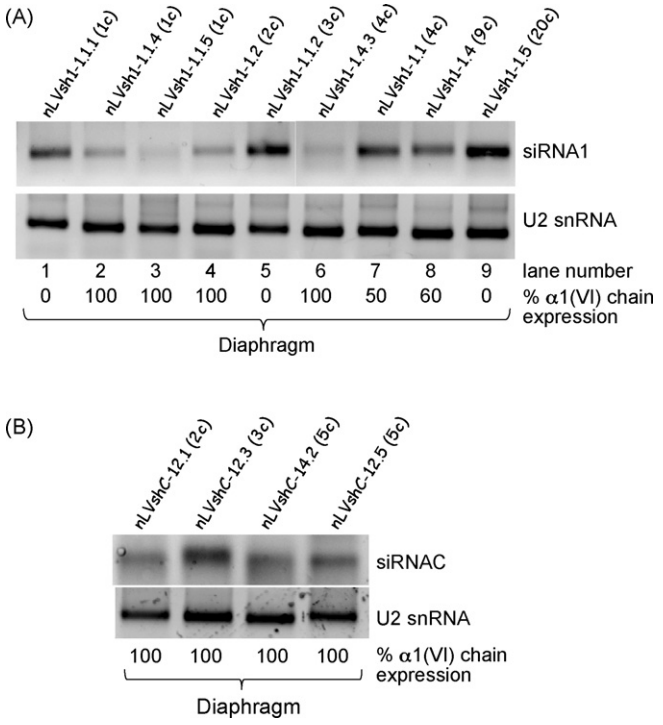

Fig. 4. The efficiency of silencing of the α1(VI) collagen chain is related to the levels of expression of shRNA expression. Detection of siRNA was carried out in the diaphragm of transgenic mice expressing the shRNA1 (A) or the shRNAC (B) sequence.

from different mice obtained with the shRNA1 sequence. In general, the degree of decline of the protein in transgenic compared to wild-type mice was dependent on the mouse line considered; thus, inhibition in line 1.4.1 was always the lowest in all muscles analyzed, whereas the decrease was stronger in lines 6.1, 1.5.1 and 1.5.2 (Fig. 5A). However, fine variations of expression could be noted in the latter lines. For example, in soleus muscle the relative intensity of α1(VI) band was lower in the 6.1 compared to the 1.5.1 line, while it was higher in diaphragm and similar in tibialis, abdominal and gastrocnemius muscles (Fig. 5A). Likewise, silencing was very effective in the heart of the same four lines, whereas it was milder in other tissues (Fig. 5B). However, the relative levels of protein expression were not the same for different transgenic lines (for example, the intensity of bands was similar for lines 6.1, 1.4.1 and 1.5.2 in heart, but was higher for 1.4.1, fainter for 6.1 and the lowest for 1.5.2 in intestine and tail). These experiments add support to the suggestion that the efficiency of collagen VI tissue specific inhibition is dependent, to some extent, on the particular pattern of insertion sites, and the consequent variation of interfering sequences expression, in each individual transgenic line. In fact, other tissue-specific effects, such as differences in H1 promoter activity or shRNA processing, would have been the same for all transgenes integrated and therefore would have not altered the relative intensity of protein levels in diverse tissues.

3.5. Phenotypic effects of silencing

Assembly and secretion of the native triple helical collagen VI molecule require the presence of all three polypeptide components (α1, α2 and α3) and lack of any one induces intracellular degradation of the other chains (Lamande et al., 1999). As a consequence, RNAi of the α1(VI) chain should reduce the levels of collagen VI in tissues and bring about phenotypic effects. Among the different

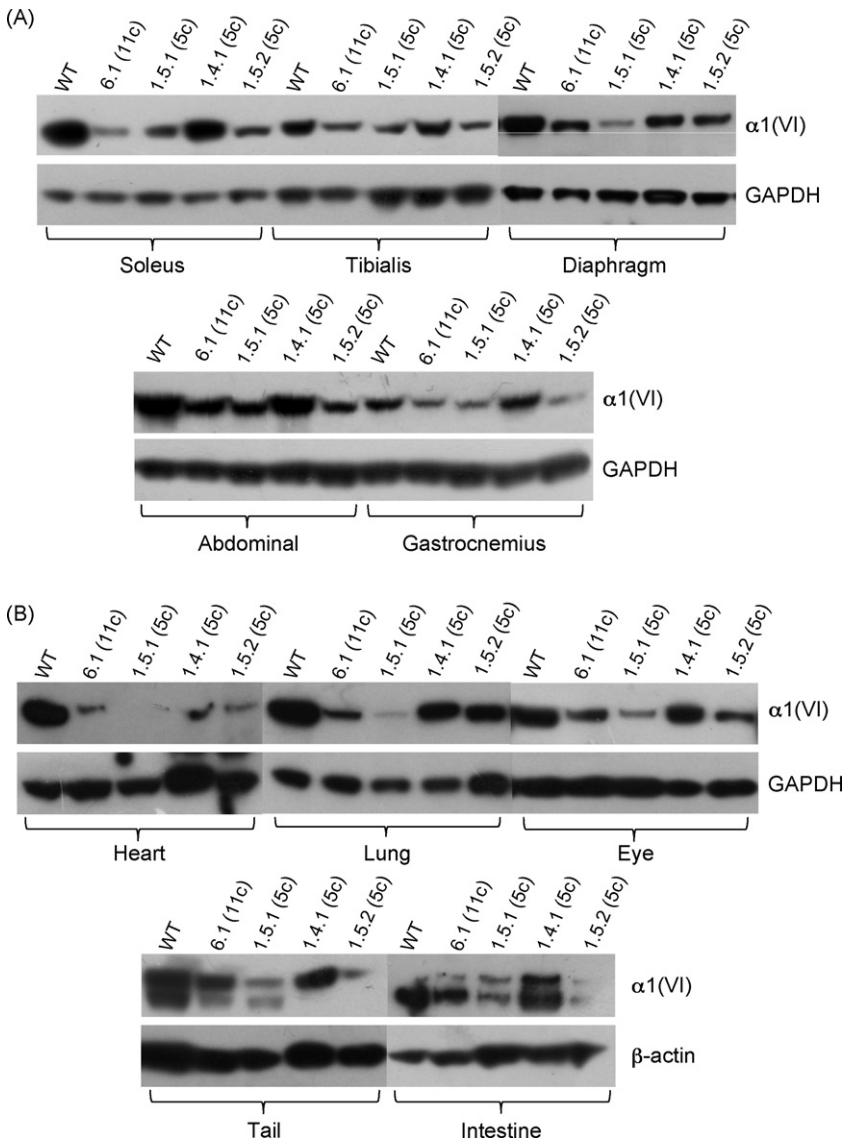

Fig. 5. Comparison of RNAi efficiency in different tissues of the same animal. The α1(VI) collagen chain was detected in a set of muscles (A) and in other tissues (B) by immunoblotting. All transgenic animals were generated with the nLVsh1 construct. WT, wild-type control.

phenotypic traits described in the muscles of collagen VI knockout mice, we chose to analyze the alterations of the mitochondrial electrochemical potential and the incidence of apoptosis (Irwin et al., 2003).

Fig. 6 shows the mitochondrial depolarization of *flexor digitorum brevis* (FDB) myofibers obtained from mice with different genotype. Only a very low proportion (3%) of myofibers from wild-type animals depolarized when oligomycine was added (Fig. 6A). This proportion increased to about 50% for myofibers derived form collagen VI null mice (Fig. 6B). Mice in which collagen VI was reduced by RNAi exhibited an intermediate percentage of depolarizing myofibers (Fig. 6C and D). The proportion of depolarizing myofibers was higher for mice in which the reduction of α(VI) collagen chain was more prominent. This can be appreciated by comparing the amount of depolarizing myofibers from line nLVsh1-1.5.2 (29%, Fig. 6C), with that of line nLVsh1-1.4.1 (8%, Fig. 6D), and the decline of α1(VI) protein detected in different muscles of the same mice (Fig. 5A). This was not an isolated finding, as lines nLVsh1-1.5.1 and nLVsh1-1.5.3, for which silencing of the α1(VI) chain was comparable to that of nLVsh1-1.5.2 (Fig. 5A and data not shown), also exhibited corresponding levels of depolarizing myofibers (30% and 24% for line nLVsh1-1.5.1 and nLVsh1-1.5.3 respectively, data not shown).

Similarly to the amount of depolarizing muscle fibers, the incidence of apoptosis in diaphragm was also dependent on the extent of decrease of α1(VI) protein expression (Fig. 7): mice in which interference was very effective exhibited a number of apoptotic nuclei comparable to those detected in Col6a1 knockout mutants (see mouse nLVsh1-1.8), while milder interference produced levels of apoptosis intermediate between those of knockout and control animals (see mouse nLVsh1-1.9).

3.6. Inducible interference

An inducible system would confer flexibility to the experimental design of investigations on the function of genes with tissue specific expression such as collagen VI. We therefore tested silencing of α1(VI) collagen expression *in vivo* using the Tet-off version of the pLVPT vectors (Szulc et al., 2006). Compared to the non-inducible vector used above (pLVTHM), the titer of viral particles obtained with pLVPT was lower (Table 1). Although the percentage of transgenic mice generated was comparable to those of the other

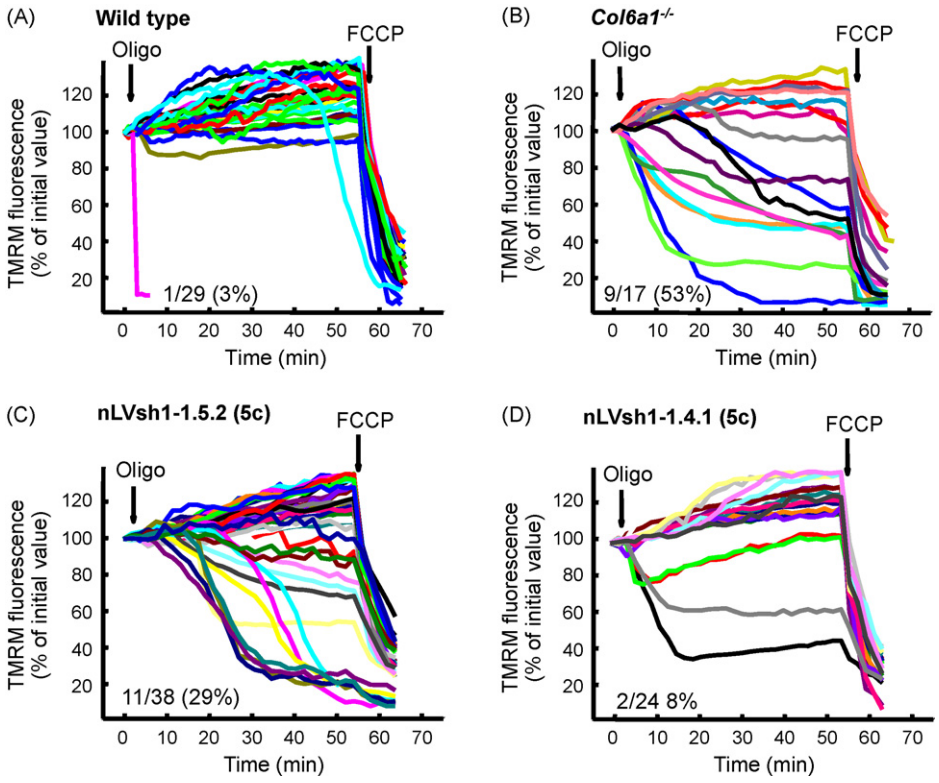

Fig. 6. Evaluation of mitochondrial potential in myofibers from *flexor digitorum brevis* muscle. The addition of oligomycine (Oligo) induces depolarization of myofibers with a frequency that is the lowest in wild-type animals (panel A) and the highest in collagen VI null (Col6a1$^{-/-}$) mice (panel B). The frequency of depolarization in myofibers with active RNAi (panels C and D) is intermediate between control and *Col6a1*$^{-/-}$ myofibers and depends on the efficiency of interference achieved in each mouse. The panels report the results obtained with myofibers from a single mouse, but the frequency was highly reproducible. At the end of each experiment, mitochondria were fully depolarized by the addition of 4 μM of the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP).

vector, the number of integrated proviral copies was inferior (from 1 to 4) (Table 1). Three animals contained 3 copies and were studied in more detail (Fig. 8). For skin and eye, tissue samples could be obtained repeatedly and therefore the synthesis of the α1(VI) collagen chain was analyzed before and after treatment with doxycycline. Both mice tested (iLVsh1-9.1.2 and -9.1.3) showed a slight increase (1.3-fold) in skin and a more evident induction (2- and 4-fold) in the eye, an indication of regulation of the siRNA expression by the drug. In the other tissues, for which the effect of the drug could not be examined in the same mouse, one animal was used as negative control (iLVsh1-9.1.1), while the remaining two were treated with doxycycline. Strong increase of expression with doxycycline (3–5-fold) was found in any tissue examined (lung, heart, soleus and diaphragm) (Fig. 8), confirming efficient inducibility of the vector. However, a few interesting subtle features should be noted. First, the intensity of silencing and the increment of expression after induction was dependent on the mouse line and the tissue examined; for example, the knockdown in the eye was more pronounced for line iLVsh1-9.1.3 than for line iLVsh1-9.1.2, while it was similar in the lung. Second, in some cases the levels of α1(VI) collagen chain achieved in transgenic animals after doxycycline induction was slightly lower than those of non-transgenic littermates (Fig. 8). This observation suggests that doxycycline treatment did not completely inhibit the production of interfering RNAs. This was indeed the case when expression of shRNA1 was measured (Fig. 9). It is worth noting in Fig. 9 that shRNA levels are higher in lung than in diaphragm (about 1.5-fold), while the levels of α1(VI) collagen mRNA detected by RT-PCR were similar (data not shown). Thus, the ratio of shRNA/mRNA may be critical for the higher reduction of the protein in the former tissues (Fig. 8).

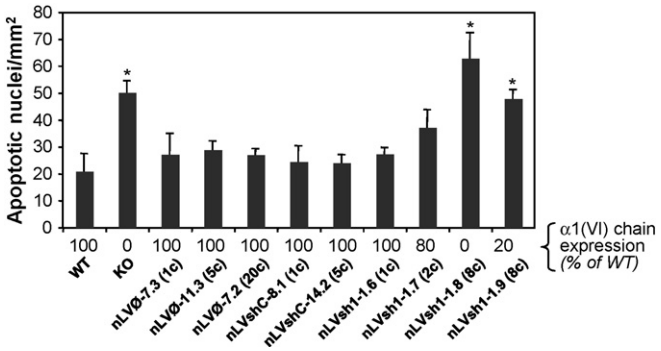

Fig. 7. Evaluation of apoptosis in diaphragm from transgenic mice. Apoptotic nuclei were detected by the TUNEL procedure. *$P < 0.05$ compared to wild-type (WT). KO, tissue from collagen VI null mouse (Bonaldo et al., 1998).

4. Discussion

In this study we have applied RNAi using lentiviral vectors to reduce *in vivo* the dosage of *Col6a1*, the gene coding for the α1 chain of collagen VI. Of the three lentiviral vectors tested *in vitro* for delivering shRNA to fibroblasts, those developed by Trono and collaborators (Szulc et al., 2006; Zufferey et al., 1997) were superior in terms of transduction and silencing efficiency and were therefore used for *in vivo* studies. Embryos transduced with these vectors at the one-cell stage express GFP in all cells, indicating that proviral integration has taken place before or during the first mitosis. This is at variance with the results of Kirilov et al. (2007) who observed mosaicism in transgenic mice derived with a lentiviral vector. These authors also observed low efficiency of transgenesis, although the

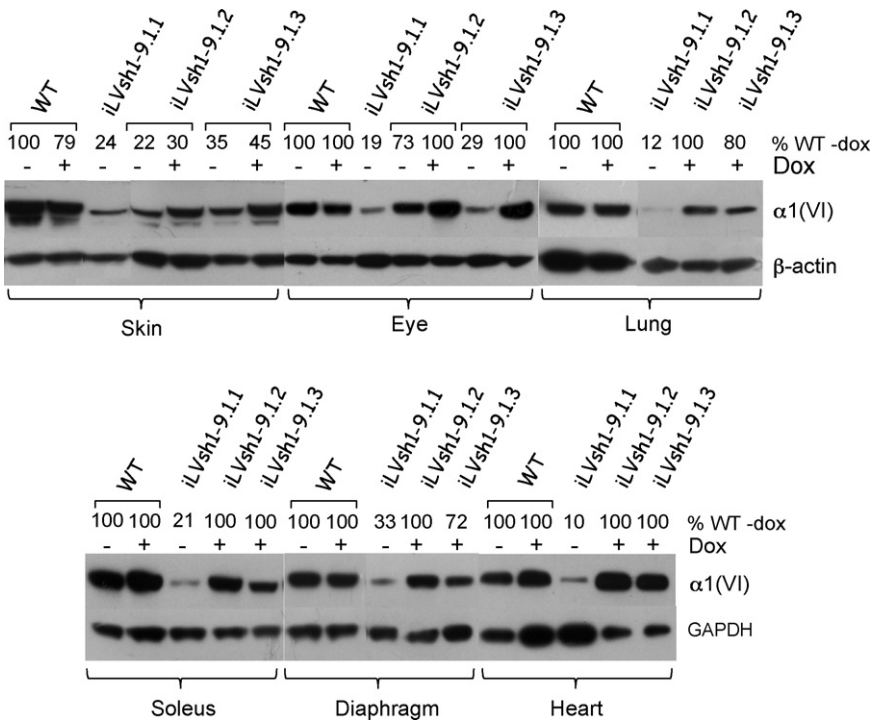

Fig. 8. Knockdown of α1(VI) collagen chain by RNAi with the inducible vector iLVsh1 analyzed by western blot. For skin and eye, biopsies were taken before and after treatment with doxycycline (Dox). For the remaining tissues two of the three mice were treated with the drug while one was not.

titer of their viral preparations were higher ($0.5–4 \times 10^9$) than those employed here ($1.0–7.0 \times 10^8$). Very likely, the discordant results are due to the different type of lentiviral vector used in the two studies. Our results point out three parameters as the major determinants of the efficiency of interference: the interfering sequence, the number of proviral copies integrated into the mouse genome and the site of insertion of the provirus.

Both sequences selected were very effective in reducing the α1(VI) collagen chain in NIH3T3 cells *in vitro* when transfected as siRNAs or produced as shRNAs from the integrated lentiviral vector; yet, the frequency of efficient gene knockdown *in vivo* was surprisingly different (3-fold, i.e. 6/13 animals for shRNA1 and 2/13 animals for shRNA2, see Fig. 1). Thus, *in vivo* conditions may impose additional stringency on the expression and the survival of shRNA, which ultimately determines the effectiveness of interference. An explanation for this observation is not available at the moment.

The number of proviral copies integrated into the mouse genome was a major factor determining the levels of silencing. This can be clearly appreciated when plotting the average amount of α1(VI) collagen mRNA measured in different animals against the copy number (Fig. 3A). A similar conclusion can be drawn from western blot analysis, in which the frequency of appreciable knockdown increased with the copy number (Fig. 3C). Considering that most animals contain low copy number of transgene, while only a small fraction has more than 5 copies (Table 2), an important improvement of the technique for gene knockdown should rely on a substantial increase of integration events. Looking at the data of Table 1, it is apparent that a major determinant of copy number is the titer of the suspension of viral particles used to inject the fertilized oocytes. Any procedure attaining an increment of the titer would therefore have a beneficial impact on the application of this technique for *in vivo* transgenesis.

The importance of the site of insertion is threefold. First, different sites allow the production of variable amounts of shRNA that, in turn, determine the degree of silencing. This is evident considering mice with one proviral copy, all of which are inefficient at reducing α1(VI) chain expression, with the exception of one (nLVsh1-1.1.1) (Fig. 3C), that also exhibits the highest levels of steady state siRNA synthesis (Fig. 4).

Second, the site of insertion influences silencing in distinct tissues, as attested by the mild variations of the relative intensity of silencing (see for example Fig. 5). This is likely due to the different epigenetic organization of chromatin in different tissues. Thus, when a copy is inserted into a genomic site that is differentially organized in diverse cell types, expression may vary. This condition is particularly important for RNAi applied to genes like *Col6a1*, whose expression is widely distributed and is regulated at the transcriptional level by different tissue specific enhancers (Braghetta et al., 1996, 2008; Fabbro et al., 1999; Girotto et al., 2000; Vitale et al., 2001). The expected result is a ratio of shRNA/mRNA distinct for each tissue with ensuing variable silencing effect. Indirect evidence supporting this suggestion comes from the observation that higher levels of shRNA in the lung compared to diaphragm (see Fig. 9) are associated with a more efficient reduction of α1(VI) protein in the former tissue (see Fig. 8).

Finally, at variance with DNA microinjected into oocytes that usually inserts as concatamers at a single site, the insertion of more than one copy of lentiviral vectors into the genome takes place at multiple sites. These copies segregate separately in the following generations, so that individuals from the same litter may contain a different number of copies. As a consequence, the interference may vary from mouse to mouse, thus allowing the analysis of the effect of different dosage of the gene of interest.

The main effect of collagen VI deficiency is apoptosis of muscle fibers as a consequence of mitochondrial dysfunction (Bernardi and Bonaldo, 2008). These traits are also found in heterozygous animals, indicating haploinsufficiency (Bonaldo et al., 1998). Both conditions were tested in muscles from lentiviral-mediated *Col6a1* knockdown mice and found to mimic the alterations described for collagen VI null animals generated by gene targeting (Irwin et al., 2003) and for patients affected by Ullrich congenital muscular dystrophy (Angelin et al., 2007). The measurement of mitochondrial

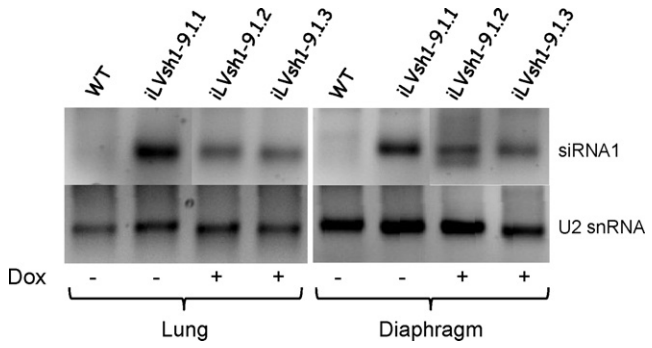

Fig. 9. Detection of siRNAs in mice expressing shRNA from the inducible vector. Tissues that did not recover completely the expression of the α1(VI) collagen chain after doxycycline (Dox) treatment (see Fig. 8) are shown.

depolarization in the presence of oligomycine was particularly valuable. In fact, this parameter appears to be a reliable indicator of the efficiency of RNAi and the consequent reduction of collagen VI produced by muscle fibers. The transgenic mice are therefore a good tool for a quantitative analysis of the effect of collagen VI on mitochondrial function. Moreover, they are a valuable model for investigating the consequences of different extents of collagen VI decrease in muscle fibers, thus helping to understand the large heterogeneity of clinical phenotypes observed in patients affected by collagen VI disorders (Bernardi and Bonaldo, 2008; Merlini et al., 2008).

In summary, our data indicate that *in vivo* gene knockdown mediated by some of the available lentiviral vectors is an efficient method even for the study of genes with complex pattern of tissue specific expression. This method can accelerate the generation of animals with substantial suppression of gene expression in an inducible way. Due to its high efficiency, the method should be considered of great potential for RNAi-based genetic screening *in vivo* (Peng et al., 2006).

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