Characterisation of transgenic mice for cardiac fibroblast-specific transgenesis

Megha Saraiya

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Vorsitzender: Univ-Prof. Dr. Hannelore Daniel

Prüfer der Dissertation:
1. Univ.-Prof. Angelika Schnieke, Ph.D
2. Univ.-Prof. Dr. Dr. Stefan Engelhardt

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ABBREVIATIONS

α-MHC  alpha-myosin heavy chain
ANOVA  analysis of variance
AMCF  adult mouse cardiac fibroblasts
AMCM  adult mouse cardiomyocytes
AU  arbitrary units
ATP  adenosine triphosphate
β-AR  Adrenoceptor
BAC  bacterial artificial chromosome
BSA  bovine serum albumin
BW  body weight
Ccdc80  coiled-coil domain containing 80
CF (s)  cardiac Fibroblast / cardiac Fibroblasts
CM  cardiomyocytes
CMV  cytomegalovirus
Cre  cyclization recombination
DAG  diacylglycerol
ddH2O  double distilled water
DMSO  dimethylsulphoxide
DNA  deoxyribonucleic acid
dNTP  deoxyribonucleotidetriphosphate
DTT  dithiothreitol
ECL  enhanced chemiluminescence
E.coli  Escherichia coli
ECM  extra cellular matrix
EDTA  ethylenediaminetetraacetic acid
EMT  epithelial–mesenchymal-transition
EndMT  endothelial–mesenchymal-transition
ERT2  mutated form of human Estrogen Receptor1
FCS  fetal calf serum
Fsp1  fibroblast specific protein-1
GFP  green fluorescent protein
Gsn  gelsolin
GW  Gateway system
HF  heart failure
HW  heart weight
Kb  kilobases
IFs  intermediate filaments
loxP  locus of X-over P1
LV  left ventricle
LVID  left ventricular inner diameter
LVPW  left ventricular posterior wall thickness
M  molar
mA  milli ampere
mGFP  membrane targeted GFP
min  minute
MI   myocardial Infraction
mTom membrane targeted tdTomato
NRCF neonatal rat cardiac fibroblasts
NRCM neonatal rat cardiomyocytes
PCR  polymerase chain reaction
pH   power of hydrogen
Postn periostin
RNA  ribonucleic acid
RT   room temperature
Rabgap1l RAB GTPase Activating Protein 1-Like
s    second
s.e.m. Standard error mean
SD   standard deviation
TAC  transverse aortic constriction
TAE  tris-acetate EDTA
Tam  tamoxifen
Tg   transgenic
TL   tibia length
UV   ultraviolet
v/v  volume to volume
Vim  vimentin
VSMCs vascular smooth muscle cells
w/v  weight to volume
°C   degree Celsius
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Introduction

1 INTRODUCTION

Cardiovascular diseases are one of the leading causes of death worldwide. Due to aging population, the estimated number of cardiovascular deaths will increase from 16.7 million in 2002 to 23.3 million in 2030\(^1\). Among the different cardiovascular diseases, heart failure (HF) is a primary health concern. Heart failure (HF) is a complex clinical syndrome, associated with impaired ability of the ventricles to fill with or to eject blood. Coronary artery disease and heart attack, high blood pressure (hypertension), faulty heart valves, cardiomyopathy (damaged heart muscles), arrhythmias (abnormal heart rhythms) or inherited mutations in structural or contractile proteins are the underlying pathologies linked to HF \(^2\). At cellular level, it is associated with cardiomyocyte hypertrophy (characterized by an increment in the cardiomyocyte size, enhanced protein synthesis, and alteration in organization of the sarcomere structure), cardiomyocyte apoptosis, alteration in the expression of genes regulating energy metabolism, calcium handling, and genes that are normally expressed in the embryonic heart \(^2\).

Besides cardiomyocytes, also non-myocyte cell populations are increasingly appreciated for their contributions in the performance of the normal and failing heart. The cardiac fibroblast (CFs) that has been recognized as the major non-myocyte cell type in the heart, contributes to multiple aspects of myocardial function and pathophysiology. Cardiac fibroblasts produce a variety of growth factors along with extracellular matrix proteins (e.g., collagens), which are involved in the intercellular signaling with cardiomyocytes. Still, the exact function of cardiac fibroblasts during adaptive responses of the myocardium remains unclear \(^3\).

1.1 The cardiac fibroblasts

Cardiomyocytes, fibroblasts and vascular cells (smooth muscle, endothelium) are the major cellular components in the heart. Originally, fibroblasts were described in the late 19th century based solely on their location and on morphological criteria\(^4,5\). They are typically identified by their spindle-shaped flattened morphology, their ability to adhere to culture plates\(^5\) and the absence of markers of epithelial, smooth muscle, endothelial, perineural, and histiocytic cells\(^5\). Their nuclei are large and euchromatic and possess prominent nucleoli. Fibroblast cells are phenotypically diverse and exhibit heterogeneity between fibroblasts from different tissues \(^6\).
1.1.1 Origin and organization of cardiac fibroblasts in the heart

Cardiac fibroblasts (CFs) are thought to be predominantly derived from the epicardium during heart development (Fig. 1.1) 7. Villous-like projections protrude from the venous pole of the developing heart to form the pro-epicardium 8,9. Cells from the pro-epicardium detach and attach on the beating ventricular surface to form the epicardium10. Subsequently epithelial cells of the epicardium undergo epithelial–mesenchymal-transition (EMT) to form mesenchymal cells that invade the developing myocardium11. A subset of these mesenchymal cells after EMT acquire migratory properties and invade the developing myofascial planes to occupy interstitial positions in between cardiomyocytes to become resident cardiac fibroblasts 12,13. In contrast to cardiac fibroblasts, valvular fibroblasts are thought to derive from the endothelium overlying the region of the cardiac cushions (site of atrio-ventricular valve formation) 14-16. The endothelium overlying the valve leaflets undergoes the endothelial-mesenchymal transition (EndMT) to generate cardiac fibroblasts that invade the valvular mesenchyme and contribute to the collagenous structure of the valve (Figure 1.1).

At the cellular level, the normal adult human heart comprises 30% cardiomyocytes and 70% nonmyocytes, of which the majority is CFs. Although CFs are the predominant cell type in number, the cardiomyocytes actually occupy the greatest volume 17,18. Unlike cardiomyocytes, endothelial cells, and vascular smooth muscle cells, CFs have no basal membrane and display multiple processes 3. Thus, CFs can be distinguished from other non-myocyte lineages upon the use of laminin or collagen IV to test for the absence of a basal membrane in CF. CFs are found throughout the heart in a 3D network surrounding myocyte19 and bridging the gaps between myocardial tissues 17,20. Myocytes are arranged in laminae bounded by endomysial collagen, and the CFs lie within this endomysial network 18,21.

In the developing murine heart, CFs are observed at stage E12.5, that is 12.5 days post fertilization (dpf). Their numbers progressively increase in postnatal life, comprising 27% of the total number of cells in the adult murine heart 22, approximately 2/3 of the total numbers of cells in the rat heart 23,22 and in humans, the non-myocyte cells comprise approximately 70% of the total number of cardiac cell types 19,24. The higher number of fibroblasts in rat and human hearts thus relates to a larger heart size, greater wall tension and consequently a need for greater production of extracellular matrix (ECM) 22.
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Figure 1.1 Cardiac fibroblasts in heart. (A) The origin of cardiac fibroblasts during cardiac development and following injury. The cardiac fibroblasts in the injured heart have diverse origins compared to a fibroblast in the developing heart (Ref: Deb A et al. 2014) 27.

1.1.2 Functions of cardiac fibroblasts

CFs are crucial in maintaining normal cardiac function, biochemical and electrical features and structure of the heart. They are into many aspects of cardiac functions, such as homeostasis and remodeling of the extracellular matrix, cell–cell interaction with cardiomyocytes, and intercellular signaling with other CFs, endothelial or smooth muscle cells via production of growth factors and cytokines. Thus, influencing several cellular events in the heart such as angiogenesis, cell proliferation, cardiomyocytes hypertrophy or apoptosis (Figure 1.2). CFs are high membrane resistance conductors19. To ensure proper contraction of the heart, CFs electrically separate the atria and the ventricle, by casting the fibrotic annulus 31. They are connected with cardiomyocytes via gap junctions, particularly connexins (Cx40, Cx43, and Cx45), which are essential to maintaining an optimal electrical conduction in the heart 32,33. Fibroblasts also interact with endothelial cells by secreting growth factors like FGF and VEGF that act on endothelial cells and stimulate angiogenesis 34. In the myocardial interstitium, CFs secretes a collection of bioactive molecules like
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cytokines (TNFα, interleukins and TGFβ) and active peptides (angiotensin II, endothelin 1), which function in autocrine and/or paracrine manners in the myocardium. CFs are the primary cell type responsible for ECM homeostasis in healthy and its remodeling in heart disease. The cardiac ECM consists of interstitial collagens (predominantly type 1 and type 3), proteoglycans, glycoproteins, cytokines, growth factors and proteases. Cardiac fibroblasts not only synthesize new matrix proteins but also express various metalloproteinases (MMPs) that degrade extracellular matrix. In the healthy heart, synthesis and breakdown of extracellular matrix are tightly regulated, but in pathological states, increased MMP expression and activity can lead to excessive ECM degradation and turnover. In pressure overload-induced cardiac hypertrophy in humans, MMP expression in the heart increases with the onset of left ventricular failure. In rodent models of pressure overload-induced cardiac hypertrophy increased expression of MMPs is associated with transition from compensation to heart failure. However following acute myocardial infarction fibroblasts not only increase the synthesis of ECM proteins at the site of injury (replacement fibrosis) but also increase ECM protein synthesis in areas remote from the injury.

1.1.3 Dynamic interaction between cardiac fibroblast and cardiomyocytes

Dynamic cross talk between cardiomyocytes and cardiac fibroblasts is a prominent feature of both development as well as injury-induced remodeling. Throughout life, cardiac fibroblasts are responsible for controlling many aspects of the heart’s microenvironments. During development, fibroblasts supports secretion of factors like platelet derived growth factor (PDGF)-β, Sox9, thymosin β4, Ets factors, and fibroblast growth factors (FGFs). These factors are conducive to cardiomyocyte proliferation and establishment of a functionally competent ventricle. They also provide the structural stability required for transitioning from pre- to postnatal life, and directly coupling to cardiomyocytes via gap junctions. The paracrine, structural, and possibly electrical interactions between fibroblasts and cardiomyocytes that underlie normal development also modulate the pathological responses to injury in the adult heart. In the failing adult myocardium, fibroblasts secrete several proinflammatory cytokines that directly promote hypertrophy of cardiomyocytes including: IL-1β, IL-6, TNF-α and TGFβ. Cardiomyocytes also secrete some of these same cytokines that induce fibroblast migration, stimulate transformation of fibroblasts into myofibroblasts (TGFβ1 in particular), and increase synthesis of several ECM components. Similarly, angiotensin II type-1 receptors on neighboring cardiomyocytes
play an important role in determining the action of cardiac fibroblasts in the early phase of cardiac remodeling. This role of activated cardiomyocytes driving fibrosis is in agreement with transgenic overexpression studies using activated forms of calcineurin or calcium-dependent signal-transducing molecules. Thus, intracellular signaling crosstalk creates an environment where cardiac fibroblasts and cardiomyocytes reciprocally influence each other’s phenotype. Fibroblasts have also been shown to have extensive electrical coupling to each other as well as to cardiomyocytes via gap junctions in vitro during both developmental stages as well as following injury; however, the functional significance of these interactions has yet to be established in vivo. Increasingly available information relating to the dynamic nature of the cardiac microenvironment further alludes to the possibility that cardiac fibroblasts may be a key regulatory cell capable of mediating in vivo communication to cardiomyocytes, which is critical for both normal heart development and facilitating pharmacological therapeutic approaches within the heart.

### 1.1.4 Cardiac fibroblasts in wound healing

Fibroblasts in the injured heart are thought to have diverse origins (Fig. 1.1 A). Resident cardiac fibroblasts at the site of injury proliferate and are thought to be the predominant pool of cardiac fibroblasts contributing to cardiac fibrosis after injury, although rigorous fate mapping studies have not been done to confirm this. Bone marrow-derived cells also contribute to cardiac fibrosis. Several studies have demonstrated that between 3 and 24% of myofibroblasts in the injured region are of bone marrow origin. These circulating fibroblast precursors have been termed fibrocytes and express hematopoietic (CD45), monocytic (CD11b) and progenitor (CD34) markers. The ability of bone marrow-derived cells to contribute to cardiac fibrosis appears to be physiologically important as inhibition of fibrocyte recruitment diminished fibrosis and had salutary effects on remodeling. In addition, after an acute cardiac injury a subset of epicardial cells undergoes EMT to generate cardiac fibroblasts, thus recapitulating a developmental program of epicardial EMT (Figure 1.1). Following EMT, epicardial-derived cardiac fibroblasts reside in the sub-epicardial space, express collagen and contribute to a pro-fibrotic repair response. Endothelial cells undergo endothelial–mesenchymal transition (EndMT) and have been reported to contribute to 30% of the cardiac fibroblasts in a murine model of pressure overload injury, but the degree to which endothelial cells contribute to fibrosis in the acutely injured heart is less certain. A small number of non-residing cells derived from other cell types (including monocytes and endothelial cells) that are functionally significant, also contributes to this CF fraction that infiltrate the heart in response to ischemia, MI or...
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pressure overload. Thus, it is apparent that resident cardiac fibroblasts are not the sole source of activated fibroblasts in cardiac remodeling.

The cardiac fibroblast plays a central role in wound healing after myocardial injury and affects various aspects of the wound healing response from deposition of extracellular matrix proteins to wound angiogenesis and scar maturation (as shown in Figure 1.2).

**Figure 1.2 Major functions of the fibroblast during cardiac development and wound healing.**

The fibroblast promotes proliferation of embryonic cardiomyocytes. It influences angiogenesis in the adult heart and regulates ECM turnover both in the adult uninjured heart and after acute injury. The fibroblast plays a pathophysiological role in scar contraction adverse remodeling and ventricular dilatation and exerts electrophysiological effects (Ref: Deb A et al. 2014) 58.

Following acute myocardial infarction, cardiac fibroblasts in the heart become activated and rapidly proliferate. In rodent hearts, their peak number is achieved within 7–14 days after permanent ligation of the left anterior descending coronary artery and within 3 days of ischemia–reperfusion injury. Activated fibroblasts at the site of injury express contractile proteins such as smooth muscle actin (myofibroblasts) and secrete ECM proteins (mainly collagens). This early fibrotic repair response is critical for maintenance of cardiac structural integrity and performance after cardiac injury. Disruption of cardiac fibroblast activation early after injury leads to impaired wound healing and worsening cardiac performance. In the later phases of wound healing (days to weeks), collagen fibers at the site of injury undergo cross-linking which increases the tensile strength of the scar. The scar subsequently contracts undergoing a reduction in surface area and myofibroblasts expressing contractile proteins are thought to contribute to scar contraction (Figure 1.2).
Scar contraction and thinning lead to adverse changes in ventricular chamber geometry and compliance thereby causing congestive heart failure. Since fibroblasts exist in close interactions with endothelial cells; they also facilitate angiogenesis via expression of angiogenic cytokines (FGF, VEGF) and fibroblast-derived matrix proteins. Thus, activated fibroblasts play a pivotal role in the wound healing response by initiating a cascade of events in order to restore tissue integrity and homeostasis.

1.1.5 Cardiac fibroblasts in regenerative processes

Developmental biology research has opened important avenues for converting fully differentiated cells into various lineages via reprogramming technologies. As a crucial player in cardiac development, the cardiac fibroblast has recently been exploited as a key target to accomplish cardiac regeneration. Fibroblasts can also be reprogrammed into different cell types, such as pluripotent stem cells, myoblasts, neurons. Expression of three transcription factors (Gata4, Mef2c, Tbx5, collectively referred to as GMT) mediated by retroviral gene transfer was shown to be sufficient to directly reprogram adult fibroblasts to become adult cardiomyocytes both in vitro, and, most significantly, in vivo. Identifying the derivatives of these reprogrammed cardiac fibroblasts was accomplished using the 3.9kb Periostin-Cre and Fsp1-Cre lineage reporters, in concert with various lacZ and fluorescent indicator mice. Importantly, it has been demonstrated that in the absence of genetic reprogramming, no cardiomyocytes expressed any lacZ either before or after myocardial infarct; however, the retroviral-induced GMT cardiac fibroblasts were able to give rise to lacZ-positive cardiomyocyte-like cells, suggesting that these cells were derived from reprogrammed cardiac fibroblasts. Not only did the reprogrammed fibroblasts differentiate into cardiomyocyte-like cells, but also these in vivo studies revealed that cellular reprogramming post-myocardial infarction resulted in improved cardiac function.

More recently, treatment with a combination of miRNAs (miRNAs 1, 133, 208 and 499) has been shown to facilitate the conversion of neonatal and adult cardiac fibroblasts into adult cardiomyocytes. While both of these techniques show promise for increasing the regenerative capacity of the heart following ischemic injury, still they have low in-vitro efficiencies.

1.1.6 Current state of gene targeting in cardiac fibroblasts

Our knowledge regarding the functions of cardiac fibroblasts in the heart, their origins during cardiac development and in disease condition, the dynamic nature of their population, how that population may be in flux during time of injury or pressure overload is
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derived from the genetic and cellular fate-mapping studies done so far. The Cre-loxP system is one of the most promising in vivo methods used to analyze the contribution of specific cell types in development and pathophysiological conditions in heart. Unlike \(\alpha\)-myosin heavy chain promoter-Cre line (which enables cardiomyocyte-specific gene-knockout strategies in heart), there is no mouse line available that specifically expresses Cre recombinase in cardiac fibroblasts. Though not organ specific, fibroblast-specific transgenic lines that express Cre under the control of a Postn promoter (Postn-Cre mice), a S100a4 [Fibroblast Specific Protein 1 (Fsp1)] promoter, and Transcription Factor 21 [Tcf21, also known as Podocyte-Expressed 1 (Pod1) combination, or a Capsulin or Class A Basic Helix-Loop-Helix 23 (bHLHa23)] promoter, have been reported. The Postn-Cre mouse contains a 3.9 kb 5′ upstream region of the mouse Periostin genomic DNA to promote the expression of an EGFP/Cre fusion expression vector. Following intercrossing with the R26R indicator mice, lacZ expression (Indicative of earlier Cre expression) is present within all non-cardiomyocyte lineages of the fetal and neonatal heart. Similar to endogenous Periostin, Postn-Cre is also expressed within a few homeostatic CFs but is robustly expressed within the CFs and myocardial infarct sites following injury. FSP1 has been used as a fibroblast-specific marker in normal and fibrotic tissues. However, a recent study documents the non-specific expression of Cre-recombinase in the heart tissue of Fsp1-Cre mice. Specifically, in a myocardial infarction or pressure overload model, Fsp1-Cre-driven gene-deleted cells were not only cardiac fibroblasts, but also hematopoietic, endothelial, and vascular smooth muscle cells. Using fate-mapping techniques, Acharya and co-workers have identified the transcription factor Tcf21 as a marker for cells that are committed to the cardiac fibroblast lineage and as an essential mediator in the development of cardiac fibroblasts. Very recently, genetic lineage tracing of CFs in pressure overload heart was done using a collagen1a1-GFP reporter line. Studies done so far using these fibroblast-specific transgenic lines have yielded remarkable insights in identifying and mapping various cell lineages that initially give rise to the developing heart and deciphering many of the key morphological events that are required for both normal heart development and the underlying causes of congenital heart defects.

1.2 Gene targeting in mice using the cre/lox system

Genetically engineered mouse lines have emerged as powerful tools not only for understanding cardio-vasculogenesis but also for understanding the pathogenesis of cardiac disease through animal modeling. The heart is composed of several cell types...
that distinguish it from other organs. Each has specific functions based on unique gene expression patterns that direct responses to its cellular, physiological, and stress environments. Approaches are therefore required to identify these functions at the animal level because this complexity cannot be fully recapitulated ex vivo. Mice are a good choice for modeling the genetic basis of mammalian cardiovascular development and disease. Mice are mammals with a 4-chambered heart, their genes can be engineered in a highly specific manner and then expressed in both inducible and non-inducible manners, the generation of genetically engineered mice is both cost and time effective relative to other mammals and inbred strains of mice are available, which allow mutations to be introduced into defined genetic backgrounds. Hereditary forms of cardiovascular disease can be modeled through germline mutations, and nonhereditary forms of the cardiovascular pathophysiology can be introduced in tissue-specific and inducible manners. Genes can be overexpressed or ablated in spatio-temporal fashion. Multiple gene defects can be combined or added in sequence through a combination of breeding and inducible systems. Polymorphisms can also be introduced in the germline or in tissue-specific and inducible manners. Similarly, the effects of microenvironment and stress can be functionally tested through gene alterations in specific cardiac cell types and through alteration of the animal’s environment and application of stress conditions. Mouse strains with complex genetic combinations and highly controlled spatial and temporal regulation of genes now predominate the modeling of cardiac disease. Advancement in molecular techniques allows the design of definite genetic modifications in the mouse. Now, along with defined nucleotide changes, also genetic switches designed to target expression or ablation of any gene (for which basic molecular information is available) to any tissue at any defined time can be engineered into the mouse genome. The most potential tool, both for the design of such genetic switches and for speeding the creation of gene-modified animals, is the Cre-LoxP recombination system.

### 1.2.1 Cre-loxP recombination system

The Cre-LoxP recombination system is a special type of site-specific recombination system from bacteriophage P1, which is particularly simple and well characterized. Cyclization recombination gene encodes a 38-kDa site-specific DNA recombinase, called Cre, which recognizes 34-bp loxP sites, and catalyzes both inter- and intramolecular recombination between two loxP sites (Fig. 1.6). The loxP (locus of X-over of P 1) is a site on bacteriophage P1 and consists of an 8-bp nonpalindromic core region flanked by two 13-bp inverted repeats. Cre-loxP mediated recombination between two directly repeated loxP sites
catalyze deletion of all DNA sequences located within the two sites. When the loxP sites are arranged in opposite directions, Cre catalyze inversion of the intervening DNA. In addition, intramolecular recombinations can be performed, also when the loxP sites are on different strands of DNA. Insertion of a DNA segment into a loxP site is also possible though the excision reaction is favored over the integration event. Since any DNA sequence introduced in between the loxP sequences (termed “floxed” DNA) is excised because of Cre-mediated recombination. Therefore, control of Cre expression in a transgenic animal, either with a tissue- or cell-specific promoter or with an inducible system, results in the spatial or temporal control of DNA excision between the two-loxP sites. In addition to conditional knockout and gene inactivation, this approach can be applied to protein over-expression. In that case, a floxed stop codon is inserted between the promoter sequence and the cDNA of interest and transgenic animals expressing Cre do not express the transgene, leading to excision of the floxed stop codon by Cre recombinase. This strategy has been successfully used to develop reporter mice that express LacZ or different fluorescent proteins like green fluorescent protein (GFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), DsRed, a red fluorescent protein (RFP) after Cre-mediated recombination. Since Cre is one of the few recombinases that do not require any additional cofactor or accessory molecules in eukaryotic cells, Cre is the first choice of investigators for introducing gene modifications into the mouse genome.

Currently, a large number of Cre transgenic mouse strains are available, allowing researchers to restrict conditional genetic modifications to particular cell types and to various other tissues of the mouse. Such Cre expressing mouse strains can be generated either by using conventional random transgenesis, by targeted insertion into a gene (knock-in), or by using a bacterial artificial chromosome (BAC) strategy. BACs are low-copy plasmids that stably maintain genomic DNA sequences, hundreds of kilobases (Kb) in length from mouse or human and can be obtained commercially. The use of BAC plasmids for transgenic gene expression is also gaining popularity over traditional proximal promoter-driven transgene expression. The main advantage of BAC is that they are most likely to contain all the important genomic regulatory elements required to recapitulate endogenous gene expression pattern. Thus, BAC can also be used for expressing Cre recombinase along with endogenous genes, by the introduction of Cre gene into defined genes encoded by the BAC via homologous recombination in bacteria. In addition to the Cre-loxP recombination system, FLP-FRT and Dre-rox systems are also available. Flp-FRT recombination is analogous to Cre-lox recombination but involves the recombination of
sequences between short flippase recognition target (FRT) sites by the recombinase (Flp) derived from the 2µm plasmid of baker's yeast *Saccharomyces cerevisiae* (Figure 1.3).

**Figure 1.3 Cre and FLP recombinase systems.** Cre and FLP recombinases recognize loxP (A) and FRT (B) sequences, respectively. These 34-bp sequences contain 13-bp inverted repeats flanking 8 bp unique sequence motifs that have directionality. When 2 loxP or 2 FRT sites line up a recombination occurs at the 8 bp motif. Depending on whether the 8-bp motifs are in the same or opposite orientation a deletion or insertion (C), inversion (D) or translocation (E) can occur (Modified from Bockamp E *et al.* 2002).
1.2.2 Tamoxifen-inducible conditional gene targeting

In order to better understand the functioning of a given gene product in a given cell type at a given developmental stage, genetic techniques have been developed intentionally allowing the introduction of defined mutations into the mouse genome, in a specific cell type and at a chosen time. Use of the site-specific recombinase Cre is the basis of current conditional gene targeting system. Clearly, the key to successful conditional gene targeting is the availability of Cre transgenic mouse strains in which Cre activity is tightly controlled in space and time. To add inducibility to the Cre/lox system, ligand-dependent chimeric Cre recombinases, so-called CreER recombinases, have been developed. These consist of Cre fused to mutated hormone-binding domains of the estrogen receptor\textsuperscript{100, 101}. Newer, more efficient versions of the ligand-dependent recombinase, termed Cre-ERT2 and ERT2-CreERT2, have since then been developed\textsuperscript{102-104}. Both, CreERT and CreERT2, do not bind to endogenous estradiol, but they bind to synthetic estrogen receptor ligand 4-hydroxytamoxifen or Tamoxifen (Tam) with high affinity (Figure 1.4). Generally, in the absence of hormone, estrogen receptors are largely located in the cytoplasm. Estrogen binding to the receptor translocates the receptor from the cytoplasm to the nucleus where it binds to DNA and regulates transcription of target genes\textsuperscript{105}. However, the CreERT recombinase are inactive and remain in the cytoplasm but can be activated and translocated to the nucleus by Tam, thereby allowing for external temporal control of Cre activity\textsuperscript{100, 106}.

A CreERT2 mouse can be a transgenic or a knockin of the CreERT2 coding region into an endogenous gene. A transgenic mouse is a biological model that has been genetically modified by the introduction of a foreign DNA sequence/fragment into the genome whereas knockin is a biological model that has a gene sequence inserted at a particular locus. ROSA26-CreERT2 mice were generated by targeting CreERT2 into the ROSA26 locus that have ubiquitous Tam-induced Cre recombination in adult mice\textsuperscript{107}. In addition, cell-type-specific CreERT2 mice such as cardiac-specific $\alpha$MHC-CreERT2 (also called $\alpha$MHC-MerCreMer) mice have been effectively used in cell-type-specific Tam-inducible gene targeting\textsuperscript{108}. In addition, Tam-inducible Cre recombination has been effectively used to modify gene function in mouse embryos in utero\textsuperscript{106,107,110}. Tamoxifen can be administered via food (custom-made chow containing 0.4–1 g/kg Tam, Harlan), water intake (0.5–1 mg/mL), or either by intraperitoneal or IP injection (100 mL of 10 $\mu$g/mL Tam) or oral gavage (200 mg/kg) for 5 consecutive days.
Figure 1.4 Inducible-Cre system.
Temporal restriction of Cre recombinase is achieved by fusing it to the tamoxifen-responsive ligand-binding domain of the estrogen receptor (Cre-ERT2). In the absence of appropriate ligand, the Cre recombinase fused estrogen receptor with mutated ligand binding domain is bound to the heat shock protein (Hsp90) and inhibited from entering the nucleus. Upon administration and binding of estrogen antagonist, tamoxifen (TM) or 4-hydroxytamoxifen (4-OHT) to the mutant estrogen receptor, Hsp90 dissociates from the CreERT2 and allows translocation of activated CreERT2 into the nucleus. In the nucleus, CreERT2 recognizes the loxP sites in the conditional allele of target genes and mediates recombination. Consequently, the reporter gene (EGFP) permanently marks the cells and appears green (Modified from Jung-Eun Kim, 2006). 

The main advantage of inducible gene expression models is the temporal control of gene expression or deletion by the external application of a drug. It is an appropriate method to overcome problems such as prenatal lethality caused by conventional or tissue-specific inactivation of genes. It also allows the control of gene expression at specific time points. This is especially attractive for studying specific gene function at specific time points during development and homeostasis. Importantly, it allows investigators to turn genes on or off at different disease stages, enabling assessment of their importance during the progression of various diseases.

This inducible system also has several limitations. Besides the problem of choosing the right method for drug application, dose determination is another pitfall when using these systems.
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Toxic side effects can result due to high dose or prolong treatment and can ruin an experiment. Another side, low doses can result in the insufficient induction of the protein of interest (e.g., Cre), causing only a partial gene knockout. Another disadvantage of the tamoxifen approach to induce constitutive or cell-type-specific gene manipulation in utero is the risk of embryo abortion and death (can occur after 4 days of treatment), so it is useful only during those 4 days \(^{106,107,110}\). Intestinal problems from Tam administration have also been reported in homozygous ROSA26-CreERT2 mice\(^{107}\). There are also some concerns that Tam can cause behavioral alterations\(^{112}\). Despite these potential problems, numerous researchers have successfully used this approach to investigate cardiac-specific gene function in the physiology and pathophysiology of the heart \(^{81,113}\).

1.2.3 Tetracycline-inducible Cre recombination

In tetracycline-inducible system, expression of the target gene is dependent on the activity of an inducible transcriptional activator. The transcriptional activator is regulated reversibly by the inducing ligand tetracycline or tetracycline derivatives such as doxycycline (Dox). This system consists of the Tet-Off system (tetracycline-controlled transactivator protein (tTA) dependent) and the Tet-On system (reverse tetracycline-controlled transactivator protein (rtTA) dependent) \(^{114,115}\). In each system, a recombinant tetracycline-controlled transcription factor (tTA or rtTA) interacts with a tTA/rtTA responsive promoter, Ptet, to drive expression of the gene of interest. In the Tet-Off system, target gene expression is turned off with the inducing ligand tetracycline or Dox. The Tet-Off system makes use of the tetracycline transactivator (tTA) protein, which is created by fusing one protein, TetR (tetracycline repressor), found in Escherichia coli bacteria, with the activation domain of another protein, VP16, found in the Herpes Simplex Virus. tTA binds to a tTA-responsive promoter (Ptet) to drive the expression of a gene in the absence of tetracycline or Dox. Addition of Dox, stops target gene expression due to formation of Dox-tTA complex with the transcription factor tTA, which cannot bind with Ptet. In contrast, in the Tet-On system, target gene expression is turned on by the inducing ligand. Addition of tetracycline or Dox, leads to binding of the transcription factor rtTA to Ptet through Dox-rtTA complex, thereby initiating the expression of gene under study. While the absence of Dox, inhibit the binding of rtTA with Ptet thus, inhibiting gene expression.

Several studies have indicated that the rtTA system is better suited for temporal control of rapid induction of gene expression \(^{116}\). Recently, many investigators have combined the Tet inducible system with the Tam-inducible Cre-loxP approach. This allows more flexibility producing more clinically relevant mouse models of human diseases\(^{116}\). Several laboratories
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have integrated the Tet-inducible and Cre-loxP approaches and developed mice based on rtTA-dependent Dox-mediated gene induction following a Cre-mediated deletion to obtain a temporal and spatial or cell-, tissue-, and organ-specific gene targeting\textsuperscript{117, 118}. Thus, gene targeting can be manipulated in specific cell types and lineages with a flexibility that is difficult to achieve with other methods. To make this system more sophisticated, a Tet-inducible CreERT2 has been targeted to the ROSA26 locus\textsuperscript{119}. By introducing Tet induction, this Tam-inducible Cre driver line permits highly selective dual Tet and Tam regulation of loxP recombination, which could open new avenues for spatiotemporally controlled gene targeting in mice.

There are pitfalls inherent in Tet-inducible technology worth mentioning. The Tet system can be leaky\textsuperscript{120}. This can occur either through weak binding of rtTA to Ptet even in the absence of Tet or Dox effectors, or through an unwanted basal activity of the Tet-responsive promoter even in the absence of rtTA. This leakiness can result in unexpected phenotypes when Tet is absent\textsuperscript{121}. Although this leakiness can be tolerated in many experimental systems in which phenotypic outcomes are desired, it can be limiting when gene function is being investigated. Use of several Cre transgenic lines could obviate some of these difficulties. Another potential problem with the Tet system is that of tissue toxicity caused by overexpression of TetR proteins\textsuperscript{120}. This can also be avoided by producing multiple transgenic lines expressing varying levels of Tet repressor. Taken together, it is very important that proper control animals are used in Tet-inducible gene targeting experiments to avoid misleading results due to these pitfalls. Overall, the ability to regulate gene activity in spatiotemporal and reversible fashions has made the Tet-inducible approach a favorite technology of numerous mouse geneticists.

Tamoxifen- and Tetracycline-inducible strategies have been applied to achieve this end\textsuperscript{122, 81,123} and have been successful in demonstrating gene function in cardiac structure and function, cardiac physiology and pathophysiology, cardio-mechanics, cardiac calcium handling, and cardiac stress response\textsuperscript{113}. The table given below summarizes the mice models validated for cardiac-specific inducible gene targeting.

<table>
<thead>
<tr>
<th>Cardiac-cell Type</th>
<th>Cre mice</th>
<th>Cre expression</th>
<th>Induction</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocardium</td>
<td>αMHC-CreERT2</td>
<td>Myocardium</td>
<td>Tam</td>
<td>108</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Endothelium, Cardiac and valvular fibroblasts</th>
<th>Cardiac neural crest cells, Epicardium</th>
<th>Cardiac conduction system</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$MHC-CrePR1, $\alpha$MHC-tTA, Tnnt2-rtTA/tetO</td>
<td>Tcf1-CreERT2, VE-cadherin-CreERT2</td>
<td>HCN4-CreERT2, Cx40-CreERT2, mink-CreERT</td>
</tr>
<tr>
<td>RU486, Doxy</td>
<td>Cardiac &amp; vascular endothelium, Tam</td>
<td>SA node, AV node, Tam</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AV bundle, bundle branches, Purkinje fibers, atrial</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cardiomyocytes, coronary vessels</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AV node, AV bundle, bundle branches</td>
</tr>
</tbody>
</table>

$\alpha$MHC indicates $\alpha$-myosin heavy chain; CreER, Cre with the mutated progesterone receptor ligand-binding.
domain; Tie2, tyrosine-protein kinase receptor TIE-2; Tcf21, transcription factor 21; Col2, collagen 2; αSMA, alpha smooth muscle actin; SM22a, SM22alpha; Wnt1, wingless-related MMTV integration site 1; Wt1, Wilms tumor 1 homolog; HCN4, hyperpolarization-activated, cyclic nucleotide-activated K_4; Cx40, connexin 40; ND, not determined; mink, Kcne1l potassium voltage-gated channel; tTA, tetracycline-controlled transactivator; Tnnt2, rat troponin T; rtTA, reverse tetracycline controlled transactivator; tetO, tetracycline operator; AV, atrioventricular, Tam, Tamoxifen; Doxy, Doxycycline.

1.3 Cardiac fibroblast-specific markers
Although CFs are widely acknowledged as prime targets for treatments of cardiac disease, our limited understanding of the details of the various roles that these cell populations play as well as how those various roles are intertwined in vivo hinder the design and application of potential therapies. The paucity of markers to faithfully identify cardiac fibroblasts at a single time point is one of the major issues challenging the cardiovascular field today. There are several well-known indicators of fibroblast phenotype, but none of them is both exclusive to fibroblasts and present in all fibroblasts. A few of the commonly used CF markers include: Discoidin domain collagen receptor (DDR)-2, Fibroblast-specific protein (Fsp1), Collagen type I, Fibroblast activation protein, Platelet-derived growth factor receptor alpha (Pdgfra), Periostin, Thy1 cell surface antigen, and Vimentin. The variable expression of the most commonly used markers at different stages of development is described in Table 2. For example, Vimentin, an intermediate filament protein has been extensively used to label cardiac fibroblasts. However, though antibodies to Vimentin label fibroblasts with great sensitivity (at this stage, it is safe to assume that all fibroblasts are Vimentin-positive), they also label various other cell types, including endothelial cells. In fact, Vimentin had first been described as an endothelial cell marker. Fibroblast-specific protein 1 (Fsp1) is another indicator of fibroblast phenotype, which was identified in a differential expression screening comparing kidney fibroblasts and kidney epithelial cells. It is also known as S100A4 and is also expressed by metastatic cancer cells. Immediate evidence suggests that it is specific for cardiac fibroblasts in the heart. However, Fsp1 antibodies detect only a subset of cardiac fibroblasts in heart. Recently it has been shown that Fsp1 is also expressed within inflammatory leukocytes and vascular cells in murine infarction and pressure overload-induced fibrosis models convoluting the future use of this marker. In the healthy adult heart, valvular fibroblasts (also called as valvular interstitial cells) express alpha-smooth muscle actin (α-SMA), but not interstitial fibroblasts of the myocardium. In cardiac fibrosis, myocardial fibroblasts start expressing α-SMA (then called as myofibroblasts), which is considered a sign of fibroblast activation. Though, there are other cell types like vascular...
smooth muscle cells and pericytes, lying in close proximity to fibroblasts, which also, found to express α-SMA. These cells can be falsely identified as fibroblasts when immunofluorescence techniques with insufficient resolution are used.

Table 2. Commonly utilized fibroblast makers are listed along with their relative expression levels at varying developmental and/or injury states.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Developmentally expressed markers</th>
<th>Adult CF resting markers</th>
<th>Myofibroblast markers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus cell antigen-1 (Thy1)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>[147,148]</td>
</tr>
<tr>
<td>Vimentin</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>[149, 150]</td>
</tr>
<tr>
<td>Periostin</td>
<td>++</td>
<td>+/-</td>
<td>++</td>
<td>[151, 74]</td>
</tr>
<tr>
<td>Ddr2</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>[37,19,4]</td>
</tr>
<tr>
<td>Fibroblast-specific protein-1 (Fsp1)</td>
<td>++</td>
<td>+/-</td>
<td>+++</td>
<td>[136, 152]</td>
</tr>
<tr>
<td>α Smooth muscle actin</td>
<td>+/-</td>
<td>+/-</td>
<td>+++</td>
<td>[65,153]</td>
</tr>
<tr>
<td>Platelet-derived growth factor receptor-α</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>[154]</td>
</tr>
<tr>
<td>Fibroblast activation protein PDGFRα</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>[37,155]</td>
</tr>
</tbody>
</table>

Discoidin domain receptor-2 (DDR2), a collagen receptor, has been used to identify and sort cardiac fibroblasts. However, DDR2 is also expressed by lymphocytic lineages and identifies only subsets of fibroblasts. Periostin, a matricellular protein is only expressed in a small subset of CFs in the quiescent adult heart but is robustly up-regulated in response to injury, therefore making it a useful marker of activated injury-site fibroblasts. Additionally, developmental studies suggest that endogenous Periostin is one of the most
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reliable markers of CFs in utero and throughout the early postnatal period making it well suited to developmental and neonatal investigations. Collagen type I is the major fibrillar component of the cardiac ECM and one of many ECM proteins produced by fibroblasts. The promoter region for the pro-\(\alpha_1(I)\) chain of type I collagen has been well characterized and it has been shown that different regions of this promoter can be used to drive expression of genes in specific collagen producing cell types including fibroblasts, osteoblasts, odontoblasts and some mesenchymal cells. A number of commercially available reporter mice (Cre, fluorescent protein expressing, inducible) have been generated using the type I collagen \(\alpha_1\) chain promoter. Type I collagen is also produced by smooth muscle cells in response to TGF\(\beta\), EGF, and angiotensin II stimulation, therefore the use of a fibroblast-specific regulatory region from the pro-\(\alpha_1(I)\) chain promoter could provide a valuable tool for marking fibroblasts within the heart. Thy1.1 (or CD90) is a membrane glycoprotein expressed on the surface of CFs but is also detectable on some endothelial cells.

Thus, the absence of comprehensive markers has inhibited our ability to study the complex interactions between CFs and the surrounding cells in vivo. It may be that there is no ideal way to identify CFs with a single marker; however, the more we are able to understand the limitations of the tools that we do have available, the more effectively we will wield them. Combining multiple markers to more conclusively identify CFs or understanding which markers are best in a particular context are footsteps that are currently being taken to improve the confidence in the interpretation of findings.

1.3.1 Vimentin

1.3.1.1 Type III Intermediate filament protein Vimentin

Vimentin is one of the most familiar members of the large protein family of intermediate filaments (IFs). It is the major protein in mesenchymal cells and it is frequently used as a developmental marker of cells and tissues. Vimentin also present a very high degree of sequence homology between species, from fish and Xenopus to humans, suggesting some important and evolutionary conserved physiological roles of this IF protein. Vimentin, a 57-kDa protein, along with microtubules and actin microfilaments make up the dynamic cytoskeleton that maintains cell shape, enables intracellular transport, and supports cell division. Studies using ras-transformed cells and transgenic mouse models have shown that vimentin regulates cell growth and differentiation. Recent studies using vimentin-deficient (Vim-/-) mice have revealed that loss of vimentin leads to failures in vascular adaptation resulting in pathological conditions, such as reduction of renal mass.
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Malformation of glia cells\textsuperscript{164}, impairment of wound healing\textsuperscript{165}, reduced resistance of arteries to shear stress\textsuperscript{166}, and disturbance of leukocytes homing to lymph nodes\textsuperscript{167}. Similar to other IF proteins, vimentin expression is often reported in a wide range of other cell types including pancreatic precursor cells, sertoli cells, neuronal precursor cells, trophoblastic giant cells, fibroblasts, endothelial cells lining blood vessels, renal tubular cells, macrophages, neutrophils, mesangial cells, leukocytes, and renal stromal cells\textsuperscript{25-30}.

1.3.1.2 Vimentin expression in heart

Two intermediate filaments, desmin and vimentin, are found in human fetal heart tissue. Desmin, an early marker expressed during cardiac embryogenesis\textsuperscript{168}, found to be expressed by cardiomyocytes and the intensity of cardiomyocytes staining for desmin increases progressively with age. On the other hand, vimentin appears during cell differentiation and is expressed by all connective tissue cells, including fibroblasts\textsuperscript{169}.

![Figure 1.5 Vimentin expressions in healthy and failing myocardium.](image)

Confocal images of immunofluorescence for fibroblast marker vimentin (red), with cell nuclei counterstained with dapi. (A) Normal myocardium with few fibroblasts and (B) In failing myocardium, increased expression of vimentin (red) in the fibrotic areas of failing heart. Scale bar represents 50\textmu m.

During cardiac muscle cell differentiation, vimentin is replaced especially by desmin; however, some authors consider that vimentin is expressed in adults during post-ischemic regenerative processes\textsuperscript{170}. During mouse development, vimentin expression begins on embryonic day 7.5 (E7.5)\textsuperscript{171} and becomes predominant in the primitive streak stage\textsuperscript{172, 173}, while in adult mice, vimentin expression was reported to be limited to connective tissue mesenchymal cells in the central nervous system and muscle\textsuperscript{174}. Alterations of the cytoskeleton have been described in many excellent studies, by Tsutsui et al\textsuperscript{175} and Tagawa et al,\textsuperscript{177} in hypertrophied and failing right ventricles of feline\textsuperscript{175} and canine\textsuperscript{180} myocardium. The activation of cardiac fibroblasts and their differentiation into myofibroblasts is of
considerable clinical interest because of their contribution to cardiac fibrosis and hypertrophy. Vimentin, the intermediate filament of fibroblasts was used as an indicator of the cellularity of the interstitium in the myocardium. Increased expression of vimentin has been demonstrated in the interstitial spaces in fibrotic heart tissues 176-179 (Figure 1.5). However, an increased expression of vimentin has also been reported in various tumor cell lines and tissues including prostate cancer, breast cancer, endometrial cancer, tumors of the central nervous system, malignant melanoma, and gastrointestinal tract tumors etc.

1.3.2 Fibroblast-specific protein-1

1.3.2.1 Fsp1/S100A4: a candidate marker for cardiac fibroblasts?

Fibroblast specific protein-1 (Fsp1) (also known as S100A4) is a member of the calmodulin S100 troponin C superfamily, and can be expressed by different cell types of mesenchymal origin. In fibrotic tissues, it is expressed by fibroblasts and has been suggested as a fibroblast-specific marker. Fsp1 has been involved in the regulation of a wide range of biological effects including cell motility181,182, differentiation183, survival184,183 and contractility. It has both intracellular and extracellular effects. Differential hybridization studies identified Fsp1 as a filament-associated protein that is not expressed in epithelial cells, mesangial cells or embryonic endoderm, but only in fibroblast136. Antibody against Fsp1 identified fibroblasts both in vitro and in vivo. Furthermore, in vitro studies also demonstrated its role in mediating epithelial to mesenchymal transition185. In experimental models of pulmonary186 and renal fibrosis136 and in biopsied samples from patients with fibrotic disorders186, Fsp1 identified interstitial fibroblasts. Based on these observations, transgenic mice with GFP expression driven by the Fsp1 promoter (Fsp1.GFP mice) have been generated and used to map cell fate in fibroblast populations20. Though, Fsp1-Cre transgenic mouse models have served as a tool to study the effects of fibroblast-specific gene deletion in fibrotic and neoplastic conditions20,187. However, growing evidence in recent years challenged the specificity of Fsp1 as a fibroblast marker, suggesting that other cell types infiltrating injured tissues, such as inflammatory macrophages188, dendritic cells189, lymphocytes190 and vascular smooth muscle cells191 may also express Fsp1.

1.3.2.2 Fsp1/ S100A4 expression in heart

Originally described as a cytoplasmic protein in mesenchymal cells, Fsp1 was found to express after embryonic day 8.5 (E8.5) in developing tissues 136. The heart of Fsp1-GFP transgenic mice displays no Fsp1-positive cells in the neonatal state, whereas only a few FSP positive cells are found in the adult heart (predominantly endothelial and perivascular cells) (Figure 1.6) 76. Increased S100A4 mRNA and protein expression in hypertrophic left
ventricles of rats, mouse and human patients is reported, apparently regardless of the underlying experimental cause of hypertrophy\textsuperscript{183,76}. Additionally S100A4 localizes to several activated, motile cell types of the injured hearts, including fibroblast-like cells, inflammatory cells, and endothelial cells, but not cardiomyocytes\textsuperscript{183, 192,76}.

![Figure 1, 6](image)

Figure 1.6 Fibroblast-specific protein-1 (FSP1) expressing cells in neonatal (n:2 wk of age) and adult (a:3–4 month of age) Fsp1-GFP reporter mouse myocardium. Neonatal hearts exhibit no FSP1 expression (A) while in adult mouse hearts, rare FSP1 cells are identified; these cells are either perivascular or endothelial (C, arrows) (Ref: Kong P et al. 2013 \textsuperscript{76}).

A recent study on the Fsp1-GFP mice in two models of cardiac remodeling (in the infarcted and pressure-overloaded myocardium) identified a large number of FSP1+ cells as endothelial cells, inflammatory leukocytes, and arteriolar smooth muscle cells \textsuperscript{76}. This raises questions regarding the use of FSP1 as a cardiac fibroblasts marker and also raises concern regarding the use of FSP1-Cre animals as a tool for fibroblast-specific gene targeting \textit{in vivo}.

1.3.3 Periostin

Periostin, also termed osteoblast-specific factor 2, is a 93.3 kDa-secreted, vitamin K-dependent glutamate-containing matricellular protein, originally isolated from a mouse osteoblast cell line \textsuperscript{193,194}. Periostin is assigned to the family of fascicilins based on its homology to fasciclin 1 (FAS1), initially identified in insects and is encoded by the Postn gene (genebank D13664) in humans. It is expressed in, vascular smooth muscle cells, cancer cells, periostium and periodontal ligament, fibroblasts, and wound-site blood
Introduction

vessels. On cell surface, periostin interacts with several integrin molecules (v1, v3, and v5), thus, providing signals for tissue development and remodeling. In addition, periostin also participates in tumor angiogenesis, metastasis, and cell migration\(^{195-198}\). Analysis of Periostin deficient mice (Postn-deficient mice) has demonstrated the importance of periostin in the development of bone, tooth, and heart valves\(^65,199\). Periostin has also shown to have another physiological role in cutaneous wound repair\(^{200-202}\). Furthermore, periostin is involved in the development of various tumors via the integrin/PI3K/Akt pathway\(^{203}\). Expression of periostin during Th2-type immune responses, in lung fibroblasts and in fibrosis of bronchial asthma\(^{204,205}\), displays its role in allergic inflammation. In the heart, periostin is physiologically expressed in embryonic cardiac valves, while it is re-expressed abundantly in adult heart after pressure overload or myocardial infarction\(^{142,73,206-209}\). During neonatal heart remodeling, peak expression of periostin induces collagen production; thereby mediating increased ventricular wall stiffness and valve functional maturation. However, periostin is downregulated in the postnatal cardiac fibroblast lineage and remains at a low level of expression. Abolishing periostin or TGF-\(\beta\) reduces both proliferation and fibrosis and improved heart function\(^{210}\). Therefore, Postn-deficient mice are more prone to ventricular rupture within the first 10 days after myocardial infarction\(^{208}\), yet survivors showed less fibrosis and better ventricular performance. Furthermore, inducible periostin overexpression not only protected mice from ventricular rupture after myocardial infarction but also induced spontaneous hypertrophy with aging\(^{207}\). Accumulation of periostin has been demonstrated to be involved in repair after vascular injury\(^{197}\) while, periostin insufficiency may contribute to valvular heart disease\(^{211,212}\), heart failure\(^{75,213}\), and atherosclerosis\(^{214}\). Increased expression of periostin in both normal and pathologic hearts is confined to the cardiac fibroblast (non-cardiomyocyte) lineages, with TGF-\(\beta\) being required for periostin expression\(^{48}\). Thus, Postn is currently being discussed as a potential target for the prevention of heart failure\(^{75,213}\).

1.4 Aim of this study

Over the years, research into the processes that control cardiac remodeling has focused on the cardiomyocyte as this cell type makes up the active beating part and also the biggest volume in the myocardial tissue. Only recently, more attention has been given to the fibroblast and a better understanding of its role in cardiac function. Though they have been recognized as active participants in the heart both in normal and disease condition; still, their exact physiological and pathological roles remain elusive, mainly due to the lack of specific markers. However, promising new techniques such as utilizing the Periostin-Cre
and Fsp1-Cre lines for lineage mapping and genetic modification of in utero and adult cardiac fibroblasts, as well as an increasing number of fibroblast markers are emerging to help address these challenges. Harnessing these new tools to examine the developmental origins of these cardiac fibroblast and their interactions with cardiomyocytes and other cell types and how they influence injury response may uncover methods of shifting pharmacologic interventions to a more proactive approach aimed at regeneration and undoing the damage caused by injury.

The overall aim of this study was to identify a cardiac fibroblast specific gene whose promoter can then be used for studying fibroblast targeted genetic interventions. This study was conducted in two parts. The first part included screening of genes enriched in CFs using microarray gene expression data. Validation of these candidate gene using qPCR-based gene expression profiling of cardiac fibroblasts and cardiomyocytes in order to identify genes enriched in cardiac fibroblasts in both neonatal as well as adult rodent hearts. Vectors enabling Cre-recombinase expression driven by selected candidate promoters were generated for validating promoter activity *in vitro and in vivo*. A transgenic mouse line that expresses Cre recombinase under Ccdc80 promoter was then generated to analyze promoter efficiency *in vivo*. Upon successful recombination after crossing with reporter mice, hearts and other tissues were studied for the promoter activity. Furthermore, another transgenic line, VimCreERT2, along with known fibroblast promoter-Cre mouse line (Fsp1-Cre) was characterised for cardiac fibroblast specific expression of Cre recombinase in both normal and disease condition in adult mouse heart and also during postnatal development.
## 2 MATERIALS AND METHODS

### 2.1 MATERIALS

#### 2.1.1 Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>J.T. Baker (Phillipsburg, USA)</td>
</tr>
<tr>
<td>Agar</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Agarose</td>
<td>Peqlab (Erlangen)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Roth (Karlsruhe)</td>
</tr>
<tr>
<td>Ammonium peroxodisulphate (APS)</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>5-bromodeoxyuridine (BrDU)</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>BDM</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
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<tr>
<td>Chloroform</td>
<td>Roth (Karlsruhe)</td>
</tr>
<tr>
<td>Complete Mini (Protease inhibitor)</td>
<td>Roche (Mannheim)</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl2)</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>Deoxynucleotide triphosphate (dTTP)</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>4,6-diamidino-phenylindole (DAPI)</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>DNase/RNase-free water</td>
<td>Gibco (Karlsruhe)</td>
</tr>
<tr>
<td>Dulbecco’s modified eagle medium (DMEM)</td>
<td>Gibco (Karlsruhe)</td>
</tr>
<tr>
<td>Dimethylsulphoxide (DMSO)</td>
<td>Roth (Karlsruhe)</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetate (EDTA)</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>J.T. Baker (Phillipsburg, USA)</td>
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<tr>
<td>Ethidium bromide</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
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<tr>
<td>Fetal bovine serum (FBS)</td>
<td>PAN (Aidenbach)</td>
</tr>
<tr>
<td>Fetal Calf serum (FCS)</td>
<td>PAN (Aidenbach)</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Fura-AM</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Glycine</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Goat serum</td>
<td>Gibco (Karlsruhe)</td>
</tr>
<tr>
<td>50% Glutaraldehyde</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Haematoxylin</td>
<td>Roth (Karlsruhe)</td>
</tr>
<tr>
<td>HEPES</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Hydrochloric acid 37% (HCl)</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Isoflurana</td>
<td>Abbott (Wiesbaden)</td>
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</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Material/Compound</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropanol</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Fluka (Seelze)</td>
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<tr>
<td>Lipofectamine™ 2000</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>PAN Biotech (Aidenbach, D)</td>
</tr>
<tr>
<td>Milk powder</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Magnesium chloride (MgCl)</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Magnesium sulphate (MgSO₄·7H₂O)</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Minimum essential medium (MEM)</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Methanol</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>Opti-MEM I</td>
<td>Gibco (Karlsruhe)</td>
</tr>
<tr>
<td>Paraffin (Paraplast)</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>Peanut Oil</td>
<td>Sigma Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>Peptone</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>Gibco (Karlsruhe)</td>
</tr>
<tr>
<td>Phenol/chloroform</td>
<td>Roth (Karlsruhe)</td>
</tr>
<tr>
<td>Phenylephrine (PE)</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
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<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>Gibco (Karlsruhe)</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Potassium bicarbonate (KHCO₃)</td>
<td>Sigma Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>Potassium di-hydrogen phosphate (KHPO₄)</td>
<td>Roth (Karlsruhe)</td>
</tr>
<tr>
<td>Potassium Ferricyanide (K₂[Fe(CN)₆])</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Potassium Ferrocyanide (K₄[Fe(CN)₆] )</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Prestained protein ladder</td>
<td>Fermentas (St. Leon-Rot)</td>
</tr>
<tr>
<td>PVDF membrane</td>
<td>Millipore (Billerica USA)</td>
</tr>
<tr>
<td>6 ROX</td>
<td></td>
</tr>
<tr>
<td>Sodium acetate (NaCH₃COO)</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>Applichem (Darmstadt)</td>
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<tr>
<td>Sodium bicarbonate (NaHCO₃)</td>
<td>Sigma Aldrich (Deisenhofen)</td>
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<tr>
<td>Sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O)</td>
<td>Applichem (Darmstadt)</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
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</tr>
<tr>
<td>Sodium hydrogen phosphate dibasic (Na₂HPO₄)</td>
<td>Sigma Aldrich (Deisenhofen)</td>
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<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>Roth (Karlsruhe)</td>
</tr>
<tr>
<td>Sodium ortho vanadate (Na₃VO₄)</td>
<td>Sigma Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>SYBR green</td>
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</tr>
<tr>
<td>Tetramethylethlenediamine (TEMED)</td>
<td>Sigma Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>Taurine</td>
<td>Sigma Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Sigma Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>X gal</td>
<td>Roth (Karlsruhe)</td>
</tr>
</tbody>
</table>
2.1.2 Kits

<table>
<thead>
<tr>
<th>Kit</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA Maxi kit</td>
<td>Qiagen (Hilden)</td>
</tr>
<tr>
<td>Plasmid DNA Midi kit</td>
<td>Qiagen (Hilden)</td>
</tr>
<tr>
<td>QIAquick Gel extraction kit</td>
<td>Qiagen (Hilden)</td>
</tr>
<tr>
<td>QIAquick PCR purification kit</td>
<td>Qiagen (Hilden)</td>
</tr>
</tbody>
</table>

2.1.3 Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuprime Pfx DNA Polymerase</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>Benzonase</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Collagenase Typ2 II Worthington</td>
<td>(Lakewood, USA)</td>
</tr>
<tr>
<td>Difco Trypsin 250</td>
<td>BD (Heidelberg)</td>
</tr>
<tr>
<td>DNase</td>
<td>Sigma-Aldrich (Deisenhofen)</td>
</tr>
<tr>
<td>Gateway BP Clonase II enzyme mix</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>Gateway LR Clonase II enzyme mix</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>Platinum Taq DNA Polymerase</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>Fermentas (St. Leon-Rot)</td>
</tr>
<tr>
<td>Restriction endonucleases</td>
<td>New England Biolabs (Frankfurt am Main)</td>
</tr>
<tr>
<td>Superscript II Reverse transcriptase</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>New England Biolabs (Frankfurt am Main)</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>Fermentas (St. Leon-Rot)</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Gibco (Karlsruhe)</td>
</tr>
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</table>

2.1.4 Bacterial strains:

Strain Genotype Manufacturer

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli</td>
<td>DH10B (electrocompetent)</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>E.coli</td>
<td>TOP10 (chemically competent)</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
</tbody>
</table>

2.1.5 Bacterial artificial chromosome (BAC) clones:

BAC clones were used for PCR amplification of the promoter sequence used for generating the constructs for studying promoter activity.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Clone #</th>
<th>BAC library</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccdc80 promoter</td>
<td>bMQ193n13</td>
<td>bMQ Mouse BAC library</td>
<td>Gene Service</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Periostin promoter</th>
<th>bMQ304b19</th>
<th>bMQ Mouse BAC library</th>
<th>Gene Service</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelsolin promoter</td>
<td>bMQ185d13</td>
<td>bMQ Mouse BAC library</td>
<td>Gene Service</td>
</tr>
<tr>
<td>Col2alpha1 promoter</td>
<td>CH-295P10</td>
<td>bMQ Mouse BAC library</td>
<td>Gene Service</td>
</tr>
</tbody>
</table>

#### 2.1.6 Plasmids

The following plasmids were available in the laboratory and were used for the experiments.

<table>
<thead>
<tr>
<th>Donor vector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDONR-221</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>pT-Rex DEST30</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td>pMIR-Reporter β-gal control plasmid</td>
<td>Ambion</td>
</tr>
<tr>
<td>pCAG-Cre</td>
<td>Addgene</td>
</tr>
<tr>
<td>pLacZ-basic</td>
<td>Clontech Laboratories, Inc.</td>
</tr>
<tr>
<td>pCALNL-GFP</td>
<td>Addgene</td>
</tr>
<tr>
<td>pCAG-ERT2CreERT2</td>
<td>Addgene</td>
</tr>
</tbody>
</table>

The following plasmids were constructed using *Gateway® Recombination* Cloning Technology (Invitrogen, Life Technology) and were used for the experiments.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLacZ-Ccdc-80</td>
<td>Ccdc80 promoter (~4Kb)</td>
</tr>
<tr>
<td>pCcdc80-Cre</td>
<td></td>
</tr>
<tr>
<td>pCcdc-80-ERT2CreERT2</td>
<td></td>
</tr>
<tr>
<td>pLacZ-Postn</td>
<td>Periostin promoter (3.9Kb)</td>
</tr>
<tr>
<td>pPostn-Cre</td>
<td></td>
</tr>
<tr>
<td>pPostn-ERT2CreERT2</td>
<td></td>
</tr>
<tr>
<td>pGsn-Cre</td>
<td>Gelsolin promoter (3kb)</td>
</tr>
<tr>
<td>pGsn-ERT2CreERT2</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.1.7 Oligonucleotide primers

The oligonucleotides were purchased in HPSF-purified lyophilized powder from MWG Eurofins (Ebersberg) and Sigma-Aldrich. The primers were dissolved in double-distilled autoclaved water (ddH2O). 20 μM working solutions were used for PCR.
**Materials and Methods**

**Oligonucleotides for amplification of promoter fragments**

Promoter sequences were PCR amplified from the respective BAC clones. The Gateway® sequences are in small letters in the primer sequences used for generating promoter constructs using Gateway recombination technology. In case of LacZ constructs restriction sites (underlined) were added before promoter primer sequence for cloning.

<table>
<thead>
<tr>
<th>Insert</th>
<th>BAC clone #</th>
<th>Constructs</th>
<th>Primer sequences (5’----3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccdc80 promoter (~4Kb)</td>
<td>bMQ193n13</td>
<td>pCcdc80-Cre</td>
<td>FP: 5’ ggggacaagtttgtacaaaaaagcaggcttcATG AGT TCC AGG ATA CCC AGG&lt;br&gt; RP: 5’ ggggaccacttctgtacaaagaagctgggtc&lt;br&gt; CAT TGT ATT ATC CAC TTG GGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pCcdc-80-ERT2CreERT2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pDONR-Ccdc80</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pCcdc-80-LacZ</td>
<td>FP: 5’ AAC GCC CCCGGG GAG TTC CAG&lt;br&gt; GAT ACC CAG GGC T (XmaI)&lt;br&gt; RP: 5’ AAC GCC GCTAGC TGT ATT ATC&lt;br&gt; CAC TTG GGG A (Nhel)</td>
</tr>
<tr>
<td>Periostin promoter (3.9Kb)</td>
<td>bMQ304b19</td>
<td>pPostn-Cre</td>
<td>FP: 5’ ggggacaagtttgtacaaaaaagcaggcttc&lt;br&gt; CTA AGG TGG ACA GTG CGG AAG AC&lt;br&gt; RP: 5’ ggggaccacttctgtacaaagaagctgggtc&lt;br&gt; CCT TCA GCC CTG AGC TCC (XhoI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pPostn-ERT2CreERT2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pDONR-Postn</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pPostn-LacZ</td>
<td>FP: 5’ CCC CCCGGG CTA AGG TGG ACA&lt;br&gt; GTG CGG AAG AC (XmaI)&lt;br&gt; RP: 5’ AAC G CTCGAG CCT TCA GCC&lt;br&gt; CTG AGC TCC (XhoI)</td>
</tr>
<tr>
<td>Gelsolin promoter (3kb)</td>
<td>bMQ185d13</td>
<td>pGsn-Cre</td>
<td>FP: 5’ ggggacaagtttgtacaaaaaagcaggcttc&lt;br&gt; CA TCA CAG ACC CTG CCT TCT&lt;br&gt; RP: 5’ ggggaccacttctgtacaaagaagctgggtcCAT&lt;br&gt; GGC GAC GGT GAG GAC CCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pGsn-ERT2CreERT2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pDONR-Gsn</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pGsn-LacZ</td>
<td>FP: 5’ AAC GCC CCCGGG GCA TCA&lt;br&gt; CAG ACC CTG CCT TCT (XmaI)&lt;br&gt; RP: 5’ AAC GCC CTCGAG GCC GAC&lt;br&gt; GGT GAG GAC CCA (XhoI)</td>
</tr>
</tbody>
</table>
| attR1-Cm®- | pT-Rex | pCre-GW (adapted for) | “Materials and Methods”

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Materials and Methods

| attR1-Cm<sup>R</sup>-ccdB-attR2 | pT-Rex DEST30 | pERT2CreERT2-GW (adapted for Gateway) | ACTAGT ATC AAC AAG TTTG TAC AAA AAA (Spel) TTT CTT GTA CAA AGT GGT TGA TCAATTG (EcoRI) |

Oligonucleotides for sequencing of the constructs

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Forward primer (5'--&gt; 3')</th>
<th>Reverse primer (5'--&gt; 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLacZ-basic</td>
<td>CGA TTT CGG CCT ATT GGT TA</td>
<td>CCGCACATATCCTGTACCTTT</td>
</tr>
<tr>
<td>pCre-GW (adapted for gateway cloning)</td>
<td>AAA AAG GGA ATA AGG GCG AC (sits on Ampicillin gene)</td>
<td>TCA GTA AAT TGG CCA TGG TG (sits on Cre gene)</td>
</tr>
<tr>
<td>pERT2CreERT2-GW</td>
<td>ACT TTC ACC AGC GTT TCT GG (sits on Ampicillin gene)</td>
<td>GGT TCC TGT CCA AGA GCA AG (sits on ERT2CreERT2 gene)</td>
</tr>
<tr>
<td>pDONR-221/pEntry</td>
<td>GTA AAA CGA CGG CCA G M13 For (-20)</td>
<td>CAG GAA ACA GCT ATG AC (M13 Rev)</td>
</tr>
</tbody>
</table>

Oligonucleotides for genotyping of Cre-transgenic mice

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Allele</th>
<th>Sequence (5'--&gt; 3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R26R (Rosa26-LacZ reporter)</td>
<td>Wild type Mutant</td>
<td>R126- AAA GTC GCT CTG AGT TGT TAT</td>
<td>WT: 500bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R127- GCG AAG AGT TTG TCC TCA ACC</td>
<td>Mut: 250bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R128- GGA GCG GGA GAA AT</td>
<td></td>
</tr>
<tr>
<td>Cccdc80-Cre</td>
<td>Cre</td>
<td>Cre 800-GCT GCC ACG ACC AAG TGA CAG</td>
<td>500bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAA TG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cre1200-GTA GTT ATT CGG ATC ATG TAC TAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cre Sense Cre -CCT GGA AAA TGC TTC TGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCG</td>
<td>400bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense Cre - CAG GTT GTT ATA AGC AAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>VimCreERT2</td>
<td>Cre</td>
<td>Sense Cre -CCT GGA AAA TGC TTC TGT</td>
<td>400bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense Cre - CAG GTT GTT ATA AGC AAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>Fsp1-Cre</td>
<td>Cre</td>
<td>Sense Cre -CCT GGA AAA TGC TTC TGT</td>
<td>400bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCG-3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense Cre - CAG GTT GTT ATA AGC AAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>R26-mTom-mGFP&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>Wild type</td>
<td>Common-CTC TGC TGC CTC GTT GCT TCT</td>
<td>WT: 330bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WT-CGA GGC GGA TCA CAA GCA ATA</td>
<td></td>
</tr>
</tbody>
</table>
Materials and Methods

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Common</th>
<th>Mut: 250bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CTC TGC TGC CTC GCT TCT</td>
<td>-TCA ATG GGC GGG GGT CGT T</td>
<td></td>
</tr>
</tbody>
</table>

Real Time PCR (for mouse)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'--&gt;3')</th>
<th>Reverse primer (5'--&gt;3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>TGG CAA AGT GGA GAT TGT TG</td>
<td>CATTATCAGCCCTGTGACCTG</td>
<td>140</td>
</tr>
<tr>
<td>RPL32</td>
<td>GCC CAA GAT CGT CAA AAA GA</td>
<td>GTC AAT GCC TCT GGG TTT</td>
<td>100</td>
</tr>
<tr>
<td>18s</td>
<td>ACC GCA GCT AGG AAT AAT GGA</td>
<td>GCC TCA GTT CCG AAA ACC A</td>
<td>63</td>
</tr>
<tr>
<td>Vim</td>
<td>CGG AAA GTG GAA TCC TTG CA</td>
<td>CAC ATC GAT CTG GAC ATG CTG T</td>
<td>111</td>
</tr>
<tr>
<td>Gsn</td>
<td>GCT TTG AGT CGT CCA CCT TC</td>
<td>TTG GGT ACC ACG TGT TTG AA</td>
<td>97</td>
</tr>
<tr>
<td>Ccdc80</td>
<td>CCA GGA GGA TCT CTG TGG TC</td>
<td>ACA CGC ATG GGT TTC TCA TT</td>
<td>100</td>
</tr>
<tr>
<td>Col8a1</td>
<td>CAA GGA CTT TGG TCC TCG AT</td>
<td>TGA TGA ACA GTA TTC CCA GCA</td>
<td>99</td>
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<tr>
<td>Postn</td>
<td>AAC CAA GGA CCT GAA ACA CG</td>
<td>GTG TCA GGA CAC GGT CAA TG</td>
<td>170</td>
</tr>
<tr>
<td>Myh6</td>
<td>GCC CAG TAC CTC CGA AAG TC</td>
<td>GCC TTA ACA TAC TCC TCC TTG TC</td>
<td>110</td>
</tr>
<tr>
<td>Rabgap1l</td>
<td>GGG CGT CTG ACA GAG TTG TT</td>
<td>CGA AGC TCT GAC CTC CAT TT</td>
<td>110</td>
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</tbody>
</table>

2.1.8 Buffers and Media

DNA loading buffer (5X)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylen-Cyanol</td>
<td>0.025g</td>
</tr>
<tr>
<td>EDTA (0.5M)</td>
<td>1.4ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3.6ml</td>
</tr>
<tr>
<td>H2O</td>
<td>7.0ml</td>
</tr>
</tbody>
</table>

DNA lysis buffer (for genotyping)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>12.1 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.87 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>11.7 g</td>
</tr>
<tr>
<td>SDS</td>
<td>0.2 g</td>
</tr>
<tr>
<td>ddH2O</td>
<td>adjust to 1 l</td>
</tr>
</tbody>
</table>

Lamelli buffer 2X

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (1M) pH 6.8</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>40 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>30 ml</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>1 ml</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>traces</td>
</tr>
</tbody>
</table>
## Materials and Methods

### Lower buffer 4X (for Western blot)
Tris 182 g  
SDS (10%) 40 ml  
ddH2O adjust to 1 l  
Adjust pH to 8.8 with HCl 37%

### PBS (10X)
NaCl 80 g  
KCl 2 g  
Na₂HPO₄·7H₂O 11.5 g  
KH₂PO₄ 2 g  
ddH₂O adjust to 1 l  

### PBST
PBS 10X 100 ml  
Tween® 20 1 ml  
ddH₂O adjust to 1 l  

### Protein lysis buffer
Tris (1M pH 6.7) 50 mM  
SDS 2%  
Na₃VO₄ 1mM  
Complete mini protease inhibitor 1 tablet per 10 ml

### Western running buffer (10X)
Tris-HCl 30 g  
Glycine 144 g  
SDS 15 g  
ddH₂O adjust to 1 l

### TAE buffer (50X)
Tris 242 g  
Acetic acid 57.1 ml  
Na₂EDTA.2H₂O 37.2 g  
ddH₂O adjust to 1 l

### Transfer buffer (for Western blot)
Tris (1M pH 8.3) 25 ml  
Glycine 11.26 g  
Methanol 100 ml  
ddH₂O adjust to 1 l
### Materials and Methods

**Tris-EDTA (TE) buffer**
- Tris: 10 mM
- EDTA: 1 mM

**Upper buffer 4X (for Western blot)**
- Tris: 61 g
- SDS (10%): 40 ml
- ddH2O: adjust to 1 l
- Adjust pH to 6.7 with HCl 37%

**Tamoxifen (20mg/ml)**
- Tamoxifen: 20mg
- Peanut oil: 1ml

**Perfusion Buffer (1X)**
- NaCl: 6.6g
- KCl: 0.35g
- KH$_2$PO$_4$: 0.082g
- Na$_2$HPO$_4$: 0.085g
- MgSO$_4$.7H$_2$O: 0.3g
- Phenol Red: 0.012g
- NaHCO$_3$: 1.01g
- KHCO$_3$: 1.01g
- Herpes Buffer (1M): 10ml
- Taurine: 3.75g
- ddH$_2$O: adjust to 1 l
- Sterile filtered the solution

**Digestion Buffer**
- Collagenase Typ2 II: 107mg
- CaCl$_2$ (100mM): 15 µl
- Perfusion Buffer: 50ml

**P1 Buffer**
- Perfusion buffer: 9ml
- FCS: 1ml
CaCl₂ (10mM) 12.5µl

**P2 Buffer**
Perfusion buffer 47.5ml
FCS 2.5ml
CaCl₂ (10mM) 62.5µl

**DMEM +++ (HEK293-Cells)**
DMEM 500ml
FBS 50ml
L-Glutamin 5ml
Penicillin/Streptomycin 5ml

**LB-Agar**
Pepton 10g
Yeast -extract 5g
NaCl 5g
Agar 15g
NaOH (1M) 1ml
ddH₂O adjust to 1 l

**LB-Medium**
Pepton 10g
Yeast -extract 5g
NaCl 5g
NaOH (1M) 1ml
ddH₂O adjust to 1 l

**NRCM incomplete medium**
MEM 10.7 g
NaHCO₃ 0.35 g
Vitamin B12 67% (w/v) 1 ml
ddH₂O adjust to 1 l
Adjust pH to 7.3 and sterile filtered the solution.

**AMCM preplating medium**
Penicillin/Streptomycin 1 ml
FCS 5 ml
BDM (500mM) 2ml
L-Glutamine (200mM) 1ml
MEM 91 ml
Materials and Methods

10% AMCF preplating medium
Penicillin/Streptomycin 1 ml
FCS 5 ml
NRCM incomplete medium 89 ml

2.1.9 Antibodies

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Host</th>
<th>Monoclonal/Polyclonal</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>-actinin</td>
<td>mouse</td>
<td>Monoclonal</td>
<td>Sigma-Aldrich (# A7811)</td>
</tr>
<tr>
<td>PECAM-1/CD31</td>
<td>Rat</td>
<td>Polyclonal</td>
<td>BD pharmingen (#550274)</td>
</tr>
<tr>
<td>Fsp1/S100A4</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Millipore (#07-2274)</td>
</tr>
<tr>
<td>GFP</td>
<td>Chicken</td>
<td>Polyclonal</td>
<td>Abcam (#ab13970)</td>
</tr>
<tr>
<td>GFP</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Life technologies (#A-11122)</td>
</tr>
<tr>
<td>P4HB/PDIA4</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Protein Tech (#11245-1-AP)</td>
</tr>
<tr>
<td>SM22alpha</td>
<td>Goat</td>
<td>Polyclonal</td>
<td>Abcam (#ab10135)</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Chicken</td>
<td>Polyclonal</td>
<td>Abcam (#ab24525)</td>
</tr>
</tbody>
</table>

Secondary antibodies

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Host</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 647 Anti-chicken IgG</td>
<td>Goat</td>
<td>Life technologies (#A21449)</td>
</tr>
<tr>
<td>Alexa Fluor® 647 Anti –Rabbit</td>
<td>Goat</td>
<td>Life technologies (#A21244)</td>
</tr>
<tr>
<td>Alexa Fluor® 647 Anti –Rat</td>
<td>Goat</td>
<td>Life technologies (#A21247)</td>
</tr>
<tr>
<td>Alexa Fluor® 488 anti-rabbit IgG</td>
<td>Goat</td>
<td>Life technologies (#A11008)</td>
</tr>
<tr>
<td>Alexa Fluor® 488 anti-chicken</td>
<td>Goat</td>
<td>Life technologies (#A11039)</td>
</tr>
<tr>
<td>Biotinylated Anti-Goat IgG</td>
<td>Goat</td>
<td>Vector laboratories (#VC-BA 9500)</td>
</tr>
<tr>
<td>Anti-mouse IgG, F(ab’)2-B</td>
<td>Goat</td>
<td>Santa Cruz (#sc-3795)</td>
</tr>
</tbody>
</table>

3 METHODS

3.1 Molecular biology methods

3.1.1 Polymerase chain reaction (PCR)

Amplification of promoter fragment
For amplification of promoter sequences from the BAC clone, Accuprime Pfx polymerase was used.
Materials and Methods

**Reaction mixture**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Accuprime Pfx buffer</td>
<td>5µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>20 pmol</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>20 pmol</td>
</tr>
<tr>
<td>Template DNA</td>
<td>100 ng</td>
</tr>
<tr>
<td>Accuprime Pfx polymerase</td>
<td>2.5 Units</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>upto 50µl</td>
</tr>
</tbody>
</table>

**PCR conditions:** A 3-step PCR cycle was used for amplification.

95°C 2min

95°C 15sec (Denaturation)

55 – 66°C 30sec (Annealing) x 30 cycles

68°C 60 sec per kb (Extension)

68°C 5min

16°C ∞

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Sequence size (Kb)</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>For Ccdc80 promoter seq with attb site in primers</td>
<td>Approx. 4.2</td>
<td>66°C</td>
</tr>
<tr>
<td>For Ccdc80 promoter seq with RE sites (Xmal (FP) &amp; Nhel (RP)) in primers</td>
<td>Approx 4.2</td>
<td>66°C</td>
</tr>
<tr>
<td>For Postn promoter seq with attb site in primers</td>
<td>3.9</td>
<td>58°C</td>
</tr>
<tr>
<td>For Periostin (Postn) promoter seq with RE site (Xmal (FP) &amp; Xhol (RP)) in primers</td>
<td>3.9</td>
<td>54°C</td>
</tr>
<tr>
<td>For Gelsolin (Gsn) promoter seq with Gsn_attb site in primers</td>
<td>Approx 6</td>
<td>66°C</td>
</tr>
<tr>
<td>For Gelsolin (Gsn) promoter seq with RE site (Xmal (FP) &amp; Xhol (RP)) in primers</td>
<td>Approx 2.8</td>
<td>55°C</td>
</tr>
</tbody>
</table>

3.1.2 Isolation and purification of PCR-amplified DNA

PCR amplified products are purified using the QIAquick PCR purification kit (Qiagen, Hilden). 5 volumes of Buffer QG were added to 1 volume of PCR product. If the color of the sample turns violet or orange, 10µl of 3M-ammonium acetate solution was added (the sample turns yellow). The samples were then applied to QIAquick column and centrifuged at 13000 rpm for 1 min. In the presence of high concentration of salts, the DNA binds to the silica membrane of the column. The flow-through was discarded and the columns were washed.
with 750μl of Buffer PE and centrifuged at 13000 rpm for 1 min. The flow-through was discarded and the empty columns were centrifuged to remove any residual solution from the column. The DNA was then eluted using 20μl of ddH2O into a fresh 1.5 ml microfuge tube.

3.1.3 Agarose gel electrophoresis

PCR amplified products were separated by gel electrophoresis in a 0.8 % agarose gel. The agarose was heated and dissolved in the appropriate amount of TAE buffer. After cooling the solution to 60°C, 10μl of ethidium bromide solution (1mg/ml) was added. This substance intercalates into DNA and illuminated under UV light of 312nm wavelength. The DNA samples were mixed with DNA loading buffer and were subjected to 120-140 volts (DC Voltage) for 30-40min for separation. As size standards, 1 kb or 100bp DNA ladders were used (New England Biolabs).

3.1.4 Gel extraction

The DNA fragments of the respective size were excised from the agarose gel using a sterile scalpel into a 2 ml microfuge tube and QIAquick gel extraction kit (Invitrogen, Karlsruhe) was used to extract the DNA. The DNA was eluted using 20 μl of autoclaved ddH2O.

3.1.5 Precipitation of DNA with sodium acetate

The samples were mixed with the 1/10th volume of 3M sodium acetate and 2.5 volumes of 100% ethanol. The samples were incubated at -80°C for 20 min, centrifuged at 14000 rpm and were then incubated at 4°C for 30 min. The pellet was washed with 1 ml of 75% ethanol, centrifuged at 14000 rpm and then kept at 4°C for 15 min. The pellet was dried at room temperature and was resuspended in 15 μl ddH2O.

3.1.6 Endonuclease digestion

Restriction endonuclease digestion was performed either to test the clones for insertion of the genes or for cloning. The restriction endonucleases were purchased from New England Biolabs and the digestion was performed in the buffers as per the manufacturers recommendations. 1 μg of DNA was digested with 1 unit of the enzyme for 1 hour at recommended temperatures. The restriction endonucleases were then inactivated by incubating the tubes at 65°C for 20 min.
Materials and Methods

3.1.7 Phosphatase treatment of DNA

After linearization of the vector with a restriction endonuclease were the only 5’ phosphate groups removed by treatment with Antarctic phosphatase to its to prevent re-ligation. For this

<table>
<thead>
<tr>
<th>Material</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested Vector</td>
<td>2 µg</td>
</tr>
<tr>
<td>Antarctic Phosphatase Reaction Buffer (10x)</td>
<td>5 µl</td>
</tr>
<tr>
<td>Antarctic Phosphatase</td>
<td>5 Units</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>adjust to 50 µl</td>
</tr>
</tbody>
</table>

The mixture was initially 30 minutes at 37°C and then for a further 30 min at 50°C incubated. By heat deactivation of the enzyme for 5 min at 65°C, the dephosphorylated vector used directly for ligation.

3.1.8 Ligation of DNA fragments

Ligation was done using the T4 DNA ligase New England Biolabs (Frankfurt am Main). The enzyme catalyzes the linkage of the 3’-hydroxy end of the 5’ phosphate end of the DNA fragments by forming a phosphodiester bond. The amount of insert was calculated using the following formula:

\[
\text{Insert mass [ng]} = \frac{5 \times \text{Vector mass [ng]} \times \text{Insert length [bp]}}{\text{Vector length [bp]}}
\]

For a reaction mixture in a final volume of 15 µl following were used:

<table>
<thead>
<tr>
<th>Material</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>10-50 ng</td>
</tr>
<tr>
<td>Insert</td>
<td>100-300 ng</td>
</tr>
<tr>
<td>Ligase buffer (5x)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

The ligation was carried out overnight at 16 °C (or available via 30 min to 1 h at room temperature).

3.1.9 Cre-expressing constructs adapted for gateway recombination reactions

For cloning promoter sequences in different Cre vectors, the vectors were modified and adapted for Gateway recombination system, where the already existing promoters sequence from these constructs (for example, CAG promoter in case of pCAG-Cre & pCAG-ERT2CreERT2 plasmids) were removed using restriction enzymes (NEB). The GatewayR adaptation cassette i.e. attR1&R2 recombination site, Chloramphenicol resistant gene and ccdB gene (attR1-Cm⁸-ccdB-attR2) was PCR amplified using primers as
mentioned in section 2.1.7 from the pTREX-Dest30 vector. The amplified PCR product was then inserted in above linearized promoterless plasmids, which then became Destination Vector for LR reaction of Gateway recombination technology (Fig 4.5 Results section)

3.1.10 Insertion of cDNA into plasmids by GatewayR™ recombination

For the cloning of the promoter fragments in front of Cre gene, the GatewayR system (Invitrogen) was used. This system ensures a higher cloning efficiency than conventional cloning and is based on a two-step homogeneous recombination strategy for creating different reporter and expression plasmids from a so-called Entry plasmid containing the relevant PCR product. To generate the homologue sequence, the characteristic attb-sites are added to the 5’ end of each primer, which is homologue to the sequence at the integration site in the Gateway DONR vector. The sequence of the two attb-sites is not identical, ensuring integration in only one direction

The sequences of the attb-sites are shown below:

| Forward primer: | 5’ GGGGACAAGTTTGTACAAAAAAGCAGGCT |
| Reverse primer: | 5’ GGGGACCACTTTGTACAAGAAAGCTGGGT |

3.1.10.1 Gateway BP reaction

The promoter fragments were amplified and purified as described above. The attb-site was added by the supplier (Invitrogen) to 5’ end of each primer. The PCR product was cloned into an Entry clone by BP recombination. For the BP recombination the following protocol was applied, as suggested by the supplier:

| pDONR221 (150 ng/μl) | 1 μl |
| PCR Product | 100 fmol |
| BP Clonase II | 2 μl |
| TE-buffer | adjust 10 μl |

The reaction was incubated overnight at 25°C. The recombination was stopped by adding 2 μl Proteinase K (Gateway-Kit®) for 10 min at 37°C.

The BP clonase catalyzes the recombination of the PCR Product with the attb-sites of the DONR vector. In between the attb-sites the ccdB Gene is expressed, encoding a toxic protein for most E.coli strains. Thus, after transformation only those E.coli will survive, which contain a plasmid comprising a successful recombination. The resulting plasmid is called Entry plasmid. It contains only the PCR fragment flanked by attb-sites and a kanamycin resistance.
The plasmids were transformed into DH10b cells and spread on a kanamycin agar plate. The Entry plasmids containing the promoter fragment were amplified and purified as described above. Before the following cloning steps, the plasmids were sequenced to assure a correct integration. Sequencing was ordered at Eurofins MWG Operon.

3.1.10.2 Gateway LR reaction

In the next step destination vectors (adapted for Gateway) was used where the promoter fragment was transferred from the pEntry plasmid to the destination vector by the LR-recombination

150 ng pENTRY
150 ng Destination vectors (pCre-GW /pERT2CreERT2-GW)
2 μl LR-Clonase II
ad 10 μl TE buffer

The reaction and transformation was performed as described for the BP reaction. To discriminate between insert carrying entry and destination vectors, the pCre-GW /pERT2CreERT2-GW vectors had an ampicillin resistance, whereas the entry vectors a kanamycin resistance. The recombination of the Entry clone with the destination vector is catalyzed by the LR clonase II. Successfully recombined plasmids are identified by antibiotic selection.

3.1.11 Transformation

3.1.11.1 Electroporation of E.coli DH10B

An aliquot of ice-thawed electrocompetent E.coli DH10B cells (50 μl) and 2μl ligation reaction or 1 μl LR or BP reaction were mixed gently and transferred to Gene Pulser 0.1 cm cuvettes (Bio-rad). A short electromagnetic pulse (1.8 kV) was applied using the Bio-rad micropulser and the electroporated cell suspension was immediately mixed with 450 μl of LB medium and transferred into a 1.5 ml tube which was incubated at 37°C and 350 rpm in a thermomixer (Eppendorf) for 1 hour. 40 –100 μl of the cell suspension was then plated on LB agar plates containing the corresponding antibiotic (33 μg/μl Kanamycin or 100 μg/μl Ampicillin). The plates were incubated overnight at 37°C bacterial incubator.

3.1.11.2 Heat shock-transformation

Chemically competent E.coli One Shot TOP10 cells (50μl) were mixed with 2 μl of LR or BP reaction or 10 μl of the ligation reaction and incubated on ice for 45 min. Heat-shock was applied by placing the samples at 42°C for 90 s and immediately transferring the tubes to ice. The cells were resuspended with 950 μl LB medium, incubated at 37°C in a thermomixer for 1 hour and were plated on LB agar plates containing corresponding antibiotic.
3.1.12 Mini culture and mini DNA purification

A single bacterial colony was picked from an agar plate into 4 ml of LB medium containing appropriate antibiotic. The culture was grown overnight in a shaker at 180 rpm in 37°C. To analyze the clones, a buffer of "Plasmid Maxi Kit" (Qiagen, Hilden) was used. 2 ml of culture was centrifuged for 30 sec at 13200 rpm. The pellet was dissolved in 250 ul of resuspension buffer (P1). RNase contained in P1 buffer degraded the bacterial RNA. After addition of 250 ul of lysis buffer (P2), the samples mixed and incubated for 5 min at room temperature, which led to the lysis of the cells in this alkaline solution. To neutralize the pH then 300 ul neutralization buffer (P3) was added and incubated for 5 min on ice. The sample were centrifuged at 13200 rpm for 10 min at 4°C, the DNA contained in the supernatant was transferred to a new tube and precipitated with 750 ul of isopropanol for 5 minutes at room temperature. The pellet was again washed with 75% ethanol, air-dried and the DNA was resuspended in 20 μl ddH2O.

3.1.13 Maxi/midi culture and purification

100 μl of the Mini-culture was incubated in 100 ml or 200 ml LB medium containing the corresponding antibiotics at 37°C and 180 rpm for 12 –16 hours for Midi- and Maxi-cultures, respectively. Maxi- and Midi- DNA purification kits were purchased from Qiagen (Hilden). The DNA pellets were resuspended in 50 μl and 150 μl ddH2O for Midi- and Maxi- DNA preparations, respectively.

3.1.14 Endofree maxi DNA purification

Endotoxins, also known as lipopolysaccharides (LPS), are cell-membrane components of Gram-negative bacteria such as E.coli. Upon lysis of the bacterial culture, the endotoxins are released from the outer membrane into the lysate. The presence of endotoxin can influence the uptake of plasmid DNA in transfection experiments and can also induce non-specific activation of immune cells such as macrophages and B-cells in animal experiments. Endofree Maxi-DNA purification kit (Qiagen, Hilden) was used to produce endotoxin-free plasmids.

3.1.15 Sequencing of plasmid DNA

2 μg of plasmid DNA was diluted in TE buffer to a final volume of 20 μl and the primers at a concentration of 10 pmol/μl in a volume of 10 μl was sent to MWG Eurofins Operon (Ebersberg) for sequencing. The sequencing results were analyzed using the SDSC Biology workbench software (http://workbench.sdsc.edu) which is available online.
3.1.16 Software

Insilico handling of DNA sequences for the cloning experiments were performed using the softwares - Gene Construction Kit (Textco, New Hampshire, USA) and MacVector 12.0.6 (Cambridge, UK).

3.2 Cell culture methods

3.2.1 Isolation of neonatal rat cardiomyocytes (NRCM) and fibroblast (NRCF)

Neonatal rat cardiomyocytes (NRCM) were obtained from 1-2 day old Sprague-Dawley rats isolated by enzymatic digestion. Whole hearts were excised and were transferred into Ca\textsuperscript{2+} and bicarbonate-free HEPES-buffered Hanks’ medium (HBSS). After removing the atria, the hearts were cut into pieces and digested with an enzyme solution containing trypsin (#215240, Becton Dickinson and PAN), and DNase under constant stirring. The suspension was collected over fixed intervals of time into tubes containing 9 ml FBS. The primary cells that were collected after passing through a 40μm cell strainer were seeded in uncoated plastic dishes for 1 hour at 37°C / 1% CO\textsubscript{2}. During the preplating time, the more rapidly adherent fibroblasts attach to the surface. The supernatant containing the cardiomyocytes was collected and either centrifuged at 900rpm for 1min to freeze the pellet at -80°C for RNA isolation or cultured in MEM containing vitamin B12, NaHCO\textsubscript{3}, BrdU and 1% FCS. The plastic dishes containing the attached cardiac fibroblasts (NRCF) were washed with PBS gently and cells were either frozen at 80°C for RNA isolation or cultured in MEM containing vitamin B12, NaHCO\textsubscript{3} and 1% FCS. A trained staff carried out the cardiomyocytes isolation from neonatal rats in a sterile condition.

3.2.2 Isolation of adult mouse cardiomyocytes (AMCM) and Fibroblast (AMCF)

Isolation of adult mouse cardiomyocytes and cardiac fibroblast was performed using a Langendorff perfusion apparatus as previously described (O'Connell TD et. al). Briefly, mice were anesthetized and the heart was rapidly excised from the thoracic cavity, cannulated via the aorta, and perfused in the Langendorff mode with calcium-free perfusion buffer for 3 minutes at a rate of 4 mL/min, followed by digestion buffer (perfusion buffer plus 25μmol/L CaCl\textsubscript{2} and Collagenase (Worthington Biochemical) at a concentration of 280 units/mg) for 10 to 11 minutes. Following digestion, atria is removed and the heart were cut into small pieces with curved fine tip forceps in 2.5 mL of digestion buffer and 2.5ml of Stop 1 buffer (10%
Materials and Methods

fetal bovine serum (FBS) in perfusion buffer plus 25μmol/L CaCl₂) in a small beaker. The suspension was filtered with 100μm nylon mesh filter into a 15 mL falcon tube (Pellet 1) and allowed to settle by gravity for 10 min at 37°C. The supernatant was transferred into another 15mL tube and marked as Cardiac fibroblast (AMCF) whereas the settled pellet (i.e. AMCM) was mixed in 10 mL of Stop 2 buffer (5% FBS in perfusion buffer plus 50μmol/L CaCl₂) and transferred to a small conical flask. A final calcium level of 1mmol/L was reintroduced through a series of resuspensions containing increasing concentrations of CaCl₂.

Cardiomyocytes were either centrifuged at 900rpm for 1min to freeze the pellet at -80°C for RNA isolation or plated on laminin coated (10 μg/mL) coverslips in CM plating media for 1 h at 37°C, 5% CO₂ for immunofluorescence. For cardiac fibroblast isolation the tube containing supernatant (labeled as AMCF above) was centrifuged at 1200rpm for 5mi and pellet was resuspended in AMCF plating medium with 5%FCS for 2h at 37°C, 1% CO₂. Plated AMCF were then washed with 1x PBS and either frozen at -80°C for RNA isolation or cultured in medium containing 10%FCS for immunofluorescence experiments.

3.2.3 Cultivation of NIH-3T3 mouse fibroblasts

NIH-3T3 mouse cells were cultured in DMEM medium containing 10% fetal bovine serum, 1% L-Glutamine and 1% Penicillin (10000U/ml)/Streptomycin (100mg/ml) in a humidified 37°C/5% CO₂ incubator. The cells grew as an adherent monolayer in culture dishes (Nunc, Langenselbold) and have a doubling time of about 24 hours. The cells were passaged to a maximal 30 times, after which new aliquots are thawed from the liquid nitrogen storage. The cells were split every 4 days. For splitting, the culture medium was aspirated from the culture dishes and the cells were washed once with DPBS (Dulbecco’s phosphate buffered saline). The cells were trypsinized using trypsin-EDTA solution (0.5 g/l trypsin, 0.2 g/l EDTA) and centrifuged for 5 minutes (1000 rpm). The supernatant was discarded and the cell pellet was re-suspended in an appropriate volume of fresh culture media for cell counting by Trypan Blue. The cells were then seeded in new culture dishes or 96 well plates.

3.2.4 Transfection of NIH-3T3 cells with promoter vectors

Transfection was carried out with Lipofectamine™2000 (Invitrogen, Karlsruhe) as per the manufacturer’s guidelines. These are specially designed cationic lipids which complexes with negatively charged nucleic acids to form liposomes in aqueous conditions. The liposomes carrying a positive charge on its surface can then fuse with the negatively charged plasma membrane thereby facilitating delivery of the nucleic acids into the cell.
3.2.5 Generation of stable cell lines

NIH-3T3 cells were grown in 10cm dishes and were transfected with pCALNL-GFP plasmids DNA using Lipofectamine (3.2.4). The expression plasmid pCALNL-GFP containing the neomycin resistance gene allows selection of cells expressing the introduced plasmid DNA stably integrated into its genome, using geneticin (G-418, Invitrogen, Karlsruhe, Germany). For this purpose, starting from 48h after transfection daily fresh medium with 0.8g/l geneticin added to the cell for two weeks. The further cultivation was carried out in medium containing 0.2-0.4 g/l geneticin.

pCALNL-GFP is a Cre recombinase-dependent expression of GFP, i.e. GFP is only expressed in the presence of Cre due to a transcriptional stop in between the loxP sites.

3.3 Methods for RNA analysis

3.3.1 Isolation of RNA

3.3.1.1 peqGOLD Trifast™

RNA from tissues was isolated exclusively with TriFast (Peqlab, Erlangen, Germany). Tissues (50-100 mg) were homogenized in 1 ml of peqGOLD TrifastTM using a turrax. The samples were then incubated at RT for 5 min. 200μl of chloroform was added to each sample, vortexed for 50 sec, and were incubated at RT for 10 min. After centrifugation at 12000 x g for 10 min at 4°C, the mixtures separated into three phases –upper aqueous phase (RNA), Interphase (DNA) and lower organic phase (protein). The upper phase containing the RNA was then transferred into a fresh 1.5 ml tube. 500 μl of isopropanol was added to the samples to precipitate the RNA. The samples were kept on ice for 10 min and then centrifuged at 12000 x g for 10 min at 4°C. The supernatant was discarded carefully and the RNA pellet was washed twice with 1 ml of 75% ethanol by vortexing gently and subsequent centrifugation at 12000 x g for 10 min at 4°C. After discarding the supernatant, the RNA pellet was air-dried to remove excess isopropanol.

3.3.1.2 MirVana Kit

RNA from cardiac cells was isolated for the mirVana kit (Applied Biosystems, (Darmstadt)) according to the manufacturer. It is a column-based technique to extract total RNA (including small RNAs). In both above isolation protocol the air-dried RNA pellet was taken up ultimately in 20μl of pre-warmed (55°C) nuclease-free water. The purity and concentration of the isolated RNA were determined using the NanoDrop spectrophotometer ND 1000 (Peqlab, Erlangen, Germany).
3.3.2 Reverse transcription

In the production of the complementary strand (cDNA), the fact that makes use of the pre-mRNAs is polyadenylated on its 3' end. After binding of the oligo (dT) primers (MWG Biotech, Ebersberg, Germany), in this so-called poly (A) tail, the reverse transcriptase synthesizes the complementary strand (cDNA) complementary to the mRNA sequence. The cDNA can be used for gene expression studies using quantitative real-time PCR (qRT-PCR) as a template then. To ensure detection of genomic DNA in the subsequent qRT-PCR, an approach with pooled RNA (four RNA samples from isolation) without reverse transcriptase (as a negative control for reverse transcription reaction) was always treated in parallel with the rest of the samples according to the protocol.

**Reaction mix**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>0.5 µg</td>
</tr>
<tr>
<td>Oligo (dT) Primer (10 mM)</td>
<td>2 µl</td>
</tr>
<tr>
<td>RNase free H2O</td>
<td>ad 11.9 µl</td>
</tr>
<tr>
<td><strong>incubation for 10 min at 70°C</strong></td>
<td>4 µl</td>
</tr>
<tr>
<td>5x First Strand Buffer</td>
<td></td>
</tr>
<tr>
<td>DTT (0.1 M)</td>
<td>2 µl</td>
</tr>
<tr>
<td>dNTPs (1 mM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNase Out/RNA Inhibitor</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>SupercriptIIReverse</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

The entire mixture was incubated for 1 h min at 42 °C and then heat inactivated (10 min, 70 °C). At the end for a final volume of 50 µl for 30 µl of water were added.

3.3.3 Quantitative real time PCR

The quantitative real-time PCR based on the conventional polymerase chain reaction, PCR that allows quantification of amplification. This is enabled by the detection of fluorescence signals (using SYBR green), which increase in proportion to the amount of PCR product. SYBR green is a non-specific fluorescent dye, which intercalates into the double-stranded DNA. The dye only fluoresces when bound with DNA. With each cycle of amplification, the emitted fluorescence intensity of the SYBR green increases as compared to the reference
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fluorophore 8-ROX (8-carboxy-X-rhodamine). The number of cycles at which the fluorescence intensity exceeds the threshold is called the cycle threshold (Ct). To quantify the gene expression for RNA of interest, it is normally normalized to the gene expression of a housekeeping gene such as GAPDH (ΔΔCt-method). This normalizes the variation in the amount and the quality of RNA between different samples. However, the expression of the reference gene needs to be similar between the samples. The real-time PCR was carried out in StepOne Plus instrument (Applied Biosystems, New Jersey) and the reagents used were from Invitrogen (Karlsruhe).

Reaction mix

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>1.25 μl</td>
</tr>
<tr>
<td>50 mM MgCl2</td>
<td>0.375 μl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.25 μl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.25 μl</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.2 μl</td>
</tr>
<tr>
<td>1X ROX</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>5X SYBR Green</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>‘Platinum’ Taq polymerase</td>
<td>0.05 μl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>6.625 μl</td>
</tr>
<tr>
<td>cDNA (2.5 ng/μl)</td>
<td>2.5 μl</td>
</tr>
</tbody>
</table>

Temperature program (StepOne Plus, Applied Biosystems, New Jersey, USA.)

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-denaturation</td>
<td>94 °C</td>
<td>120 sec</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>20 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>56 °C</td>
<td>20 sec</td>
<td></td>
</tr>
<tr>
<td>Elongation</td>
<td>65 °C</td>
<td>35 sec</td>
<td></td>
</tr>
<tr>
<td>Final Elongation</td>
<td>65 °C</td>
<td>1 min 30 sec</td>
<td></td>
</tr>
<tr>
<td>Dissociation</td>
<td>16 °C</td>
<td>∞</td>
<td>X 40 Cycle</td>
</tr>
</tbody>
</table>
3.4 Methods for protein analysis

3.4.1 Preparation of protein lysates
Tissues (50-100 mg) were homogenized in 800 μl of protein lysis buffer using a turrax. In the case of adherent cells such as NRCM and NRCF, the stimulated cells were washed with PBS and 250 μl of cold protein lysis buffer was added to the plates. The cells were scraped using a cell scraper and the lysates were transferred into a 1.5 ml tube. The cell lysates were then sonicated. The lysates were then incubated with 1/10th volume of 5% (v/v) Benzonase at RT for 10 min. Benzonase is a genetically engineered endonuclease which degrades all forms of nucleic acids (DNA, RNA). The tubes were then placed in an ultrasonic bath at 4°C for 5 min. The samples were then centrifuged at 12000 rpm for 20 min at 4°C to clear the lysates and were stored at -80°C until use.

3.4.2 BCA protein quantification
The concentration of the protein lysates was determined by the Bicinchoninic acid colorimetric assay using the BCA protein assay kit (Thermo Scientific, Rockford USA). The peptide bonds in the protein reduce the divalent copper ions to monovalent ions, which then chelates with the bicinchoninic acid to form a purple coloured complex with maximum absorption at 562 nm. The absorbance of the samples at 562 nm was measured using an Infinite 200 spectrophotometer (Tecan, Männedorf). The protein concentration was then evaluated with reference to internal calibration standards such as bovine serum albumin.

3.4.3 Western blot
Depending on the size of the detected proteins, 8 – 12% polyacrylamide gels were used.

<table>
<thead>
<tr>
<th></th>
<th>Stacking gel</th>
<th>Running gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/Bisacrylamide 30%/0.8% (v/v)</td>
<td>0.5 ml</td>
<td>4ml</td>
</tr>
<tr>
<td>Lower buffer (4X)</td>
<td>-</td>
<td>3.8ml</td>
</tr>
<tr>
<td>Upper buffer (4X)</td>
<td>1.25ml</td>
<td>-</td>
</tr>
<tr>
<td>H2O</td>
<td>3.2</td>
<td>4.7 ml</td>
</tr>
<tr>
<td>Glycerol (80%)</td>
<td>-</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>6 μl</td>
<td>12 μl</td>
</tr>
<tr>
<td>APS (10%)</td>
<td>48 μl</td>
<td>72 μl</td>
</tr>
</tbody>
</table>
The gels were casted on Mini-PROTEAN casting stand (Biorad, München). The samples were prepared by boiling at 95°C for 5 min. The gel electrophoresis chamber was filled with 1X running buffer, samples were loaded into the wells and 30 mA current per gel was applied at maximum voltage. Under denaturing conditions, the proteins are separated based on their molecular weight. The proteins were then transferred onto a PVDF membrane (Millipore, Billerica USA) using wet transfer (Mini-PROTEAN transfer system, Biorad, München). The PVDF membrane was cut to the size of the mini-gel and was activated using methanol. The membrane was placed on the cassette facing the anode and the gel was placed facing the cathode side. The membrane and the gel were sandwiched between 1-2 layers of filter paper. The transfer was carried out at 350 mA current at the maximum voltage for 90 min. The membrane was blocked with 10% non-fat milk for 1 hour at RT on a horizontal shaker and then incubated with the primary antibody for overnight at 4°C. The following antibodies diluted in 5% milk block buffer were used: anti-Cre (Millipore, 1:1000), anti-Beta-gal (Abcam, # ab616), anti-HSP90. The membrane was washed thrice with 1X PBST for 10 min at RT. The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies (1:10000) at RT for 90 min. The membranes were again washed with the PBST buffer. The proteins were detected by chemiluminescence. ECL Plus (GE Healthcare, München) was applied to the membrane according to the manufacturer’s instructions. The signal was visualized using a Fujifilm LASmini4000 instrument (Fujifilm, Düsseldorf). The blots were then analysed using the Multigauge software (Fujifilm, Düsseldorf).

3.5 Immunofluorescence

3.5.1 In tissues

Heart tissues were processed in two ways:

(A) **4% PFA perfused hearts tissues for visualizing mTomato and mGFP direct fluorescence**- Hearts tissue was isolated from anesthetized mice perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer saline (PBS), fixed for 2 hours in 4% PFA at 4°C, followed by overnight incubation in 30% sucrose, and embedded in OCT (Tissue Tek). 5mm sections were cut. The slides were shortly rinsed with PBS and mount with Vectashield fluorescence mounting medium containing DAPI and imaged at a confocal microscope for mTomato and mGFP direct fluorescence.

The PFA perfused heart sections of 7 days old and 6 weeks old mice were also used
for single staining with a different antibody (mentioned below) in case of vimentin promoter study after maternal injection.

(B) **Sucrose section for immunostaining**- Heart tissues was dissected and cryoprotected in 30% sucrose overnight at 4 degrees, followed by embedding in OCT. The cryosections (5mm) were fixed either with 4% PFA or cold methanol, followed by permeabilization (only in case of PFA fixation) with 0.2% Triton X-100 or 0.05% PBST and blocking with 10% Goat serum or BSA for 1 h at room temperature. The sections were then incubated with either with primary antibody against GFP (Abcam 1:1000) alone or with cocktail of antibody against vimentin (Abcam, 1:200), CD31 (1:200, Pharmingen, San Diego, CA), SM22α (Abcam 1:200), P4HB (1:50, Proteintech) and ACTN (α-Actinin) (Sigma, 1:600) for overnight at 4 °C followed by secondary Alexa Fluor 647 goat anti-rabbit, goat anti-rat, goat anti-mouse, donkey anti-goat or Alexa Fluor 488 goat anti-chicken (Invitrogen, Carlsbad, CA). (1:200) for 1h at RT. Confocal microscopy (a Zeiss LSM 510 system and Leica SP5) was performed using a 40X oil immersion lens.

Brief information about the double antibody staining (co-staining) protocol used for the study is given in below in tabular format

<table>
<thead>
<tr>
<th>Co-staining</th>
<th>Fixation</th>
<th>Blocking</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin and GFP</td>
<td>4% PFA for 10min at RT, 5-10min permeabilization with 0.2% triton X100 in PBS for at RT</td>
<td>10% goat serum</td>
<td>Cocktail o/n at 4°C of vimentin (1:200) and GFP (1:1000)</td>
<td>Cocktail for 1h at RT Alexa Fluor® 647 Goat Anti-chicken IgG (H+L) (1:200) Alexa Fluor® 488 Goat Anti-rabbit IgG (H+L) (1:500)</td>
</tr>
<tr>
<td>P4HB and GFP</td>
<td>Methanol (cold) for 10min at -20°C. Washing with 0.05% PBST reduces the background</td>
<td>10% goat serum</td>
<td>Cocktail o/n at 4°C P4HB (1:50) GFP (1:1000)</td>
<td>Cocktail for 1h at RT Alexa 647 Anti –Rabbit (1:200) &amp; Alexa 488 anti-chicken (1:500)</td>
</tr>
<tr>
<td>CD31 and GFP</td>
<td>4% PFA for 10min at RT, 5-10min permeabilization with 0.2% triton X100 in PBS at RT</td>
<td>10% goat serum</td>
<td>Cocktail o/n at 4°C CD31 (1:100) GFP (1:1000)</td>
<td>Cocktail for 1 h at RT of Alexa 647 Anti–Rat (1:200) &amp; Alexa 488 anti-chicken (1:500)</td>
</tr>
<tr>
<td>SM22α and GFP</td>
<td>4% PFA for 10min at RT, 5-10min</td>
<td>10% BSA</td>
<td>1step: SM22alpha (goat) (1:100)</td>
<td>Cocktail for 1h at RT Biotinylated IgG (anti-goat) (1:200) &amp; Alexa 488 anti-chicken (1:500)</td>
</tr>
</tbody>
</table>
permeabilization with 0.2% triton X100 in PBS at RT | o/n at 4°C | chicken (1:500) Followed by additional incubation (30min) with Streptavidin Alexa-647 conjugate (1:300) for SM22a at 37°C.

2step: GFP Rabbit (1:600) at 37 for 2hrs | Followed by additional incubation (30min) with Streptavidin Alexa-647 conjugate (1:300) for SM22a at 37°C.

| 3.5.2 In isolated cells |

For visualizing mTomato and mGFP signals the isolated cardiomyocytes (CM) were plated on laminin coated coverslip for 1hrs and the cardiac fibroblasts (CF) for 48hrs on normal coverslips were fixed with 4% paraformaldehyde for 10 min, washed shortly with PBS and mount with 50% glycerol containing DAPI. However, for co-staining the CF were fixed with 4%PFA, permeabilized with 0.2% Triton X-100 for 5 min and then incubated with primary antibodies as used above against GFP, Vimentin, P4HB, CD31 and α-actinin for 1 h at 37°C incubator. Following three washes in PBS, coverslips were incubated with secondary antibodies conjugated to Alexa Fluor dyes as above (Invitrogen; 1:200) for 35 min at 37°C. The coverslips were then washed three times in PBS, mounted onto glass slides using mounting medium containing Dapi and subjected to automated fluorescent microscopy (20X or 10X).

| 3.6 Staining |

3.6.1 Detection of β-Galactosidase activity (X-gal staining)

For Xgal staining of cryosections, whole hearts were harvested, cryoprotected in 30% sucrose overnight at 4°C and were embedded in Tissue-Tek OCT. Cryosections (5mm) were prepared and stained for β−galactosidase activity. Prior to staining, sections were fixed in cold PBS containing 0.2% glutaraldehyde, 5mM EGTA (pH 7.3) for 10 min. Sections were washed three times for 5 min in X-gal wash buffer (2mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 (NP-40) in PBS) and then stained in Xgal staining solution (1 mg/ml Xgal, 5mM potassium ferrocyanide, and 5mM potassium ferricyanide in washing buffer) at 37°C for 3-4hours. Sections were rinsed in PBS, counterstained with eosin, moved through a graded ethanol series for dehydration, incubated in toluene for 3min and then mounted with DePEX. X-gal staining was used to check whether a cell expresses the β-galactosidase enzyme, which is encoded by the lacZ gene. β-galactosidase cleaved X-gal by yielding galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter is then oxidized into 5,5′-dibromo-4,4′-dichloro-indigo, which is an insoluble blue product. X-gal itself is colorless, the presence of blue-colored product therefore used as a test for the
presence of an active β-galactosidase (or LacZ gene).

### 3.6.2 Fast green/Sirius red staining for cryosections

This method was used for measurement of collagen contents in heart tissues. The total amount of collagen and non-collagenous proteins in tissue sections is determined by differential staining with two dyes, sirius red and fast green. Sirius Red binds to all types of collagen, whereas fast green stains non-collagenous proteins. For this, the sections were incubated in preheated Bouin’s Solution at 58°C for 1 hour, washed in running tap water and stained with fast green dye for 20 min and Sirius red solution for 30 min, RT and then washed briefly in ddH2O (10 sec). The sections were subjected to an ascending ethanol series (70%: 1 min, 100%: 1 min), incubated in toluene (3 min), covered with DEPEX and dried overnight at RT. The quantification was performed using the MetaMorph Basic Imaging software package (Molecular Devices, Downingtown, USA).

### 3.7 Microscopy

#### 3.7.1 Confocal microscopy

Confocal images were taken using two confocal Microscope—(A) Zeiss LSM 510 META where, Alexa-488, mTomato, Alexa 647 and DAPI were excited at 488nm, 543nm, 633nm and 364nm laser lines, respectively and 40X oil immersion objective was used. (B) Leica SP5 confocal microscope where, mGFP/Alexa 488, mTomato, Alexa 647 and DAPI were excited at 488nm, 561nm, 633nm and 405nm laser lines and 63X glycerin objective was used. The images were processed using Adobe Photoshop software.

#### 3.7.2 Automated fluorescent microscopy

Images were acquired automatically using a 10X or 20X objective on an AxioObserver.Z1 (Zeiss, Jena), a motorized scanning stage (Märzhäuser, Wetzlar), Lumen200 fluorescence illumination system (Prior, Cambridge UK) and Retiga4000 CCD fluorescence camera (QImaging, Surrey Canada). Metamorph imaging software (Molecular Devices, Downingtown USA) was used to drive the microscope automatically and also for subsequent analysis of the acquired images.

### 3.8 Methods for animal experiments

#### 3.8.1 Generation of a Ccdc80-Cre mouse line

The Ccdc80-Cre targeting vector was prepared amplifying promoter sequence (4.2kb) from BAC clone (Gene Service) that was cloned in Cre expressing Vector through Gateway
Recombination Technique (Invitrogen). Ccdc80-Cre vector was first amplified in bacteria and purified with the Endotoxin free Maxi Kit (Qiagen) according to the manufacturer’s instructions. 50-100 ug of the vector were then linearized with the Aval restriction enzyme (NEB) incubation (Section 3.1.6). Linearized vector was sent to the Institute of Laboratory Animal Science, the University of Zurich for generation of transgenic mice through pronuclear injection.

**Establishing Ccdc80-Cre founder lines:** The transgenic technology facility returned forty pups’ ear punches from eleven foster mothers for screening of transgene. 13 of 40 pups were positive for the Cre gene. Genotype diagnostics were carried out using PCR amplification followed by gel electrophoresis with DNA isolated from ear punches. Four chosen founders were returned from the transgenic technology facility that was then crossed with the R26R-lacZ reporter line to histologically determine the expression of promoter activity. One out of those four founders was able to show active promoter activity driving cre expression through LacZ staining in heart and other tissues.

**3.8.2 Isolation of genomic DNA**

Genomic DNA was isolated from tail biopsies incubated overnight at 55°C and 1100 rpm in 500 μl of DNA lysis buffer (for genotyping) containing 2.5μl of ‘Fermentas’ Proteinase K (20mg/ml). To the tubes, 500 μl of ‘Roth’ phenol-chloroform was added and were centrifuged at 14000 rpm for 10 min. The upper phase of the cleared lysates was then transferred into fresh 1.5 ml microfuge tubes. The DNA was precipitated by addition of 200 μl of isopropanol and subsequent centrifugation at 14000 rpm at 4°C for 10 min. The supernatant was discarded and the pellet was washed with 500 μl of 70% ethanol. After centrifugation at 14000 rpm at 4°C for 5 min, the pellets were air-dried and resuspended in 20 μl ddH₂O.

**3.8.3 Genotyping PCR**

The PCR reaction was set up as follows:

1. **Rosa (R26R) PCR**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genescript Taq Buffer (10X)</td>
<td>2μl</td>
</tr>
<tr>
<td>RF126 (25pmol/μl)</td>
<td>0.3μl</td>
</tr>
<tr>
<td>RF127 (25pmol/μl)</td>
<td>0.3μl</td>
</tr>
<tr>
<td>RF128 (25pmol/μl)</td>
<td>0.3μl</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>0.4μl</td>
</tr>
</tbody>
</table>
Materials and Methods

Taq Polymerase 0.1µl
H2O 15.6µl
gDNA 1µl

2. Cre Gene

<table>
<thead>
<tr>
<th>Substance</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genescript Taq Buffer (10X)</td>
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</tr>
<tr>
<td>Sense Cre (20pmol/µl)</td>
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</tr>
<tr>
<td>Antisense Cre (20pmol/µl)</td>
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</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>0.4µl</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.2µl</td>
</tr>
<tr>
<td>H2O</td>
<td>15.5µl</td>
</tr>
<tr>
<td>gDNA (50ng/µl)</td>
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</tbody>
</table>

3. mTom-mGFP Gene

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<tr>
<td>oIMR 7318 (20pmol/µl)</td>
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</tr>
<tr>
<td>oIMR 7319 (20pmol/µl)</td>
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</tr>
<tr>
<td>oIMR 7320 (20pmol/µl)</td>
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</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>0.4µl</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.1µl</td>
</tr>
<tr>
<td>H2O</td>
<td>11µl</td>
</tr>
<tr>
<td>gDNA (50ng/µl)</td>
<td>1µl</td>
</tr>
</tbody>
</table>

The subsequent amplification reaction was performed in a thermocycler (Mastercycler pro, Eppendorf, Hamburg, Germany) with the following temperature program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>Before denaturation</td>
<td>94°C</td>
<td>120-300s</td>
</tr>
<tr>
<td>Stage 2</td>
<td>Denaturation</td>
<td>94°C</td>
<td>30 s</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Hybridization</td>
<td>Depending on primer sequences</td>
<td>30-35x</td>
</tr>
</tbody>
</table>
Materials and Methods

<table>
<thead>
<tr>
<th>Stage 4</th>
<th>Elongation</th>
<th>Depending on the template length (1 min / 1000 nucleotide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 5</td>
<td>Final elongation</td>
<td>72°C 180-300 s 1x</td>
</tr>
</tbody>
</table>

3.8.4 Animal models

3.8.4.1 Reporter Mice used for study

Two reporter mouse models are used for the study.

(A) Rosa26-lacZ reporter mice (Soriano, 1999): The R26R 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 (stock numbers 003309 and 003310). Rosa transgenic mice contain a lacZ reporter gene that is transcriptionally silenced by a floxed stop sequence immediately upstream. Upon expression of Cre-recombinase in target tissues, the stop sequence is cleaved, and the beta-galactosidase reporter is transcribed.

(B) mTomato-mGFP (mT/mG) dual fluorescent reporter mice (Muzumdar, 2007): ROSA mT/mG is a cell membrane-targeted, two-color fluorescent Cre reporter allele; expressing cell membrane-localized red fluorescence in widespread cells/tissues prior to Cre recombinase exposure, and cell membrane-localized green fluorescence in Cre recombinase expressing cells (and future cell lineages derived from these cells). It is also available at Jackson Laboratory (stock number 007576).

3.8.4.2 VimCreERT2 transgenic mice

VimCreERT2 transgenic mice were generated using BAC recombination in Dr Robert F. Schwabe Laboratory, New York. These transgenic mice express tamoxifen inducible Cre recombinase under the control of mouse vimentin promoter. The mice received as a gift from Dr Schwabe and Dr Micheal Quante (Technical University in Munich) for the study.

3.8.4.3 Fsp1-Cre (or S100A4-Cre)

These transgenic mice express Cre recombinase under the control of the mouse S100 calcium binding protein A4 (S100A4) promoter. Cre recombinase expression is detected specifically in stromal fibroblasts of tissues such as the prostate, forestomach and mammary gland. Mice that are homozygous for the transgene are viable, normal in size and do not display any gross physical or behavioral abnormalities.

3.9 Methods for cardiovascular phenotyping of mice

The following methods were carried out by trained personal.
Materials and Methods

3.9.1 Echocardiography

To be tested, mice were anesthetized by inhalation anesthesia (a mixture of 2 % isoflurane and 98 % oxygen) and were fixed on a hot plate (40 °C). For ultrasound examination of the Vevo 700 (Visual Sonics, Ontario, CAN) was used. After shaving off the chest, the ultrasound probe was parasternal placed on them and the left ventricle in the long axis (B-mode) optimally aligned for measurement. For the recording of the echocardiogram in the short axis (M-mode) of the transducer was rotated 90°C. Following parameters in diastole (d) and systole (s) were read from the echocardiogram: interventricular septum thickness (IVS), left ventricular internal diameter (LVID) and left ventricular posterior wall (LVPW). Other parameters (fractional shortening (FS), ejection fraction (EF)) were quantified with the corresponding formulas.

3.9.2 Transverse aortic constriction (TAC)

TAC was performed as described previously (Rockman HA, et al). Briefly, mice (8 weeks old) were sedated by 2% isoflurane and 600 mL/min O₂. The animals were then placed in a supine position, an endotracheal tube was inserted and the chest cavity was exposed by cutting open the proximal portion of the sternum. After the aortic arch between the innominate and left common carotid arteries was isolated, it was constricted with a 6-0 silk suture tied firmly 2 times against a 27-gauge blunted needle. Sham-operated mice underwent the identical surgical procedure, including isolation of the aorta, but without placement of the suture. Animals were anesthetized and euthanized 21 days after TAC for histological studies.

In case of vimentin promoter study six weeks old Vim-CreER;mTom/mGFP⁶/⁺ mice were treated with vehicle and tamoxifen (2mg/mouse/day) i.p. for 6 consecutive days prior to TAC.

3.9.3 Statistics

Average data are presented as mean±SEM. Statistical analysis was performed using the Prism software package (Graph Pad, San Diego, U.S.A.). To compare two groups, unpaired student t-test was performed. Statistical significance was evaluated using one-way ANOVA followed by Bonferroni test or 2-way ANOVA/ Bonferroni posttest. Differences were considered significant if P< 0.05 and were represented P<0.001 (***) or P<0.01 (**) or P<0.05 (*).
4 RESULTS

4.1 Generation and characterisation of a Ccdc80-Cre transgenic mouse line

4.1.1 Identification of cardiac fibroblast specific gene and validation of candidates

Figure 4.1 Screening of cardiac fibroblast-specific genes using mouse microarray database. Scatter plot illustrating the expression of 1371 genes enriched in CFs as compared to heart (>6 fold) and CM (>16 fold). The selected 9 candidate genes (marked in red) were then used for further validation.

In order to search for cardiac fibroblast-specific genes, a microarray database was generated, which includes gene expression data from CFs & CM of adult mouse and neonatal rat, CFs of β1-AR transgenic mice and fibroblast from lung, liver, skin, kidney of neonatal rats. β1-transgenic mice were generated by overexpression of β1-adrenergic receptor under the control of heart-specific α-MHC promoter, and was used as the heart
failure models in the study. From this database, 1371 genes that are strongly expressed in mouse CFs were pre-selected. Then the expression of these pre-selected genes in CFs were compared to their expression in the whole heart (>6 fold) & in CM (>16 fold) where 275 genes were left out (Figure 4.1).

To further assess the expression of these 275 genes in different tissues, another published microarray mouse data of Henrichsen et al. (2009) was included in the microarray database. The microarray data of Henrichsen et al. analyzed the transcriptome in different tissues of the mouse. To constrain the search for the gene, the 275 genes were related to the Henrichsen et al., mouse data where genes with a preferential cardiac expression as compared to other tissues were searched out. This analysis resulted in 47 genes (as mentioned in 8.1 in appendix section). Finally the cardiac expression of these 47 genes was confirmed using publically available databases GENECARD that results in leaving only nine candidate genes (as shown in Figure 4.1).

In order to determine the expression of nine candidate genes in different tissues, total RNA was isolated from six different tissues (lung, liver, kidney, heart, brain, skeletal muscle) of 3 months old FVB/N mice. Reverse transcription (using Invitrogen RT-Kit) and real-time PCR analysis were carried out. Here only four out of nine genes showed a high level of expression in the heart as compared to other tissues as shown in Figure 4.2 A.

Several scientific groups reported increased expression of periostin and gelsolin in a diseased heart. Further investigation was done for the expression of these four genes in disease condition in the heart. For this total RNA was isolated from non-failing hearts, (here a wild-type mouse is a model for a non-failing heart) and failing hearts (β1-adrenergic receptor transgenic mice as a heart failure model). Reverse transcription and real-time PCR was carried out. Ccdc80 gene showed a significant up-regulation (around two-fold) in failing heart as compared to non-failing heart while Rabgap1l showed a slight increase in failing heart that was non-significant (Figure 4.2 B). Similar to previous findings, periostin and gelsolin expression levels were up-regulated in failing heart (Figure 4.2 B). In order to further validate the cell-specific expression of these candidate genes, real-time PCR was performed on RNAs isolated from CM and CFs of neonatal rat (NR) and of an adult mouse(AM). In the neonatal rat, Ccdc80 had two-fold expression and periostin three-fold expression in CFs compared to CM while gelsolin was found to be expressed in CM. There was no expression detected for Rabgap1l gene in any of the two cell type (Figure 4.2C).
Similarly significantly increased expression of Ccdc80 and perlostin gene was observed in AMCF compared to AMCM. However, we also observed an increased
expression of gelsolin this time in AMCF instead of AMCM but this increase was not significant (Figure 4.2 C).

4.1.2 Cloning and validation of candidate promoters \textit{in vitro}

Putative promoter sequences (5-7Kb upstream) of two respective candidates genes Ccdc80 (4.2Kb) and Periostin (3.9Kb) were amplified from BAC clones (mentioned in Materials section) using PCR.

Figure 4.3 BAC clone (bMQ193n13) having genomic region (upstream and downstream) of Ccdc80 gene at Chromosome16: 45,076,911–45,145,018 (as represented by blue line), was used for amplifying Ccdc80 promoter sequence (5000bases upstream) (Ref. www.ensembl.org.)

The BAC clones used for amplification of promoter sequences were ordered from Mouse bMQ BAC library as displayed on the Ensemble genome browser within a DAS (Distributed
Annotation Server) for desired genomic region. Figure 4.3 showed a snapshot of ensemble browser showing the BAC clone for Ccdc80.

In order to verify that these promoters have indeed a CFs-specific expression, we used a LacZ reporter approach first. Moreover, as our main goal was to be able to specifically target genes in the cardiac fibroblast in vivo, promoter constructs to drive Cre-recombinase were also generated.

4.1.2.1 Cloning and analysis of candidate promoter activity using a LacZ reporter vector

The well-characterized bacterial E.coli lacZ gene, which encodes b-galactosidase (b-gal), has become a standard tool for following localized transgene expression in many organisms, including mammalian cells and transgenic animals. Detection of the encoded β-galactosidase activity is achieved either by direct enzymatic assay using fluorogenic or chromogenic substrates or by visualization in situ using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Because X-gal forms an insoluble blue precipitate upon hydrolysis by β-galactosidase, it allows in situ detection of lacZ transgene expressing cells in vitro and facilitates spatial determination of reporter gene expression in transgenic animals in vivo.

In order to analyze the amplified promoter sequences for its transcriptional activity in vitro, the respective promoter sequences (Cccdc80 (4.2Kb) & Periostin (3.9Kb)) were cloned into the promoterless-LacZ vector (Clontech, GenBank Accession No.U13184) (Figure 4.4 A&B). To assess the expression of LacZ reporter gene under the control of these respective promoters, standardization of β-Galactosidase (β-Gal) staining protocol was done using a β-Gal control vector (Ambion) as a positive control. This vector consists of CMV promoter that drives the LacZ gene expression. The promoter activities were then analyzed in vitro by transfecting fibroblast cell line (NIH-3T3 cells) and by performing a β-Gal staining 24 hours post-transfection. As shown in Figure 4.4 C, cells stained in blue were observed after transfection with the CMV-LacZ control vector (β-Gal control vector), and also with Cccdc80-LacZ and Periostin-LacZ (Postn-LacZ) construct. Thereby, demonstrating the efficient activity of both promoter sequences in vitro. However, low transfection efficiency was observed with both the promoter-LacZ reporter constructs, but this may be due to their larger sequence sizes.

Similarly, electroporation of neonatal rat cardiomyocytes (CM) and cardiac fibroblast (CFs) with these promoter-LacZ reporter constructs (Cccdc80-LacZ & Postn-LacZ) was done in order to analyze the preferential CFs specificity in vitro. There also very low transfection
efficiency observed (data not shown).

Figure 4.4 Analysis of amplified promoter sequences for transcriptional activity in vitro. (A) PCR Amplified Ccdc80 (4.2Kb), Gelsolin (Gsn) and persiotin (3.9Kb) promoter sequence on 0.7% agarose gel. (B) Schematic diagram showing cloning of amplified Ccdc80 promoter sequence having restriction site (Xmal & Nhel) and periostin promoter sequence (Xmal & Xhol) into the multiple cloning sites (MCS) of promoterless pLacZ –basic vector (Ref. MacGregor GR et al. 1987). (C) β-Gal staining of transfected fibroblast cell line with Promoter-LacZ constructs. Scale bar represents 100µm.

4.1.2.2 Generation of vectors enabling cre-recombinase expression driven by the candidate promoters

Mouse strains expressing the site-specific Cre recombinase facilitate conditional ablation of genes when loxP sites flanked one or several exons of the gene of interest. In order to genetically modulate gene expression specifically in cardiac fibroblasts in vivo, several constructs were generated allowing the expression of the Cre driven by the candidate promoters.

Cloning of the promoter sequence in Cre recombinase expression vectors
Due to the non-availability of a promoterless-Cre construct, the pCAG-CRE construct (Addgene) was first adapted as a destination vector (pCre-GW) for gateway cloning. The

**Figure 4.5 Cloning strategies for generation of promoter-cre Constructs.** 1. Amplification of promoter sequences having attB sites from BAC clones using PCR and cloning of
Results

Promoter sequence to pDonR221 vector in order to generate Entry vector for the promoter sequences. 2. pCAG-Cre vector adapted as a destination vector (pCre-GW) for LR reaction of gateway cloning. 3. LR reaction between the different promoter-pEntry vector and pCre-GW (destination vector) for generation of Promoter-Cre constructs.

pCAG-Cre was adapted by removing its promoter region by restriction enzymes (SalI & XbaI) and ligation of the attb-ccdb–attb fragment (from pTREX-Dest30 vector) into the vector (Figure 4.5).

The Gateway System (Invitrogen) ensures a higher cloning efficiency than conventional cloning and is based on a two-step homologous recombination strategy for creating different reporters and expression plasmids (i.e. Destination vector) from a so-called Entry plasmid containing the relevant PCR product. Therefore, the two promoter fragments (Ccdc80 & Postn) were then amplified as described above (Figure 4.5). On each 5' end of the two primers, the attb-sites were added. The PCR product was cloned into an Entry clone by BP recombination and then into the above pCre-GW vector (destination vector) by LR recombination (Figure 4.5).

The correct integration and orientation of Ccdc80 and periostin promoter fragment into the adapted Cre vector was assured by analytical digestion with different restriction enzymes (Figure 4.6). Correctly oriented insert in pCcdc80-Cre vector lead to a digestion of the plasmid into a 3.6 kb, a 3.3 kb, and a 1.6 kb fragment while incorrect integration lead to a 3.5 kb, a 2.1 and a 1.2 kb fragment, as shown in Figure 4.6 A. On the other hand correctly oriented insert in pPostn-Cre vector leads to a 5kb and a 3 kb fragments when digested with two restriction enzymes (Nhel & Clal) while with no integration there was linearized 6kb fragment of empty pCre-GW vector (Figure 4.6 B). Positive clones of both vectors were selected, amplified in E. coli and purified. The correct junction from the promoter to the Cre gene and an unmutated transcription start site was confirmed by sequencing the plasmid.

To assess the efficiency of pCcdc-80-Cre and pPostn-Cre vectors, the vectors were transfected with an NIH-3T3 cell line that stably expresses the reporter pCALNL-GFP construct 217. Both promoter sequences were capable of inducing efficient Cre recombination as seen by the GFP fluorescence in the cells similar to positive control (pCAG-Cre) (Figure 4.7 A).
Results

Figure 4.6 Analytical digestion of Ccdc80 and periostin promoter Cre constructs. (A) 9 colonies were picked and plasmids were digested with Sall and Clal together (marked as Double digestion Dd) & with Sall alone (marked as S). Positive clones for Ccdc80-cre showed band with 3.6kb, 3.3kb, and 1.6kb. (B) Agarose gel of digested plasmids with Nhel & ClaI together (Dd), with Nhel alone (marked as N) and with ClaI alone (marked as C) along with undigested plasmids (U). Digested empty vector (pCre-GW) was loaded as control. Only one clone was positive clone for Postn-Cre showed band with 5kb and 3Kb. The positives clones in the figure shown were labeled with an asterisk.

In order to control the time of Cre induction in CFs in vivo, tamoxifen-inducible Cre vectors were also generated. Temporal control of Cre recombinase activity in transgenic mice has been demonstrated utilizing Cre recombinase fused with the mutated hormone-binding domain of the estrogen receptor (ER<sup>T</sup>). Such transgene can be activated by the synthetic estrogen analog like tamoxifen or 4-OHT, but not by the physiological ligand 17β-estradiol. Therefore, such an inducible Cre recombinase system is further able to facilitate conditional gene knockout analysis in transgenic mice. It also permits the analysis of gene function at specific time points in a highly controlled manner. In vitro efficiency of tamoxifen-inducible Cre vectors (pCcdc80-ERT2CreERT2 & pPostn-ERT2CreERT2) was tested using stably transfected pCALNL-GFP reporter NIH-3T3 cells as above. Solely upon stimulation with tamoxifen, a significant induction of GFP was observed with both the Cre constructs (Figure 4.7 B), demonstrating efficiently induced recombination by these two constructs in vitro.
Results

Figure 4.7 Verification of promoter vector for efficient expression of Cre recombination in vitro. (A) Photograph of the representative field showing NIH-3T3 stable cell line expressing GFP when transfected with pCcdc80-Cre, pPostn-Cre & pCAG-Cre vectors. The presence of endogenous GFP fluorescence accounts for the successful promoter activity in driving Cre expression in vitro. (B) GFP fluorescence observed in GFP-expressing stable NIH-3T3 cell line when transfected with promoter vectors expressing tamoxifen inducible-Cre recombinase in the presence (left panel) or in the absence (right panel) of 200nM 4-Hydroxy tamoxifen 48 hours post transfection. (a) GFP channel (b) overlay of GFP fluorescence and bright field image. Non-transfected cells were treated as control. Magnification: X20; Scale bar represents 50µm.

4.1.3 Generation of a mouse line that expresses Cre recombinase under the control of the Ccdc80 promoter

As the ultimate goal was to generate transgenic mice to specifically knockout or overexpress a specific protein in cardiac fibroblasts, mice expressing Cre gene under the control of the Ccdc80 promoter were generated. For this study, Ccdc80 promoter was
chosen over periostin as periostin promoter (3.9Kb) had already been shown to drive tissue-specific expression in the neural crest-derived Schwann cell lineage and in a subpopulation of periostin-expressing cells in the cardiac outflow tract and endocardial cushions. In addition, increased expression of periostin has been shown in the embryonic heart, restricted to mesenchymal cells, in pathological conditions and very low in adult hearts. It is not feasible to have such a promoter whose activity is age dependent or condition dependent and can only be used for lineage mapping. The gene expression data and promoter sequence validation in vitro, so far (shown in Figure 4.2) showed Ccdc80 as a more promising candidate for the study.

To obtain a linearized construct for integration in the mouse genome and also to get rid of the extra unnecessary fragment, the pCcdc80-Cre plasmid was digested in front of the Ccdc80 promoter and after the Cre gene. The linearized fragment was then purified by dialysis. The construct was then integrated into the mouse genome by pronuclear injection and subsequent homologous recombination. The F0 generation of the transgenic mice was genotyped for the presence of the Cre gene. 5 founders were received from our partner transgenic facility where only one animal was identified as transgenic having intense amplicon for Cre gene (Figure 4.8). The transgenic mice were bred with FVB mice to give rise to independent transgenic mouse lines.

Figure 4.8 Genotyping of Ccdc80-Cre founders by PCR. Litters were genotyped after microinjection using primers for Cre gene. Out of five transgenic founders received only one founder showed intense band for Cre gene. The animal identified gave rise to the transgenic line (Tg4) for further study.

4.1.4 Efficacy of the Ccdc80-Cre transgene in vivo

To analyze the Ccdc80 promoter efficiency in vivo, Ccdc80-Cre mice were bred with the homozygous Rosa26LacZ reporter mice. The Rosa26LacZ reporter mice carries a bacterial β-
galactosidase gene (lacZ) flanked by LoxP sites. When crossed with Cre-driver mice, lacZ is expressed in cells/tissues where Cre is expressed. β-Gal staining was performed, to investigate the site of Cre recombinase activity in frozen heart section of 6-7 weeks old Ccdc80-Cre<sup>tg/0</sup>; Rosa26<sup>LacZ</sup> mice. Cells stained in blue were observed indicating successful Cre recombination in the heart tissue (Figure 4.9).

![Image of β-Gal staining in Ccdc80-Cre<sup>tg/0</sup>; Rosa26<sup>LacZ</sup> bi-transgenic mice](image)

**Figure 4.9 Cre recombination in Ccdc80-Cre<sup>tg/0</sup>; Rosa26<sup>LacZ</sup> bi-transgenic mice.** (A) Representative image of β-Gal staining in a transverse section from an adult mouse heart Ccdc80-Cre<sup>tg/0</sup>; Rosa26<sup>LacZ</sup> and its littermate control (Ccdc80-Cre<sup>0/0</sup>;Rosa26<sup>LacZ</sup>) obtained after crossing Ccdc80-Cre<sup>tg/0</sup> and Rosa26<sup>LacZ</sup> mice. The tissue was counterstained with eosin (red) for contrast. Ccdc80-Cre expression was observed in cardiomyocytes in the myocardium (as shown by an arrow) based on anatomical and morphological characterization. Scale bar represents 50 µm.

However, unexpectedly, the β-gal staining was observed in the cardiomyocytes (blue cells) and not in the interstitial spaces in the heart as shown in Figure 4.9.
4.2 Characterization of VimCreERT2 transgenic mice expressing Cre recombinase in the heart

4.2.1 Vimentin promoter activity in heart tissue in comparison to Fsp1 promoter activity

Vimentin (the intermediate filament protein) has been extensively used to label fibroblast (in heart, all the CFs are positive) although they also label various other cell types including endothelial cells. In order to validate the vimentin promoter activity in the heart, a Vim-CreER BAC transgenic mouse line was provided by our collaborator Robert F. Schwabe, MD (Columbia University, New York). This mouse line has a CreERT2 cassette inserted in a BAC containing the mouse vimentin locus (Figure 4.10).

![Diagram of BAC recombination](image)

Figure 4.10 Shown is the construct for generation of VimCreERT2 transgenic mice by BAC recombination (Ref: Troeger JS et al. 2012).

Using the mTom-mGFP (mT/mG) dual fluorescent reporter mouse, VimCreER marked cell population was analyzed in the heart with respect to known fibroblast promoter (Fsp1-Cre). The mT/mG reporter mouse has a consecutive expression of a membrane-targeted tdTomato (mTom) gene in cells without Cre activity and membrane-targeted EGFP (mGFP) expression in cells with Cre activity (Figure 4.11 A). For analyzing Cre-mediated
Results

Figure 4.11 Validation of VimCre promoter activity in frozen heart section after tamoxifen injection compared with described fibroblast-Cre mice. (A) Schematic diagram showing mTom -mGFP reporter gene expression after Cre mediated recombination in Fsp1-Cre mice and tamoxifen and vehicle-treated VimCreER mice. (B) Vim-CreER BAC transgenic mice were crossed to mTom -mGFP reporter mice and injected 6 times with tamoxifen to visualize Cre-mediated recombination. Shown are the representative longitudinal section of the whole heart with Cre-mediated recombination visible as green (GFP) signals enhanced using anti-GFP antibody in tamoxifen-treated VimCreER;mT/mG<sup>fl<sup>ox/+</sup></sup> bitransgenic mice with respect to the vehicle (peanut oil) treated control and Fsp1Cre;mT/mG bitransgenic mice. Confocal microscopy for direct fluorescence of mtomato signals and indirect immunofluorescence of GFP signals in frozen heart sections of (C) tamoxifen-injected or vehicle-injected VimCreER; mT/mG<sup>fl<sup>ox/+</sup></sup> mice & (D) Fsp1-cre;mT/mG<sup>fl<sup>ox/+</sup></sup> mice with its littermate control mT/mG<sup>fl<sup>ox/+</sup></sup> mice.
Scale bar = 100µm. The Vimentin marked cell population expresses GFP in the interstitial spaces of heart section and not in cardiomyocytes. Fsp1-Cre tagged cell also shows the GFP positive cells restricted to interstitial spaces, but the amount of cells expressing GFP was less as compared to tamoxifen induced VimCreER tagged cells.

recombination cryosections of heart tissue from 6-7 weeks old VimCreER;mT/mG bitransgenic mice were examined by immunofluorescence using the anti-GFP antibody. GFP-positive cells were observed in the interstitial spaces of frozen heart section after six-tamoxifen injection in VimCreER;mT/mG bitransgenic mice with respect to the vehicle (peanut oil) injected VimCreER;mT/mG mice (Figure 4.11 B&C). Due to non-availability of Fsp1 inducible Cre mouse line, Fsp1-Cre, the known fibroblast-Cre mouse line was used for comparison. Immunofluorescence staining in frozen heart section of Fsp1-Cre; mT/mG bitransgenic mice also show immuno-reactivity for GFP in interstitial spaces as compared to its littermate control (Figure 4.11 B&D). In spite of consecutive expression of Cre drove under Fsp1 promoter, the recombination observed in these bitransgenic mice were lower than tamoxifen-treated VimCreER;mT/mG bitransgenic mice (Figure 4.11 B&D) targeting around 20% of the interstitial cells in whole heart sections.

4.2.2 VimCreER marks non-myocytes in the heart.

In order to characterize VimCreER lineage- tagged cells, dual antibody immunofluorescent labeling was performed on frozen heart sections of tamoxifen or vehicle-injected VimCreER;mT/mG bitransgenic mice. For dual antibody immunofluorescent labeling different cell markers were used: P4HB and Vimentin (fibroblast marker), SM22a (smooth muscle cell marker) & CD31 (endothelial cell marker) along with GFP. As known from the literature, vimentin is found to be expressed by all fibroblasts and at a lower level by endothelial cell, we observed the GFP-positive cells in tamoxifen-injected VimCreER;mT/mG bitransgenic mice co-stained with fibroblast markers (Figure 4.12 A) and this overlay was almost 80%. We also observed some GFP-positive cells were co-stained with endothelial cell marker (CD31) (Figure 4.12 E), and most of them were seen lying in close proximity to CD31 positive cells. Several previous Immunofluorescence studies have demonstrated endogenous vimentin expression in blood vessels of human\textsuperscript{220, 222} and of rabbit\textsuperscript{221}. Consistently, the data presented indicate an overlay between GFP-positive cells and smooth muscle cell marker in the blood vessels of tamoxifen-injected VimCreER;mT/mG mice (Fig 4.12 C). In comparison to tamoxifen-injected VimCreER;mT/mG mice and its littermate control mT/mG\textsuperscript{fox/+} mice, the GFP positive cells in Fsp1-Cre;mT/mG mice were co-stained with fibroblast marker (Fig 4.12 B) and with endothelial cell marker but not with smooth muscle...
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cell markers (Fig 4.12 D, F).

Figure 4.12 Characterization of VimCreER lineage-tagged cells in heart sections by immunofluorescence. Confocal microscopy of frozen heart section of tamoxifen or vehicle-injected VimCreER;mT/mGflox/+ bitransgenic mice and Fsp1-Cre; mT/mGflox/+ along with its littermate
control mT/mG^fox/+ mice, co-stained for (A&B) Fibroblast marker (Vimentin & P4HB); (C&D) Smooth muscle marker (SM22α) & (E&F) Endothelial marker (CD31) along with anti-GFP antibody. Scale bar =50µm. VimCreER marked cells expressing GFP co-stains with fibroblast markers (Vim & P4HB), smooth muscle cell marker (in some big vessels as shown above), and not with endothelial cells (except for few cells). However, the Fsp1-Cre marked cells were co-stain with fibroblast markers and with endothelial cell marker but not with smooth muscle cell marker as compared to its littermate control mT/mG^fox/+ mice.

4.2.3 Cardiac fibroblast specific Cre recombination in VimCreER bitransgenic mice

In order to verify that vimentin promoter has indeed a CF-specific expression, CFs and CM were isolated from tamoxifen-injected VimCreER;mT/mG and Fsp1-cre;mT/mG bitransgenic mice.

Cre-mediated recombination was observed by direct fluorescence for mGFP signals. The majority of cultured CFs (32hrs cultured) from tamoxifen-injected VimCreER;mT/mG

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Figure 4.13 Cell-specific expression of Vimentin promoter compared to described fibroblast promoter. (A) mTomato and mGFP signals detected in isolated and 32hrs cultured cardiac fibroblast (CF) and cardiomyocyte (CM) from the two bitransgenic mice. Images captured using a fluorescent microscope. Magnification 20X; Scale bar =50µm. Vimentin promoter activity is specifically in CF and not in CM isolated from tamoxifen-treated VimCreER;mT/mG mice with respect to vehicle-treated mice. Only a few CF appeared green in case of Fsp1-cre; mT/mG that accounts for weak Fsp1 promoter activity.
bitransgenic mice had mGFP signals as compared to Fsp1-cre;mT/mG bitransgenic mice, where only a few cells had mGFP signals (Fig 4.13 A). This observation was similar to whole heart tissue. In contrast, in the CM, isolated from both bitransgenic animals had only mTomato fluorescence. This demonstrated that Cre-mediated recombination is restricted to CFs and not to CM in both the bitransgenic animals (Fig 4.13 B).

To further confirm that the GFP-expressing cultured CFs were fibroblast cells and not any other cell type, co-staining was performed for different cell markers along with anti-GFP antibody. Cultured CFs positive for GFP were co-stained only with the fibroblast marker (Vimentin and P4HB) (Figure 4.14 A) and not with endothelial cell
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marker (Figure 4.14 B) or myocytes marker (data not shown). There was no mGFP fluorescence detected in cultured CFs, isolated from vehicle-treated control mice. This accounts for the tight regulation of vimentin promoter activity to drive tamoxifen inducible Cre expression in CFs. Also co-staining with fibroblast marker confirmed that the cultured CFs as fibroblast cells.

Thus, concluding that presence of bright mGFP fluorescence restricted to cultured CFs signifies successful and specific vimentin promoter activity in driving Cre expression in vivo.

4.2.4 Vimentin promoter activity in the TAC model for chronic cardiac pressure overload

TAC in the mouse is a commonly used experimental model of pressure overload-induced cardiac hypertrophy and fibrosis. In order to determine whether vimentin promoter has altered activity and specificity after cardiac injury, the endogenous level of Vimentin and Fsp1 genes under pathological cardiac hypertrophy were first analyzed. For this analysis, C57BL/6N mice were randomized for transverse aortic constriction (TAC) and sham surgery. Expressions of Vimentin (Vim) and Fsp1 genes were then quantified in CFs and CM isolated from C57BL/6N mice that were challenged with TAC for 6 weeks along with Sham-operated mice as the control (Figure 4.15 (A). Increased expression of vimentin and Fsp1 was observed in isolated CFs of C57BL/6N wild-type mice as compared to CM. Moreover, this difference was significant for vimentin expression between CFs and CM isolated from TAC-operated mice. Though there was no significant difference observed in the expression of vimentin between CFs isolated from Sham and TAC operated mice. On the other hand, a difference in Fsp1 expression was significant between CFs from Sham and TAC operated mice Figure 4.15 (A). In addition, the expression of Fsp1 was more than Vimentin in CFs isolated from sham and TAC operated mice.

To further investigate the vimentin promoter activity in pressure overload myocardium, the 6-week-old VimCreER;mT/mG mice were first injected with vehicle (peanut oil) and tamoxifen (2mg /mouse /day dissolved in peanut oil) i.p. for 5 consecutive days and then subjected to thoracic aortic constriction (TAC; causing chronic pressure overload) or control surgery (sham) for 3 weeks. The animals were then sacrificed and hearts sections were used for immunofluorescence staining. Sham-operated mice hearts were treated as the control in each case. Confocal microscopy of immunostaining with the anti-GFP antibody on the heart sections showed an increased number of GFP-positive cells in the TAC-operated tamoxifen
Fig 4.15 Vimentin promoter activity in TAC-operated heart. Comparison with described fibroblast promoter (Fsp1). (A) Relative gene expression data of Vimentin and Fsp1 in isolated cardiac fibroblast (CF) and cardiomyocytes (CM) from C57BL/6N mice challenged with TAC for 6 weeks. Gene expression data of Myh6 and Sham-operated mice are taken as control for
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analysis. Sham and TAC (n=3 each). All data were evaluated by 1-way ANOVA/ Bonferroni paired test. Statistical significance is shown as *P<0.05, **P<0.01, ***P<0.001. (B) Confocal microscopy of frozen heart sections for mTomato-mGFP signals after 3 weeks of TAC in the two bi-transgenic mice with respect to the vehicle-treated (Sham and TAC) control. (C&D) Confocal microscopy images of frozen heart section of TAC-operated bitransgenic mice with respect to TAC Vehicle-treated control mice co-stained with fibroblast marker (anti-Vimentin & anti-P4HB). Scale bar =50μm.

injected VimCreER;mT/mG bitransgenic mice with respect to the Sham & TAC-operated vehicle-injected VimCreER;mT/mG control mice (Figure 4.15 B). The TAC-operated Fsp1Cre: mT/mG mice also showed an increase in the number of GFP-positive cells compared to its sham control group. Although this increase was not as much as observed in the case of TAC-operated tamoxifen-injected VimCreER;mT/mG bitransgenic mice (Figure 4.15 B).

Fibroblasts in the injured heart are thought to have diverse origins. Recent studies on organs such as the kidney, lung and liver, heart and on metastatic tumors showed that during fibrosis, in addition to the proliferation of resident fibroblasts, bone marrow–derived fibroblasts, epithelial cells contribute to fibroblast accumulation through an epithelial-mesenchymal transition (EMT)\textsuperscript{55}. Likewise, endothelial cells contribute to fibroblast via Endothelial-mesenchymal transition (EndMT)\textsuperscript{20}, which is also a form of EMT as observed during formation of the atrioventricular cushion in an embryonic heart. To determine whether the GFP expression in the hearts of TAC-operated VimCreER;mT/mG mice was limited to fibroblasts, co-staining was performed on frozen hearts sections for fibroblast or endothelial cell markers along with anti- GFP antibody. In comparison with TAC-operated Fsp1-Cre;mT/mG and TAC-operated vehicle control mice, a majority of the GFP-positive cells were identified as fibroblasts in TAC-operated, tamoxifen-injected VimCreER;mT/mG mice hearts (Figure 4.15 C&D). There was also no overlay observed with the endothelial cells marker (CD31) (Figure 4.16). In contrast, in TAC-operated Fsp1-Cre;mT/mG mice, the GFP-positive cells showed a co-localization both with the fibroblast marker (Figure 4.15 D) and the endothelial cell marker (Figure 4.16).
Echocardiography was conducted to assess the cardiac phenotype in tamoxifen-injected Vim-CreER;mT/mG mice compared to vehicle-injected control mice post TAC (Figure 4.17 A). Change in the ratio of heart weight to body weight or heart weight to tibia length is one of the parameters for measuring cardiac hypertrophy in response to pressure overload. Here we observed an insignificant change in the ratio of heart to body weight & heart weight to tibia length in Vim-CreER;mT/mG bitransgenic mice post TAC. Equally, we could not find any significant differences in contractility, as measured by the change in the ejection fraction (%) or fractional shortening (%) (Figure 4.17B). Likewise, in the case of Fsp1-cre;mT/mG mice post TAC no significant changes were observed in the heart to body weight & heart weight to tibia length compared to its littermate mT/mG<sup>+/+</sup> control (Figure 4.17 C). This could be because a lower number of both types of animals were used in this study. However, an increase was noticed in HW/BW and HW/TL after TAC compared to Sham-operated mice, which account for cardiac hypertrophy induced in response to TAC in these mice. A significant difference in the percentage of fractional shorting and ejection fraction in TAC-operated Fsp1-Cre bitransgenic mice with respect to its TAC-operated control mice indicate a loss of function or failing condition in hearts of Fsp1Cre mice. No change in the percentage of ejection fraction and fractional shorting was observed in TAC and Sham-operated mT/mG<sup>+/+</sup> control mice. This indicates a delay in loss of function or occurrence of some compensation in these control animals.
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Figure 4.17 Cardiac phenotyping in VimCreER mice post TAC. (A) Experimental strategy for TAC study. The 6-week-old VimCreER;mT/mG mice were injected with vehicle (peanut oil) and tamoxifen (2mg /mouse /day dissolved in peanut oil) i.p. for 5 consecutive days and then subjected to thoracic aortic constriction (TAC; causing chronic pressure overload) or control surgery (sham). At week 11, echocardiographic measurements were taken, and animals were euthanized for further analysis (B) Ratio of the heart weight (HW) to body weight (BW) and heart weight (HW) to tibia length (TL) in vehicle-injected and tamoxifen-injected VimCreER;mT/mG mice post TAC. Sham and TAC n=3 each group. Ejection fraction (%) and left ventricular shortening fraction (fractional shortening (%)) as two parameters of the echocardiographic analysis post TAC. Sham (n=3 each) & TAC (n=5 each). (C) The ratio of HW to BW and HW to TL in Fsp1-cre: mT/mG and littermate control mice post TAC. Sham (n=3 each) & TAC (n=4-5 each). Left ventricular shortening fraction (fractional shortening (%)) and ejection fraction (%) calculation was done based on Sham (n=3) & TAC (n=4-5) mice. All data were evaluated by 2-way ANOVA/ Bonferroni posttest. Statistical significance is shown as *P<0.05, **P<0.01, ***P<0.001.
4.2.5 Tamoxifen-independent recombination in the Vim-CreER mouse heart

In order to determine the tamoxifen-independent recombination, cryosections of heart tissue from 3- and 4-month-old vehicle-injected VimCreER;mT/mG bitransgenic mice were examined by immunofluorescence staining using the anti-GFP antibody. In principle, in a progeny containing both (VimCreER & mT/mG) transgenes, the mGFP expression in the cells should be detected only upon tamoxifen-induced removal of the floxed mTomato Stop cassette (Figure 4.18 A).

However, positive reactivity for GFP was detected in heart tissues of some vehicle-injected mice (Figure 4.18 B) similar to tamoxifen-injected VimCreER;mT/mG bitransgenic mice (Figure 4.18 A) demonstrating tamoxifen independent Cre recombination in these mice. While some vehicle-injected mice were negative for GFP (Figure 4.18 C). The specificity of this labeling was confirmed by the absence of immunoreactivity for GFP in heart sections of mT/mGfl/+ reporter and BL/6N wild-type control mice (Figure 4.18 D, E).

![Figure 4.18 Tamoxifen-Independent Cre recombination in VimCreER;mT/mG bitransgenic mice.](image)

Immunofluorescence microscopy for GFP in frozen heart sections from 4-month-old (A) tamoxifen-injected, (B-C) vehicle-injected, (D) mT/mGfl/+ & (E) BL/6N wild-type control mice. GFP fluorescence detected in frozen heart section of vehicle-injected mouse (B) compared to vehicle-injected mouse (C) demonstrates tamoxifen-independent recombination in these mice. A similar effect was observed in the case of an untreated mouse (D). The absence of GFP signals in mT/mGfl/+ account for proper regulation of tamoxifen inducible Cre. Scale bar =50µm.

Additionally, other tissue sections from these vehicle-injected bitransgenic mice were also examined for tamoxifen independent recombination where except for lung, other tissue
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sections had no mGFP fluorescence compared to tamoxifen-injected VimCreER;mT/mG mice and mT/mG reporter mice as control (Figure 4.19).

![Image of fluorescence signals in different tissue sections of VimCreER;mT/mG mice.](image)

**Figure 4.19 Direct fluorescence for mTom-mGFP signals in different tissue sections of VimCreER;mT/mG mice.** Microscopy images of direct fluorescence of mGFP and mTom signals in fixed tissue sections from various organs after tamoxifen injection, vehicle injection in adult VimCreER; mT/mGflox/+ mouse along with mT/mGflox/+ reporter mouse. Tamoxifen injection in adult VimCreER;mT/mGflox/+ mouse results in mosaic expression of mTom and mGFP in multiple tissue types while vehicle injection results in ubiquitous mTom expression in all tissues except for lung tissue section having tamoxifen independent mGFP expression. Fixed tissue sections of various organs from an adult mT/mGflox/+ reporter mouse demonstrate ubiquitous mTom labeling as a control. Scale bar =50µm.

4.2.6 Recombination in the heart of VimCreER;mT/mG mice during postnatal development

To further investigate feasibility of tamoxifen-inducible recombination during early and late postnatal development, and to elude the tamoxifen independent recombination in VimCreER;mT/mGflox/+ bitransgenic mice, VimCreER<sup>tg/0</sup> males were crossed with mT/mGflox/flox females. Lactating mothers (mT/mGflox/flox) were then injected with tamoxifen (1mg/mouse/day) for five consecutive days starting at postnatal day 1 (P1; Figure 4.20 A).

It was predicted that the tamoxifen would be provided to the nurtured offspring through the milk. Intraperitoneal injections were well tolerated by lactating mothers and all their offspring were alive during the tamoxifen treatment. One-week (P7) old VimCreER;mT/mGflox/+ and its littermate control (mT/mGflox/) neonates were then analyzed for cell-specific expression of mGFP fluorescence and generalized expression of mTomato. As expected, bright mGFP fluorescence was observed in the interstitial spaces in the myocardium of VimCreER;mT/mGflox/+ neonates indicating successful Cre recombination. While the littermate control neonates (mT/mGflox/) have only mTomato fluorescence...
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in heart, indicating an absence of Cre recombination in these pups (Figure 4.20 B).

In conclusion, administration of tamoxifen to lactating mothers allows sufficient recombination during early postnatal development.

Figure 4.20 Administration of tamoxifen (TM) to lactating mT/mG\textsuperscript{flox/flox} mice leads to efficient recombination in the nourished pups. (A) Scheme of administration protocol and subsequent analysis. Lactating mothers (mT/mG\textsuperscript{flox/flox}) were injected with 1 mg of TM daily for 5 consecutive days starting on the day of birth of the litter. VimCreER; mT/mG\textsuperscript{flox/} bi-transgenic litters were analyzed at the age of postnatal day 7 and at 6 weeks for expression of mGFP. (B) Confocal Images of direct fluorescence of mGFP signals along with mTom in the frozen heart sections at 7-day-old neonate and 6 weeks old VimCreER; mT/mG\textsuperscript{flox/flox} mice along with their littermate control. Scale bar = 100µm.
**Figure 4.21 Immunofluorescence for fibroblast cell markers at postnatal day 7 (P7).**
(A) Confocal images showing colocalization of membrane tagged GFP (mGFP) expressing cells with vimentin & (B) P4HB in the frozen heart sections of seven-day-old VimCreER; mT/mGflox/+ neonates along with their mT/mGflox/+ littermate control. Scale bar = 100µm.

As reported in previous studies, vimentin is found to expressed from embryonic day E7.5 onwards (Scherholz *et al.* (2013)) \(^{171}\) and shown to localize in interstitial spaces in later stages of heart development (Bennett *et al.* (1979) \(^{223}\); Gard and Lazarides, (1980); Speiser *et al.* (1992), Kim *et al.* (1996) \(^{169}\)). We hypothesized that activating vimentin promoter at an
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early stage will have mGFP expression restricted to fibroblasts in the heart. Immunostaining on frozen heart sections of neonates (P7) for fibroblast marker was performed. Colocalization of the GFP positive cells with fibroblast cell markers (Vimentin and P4HB) confirmed that the recombination occurred in fibroblast cells (Figure 4.21 A&B). Consistent with previous studies demonstrating expression of vimentin in smooth muscle cells of blood vessels, mGFP-expressing cells around the vessels were observed in 7-day-old pups during the study. However, reduced number of the mGFP-expressing cells was observed in the heart sections of 6-weeks old animals (Figure 4.22).

Figure 4.22 Immunofluorescence staining for vascular smooth muscle cell marker in postnatal day 7 and 6 weeks old hearts. Confocal images showing co-localization of membrane tagged GFP (mGFP) expressing cells with SM22α antibody in frozen heart section of postnatal day 7 (P7) & of 6-week-old VimCreER;tmT/mGflox/+ bitransgenic animals along with their mT/mGflox/+ littermate control. Scale bar = 100µm

It has been shown through in situ hybridization that SM22α transcripts were first expressed at about embryonic day (E) 9.5 in vascular smooth muscle cells and thereafter continued to

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express in all smooth muscle cells in adulthood\(^{224}\). In contrast to its smooth muscle specificity in adult tissues, SM22 \(\alpha\) was expressed transiently in the heart between E8.0 and E12.5 and in skeletal muscle cells in the myotomal compartment of the somites between E9.5 and E12.5. The expression of SM22 \(\alpha\) in smooth muscle cells, as well as early cardiac and skeletal muscle cells, suggests that there may be commonalities between the regulatory programs that direct muscle-specific gene expression in these three myogenic cell types. Further analysis of GFP-expressing cells near the vessel is done by staining for smooth muscle cell marker (SM22\(\alpha\)). An overlay between mGFP-expressing cell and SM22\(\alpha\) around the vessels in 7-day old pups was observed but in 6-weeks old VimCreER; mT/mG animal the number of cells was very low. This suggests that, in later stages of development, the vimentin promoter is mainly active in fibroblasts and to significantly lower extent in smooth muscle cells.

To test for vimentin promoter activity in endothelial cells, immunostainings with an antibody against the surface marker CD31 was carried out. This analysis revealed colocalization of mGFP-expressing cells (around 80\%) with CD31 not only in 7-day-old pups (P7) but also in the later stage in 6 weeks old adult VimCreER; mT/mG bitransgenic mice hearts (Figure 4.23 A&B). This indicates that the recombination had also occurred in the endothelial cells along with fibroblasts during development, and somehow the vimentin promoter activity was not restricted to cardiac fibroblasts in the heart when induced in an early stage as observed in the case of adult recombination.

In conclusion, insubstantial recombination occurred in the cardiac fibroblasts of VimCreER; mT/mG offspring from tamoxifen-injected lactating mothers during early and postnatal development.
Figure 4.23 Cre mediated recombination in endothelial cells in postnatal day 7 and 6 weeks old hearts. Confocal images showing co-localization of membrane tagged GFP (mGFP) expressing cells with CD31 antibody in frozen heart section (A) of postnatal day 7 (P7) & (B) of 6-week-old VimCreER;mT/mGfox+ bitransgenic animals along with their mT/mGfox+ littermate control. Scalebar =100μm.
5 DISCUSSION

5.1 Screening for cardiac fibroblast specific genes

A primary goal of this project was to search for and to characterize gene promoters that are specifically active in cardiac fibroblasts (CFs). CFs represent one of the largest cell population in the heart, are a key source of components of the extracellular matrix (ECM) that regulates the structure of the heart and hence mechanical, chemical and electrical signals between the cellular and non-cellular components. More recently their active involvement in both normal cardiovascular biology and many other aspects of cardiac pathophysiology besides fibrosis have been recognized. Though cardiac fibroblasts play a critical role in the maintenance of normal cardiac function in the heart, the analysis of cardiac fibroblasts in heart tissue has been hampered by the absence of a fibroblast-specific surface marker.

Several fibroblast-specific transgenic lines, although not organ specific, have been established that express Cre and are driven by a Postn promoter (Postn-Cre mice)48,67, a S100a4 [Fibroblast Specific Protein 1 (Fsp1)] promoter 67,70, and Transcription Factor 21 [Tcf21, also known as Podocyte-Expressed 1 (Pod1) combination, or a Capsulin or Class A Basic Helix-Loop-Helix 23 (bHLHa23)] promoter, have been reported71 but none of them seems to be specific for cardiac fibroblasts. Here we studied in two parts the promoters of candidate genes with potential specificity for cardiac fibroblasts, both in vitro and in vivo.

The first part was based on microarray database screening approach where we found out some cardiac specific genes based on their enrichment in heart and specifically in CFs in comparison with other tissue and CM. In this screening, we found Ccdc80 as a potential candidate for the study and also included Periostin, which has been used as a marker of activated fibroblasts in the remodeling myocardium66,76. Periostin, an extracellular matrix protein is highly expressed in embryonic myocardium73 and is absent from cells of cardiomyocyte lineage, but is localized in cardiac mesenchymal cells65,72,74. In this study we demonstrated enrichment of an adipocyte-secreted protein Ccdc80 (also known as Cl2, DRO1, equarin, Ssg1, and Urb) in heart tissue as compared to other mice tissues that goes well with previous findings in literature about distribution or expression of Ccdc80 in heart tissue in mice, rat and human225,226. Consistent with previous findings where periostin (Postn), have been found to be expressed, both at the mRNA and protein level by majority of normal adult tissues, including the aorta, stomach, lower gastrointestinal tract, placenta, uterus, adrenal glands, lung, thyroid, stomach, colon, ovary, testis and prostate and...
breast\textsuperscript{227, 228}. We also found periostin to be expressed more in the lung than in mouse heart tissue when quantified using qPCR.

5.2 Ccdc80 promoter activity in cardiomyocytes \textit{in vivo}

In last ten years, there are only a few studies published on Ccdc80 function. Ccdc80 (also known as CI2, DRO1, equarin, Ssg1, and Urb) was initially identified as an estrogen-induced gene in rat uterus and mammary gland\textsuperscript{229}. It is expressed in a number of tissues in both the embryo and adult\textsuperscript{229, 230, 231}. It is a novel adipocyte-secreted protein\textsuperscript{237, 225, 230} and revealed to be abundantly expressed in adipose tissues\textsuperscript{231}. Ccdc80 is deregulated in obesity\textsuperscript{236} thereby, regulating adipogenesis through the down-regulation of Wnt/b-catenin signaling and induction of C/EBP\textalpha{} and PPAR\gamma{}\textsuperscript{237}. It is down-regulated in thyroid, ovarian, pancreatic, colon cancer cell lines and tumors\textsuperscript{236}. Overexpression of Ccdc80 in colorectal and pancreatic cancer cell lines inhibits malignant growth and suppresses anchorage-independent growth\textsuperscript{232}, suggesting that Ccdc80 may be a tumor-suppressor. In addition, Ccdc80 is also expressed in dermal papilla cells\textsuperscript{234}, in bone marrow stromal cells\textsuperscript{235} and in eye formation\textsuperscript{230}. During mouse development, Ccdc80 RNA is barely detectable in 9dpc embryos. Though, in later stages, its expression is increased. The temporal and spatial expression pattern of Ccdc80 suggests its role in mouse skeletogenesis\textsuperscript{225}. Ccdc80 has also been proposed to be a component of the extracellular matrix owing to its ability to bind various extracellular matrix proteins and promote cell adhesion\textsuperscript{238}. A recent study showed Ccdc80 knockout (KO) mouse are hyperglycemic and glucose intolerant and display impaired insulin secretion in vivo when fed a HF diet thereby suggesting Ccdc80 as a novel modulator of glucose and energy homeostasis in mice\textsuperscript{226}.

Since there is no literature published so far reporting, the function of Ccdc80 in the heart tissue, we are the first one to study the expression of Ccdc80 in the two major cell types (isolated CFs and CM) in the heart. Here we found the expression of Ccdc80 was significantly enriched in isolated CFs as compared to isolated CM, in both neonatal rat and adult mouse hearts. This also confirms our microarray expression data. Most of the cardiac diseases are coupled with fibrosis in the heart. In general, Fibrosis is a scarring process, which is characterized by fibroblast accumulation and accumulation of extracellular matrix (ECM) proteins that lead to distorted organ framework and function\textsuperscript{137}. Ccdc80 has also been reported to be a component of the extracellular matrix owing to its ability to bind various extracellular matrix proteins, and promote cell adhesion\textsuperscript{238}. An up-regulation of Ccdc80 gene expression in failing heart with respect to the healthy heart and also in
isolated CF indicates that Ccdc80 gene is expressed by cardiac fibroblast in the heart in both normal and diseased conditions.

**In vitro** validation of Ccdc80 promoter sequence upstream (approx. 4.2kb) by β-gal staining or by Cre recombination suggested that the promoter sequence amplified was efficient enough to drive the expression of Cre in transfected fibroblast cell line (NIH-3T3 cells). On the other hand, **in vivo** validation of Ccdc80-Cre transgenic mice upon crossing with Rosa26\textsuperscript{LacZ} reporter mice demonstrated Cre recombination under Ccdc80 promoter in cardiomyocytes and not in cardiac fibroblast of heart tissues. However, the promoter activity was active only in a small population of CM in the heart where only 20% of CM appeared blue after β-gal staining (Figure 4.9). We also found a strong and stable Ccdc80 promoter activity in other organs like Lung, kidney, and liver of Ccdc80-Cre\textsuperscript{tg0};Rosa26\textsuperscript{LacZ} double transgenic mice (data not shown). To our understanding the reason for such a different activity of Ccdc80 promoter in heart targeting CM **in vivo** can be because of the pronucleus microinjection technique used for generation of transgenic mice which is an old method and also include frequent random integration of multiple copies of a transgene that can results in silencing of our transgene, probably because of a positional effect and/or repeat-induced gene silencing. Another reason can be the promoter sequence, maybe it would have been better to use a BAC construct having all the important regulatory elements that are required to recapitulate endogenous Ccdc80 gene expression.

### 5.3 Vimentin promoter activity in non-myocyte cells in heart tissue

The second part of this study characterized VimCreERT2 transgenic mice as a candidate CF-specific Cre-expressing mouse line in comparison with Fsp1-Cre mouse line, which has been reported as a CF-specific line. Current analyzes of the origins of CF lineages during development and in disease have been established without the use of specific markers or systematic quantitative analysis of fibroblast lineages present in the heart. Markers used to identify fibroblasts, such as fibroblast specific protein 1 (Fsp1), not only marks only a subset of fibroblasts but, is also expressed by several other cell types, including endothelial and immune cells. Nowadays, vimentin has been extensively used to label cardiac fibroblasts in the heart. It is a major structural component of intermediate filaments (IFs) in many cell types and plays an important role in vital mechanical and biological functions in cells such as cell contractility, migration, stiffening, and proliferation. It is shown that the primary fibroblast derived from a vimentin-deficient mouse embryo compared with those from wild-type mouse embryo exhibit decreased motility, chemotactic migration, and
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delayed wound healing. More studies on Vimentin-deficient (Vim-/-) mice have revealed that loss of vimentin leads to failures in vascular adaptation resulting in pathological conditions, such as reduction of renal mass, malformation of glia cells, reduced resistance of arteries to sheer stress, and disturbance of leukocytes homing to lymph nodes. During the past 2 decades, advances in the use of site-specific recombinase have added greatly to our ability to manipulate cells and gene expression. These site-specific recombinases bind to and recombine specific sequences of DNA, allowing researchers to hereditarily label cells, conditionally inactivate or activate genes, and even ablate cells based on their gene expression. Further use of dual-fluorescent reporter system that permits direct live visualization of both recombined and non-recombined cells at single cell resolution, offers an internal control for phenotypic analysis of Cre-induced mosaic mutants. Thereby, also providing a second marker for lineage tracing applications. Here by using tamoxifen-inducible Cre system and dual fluorescent reporter mice (mTomato-mGFP), we have demonstrated through Cre recombination the vimentin promoter activity in heart tissue of the VimCreER:mT/mG bitransgenic mice. Immunofluorescence staining for recombined cells (i.e. GFP positive cells) in tamoxifen-injected VimCreER;mT/mG bitransgenic mice heart tissues showed the vimentin promoter activity restricted to cardiac interstitium, targeting nearly 80% of cells of interstitial spaces (Figure 4.11). Co-labeling with GFP and two fibroblast cell markers identified the majority of GFP-positive cells as fibroblasts (Figure 4.12).

A number of studies have reported an expression of vimentin within endothelial cells, but to a lower extent. Here we also found very few VimCreER marked cells (GFP positive cells) positive for endothelial cell markers (CD31) in VimCreER:mT/mG bitransgenic mice heart sections following co-staining. In our observations, the majority of GFP-positive cells were lying in close proximity to CD31 positive cells suggesting that the vimentin promoter is active only in a very small population of endothelial cells in VimCreER:mT/mG bitransgenic mice.

The smooth muscle cell is the most abundant cell type in the blood vessel walls. It occurs in all vessels except capillaries and pericytic venules. Vascular SMCs have been divided into (at least) two distinct states of differentiation, usually referred to as synthetic and contractile phenotypes, the latter being predominant in the blood vessels of adult organisms. Previous studies show vimentin and desmin, as major constituents of the network in the smooth muscle cells and tissues. Vimentin insufficiency impairs contractile ability of various smooth muscle preparations, implying their important role for smooth muscle force...
Vascular smooth muscle cells mainly express vimentin that is widely distributed in various blood vessels ranging from the elastic arteries to micro arteries. Immunofluorescence studies demonstrate the presence of vimentin on vascular smooth muscle cells of blood vessels in different organs and species. Consistent with immunofluorescence studies, we also found an overlay between VimCreER marked cells (GFP+) and smooth muscle cell marker (SM22α) in the myocardial arteries and the vessels of VimCreER:mT/mG bitransgenic mice hearts (Figure 4.12).

**Figure 5.1 Vimentin is expressed in the blood vessels in the heart.** Immunostaining against vimentin (Green) in frozen heart sections of C57BL/6N mice demonstrates the presence of vimentin expressing cells in blood vessels. Scale bar =50µm, 100µm.

C57BL/6N wild-type mice heart tissues stained for anti-vimentin (Fig 5.1) also demonstrates and confirms our finding along with previous findings regarding the expression of vimentin in the blood vessel-associated vascular smooth muscle cells in heart tissue.

In agreement with previous reports using FSP1 as a known fibroblast promoter, we found FSP1 marked cells (GFP positive cells) to be rare in the normal adult myocardium of Fsp1-Cre;mT/mG bitransgenic mice suggesting the limited activity of Fsp1 promoter in the heart (Figure 4.11). Also co-labeling with different cell markers demonstrates Fsp1-cre marked cells (GFP positive cells) mostly as endothelial cells or fibroblasts cells.
5.4 Cardiac fibroblast-specific Cre recombination in VimCreER; mT/mG bitransgenic mice

Confirmation of CF-specific expression of vimentin promoter in comparison with Fsp1 promoter activity has been demonstrated by detecting the direct fluorescence for mGFP and mTomato signal in isolated CM and CFs of the two-bitransgenic mice. Here we have found mostly all CFs isolated from tamoxifen-treated VimCreER;mT/mG bitransgenic mice underwent Cre-mediated recombination (as shown in Figure 4.13) and had mGFP fluorescence thereby confirming efficient vimentin promoter activity. Whereas, the number of CFs isolated from Fsp1-Cre; mT/mG bitransgenic mice showing mGFP fluorescence were low. In addition, the CM isolated from both the bitransgenic mice has only mTomato fluorescence confirming strict cell-specific activity of both promoters (Figure 4.13).

Further double-staining with GFP and fibroblast cell markers (Vimentin and P4HB) showed a 100% overlay between the GFP-positive cell and the fibroblast cell markers in tamoxifen-injected VimCreER;mT/mG and Fsp1-cre:mT/mG mice (Figure 4.14), thereby confirming that the cells isolated and cultured were purely CFs and not contaminated with any other cell type, as we have also not found any cell positive for CD31 antibody in these cultures. There was also no overlap between GFP and myocytes marker (ACTN) in this culture (data not shown). All these data suggest that the vimentin promoter efficiency and CF-specific Cre-mediated recombination is much higher in VimCreER mice then Fsp1-Cre mice.

5.5 Vimentin promoter activity in the pressure-overloaded myocardium

Fibroblast activation and expansion of the cardiac interstitium through deposition of matrix proteins are hallmarks of the myocardial response to pressure overload. Increased expression of vimentin has been demonstrated in the interstitial space in fibrotic heart tissues. Several lines of evidence suggest that cardiac fibroblasts are a heterogeneous population and derive from various distinct tissue niches in physiological and pathological conditions. Conventionally, adult fibroblasts considered to be derived directly from resident embryonic, mesenchymal cells and epithelial-mesenchymal transition (EMT), and their increase in number is only as a result of the proliferation of resident fibroblasts. Zeisberg and colleague have revealed that endothelial cells undergo the Endothelial-mesenchymal transition (EndMT) and contribute to the total pool of cardiac fibroblasts during fibrosis. On the contrary, a very recent study showed that fibroblast accumulation associated with pressure overload hypertrophy resulted from activation and proliferation of these resident
lineages and not EndoMT, hematopoietic progenitor recruitment, or epicardial activation\textsuperscript{78}. In line with a previous study, an increase in the number of cells expressing GFP in the cardiac interstitium of VimCreER:mt/mG bitransgenic mice post TAC was observed with respect to sham control (Figure 4.15 B). Further analysis of these accumulated GFP-expressing cell in the fibrotic area of pressure overload myocardium of VimCreER:mt/mG bitransgenic mice showed no co-localization between endothelial cell marker (CD31) and GFP-positive cells suggesting that the increased VimCreER marked cells are not of EndMT origin (Figure 4.16). Rather co-labeling with different fibroblast markers (Vimentin or P4HB) along with GFP showed double positive cells for GFP and fibroblast markers (Fig 4.15 C&D) in these mice, thereby confirming the accumulated GFP-expressing cells as fibroblasts.

Several high-profile studies have used Fsp1 as a fibroblast-specific marker to investigate the role of fibroblasts in the pathophysiology of disease through the development of fibroblast-specific knockout mice\textsuperscript{187} or to study the origin of fibroblasts in fibrotic conditions\textsuperscript{20}. However, Fsp1 was found to be expressed in other cell types entering injured tissues, such as inflammatory macrophages\textsuperscript{188}, dendritic cells\textsuperscript{189}, lymphocytes\textsuperscript{190}, and vascular smooth muscle cells\textsuperscript{191}. Because of the foregoing issues with Fsp1 as a fibroblast marker, we analyzed the Fsp1 promoter activity in fibrotic areas in heart sections of Fsp1-cre: mt/mG bitransgenic mice post TAC. We found there was a slight increase in the number of GFP-expressing cells in interstitial spaces, but this was still lower than what was observed in case TAC operated VimCreER bi-transgenic mice. Co-labeling of these GFP-expressing cells in pressure overload myocardium of Fsp1Cre bi-transgenic mice with fibroblast markers confirmed some of the GFP-expressing cells as fibroblasts. Also presence of GFP and CD31 double positive cells indicate that a population of the GFP-expressing cells were endothelial cells that might have the EndMT origin in Fsp1-cre mice. Our results were consistent with the recent findings of Kong et al. (2014)\textsuperscript{76}, where a large number of Fsp1 positive cells were identified as endothelial cells, inflammatory leukocytes, and arteriolar smooth muscle cells in pressure overload myocardium of Fsp1-GFP reporter mice\textsuperscript{76}.

In terms of cardiac phenotype, we have observed decreased ejection fraction and fractional shorting in case of Fsp1-Cre mice post TAC with respect to its littermate control. On the other hand, insignificant change and difference in cardiac functions were observed in tamoxifen or vehicle-injected VimCreER mice post TAC. Thereby, suggesting that following TAC VimCreER mice are less susceptible to heart failure than Fsp1-Cre mice.
5.6 Vimentin promoter activity in endothelial cell of VimCreER; mT/mG bitransgenic mice during postnatal development

In the developing murine heart by E12.5 days post fertilization (dpf), cardiac fibroblasts were observed, and their numbers progressively increase throughout development. Using flow cytometry, Banerjee et al. (2007) estimated that cardiac fibroblasts comprise approximately 14% of all murine heart cells at E18.5 dpf and their number progressively increases in the heart in postnatal life comprising 27% of the total number of cells in the adult murine heart. Similarly during murine development, vimentin expression commences on embryonic day 7.5 (E7.5) and becomes predominant in the primitive streak stage, while in adult mice, vimentin expression was reported to be limited to connective tissue mesenchymal cells in the central nervous system and muscle. We determined the vimentin promoter activity during early and late postnatal development hypothesizing that activation of vimentin promoter at the earlier stage would have more specific and restricted recombination in cardiac fibroblast in the heart. In addition, the tamoxifen-independent Cre recombination observed was eluded. Here we found successful recombination induced in cells of cardiac interstitium but not in the cardiomyocytes in heart sections of VimCreER; mT/mG pups at postnatal day 7 (P7) and 6 weeks old adults as measured by the mGFP direct fluorescence (Figure 4.20). Further immunostaining with different fibroblast markers confirmed a high number of the GFP-expressing (i.e recombined) cells as fibroblasts. However, a large fraction of these GFP-expressing cells was also identified as endothelial cells by staining with CD31 (endothelial cell marker) both at P7 and in 6 weeks old mice hearts.

Cellular switching from an epithelial-to-mesenchymal (EMT) phenotype, and conversely from a mesenchymal-to-epithelial (MET) phenotype, are important biological programs that are functioning throughout the life of a mammalian organism. The heart forms via a remarkable series of sequential waves of EMT/MET. All cells in the heart arise from one or more EMTs. During heart development, cardiogenic mesodermal cells give rise to two types of heart cells, myocardial and endocardial cells. Most of the endocardial cells express endothelial markers, such as VE-cadherin and CD31. A population of endocardial cells in the atrioventricular canal differentiates into the mesenchymal heart cushion cells, forming cardiac septa and valves. Similarly, several studies have shown that endocardial–endothelial cells might transdifferentiate into mesenchymal cells during the formation of endocardial cushion tissue in the early embryonic chick heart. Since endothelial cells undergoing
EndoMT during embryonic heart development they have been shown to expresses markers of mesenchymal cells, this could be one of the explanation for activation of vimentin promoter in endothelial cells in VimCreER; mT/mG bitransgenic mice during postnatal development. Another reason could be the expression of vimentin in a wide range of cells. Since our objective was to check for the maintenance of specificity by vimentin promoter when activated early during mouse development we discovered that the promoter was active in the vimentin expressing endothelial cells along with fibroblasts. In conclusion, significant recombination efficacies could not be attained in VimCreER; mT/mG bitransgenic offspring from tamoxifen-injected lactating mothers as shown in adult recombination (70%).

5.7 Challenges and limitations of study

In our understanding, it may be that the diverse origins of cardiac fibroblasts preclude the discovery of a universal “one size fits all” marker. But by better understanding which markers are reliable under certain conditions and which combinations of markers best encompass the normal, quiescent cardiac fibroblast versus the transient, activated fibroblast, we may begin to probe this intricate system in greater detail and with increased precision. All fibroblast-specific transgenic mouse lines studied so far have considerable limitations. The recently identified fibroblast promoter fibroblast-specific protein 1 (FSP1, also known as S100A4), labels only a subset of fibroblasts and is expressed by several other cells including endothelial and immune cells which is also confirmed by our study along with other recent studies. Periostin, a matricellular protein, and another mesenchymal cell marker are found to be expressed in connective tissues including the periodontal ligament, tendons, skin and bone, in neoplastic tissues, cardiovascular disease, as well as in connective tissue wound repair. At present, Postn-Cre66 and inducible Tcf21 (iCre) MerCreMer71 transgenic mouse lines are two of the most promising tools for lineage mapping and genetically manipulating CFs, and particularly cardiac fibroblasts. However, considering the negligible expression of periostin in normal hearts, these animals are of limited value for targeting gene expression in normal cardiac fibroblasts.

Vimentin expression, on the other hand, is often reported in a wide range of other cell types including endothelial cells, vascular smooth muscle cells lining the blood vessels, macrophages, neutrophils, and leukocytes25. Despite this drawback here we found Vimentin promoter as more promising promoter and VimCreER100 mouse line as another genetic means to investigate the function of cardiac fibroblast cells in vivo. As compared to other identified fibroblast-specific transgenic mouse lines expressing Cre-recombinase driven under Periostin (Postn) or Fibroblast specific protein-1...
Discussion

(Fsp1), VimCreER<sup>tg/0</sup> mouse line had Cre recombination in the cells of interstitial spaces mostly cardiac fibroblast in heart tissue of adult mouse and also in pressure overload myocardium of these mice. This specificity was also observed in isolated cells (CFs) from this transgenic mouse. In addition, unlike Fsp1-Cre mice, VimCreER<sup>tg/0</sup> mice also showed fibroblast accumulation associated in response to TAC that might result from activation and proliferation of the resident CFs lineages and not EndoMT. Some missing experiments in this study have become one of its limitations. It would have been useful if through FACS further analysis of GFP-expressing cells as hematopoietic progenitor recruitment, or as epicardial activated cells can be done which can be helpful in confirming the origin of these accumulated fibroblasts in TAC-operated VimCreER mice. Another limitation was the tamoxifen independent recombinase activity observed in heart tissue of some animals from this transgenic mouse line. The possible explanations for this discrepancy is that maybe after many generations of backcrossing to the C57BL/6 background, the expression of the randomly integrated VimCreER transgene is enhanced, thereby increasing its probability of nuclear entry, even in tamoxifen untreated cells or it’s the old CreERT2 system which has disadvantage of showing some leak in the animals as published by few authors. Maybe the use of ERT2CreERT2 system instead of CreERT2 would ensure tight regulation. As ERT2CreERT2 double fusion has a higher affinity for Hsp90 to form a tighter complex. It is having less activity due to the double fusion, and thus, less background activity. It is also possible that degradation of CreERT2 results in the generation of “active Cre” lacking the regulatory domain, whereas ERT2CreERT2 is still inactive even after losing one regulatory domain. Thus, use of ERT2CreERT2 system would have been a better option for studying inducible Cre activity.

5.8 Conclusions

Past several years have yielded remarkable insights and progress in identifying and mapping the various cell lineages which initially give rise to the developing heart and deciphering many of the key morphological events that are required for both normal heart development and the underlying causes of congenital heart defects. Despite this recent progress, our understanding of the mechanisms of induction and lineage specification of early non-cardiomyocyte cell fate is still rudimentary, and the signals that instruct key precursors to select a CFs cell-lineage remains unclear. In this study, we analyze the Vimentin promoter activity in heart tissue. In our understanding, vimentin promoter can serve as a useful tool for studying CFs in both physiological and pathophysiological conditions. Characterization of VimCreER<sup>tg/0</sup> mouse line demonstrated vimentin promoter
drive Cre recombination in the interstitial cells in heart specifically in the majority of CFs. However, recombination was also documented in other tissues including Liver, lung, kidney and skeletal muscle. Recombination in endothelial cells along with fibroblast in VimCreER\textsuperscript{10/0} mice during postnatal development somehow hinders the use of this mouse line for development studies. With some drawbacks and in comparison with other known fibroblast-Cre mouse line, this mouse line is still highly efficient and specific to cardiac fibroblast in the heart.
6 SUMMARY/ ZUSAMMENFASSUNG

Cardiac fibroblasts comprise a substantial component of the mammalian heart and are intimately involved in both normal cardiac development and injury through paracrine, mechanical, and potentially electrical interactions with cardiomyocytes. While there has been a steady increase in research investigating these interactions, further in vivo work is critical for addressing the functional contribution of each element both in utero and following injury to more aptly describe the dynamic roles of cardiac fibroblasts in development and disease. Obstacles such as the absence of a comprehensive cardiac fibroblast marker have hindered in vivo analysis of these interactions to date; however, new techniques such as utilizing the 3.9kbPeriostin-Cre and Fsp1-Cre lines for lineage mapping and genetic modification of in utero and adult cardiac fibroblasts, as well as an increasing number of fibroblast markers are emerging to help address these challenges. Here we tried to study the CF-specific transgenesis by characterizing two transgenic mouse lines (Ccdc80-Cre<sup>tg0</sup> and VimCreER<sup>tg0</sup>). The study was conducted in two parts. The first part of the study was based on microarray database approach where Ccdc80 gene was found out as a promising candidate in search for CF-specific genes. Validation of Ccdc80 promoter sequence constructs confirms the promoter activity in vitro. Further, a mouse line where Ccdc80 promoter consecutively drives the expression of Cre recombinase was generated. Furthermore, validation of promoter activity in frozen heart section of Ccdc80-Cre<sup>tg0</sup>;Rosa26<sup>LacZ</sup> bitransgenic mice through β-gal staining showed Cre recombination in a small subset of CM instead of CF that somehow question about the different in vitro and in vivo promoter activity of Ccdc80.

In the second part of the study, we characterised the VimCreER<sup>tg0</sup> mouse line as CF-specific in comparison with Fsp1-Cre<sup>tg0</sup> mouse line (a known fibroblast promoter) by using dual fluorescent reporter mice (mTom/mGFP<sup>fox/+</sup>). In comparison with Fsp1-Cre;mT/mG<sup>fox/+/</sup> bitransgenic mice, VimCreER;mT/mG<sup>fox/+</sup> bitransgenic mice upon tamoxifen injection showed Cre recombination (i.e GDP-expressing cells) in a majority of interstitial cells in the myocardium. Co-staining of these VimCreER marked cells with different cell markers along with GFP both in heart tissue and in isolated cardiac cells confirms them as fibroblasts. Furthermore, validation of vimentin promoter activity in response to pressure overload (TAC) showed an increase in the VimCreER marked cells in the cardiac interstitium in these mice where a majority of them were stained for fibroblast markers. On the other hand, TAC-operated Fsp1-Cre;mT/mG<sup>fox/+/</sup> bitransgenic mice showed a slight increase in the number of
Fsp1-Cre marked cells in these mice, and most of these cells were labeled as endothelial cells along with fibroblasts. Cre recombination in early and postnatal development in VimCreER<sup>tg<sup>0</sup> mice is somehow not able to attain the significant efficiency as observed in adult recombination. In our understanding and also in comparison with other fibroblast-specific established transgenic mouse line expressing Cre-recombinase driven under Fibroblast specific protein-1 or Periostin promoters, VimCreER<sup>tg<sup>0</sup> mice may represent as another genetic mean to investigate the function of cardiac fibroblast cells both in physiological and pathophysiological conditions <i>in vivo</i>. 

In dieser Arbeit wurden zwei transgene Mauslinien auf ihre Spezifität für kardiale Fibroblasten charakterisiert: Ccdc80-Cretg/0 and VimCreER<sup>tg/0</sup>. Zunächst wurde basierend auf einer Microarray Datenbank nach einem Gen gesucht, das spezifisch in kardialen Fibroblasten exprimiert wird. Ccdc80 erwies sich als vielversprechender Kandidat. Der Ccdc80-Promotor war in vitro aktiv. Im Folgenden wurde eine Mauslinie generiert, in der der Ccdc80-Promotor, wenn aktiv, konstitutiv die Expression der Cre-Rekombinase induziert. Die Validierung des Promotors in β-gal gefärbten Herz-Gefrierschnitten von Ccdc80-Cre<sup>tg/0</sup>;Rosa26LacZ Mäusen ergab eine Cre Expression in wenigen Kardiomyozyten und nicht in Fibroblasten, was eine kritische Beurteilung der Übertragbarkeit der Aktivität des Ccdc80-Promotors von in vitro nach in vivo aufwirft.

In einem zweiten Teil wurde die VimCreER<sup>tg/0</sup> Mauslinie mit der Fsp1-Cre<sup>tg/0</sup> (einem bekannten Fibroblastenpromotor) im Hinblick auf die Spezifität für kardiale Fibroblasten verglichen. Hierzu wurde die duale Fluoreszenz-Reporterlinie mTom/mGFP<sup>flx/flx</sup> verwendet. Die VimCreER;mT/mG<sup>flx/flx</sup> Mäuse zeigten nach Injektion von Tamoxifen Cre-Rekombination (i.e. GFP-positive Zellen) in der Mehrheit der interstitiellen Zellen im Myokard. Co-Färbungen dieser GFP-positiven Zellen mit Markern für verschiedene Zellarten zeigte, dass es es sich dabei sowohl im Herzhewebe als auch in isolierten kardialen Zellen um Fibroblasten handelte. Darüber hinaus stieg die Zahl der positiven Zellen im Interstitium nach induzierter Aortenkonstruktion (TAC). Auch hier war die Mehrheit positiv für Fibroblastenmarker. Dagegen zeigten Fsp1-Cre;mT/mG<sup>flx/flx</sup> Mäuse nach TAC nur einen leichten Anstieg in der Zahl an GFP-positiven Zellen, wobei die meisten davon Endothelzellen waren.
In der frühen und postnatalen Entwicklungsphase in VimCreER^{tg/0} ist die Rekombination signifikant weniger effizient als in adulten Tieren. Im Vergleich zu anderen etablierten Fibroblasten-spezifischen transgenen Cre-Rekombinase exprimierenden Linien wie Fsp1-Cre scheint VimCreERtg/0 gut geeignet, um die Funktion kardialer Fibroblasten in vivo unter physiologischen und pathophysiologischen Bedingungen zu untersuchen.
7 REFERENCE


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## Tabular result of the Microarray analysis

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<td>cell cycle exit and neuronal differentiation 1</td>
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8.2 Map of Ccdc80 and Periostin promoter constructs used for study
8.3 DNA sequence of Ccdc80-Cre targeting Vector

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   GTAAGGAGAC
0061 CCTGTCTCAA AAGACCAACC ACAAAATCAA ACAACCCTAC TAAATTAATC
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0121 TTTCCCTCTG GCTGAGTACA CACATCAACC AGGCTCAGTA TCTCCTCTGG
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0181 CCCCCATCAAA GTCTTTCTTG GGGTGCAAGA AAATGGGCTG GTCTAGATGAG
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0241 AAATAGAAA TCCAGATCTT CATAAGCCAC AAATCATTCT AGGTCGCCCC
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   ACATCCCCG
0361 AGAGCTTTTTG TGAGACCAACA GTTAGGGTTT AGACAGCAAG ATGGAAAACCT
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0481 GTTTAATGCGG CCAACCTACTG ACATGGGATG TCAGAATGAAG AGTGAGCCT
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0541 GAAGAGAAAAG GCAGTCAGGG TGGTTGTTGTG TCAGTGCTTTC CCCCTCTGGA
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0601 CAGCCTTCTTG TTATAACTTC TCTCAACTCT ACTCCCTCAC GGAGCACCGGG
   TGTCCAGTAC
0661 TGTTCTATT GTTGGGAGG GGGTGATGAAAA TACATGAGAA TTCAGGAAAT
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0721 TTTTAAGGAA CTACCGAGAA AGGCAAAAGG TAAGCTACAC GATTGTTTTA
   GAAAGCAGAG
0781 CTCCAGAAGG ATAACTTTGC TCTGGCTGAA CAAATCAACG AGAGATCCTT
   TATGAAATCT
0841 TTAATTGAAAG GGGGAAACAC AAGAACCCTA AAGCATATTT TAAAAAGGGG
   ACAGTGTTGG
0901 CAGAAATAGGG AAATGAAGG CAGGCCAACCT CACTGCAGGA TACCACGTCT
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0961 GCCACCCCCG TTAATTATTT GTGGAATAC GAGGAATGTA TGGAAACATT
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1021 GACCAGCTTT AATGGAAGGA TGGGACTGTA CCAAGCTCAGT CCAGGGCTG
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1081 GAGTCTCTCA AAGTGGCAAG CCCTTGAAAA TACGTAGTAA ACTGATATCT
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1141 GGGAAAAAAC TAGAAGAAAA TGGCAACACC AAATAAGAGG AGAAGGCTG
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1201 TGTGAACCAT ATGGGGAAAT AATGAATAAC ACACAGAGAC TCTATTACCG
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126
1261 ACTAATGAGC TTTGCGGTTT GGATGAAATG GGATTTGTGA TGGTGGGCAT CATTCCTGTT
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1441 GAGGGATGGA GCTGGTTGTG GGATGGATGC AACTAGGGAT GAGGTTTTTCA CCATCCAGG
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1561 CGACAGTTAA TATAATTTTG GAGAGTTTGG GAGAAATGCC CACAAGGACCT GAGATTCTT
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1681 ATAGTCTCTCT CTGTAAGGCC TTATATGACT ATTTCTTTTG GTGTGGTGTG GCCGCTGGTG
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1921 TCATGAACAC ACACACACAG GCAATGTCTG GTGACTGCAG AAGTCAGAAC GAGATATCTT
1981 GCTGATGTGA GTAATATTCC TCAGCCACTC TCTACCTTAT TTATTAATAG TGTATGAAG
2041 GTCTTCTTCA AGAGCAACTG ATGCTCTTTA CCAATGAGTC CGACTACGCC CTCCACCTTAA
2101 CTTTTTGTGAG ACAGGGGCTC CCATGCACTC TAGAGCTGTT TGTATCAGCT AGAAAGGCTG
2161 AGAGTAGGAGT TCCAGTGACC CACCTGCTCC TGCTCCTTTT CCCTCAGTGC CAGAGGCTA
2221 GCCTCAGTGC CCAATTTTTC TGTAATAGCT GGGATCTGTA ATTCAGGCCT TCATCTGGCG
2281 AGAAGCAAAAG ACTTATCCAG CTGAGGCACC TTCCACCTAA AGCATAACAC TACTTCAGG
2341 GACTGACATAA GCATTATTAGG TTCTTAAAGC ATCTGGAGAG CTGAATATTG TAACACACAC
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Appendix
Appendix

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CCCAATTTAGC
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AACTCAAAGC
2701 ATAAAGCAGG CAGAAAGAGA TAAAATATATA CACGTGGAAC TATAAATAGA
TGCTACATGT
2761 CTGCAAATAGC AATGCAAATA TCTGTTGCCC AAATGAGCAG CTGTCTCTTAG
GAACCCAGT
2821 CAGATGGACC AACCTGACGA CTTTCCCCTT CTCAAACCTGT AGTGTGAGAA
TCCACAGGAG
2881 GGGGAAGGTC CGGCCCTATG GCTCGGTACG GAAGGGGTTA AGTCCTCAGT
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2941 TGAGGTTGTATT TACAATGATG TGTAAGGTTTT CAAAACCTTTTAG CCAAGCCCCA
GCCAGCTCCT
3001 CCCTCTTCCTCC CGGCGTTTACA CAGACTCTCC ATGTACTGAG AGGGGAAGGAA
GCGGTTT
3061 GCTGATCCTGT TAAATCTTTA GTGAAGGTCTT CTGGATTCTT TTTGATTGTTG
TTT
3121 GTTTGAGCAGGA ACCTGAAGGT AGCCCTGACA TTTCTGGGAT GGAACCCAGA
TGAGGAACCAAA
3181 ATAGAAGGAGG AAGGGGGAA AAAGGGAGAA GAGAAGGTTG GGGGAATAGAA
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3241 GACCGGAGGG AAGGGAAAA TTTGCCTCTT TACACATCAG GCTCCTCCTTG
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3301 CGCTGAAACCC ATATGAAATCC CAGACGTTTTTT TTTTTTTTTTTTTTTTTTT
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3361 CGCTCGGAAA ACAAAGGACT CGGCCAGACT GCAGGAGGG GAGGTTGATA
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CAGAGAATGAA
3601 AAGACACAGC CAGCAAAAGG AATCGGCCCC GGTGGGGCTCTC CCTGGGGCGT
CTTGGCAGAC
3661 AGACAGAGAG ACGAGAGGCA GGAGGAGAAC CATCTACCTG AGGAAAAGCA
CCTGAGGACC
3721 AACCTTCACG TTTCTGTGGA AAGCCCTCTGC AAGCATCTCC AGTGAACACTC
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3781 GCCAACCTTC CTCTCTGCTG TTCGTTTCTT TTGGCTTGCT TTGAAGACAT
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3841 TGCACATTCA CAGTACAAAT TCATAATTAG CATGCTCAGT TTACACAGTC
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Appendix

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4201 CcCTcTGcGCcG cTccccAAcTG cATAcTAcAG cACcAACcGc ATCTTTGcTAcA
4261 TGcGGGCcGCcG CTCTAGcCTAG A GCCTcCTGcCT AACcAGTCtC ATGCcTTcCTT
4321 cAGcTTcCTGC cGCAcAGGTcG GttATGCtGT cGTCCTcATcC A TTTGGcCAcA
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Appendix

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5341 GTAAATATTG TCATGAACTA TATCGAATAC CTGGATAGTG AAACAGGGGC AATGGTGCGC
5401 CTGCTGGAAG ATGGCGATGG ACCGGTGGAA CAAAACTTA TTTCTGAAGA AGATCTGTGA
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5521 TGCCCTGGCT CACAAATACC ACTGAGATCT TTTTCCCTCT GCCAAAAATT ATGGGACAT
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5761 ATGAACAAAG GTGGCTATAA AGAGGTCATC AGATATGAA AGCAGCCCTT GCTGCTGGAC
5821 CTTTATCCTA TAGAAAAGCC TTAGACTTGG GTTAGATTTT TTTTATATT TGTGTTGATGT
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6481 GCATCTCTAGG TGTTGGTTGG CCAAACTCAT TATGTGATCT TATGTGCTG TGGATCCGCTG
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7921 GTTACATGAT CCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC
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7981 GTCAAGAAGTA AGTTGGCCGC AGTTTTATCA CTCATGTTA TGGCAGCACT
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8341 CAAAATGGCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT GTTGAATACT
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8401 CTTTTTCAAT ATTATTGAG CATTTATCAG GGTTATGTC TCAAGAGCG
    ATACATATT
8461 GAATGTATTT AAAAAATCAA ACAAATAGGG GTTCCGGCGA CATTTCCCC
    AGAAATGCCA
8521 CCTGGGTCGA CTAAGGATC CCTACCGGTG ATATCCCGGA GCCCATCAA

Legend

ACAA ----- attB1 and attB2 gateway cloning site
TCAG ----- Ccdd80 promoter sequence forward and reverse
primes
TGCG ----- Ccdd80 promoter sequence
AGAA ----- Cre_NLS sequence
8.4 Acknowledgement

First and foremost I would like to thank my supervisor Prof Dr. Dr. Stefan Engelhardt whose guidance, support and encouragement helped me to accomplish this work. I appreciate all his contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating. I would like to thank Prof Angelika Schnieke for being my first supervisor. My heartfelt thanks to Robert F. Schwabe, M.D., PD Dr. med. Michael Quante for providing us with VimCreERT2 mice and with the useful information time to time. I would like to acknowledge Dr Bernhard Laggerbauer for reviewing and for his valuable comments and support during my thesis writing. I would like to thank all former and present group members for the constant willingness to help and support in the daily laboratory work as well as for the really great atmosphere in the team. My special thanks to Isabell Flohrschtuz and Lucia Koblitz for the neonatal rat and mouse cardiomyocytes and cardiac fibroblast cells isolation. Korneliya Sakac and Pascal for TAC operations, which represented an essential basis for numerous experiments. Andrea Ahles (Ph.D) for her immense support and useful guidance in understanding confocal microscopy and also for numerous joint ventures. Astrid Vens and Sabine Brummer for the professional collaboration and the great time in the laboratory and on private trips. Deepak Ramanujam (Ph.D), Simon Leierseder (Ph.D), Jaya Ganesan (Ph.D), Kathleen Meyer, Yassine Sassi (Ph.D.), Michael Regn, Katrin Domes (Ph.D), Xavier Loyer (Ph.D.) have also been very supportive of me in many ways and always had an open ear, which I am very grateful.

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8.5 Curriculum vitae

Name: Megha Saraiya
Geburtstag/-ort: 27.03.1981 in Berasia, India
Adresse: Hansjakobstraße 95, 81825 München
Telefon: 017632041581
Email: saraiyamegha@gmail.com

Studium und Praktika
2003-2005 M.Sc. (Microbiology), Barkatullah University, India

Betreuer: Dr. A.K. Shasany and Dr.M.P.Darokar,

2001-2003 B.Sc. (Biotechnology) Barkatullah University, India

Advanced Instrumental Training at Central Laboratory Center of M.P Council of Science & Technology, Bhopal, MP (2001).

Arbeit
01/2006–02/2008 Als Projektassistentin im Projekt gearbeitet
Title: Spermato-transgenesis in Primates for developing animal model to study HIV/AIDS.
Betreuer: National Institute of Immunology, New Delhi.

Promotion
10/2009- heute Doktorarbeit am Institut fur Pharmakologie und Toxikologie der Technische Universität München.
Title: Cardiac-fibroblast specific transgensis.
Betreuer: Prof. Dr. Dr. Stefan Engelhardt.

04/2013-04/2014 Mitglied der TUM Graduate School der Technische Universität München.

Honor & Award
10/2009 –03/2013 Awarded DAAD (Deutscher Academischer Austauch Dienst) Scholarship for Doctoral studies in Germany
Publications
