MECHANISTIC ANALYSIS OF SRF AND THE MYOCARDIN FAMILY OF COACTIVATORS DURING MUSCLE DEVELOPMENT

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To My Mom and Dad,

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To My Wife,

Shurong Chang

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MECHANISTIC ANALYSIS OF SRF AND THE MYOCARDIN FAMILY OF COACTIVATORS DURING MUSCLE DEVELOPMENT

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The precise mechanism of how specification and differentiation of different muscle types are controlled by a large number of transcription factors has been a long-standing question in developmental biology. Using animal models with tissue-specific deletions of various transcription factors, coupled with biochemical studies, the molecular mechanisms regulating muscle development and growth are being elucidated.

Serum response factor (SRF), a muscle-enriched transcription factor, activates the expression of numerous muscle genes by recruiting a variety of partner proteins. The function of SRF in each muscle type *in vivo* is clouded by the fact that SRF mutant mice die before

gastrulation without the formation of mesoderm. Generating a tissue-specific deletion of the *SRF* gene, I found that SRF is required for skeletal muscle growth and maturation.

Myocardin was identified as a cardiac and smooth muscle-specific transcriptional coactivator of SRF. Mice lacking *myocardin* die during early embryogenesis due to cardiovascular defects, which are caused by the failure of vascular smooth muscle to differentiate. Together with the data that overexpression of myocardin in non-muscle cells can activate the smooth muscle gene program, we demonstrate that myocardin is both required and sufficient for smooth muscle differentiation. Two Myocardin Related Transcription Factors, referred to as MRTF-A and B, which also interact with SRF and stimulate its transcriptional activity, are expressed in numerous embryonic and adult tissues, implying their potential to modulate SRF target genes in a wide range of tissues. Consistent with the role of SRF during skeletal muscle development, a dominant-negative form of MRTF-A interferes with skeletal muscle development in transgenic mice.

To further elucidate MRTF-A's function, I generated *MTTF-A* mutant mice by gene homologous recombination. Female *MRTF-A* mutant mice fail to nurture their offspring due to mammary defects. While milk is produced at a normal level, mammary myoepithelial cells, which are similar to smooth muscle cells and required for milk ejection, fail to differentiate and undergo programmed cell death during lactation.

Taken together, these data indicated that SRF regulates specification or maturation of different muscle types by interacting with various members of the myocardin family of coactivators.

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ANF	atrial natriuretic factor
bHLH	basic helix-loop-helix
CALLA	common acute lymphoblastic leukaemia antigen
cDNA	complementary DNA
CK	cytokeratin
DMEM	Dulbecco's Modified Eagle's Medium
DN	dominant-negative
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GST	glutathione S-transferase
HAT	histone acetyltransferase
HDAC	histone deacetyltransferase
IP	immunoprecipitation
MADS box	MCM1, Agamous, Deficiens, and SRF box
MAP (kinase)	mitogen activated protein kinase
MRTF	myocardin-related transcription factor
MEF2	myocyte enhancer factor 2
NLS	nuclear localization signal

OTR	oxytocin receptor
PBS	phosphate-buffer saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SM	smooth muscle
SMC	smooth muscle cell
SM-MHC	smooth muscle myosin heavy chain
SM-MLCK	smooth muscle light chain kinase
SM22	smooth muscle 22 kDa protein
SRF	serum response factor
TAD	transcriptional activation domain
TCF	ternary complex factor
Tg	transgenic
WAP	whey acidic protein
WT	wild-type

Chapter I

Introduction: Transcriptional Control of Muscle Development

Introduction

There are three types of muscles in vertebrates, skeletal muscle, cardiac muscle and smooth muscle. These different muscles share common properties, mainly supporting the body, providing contractility and excitability, and express overlapping sets of muscle structural genes. However, each of them is unique in many respects, including the spectrum of muscle gene isoforms expressed, morphology, contractile properties and the ability to divide.

During vertebrate embryogenesis, skeletal, cardiac and smooth muscle cells arise from distinct mesodermal precursors in different regions of the embryo [1]. Skeletal muscle arises from the somites which form in a rostrocaudal progression by segmentation of the paraxial mesoderm lateral to neural tube. Newly formed somites appear as epithelial spheres, which subsequently compartmentalize to form dermamyotome and sclerotome. The sclerotome is comprised of mesenchymal cells that eventually give rise to ribs and vertebrae. Cells from dorsomedial region of the dermamyotome adjacent to neural tube later form the myotome, which gives rise to the vertebral and back muscles. Cells from the ventrolateral region of the dermamyotome migrate out to form limb muscle and body wall muscle [2]. Cardiac muscle is derived from cells in the anterior lateral plate mesoderm which are committed to the cardiogenic fate soon after gastrulation. These precardial cells subsequently form the primitive heart tube at the central midline, which undergoes looping and chamber maturation to form the mature multi-chambered heart [3].

Unlike cardiac and skeletal muscles, which arise from distinct embryonic origins, smooth muscle arises from multiple, not very well defined areas throughout the embryo. For

example, portions of smooth muscle cells in the great vessels (systemic aorta, pulmonary arteries and carotid arteries) come from neural crest cells; the coronary artery smooth muscle cells arise from the proepicardial organ; and majority of smooth muscle cells, including those of visceral organs, are derived from local mesenchymal cells [4, 5].

Recently, there has been dramatic progress toward understanding the molecular mechanism controlling skeletal muscle development, and to some extent, cardiac muscle development, in contrast, little was known about the transcriptional control of smooth muscle development.

Skeletal Muscle Development

During vertebrate embryogenesis, the paraxial mesoderm will give rise to somites, blocks of mesodermal cells on both sides of the neural tube, which will produce many of the connective tissues of the body, including bone, muscle, cartilage and demis. Muscle cells come from two cell lineages in the somite, the hypaxial myotome and the epaxial myotome. Paracrine factors instruct these myotome cells to become muscles. Wnts proteins (Wnt1 and Wnt3a) from dorsal neural tube, in combination with low concentrations of Sonic hedgehog from the notochord and floor plate, induce the epaxial myotome. While Wnt proteins from the epidermis, in conjunction with BMP4 and FGF5 from the lateral plate mesoderm, are thought to induce the hypaxial myotome [6, 7]. The myotome cells will produce myogenic bHLH transcription factors and become committed muscle cell precursors ─ myoblasts. These cells then align with each other and fuse to form the multinucleated myotubes characteristic of muscle tissue. Thus the multinucleated myotube cells are the product of

several myoblasts joining together and fusion of their cell membranes[8, 9]. The transcription factors that play important roles during myogenesis are discussed below.

Myogenic bHLH proteins The myogenic regulatory factors (MRFs) are part of a superfamily of basic helix-loop-helix (bHLH) transcription factors. The MRF subfamily consists of MyoD, myogenin, myf5 and MRF4, which are expressed exclusively in skeletal muscle [10]. The MRF proteins contain a conserved basic DNA-binding domain and a helix-loop-helix motif required for heterodimerization. These proteins form heterodimers with ubiquitously bHLH proteins known as E-proteins, to bind to a consensus DNA sequence CANNTG (E-box), which is found in the control regions of most skeletal muscle genes. These myogenic proteins have the striking ability to activate the entire skeletal muscle differentiation program when introduced into a variety of non-muscle cells, including cells from all three germ layers. Thus they are considered to be the "master regulators" of skeletal muscle differentiation. Their roles in muscle development have been confirmed by gene knockout studies. MyoD and myf5 play redundant roles in the generation of myoblasts, whereas myogenin, as well as MRF4 controls myoblast differentiation [1].

MEF2 proteins Another family of transcription factors that play essential roles in skeletal muscle development is the MEF2 family of proteins, including MEF2 A-D in vertebrate, which are highly enriched in all three muscle lineages [11, 12]. MEF2 proteins belong to the MADS box family of transcription factors. MADS box is named after the first four proteins in which this domain is identified, including MCM1, which regulates mating type-specific gene expression in yeast; Agamous and Deficiens, which act as homeotic factors that control flower development; and Serum Response Factor (SRF), which controls

serum-inducible and muscle-specific gene expression [13]. The MADS box of MEF2 proteins binds to a consensus DNA sequence YTA(A/T)$_4$TAR, termed the MEF2 site, which has been found in a variety of muscle-specific promoters, and in many cases to be essential for muscle-specific expression. The definitive proof of the requirement of MEF2 for muscle development comes from the inactivation of the only MEF2 gene, *D-mef2* in flies. In the mutant fly, the precursor cells for all three muscle lineages are specified and positioned normally, but failed to form normal differentiated muscles, suggesting an obligatory role of MEF2 in muscle differentiation [14].

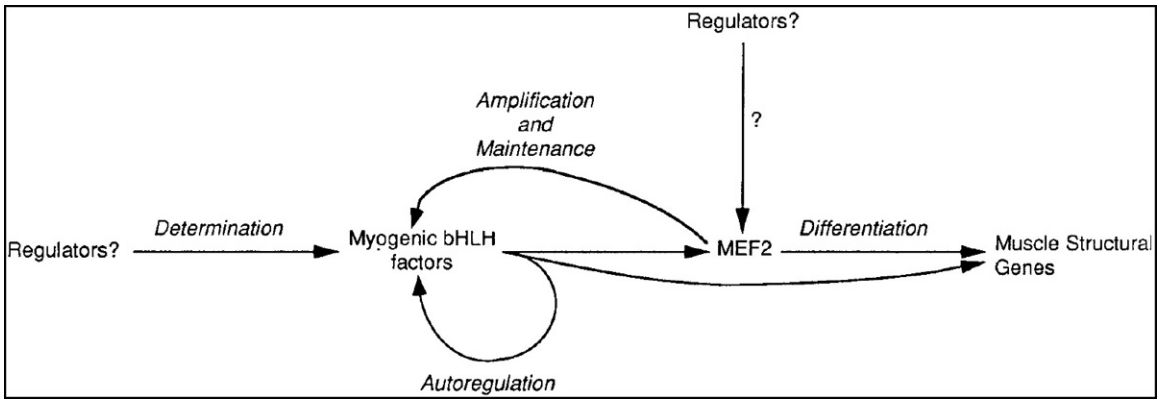

Fig.1.1. Myogenic bHLH proteins and MEF2 factors in the skeletal muscle lineage. Early mesodermal regulators induce the expression of myogenic bHLH factors during myoblast determination. Myogenic bHLH factors autoregulate their expression and induce the expression of MEF2, which binds the promoters of several myogenic bHLH genes, amplifying and maintaining their transcription. MEF2 and myogenic bHLH factors collaborate to induce muscle structural genes during differentiation. While MEF2 can be induced by myogenic bHLH factors in tissue culture, this has not yet been demonstrated in vivo. Other regulators might also initially induce MEF2 expression in skeletal muscle cells independent of myogenic bHLH factors. (Adapted from Olson et al., 1995)

Importantly, the myogenic bHLH proteins and MEF2 proteins physically interact and the interaction precisely correlates with their myogenic activity [15]. Furthermore, myogenic bHLH proteins upregulate their own expression and that of MEF2 factors. MEF2 proteins then feedback on myogenic bHLH factors and themselves to amplify and maintain their expression in committed muscle cell, thus establishing a mutually reinforcing regulatory circuit for skeletal myogenesis (Fig. 1.1) [1].

Heart Formation and Cardiac Muscle Development

The circulatory system, consisting of a heart, blood cells and an intricate system of blood vessels, is the first functional unit in the developing embryo, and the heart is the first functional organ. Studies in model organisms have revealed an evolutionarily conserved program that controls the genesis of cardiomyocytes from mesodermal stem cells and the subsequent activation of genes responsible for cardiac contractility and morphogenesis. Cardiomyocytes originate in the anterior lateral mesoderm soon after gastrulation [16]. They are produced in response to protein factors, including bone morphogenetic proteins, which are secreted from adjacent endoderm [17]. Soon after their specification, cardiac muscle cells converge along the ventral midline of the embryo to form a beating linear heart tube composed of distinct myocardial and endocardial layers separated by an extracellular matrix. The linear heart tube then undergoes rightward looping, which is essential for proper orientation of the pulmonary (right) and systemic (left) ventricles, and for alignment of the heart chambers with the vasculature. Each cardiac chamber balloons out from the outer

curvature of the looped heart tube in a segmental fashion. The four chambers then form and are separated by atrial and ventricular septa [3, 18].

In contrast to skeletal muscle—in which a single transcription factor, MyoD, is sufficient to activate the entire program of muscle differentiation—cardiac muscle differentiation is dependent on combinations of transcription factors [19, 20].

Homeobox proteins The homeobox gene tinman is required for the formation of the primitive heart in the fruit fly. A mammalian ortholog of tinman, called Nkx2-5 or Csx, is expressed in cardiac muscle cells from the onset of embryonic heart formation until adulthood. Tinman in flies is necessary for specification of cardiac lineage and directly activates D-mef2 expression [21, 22]. But in contrast to tinman, Nkx2.5 in mice, although highly conserved and restricted to the cardiac lineage, is not necessary for cardiac specification, instead it is required for proper cardiac looping and left ventricle development [23, 24], suggesting that other homeobox genes may play redundant roles.

GATA factors Tinman and Nkx2.5 interact with zinc finger transcription factors of the GATA family to activate cardiac gene expression [25]. These two classes of transcription factors also regulate each other's expression through mutually reinforcing positive feedback loops [26]. The fly GATA gene *pannier* is required for cardiogenesis [27]. In mice, loss of *GATA-4* leads to bilateral heart tubes and a reduced number of cardiomyocytes [28]. The less severe phenotype in mice can be partly attributed to the expression of other GATA factors (GATA 5 and 6) in the mouse heart.

MEF2 proteins MEF2 proteins have been found to interact with Nkx2.5 and GATA factors to synergistically activate expression of a variety of cardiac genes [12]. Mutation of

the only MEF2 gene in flies, *D-mef2*, led to differentiation defects in all three muscle lineages. This is partly recapitulated by the deletion of the *MEF2C* gene in mice, which develop hypoplasia of the right and left ventricles and vascular defects, leading to early embryonic lethality [29]. Deletion of the *MEF2A* gene in mice leads to a mitochondrial deficiency and cardiac sudden death [30].

HAND proteins During mouse heart development , the related bHLH transcription factors dHAND/HAND2 and eHAND/HAND1 are expressed predominantly in the right and left ventricle, respectively [31]. Deletion of *dHAND/HAND2* in the heart results in hypoplasia of the right ventricle [32]. *eHAND/HAND1* has also been implicated in left ventricle development, although early placenta defects precluded a detailed analysis of its role in the heart [33, 34]. This is corroborated by a conditional knockout of *eHAND/HAND1* in the heart [35]. The role of HAND proteins in heart development is further confirmed in zebrafish. Loss of the only *HAND* gene in zebrafish abolishes ventricle development [36].

More and more transcription factors have now been implicated in the network of regulating cardiac gene expression. It is increasingly evident that it is the combination of different factors that determines the specificity and intensity of gene expression. However, so far no single factor or a combination of factors is able to activate the entire cardiac gene program in non-muscle cells.

Blood Vessel Formation and Smooth Muscle Development

Although the heart is the first functional organ of the body, it does not even begin to pump until the vascular system of the embryo has established its first circulatory loops of

blood vessels. Blood vessels are constructed by two processes, vasculogenesis and angiogenesis. During vasculogenesis, blood vessels are created de novo from the lateral plate mesoderm. The splanchnic mesoderm cells are specified to become hemangioblasts, the precursors of both the blood cells and the blood vessels. These cells condense into aggregations that are often called blood islands. The inner cells of these blood islands become hematopoietic stem cells, while the outer layer cells become angioblasts, the progenitor cells of the blood vessels. The angioblasts then multiply and differentiate into endothelial cells, which form the lining of the blood vessels. Finally the endothelial cells form tubes and connect to form the primary capillary plexus, a network of capillaries. This process of primary vascular network formation occurs both within the embryo and in extra-embryonic tissue, the yolk sac [37-39].

After the phase of vasculogenesis, angiogenesis begins. New vessels form by sprouting from the capillaries or splitting an existing vessel. The capillaries also fuse with each other to form wider vessels, the arteries and veins. Vessel maturation requires the subsequent recruitment of surrounding mesenchymal cells and their differentiation into vascular smooth muscle cells. This process has been shown to involve the interaction of endothelial cells with mesenchymal cells and the release of specific growth factors such as platelet-derived growth factor [40, 41].

Therefore, endothelial differentiation is an early event followed by the formation of primitive tubes. The subsequent recruitment and differentiation of vascular smooth muscle cells is a later event leading to the formation of stable blood vessels. Growth factors including platelet-derived growth factor, βFGF, VEGF, angiopoietin-1, and transforming growth

factor-ß (TGF-ß) are key mediators of these events promoting proliferation, differentiation and migration of these cells. A series of transcription factors are either key regulators of the expression of either the growth factors or their receptors, or mediators of the cellular responses to these growth factors, as summarized in Fig. 1.2 [42].

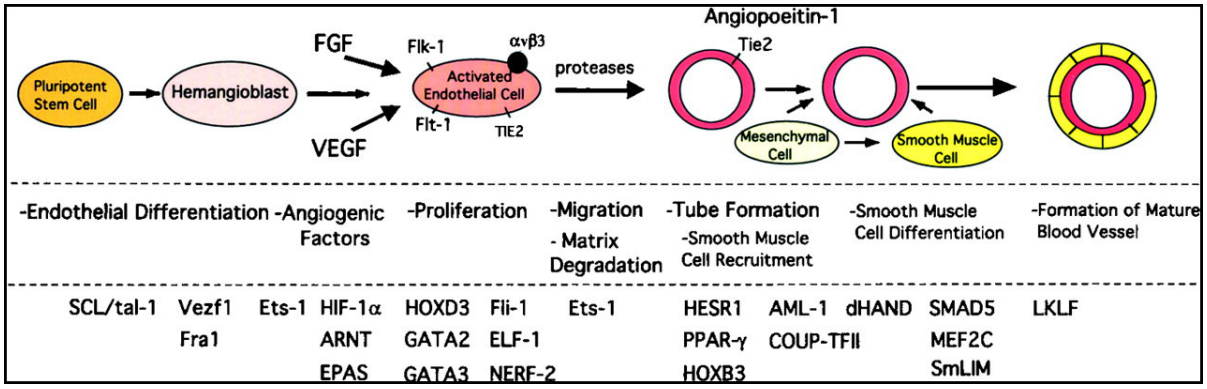

Fig.1.2. Role of transcription factors during different stages of vascular development. (Adapted from Oettgen, 2001).

Vascular smooth muscle cells originate from several sources, such as splanchnic mesoderm, epicardial cells and neural crest cells. One family of transcription factors that is crucial for muscle development, in general, is the MADS-box transcription factor family. For example, two members of this family, SMAD5 and MEF2C, have recently been shown to be important in vascular development and in smooth muscle cell differentiation. Targeted disruption of SMAD5 in mouse leads to vascular defects resulting in embryonic lethality at day 10.5 to 11.5. Similarly, the targeted disruption of MEF2C leads to abnormalities in smooth muscle cell differentiation and the inability of endothelial cells to form into vascular structures [43, 44]. However, the breakthrough in revealing the mechanism of smooth muscle differentiation is identification of the *cis*-elements that control smooth muscle-specific gene

expression. Using a transgenic mouse approach, virtually every smooth muscle specific gene analyzed to date contains two or more CArG boxes in its control region, and mutation of these CArG elements abolishes smooth muscle specific gene expression (reviewed in (Miano, 2003 #61). The CArG box sequence $CC(A/T)_6GG$ is the binding site for Serum Response Factor (SRF), which is also a MADS box transcription factor.

Serum Response Factor and its Role during Muscle Development

SRF was first identified for its ability to bind CArG box and confer serum inducibility to the growth-responsive gene *c-fos* [45]. Later it was found to regulate other immediate early genes, such as *fosB, junB* and *egr-1*, and numerous muscle-specific genes, such as α-*myosin heavy chain, cardiac and skeletal* α-*actin*, etc. [46]. SRF protein contains a DNA binding domain, a transactivation domain and several phosphorylation sites. Overexpression of SRF in the heart causes hypertrophic cardiomyopathy in transgenic mice, and inhibition of SRF by microinjection of SRF antibody or expression of antisense RNA suppressed muscle gene expression and blocks differentiation of myoblasts to myotubes [47, 48]. The requisite role of SRF in smooth muscle (SM) gene expression has been demonstrated by the ability of dominant negative SRF mutants to prevent smooth muscle cell (SMC) differentiation. [49] However, *SRF* null mice die at early gastrulation and fail to form mesoderm, prohibiting a detailed analysis of its role in the development of each muscle lineage [50]. Moreover, how ubiquitously expressed SRF achieves muscle-specific gene activation, how SRF coordinates the activation of both growth genes and muscle genes, which normally belong to exclusive

gene programs, and how SRF potentiates gene transcription with its weak transcription domain, are still outstanding puzzles.

One of the explanations for these questions lies at the ability of SRF to interact with different cofactors to regulate different sets of genes. For example, SRF interacts with members of ternary complex factor (TCF) family of the Ets domain transcription factors to activate *c-fos* gene expression upon growth factor stimulation [51]. On the other hand, it cooperates with cardiac-restricted transcription factor GATA4 and Nkx2.5 to activate cardiac specific gene expression [52, 53]. However, so far no smooth muscle or skeletal muscle specific SRF cofactors have been found.

Identification of Myocardin

Myocardin, named after its highly specific expression in the myocardium, was discovered in an *in silico* screen for novel genes expressed specifically in the heart by Da-zhi Wang, a former postdoctoral fellow in Dr. Eric Olson's lab. Myocardin contains a SAP domain, found in a variety of proteins that influence nuclear architecture and transcription [54]. Other notable features of the protein include a basic region, a putative leucine zipper domain and a stretch of glutamine (Q) residues (Fig.1.2.A).

Northern blot analysis of adult mouse tissues reveal multiple myocardin transcripts specifically in the adult mouse heart. There is no detectable expression of myocardin in any other adult tissues examined. *In situ* hybridization to staged mouse embryos showed that myocardin transcripts were first detected in the cardiac crescent at E7.75, concomitant with expression of the homeobox gene *Nkx2.5*, the earliest known marker for cardiogenic

specification [55]. Thereafter, myocardin transcripts are detected specifically in the linear heart tube at E8.0, and throughout the developing atrial and ventricular chambers until birth. Myocardin is also expressed in a subset of embryonic vascular and visceral smooth muscle cells. At E13.5, myocardin expression was evident within smooth muscle cells lining the walls of the esophagus and aortic arch arteries, as well as the pulmonary outflow tract (Fig. 1.3.A). Expression of myocardin in these smooth muscle cell types is still apparent, but decreased, by E15.5. Myocardin expression is also detected in smooth muscle cells within the lung and gut, as well as in head mesenchyme, which may serve as a source of smooth muscle precursors. Myocardin is not expressed at detectable levels in skeletal muscle.

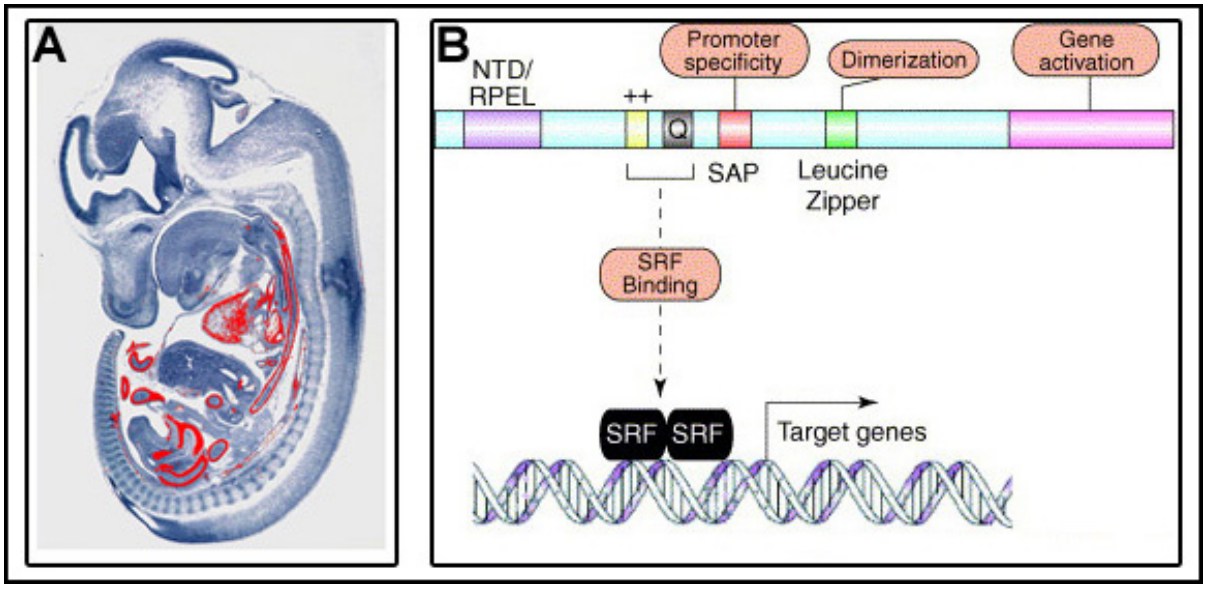

Fig. 1.3. Expression Pattern, Functional domains and mechanism of gene activation of myocardin. *A*. Cardiac and smooth muscle specific expression of myocardin in a E13.5 mouse embryo (RNA in situ hybridization). *B*. Schemetic representation of myocardin protein and the mechanism that myocardin activates gene transcription through interaction with SRF.

Myocardin is a highly potent transcriptional activator and the transactivation domain resides at the C-terminus of the protein. Transcriptional activity of myocardin requires at least two CArG boxes, which is the SRF binding site, within the responsive promoter region. Myocardin and SRF forms ternary complex with DNA fragments containing a CArG box, and the interaction of myocardin and SRF is also detectable by co-immunoprecipitation assay. Therefore, myocardin is a cardiac and smooth muscle specific SRF co-activator with strong transcriptional activity [56, 57]. The functional domains and the mechanism of how myocardin activates gene transcription are summarized in Fig. 1.3.B.

The highly muscle-specific expression pattern and the potent ability to activate SRF dependent promoters make myocardin a very interesting molecule. The goal of this thesis work is to mechanistically analyze the functions of SRF and myocardin during muscle development *in vivo*, specifically:

(A) Examine SRF's role during skeletal muscle development *in vivo*.

(B) Examine myocardin's function during embryogenesis *in vivo*.

(C) Identify and characterize myocardin related transcription factors.

(D) Investigate the functions of myocardin related transcription factors *in vivo*.

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Chapter II

Requirement of Serum Response Factor during Skeletal Muscle Growth and Maturation Revealed by Tissue-specific Gene Deletion in Mice

Abstract

Serum response factor (SRF) controls the transcription of muscle genes by recruiting a variety of partner proteins. Mice lacking SRF fail to form mesoderm and die before gastrulation, precluding an analysis of the roles of SRF in muscle tissues. To investigate the functions of SRF in skeletal muscle development, I conditionally deleted the *Srf* gene in mice by skeletal muscle-specific expression of Cre recombinase. In mice lacking skeletal muscle SRF expression, muscle fibers formed, but failed to undergo hypertrophic growth after birth. Consequently, mutant mice died during the perinatal period from severe skeletal muscle hypoplasia. These findings reveal an essential role for SRF in the control of skeletal muscle growth and maturation *in vivo*.

Introduction

Skeletal muscle development involves a precisely orchestrated series of steps that begins when mesodermal precursor cells become committed to the skeletal muscle lineage, giving rise to proliferating myoblasts. In response to extracellular cues, myoblasts withdraw from the cell cycle and fuse to form multinucleated myotubes that express an array of muscle-specific genes encoding proteins that mediate the specialized contractile, metabolic, and structural functions of the muscle fiber. Subsequent hypertrophic growth of the muscle fiber through the assembly of sarcomeres and increased diameter of the fiber is required to enhance contractile force to meet the functional demands associated with postnatal life.

The early steps in skeletal muscle development are controlled by combinatorial interactions between members of the MyoD family of basic helix–loop–helix transcription factors (MyoD, myogenin, Myf5, and MRF4) and the myocyte enhancerfactor-2 (MEF2) family of MADS (MCM1, Agamous, Deficiens, serum response factor) box transcription factors [1]. MyoD and Myf5 play redundant roles in specification of muscle cell fate, whereas myogenin and MRF4 act together with MEF2 factors to activate and sustain the muscle differentiation program [2, 3].

Serum response factor (SRF), a MADS box transcription factor related to MEF2, also regulates skeletal, as well as cardiac and smooth muscle genes by binding a DNA sequence known as a CArG box [4, 5]. Like MEF2 and other MADS box transcription factors, SRF activates transcription by associating with a variety of signal-responsive and cell type-restricted cofactors [6]. A requisite role for SRF in skeletal muscle development has been inferred from experiments in cultured muscle cells in which injection with anti-SRF antibody

or expression of a dominant negative SRF mutant blocks myoblast fusion and differentiation [7-9]. However, knockout mice lacking SRF die before gastrulation, precluding the analysis of potential functions of SRF in muscle development *in vivo* [10]. Several groups recently have generated conditional *Srf* null alleles allowing for temporal and spatial specificity of gene deletion in the mouse [11-13]. Cardiac-specific deletion of *Srf* results in embryonic lethality from cardiac defects [13], and deletion of the gene in smooth muscle results in embryonic lethality from a deficiency of differentiated smooth muscle cells [12].

To determine the function of SRF in developing skeletal muscle, I conditionally deleted the *Srf* gene in mice by using skeletal muscle-specific transgenes encoding Cre recombinase. Mice lacking skeletal muscle expression of SRF died during the first few days after birth with a severe skeletal muscle myopathy characterized by a deficiency in muscle growth. These findings reveal an essential role for SRF and in the control of muscle fiber growth and maturation.

Materials and Methods

Transgenic Mice

To create a muscle-specific Cre recombinase transgene, a Cre recombinase expression cassette was placed under the control of the 1.5-kb mouse *myogenin* promoter [14] and the 1-kb mouse *MEF2C* enhancer [15], yielding a transgene called *Myo-Cre* (Dr. Michael Czubryt) Transgenic mice were generated by oocyte injection according to standard procedures. The *MCK-Cre* transgenic line and the *ROSA26-lacZ* indicator line have been described [16, 17]. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center.

Skeletal Muscle-Specific Deletion of Srf

The conditional *Srf* allele (Srf^{flex1}), which contains loxP sites in the 5' UTR and first intron of the gene, has been described [11]. Mice homozygous for this allele are viable and fertile. Cre:loxP recombination results in deletion of part of exon 1, which encodes the start codon and the DNA binding domain of SRF. Breeding of Cre transgenic mice heterozygous for the floxed *Srf* allele ($Cre{:}Srf^{flex1/+}$) with $Srf^{flex1/flex1}$ mice yielded $Cre{:}Srf^{flex1/flex1}$ mice. Breedings were performed in the 129SvEv and C57BL/6 mixed backgrounds. DNA prepared from tail biopsies was used for genotyping by PCR, using two primers (SRF-L and SRF-R) as described [11]. This process allowed amplification of a 1.34-kb fragment from the undeleted Srf^{flex1} allele and a 380-bp DNA fragment from the $Srf^{\Delta x}$ allele obtained when floxed Srf^{flex1} alleles had been recombined by Cre recombinase.

RT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen). Briefly, muscle tissues were homogenized in Trizol reagent. After a 5 minutes incubation at room temperature, 1/5 volume of chloroform was added and the mixture was vigorously shaken for 15 seconds. After centrifugation at 12,000 g for 5 minute at 4 ^{o}C, the upper phase was transferred to a new tube and 500 ul of isopropanol was added. After 10 minutes of incubation at room temperature, the mixture was centrifuged at 12,000 g for 10 minutes at 4 ^{o}C. After washed with 70% ethanol, the pellet was dissolved in TE buffer containing 0.1U/ul of RNase inhibitor.

After treatment with DNaseI, 1ug of RNA was used as a template for reverse transcription with random hexamer primers (Invitrogen). Briefly, 1 ug of RNA was mixed with 1x DNase buffer, 1 ul DNase (Invitrogen) and ddH_2O in a 10 ul reaction volume. After 15 minutes of incubation at room temperature, 1 ul of 25 mM EDTA was added and the DNase was inactivated by 10 minutes incubation at 65 ^{o}C. 150 ng of random primers and 2 ul of 5 mM dNTP mix were added to the mixture and incubated at 65 ^{o}C for 5 minutes. After a quick chill on ice, 4 ul of 5x first strand buffer, 2 ul of 0.1 mM DTT and 1 ul of RNase inhibitor and 1 ul of reverse transcriptase and ddH_2O were added to make a 20 ul reaction. After 10 minutes of incubation at room temperature, the reaction was incubated at 42 ^{o}C for 50 minutes to complete the reverse transcription. The final cDNA products were stored at -20 ^{o}C. All PCR products span intron regions of the genes. RT-PCR reactions were performed under conditions of linearity with respect to input RNA. The primer sequences for the specific genes are listed in table 2.1.

Histology and β-Galactosidase Staining

Skeletal muscle was dissected from the hind limbs of WT and mutant mice and fixed with paraformaldehyde. Embedding of tissues, histological sectioning, and staining with hematoxylin and eosin (H&E) were performed by standard procedures. Staining of embryos for β-galactosidase was performed as described [14]. Briefly, the embryos were dissected out free of yolk sac and amnion. After 2 washes in cold PBS, they were fixed with cold PBS containing 4% paraformaldehyde and 0.2% glutaraldehyde. Embryos younger than E9.5 were fixed for 30 minutes; E10.5-E13.5 embryos were fixed for 1-1.5 hrs. After fixation, the embryos were washed with cold PBS for 3 times, 10 minutes each. Then they were stained in the dark overnight at room temperature with staining solution. The staining solution contains: 4 mM Ferrocyanide, 4 mM Ferricyanide, 2 mM $MgCl_2$, and 1 mg/ml X-gal in PBS. The X-gal stock is 40 mg/ml in Dimethylformamide. After staining, the embryos were washed with PBS for 3 times and post-fixed with the same fix solution overnight at 4 ^{o}C.

Electron Microscopy

For electron microscopy, skeletal muscle was fixed overnight in 2% glutaraldehyde in PBS at 4°C, then postfixed in 1% OsO_4, and dehydrated in an ethanol series. Samples were then embedded in Spurr resin (Ted Pella, Inc., Redding, CA), stained with uranyl acetate and lead citrate, and sectioned at 80 nm.

Table 2.1. Sequences of RT-PCR primers:

Mouse

GAPDH: GCA GTG GCA AAG TG GAGA TTG; TTT GGC TCC ACC CTT CAA GTG

Cardiac-α-MHC: ATG GCT GAG GAG CTG AAG AAG G; GTT GAC CTG GGA CTC GGC GAT G

Cardiac-α-Actin: AGA GTA TGA TGA GGC AGG CC; ATG ACT GAT GAG AGA TGG GG

MyoD: AGC ACT ACA GTG GCG ACT CAG AC; TTT GAG CCT GCA GGA CAC TGA GG

Myogenin: TGG AGC TGT ATG AGA CAT CCC; TGG ACA ATG CTC AGG GGT CCC

MEF2C: CAT GCC GCC ATC TGC CCT CAG; CCC TTT CGT CCG GCG AAG GTC

Skeletal-α-Actin: CAG AGC AAG CGA GGT ATC C; GTC CCC AGA ATC CAA CAC G

Skeletal-MHC (emb): CAC CTG GAG AGG ATG AAG AAG AA; AAG ACT TGA CTT TCA CTT GGA GTT TAT C

Skeletal-MHC (perinatal): AAC AGA AAC GCA ATG CTG AGG CTG; TTT GGT GTG AAC CTC TCG GCT CTT

Skeletal-MHC (adult): AAC AGC TTG GAA CAG CTG TGC ATC; TTG CCT CTG CTT CAG CAG TTT GTG

MCK: CAG ACC TCA GCA AGC ACA ACA ATC AC; GCG GAG GCA GAG TGT AAC CCT TG

SM-α-Actin: GAA CGC TTC CGC TGC CCA GA; CGT TCA CAG TTG TGT GCT AGA G

SM22: GGT GAA CAG CCT GTA TCC TG; GCA GTT GGC TGT CTG TGA AG

SM-Calponin: GAT ACG AAT TCA GAG GGT GCA GAC GGA GGC TC; GAT ACA AGC TTT CAA TCC ACT CTC TCA GCT CC

SM-MLCK: GGA AGA CTG TCC TCT ATG GCA ATG; CTT CCT CCA TGG TTT CCA CAA TG

SM-MHC: CTG GAG GCT TTC GGC AAT GC; GAC AAT GTT TCC AAG CTG AAG G

SRF: AGT TCA TCG ACA ACA AGC TGC GG; TGC CTG TAC TCT TGA GCA CAG TC

MRTF-A: CAC TGT GAC CAA TAA GAG TGC; GCC TGT GGA GGT CAT CAA TG

MRTF-B: AAG CCA TCC CAA GAA TCC AAA C; TCT GCC TTG AAA GTG GAC ACA G

c-fos: GAT GTT CTC GGG TTT CAA CG; TGA TCT GTC TCC GCT TGG AG

a-lactalbumin: CAT CTG TGG CAT CTC CTG TGA CAA G; TAG AGT CCG GTG GTG TCA CTA CAG

b-casein: TTT CTG CAG CAA GTA GCA CCC TTC C; GAG AAG CAT ATA GAG TCC ATG GGT CG

WAP: TGA GGG CAC AGA GTG TAT CAT CTG C; CTG TAT AGA CTT GGG CTG GTC ACT C

CK-18: CCA GAC CTT GGA GAT TGA CTT GGA C; TTC TCT GCC TCA GTG CCT CAG AAC

CK-14: AGT CCC AGC TCA GCA TGA AAG CAT C; CCT GAG CAG CAT GTA GCA GCT TTA G

CALLA: GGA GTC GAT TTT AGG TGA TGG GAA G; TTA CTG TAT CGG GAA CTG GTC TCG G

Results:

Creation of a Skeletal Muscle-Specific Cre Transgene

To enable the skeletal muscle-specific deletion of a floxed *Srf* gene, Dr. Michael Czubryt, a former postdoc fellow in the lab, created a transgene in which Cre recombinase expression was controlled by the mouse *myogenin* promoter and the skeletal muscle-specific enhancer of the mouse *MEF2C* gene. Both of these regulatory elements are active only in the skeletal muscle lineage from embryonic day (E) 8.5 to adulthood [14, 15]. The expression pattern of this transgene, referred to as *Myo-Cre*, was determined by crossing mice harboring the transgene into the *ROSA26R* heterozygous background, which contains a "floxed" *lacZ* allele that is activated in the presence of Cre [16]. As shown in Fig. 2.1.A, the *Myo-Cre* transgene directed the expression of lacZ specifically in skeletal muscle cells within the somite myotome at E9.5. Expression was initiated in the anterior somites at ~E9.0 (data not shown), and strong expression throughout skeletal muscle was maintained throughout embryogenesis (Fig. 2.1. A). Serial histological sections through stained embryos at multiple stages confirmed the skeletal muscle specificity of lacZ expression and showed that all skeletal muscle cells were stained for lacZ (data not shown).

Skeletal Muscle-Specific Deletion of Srf with the Myo-Cre Transgene

To delete *Srf* specifically in skeletal muscle, I used a conditional *Srf* allele (called Srf^{flex1}) harboring loxP sites flanking exon 1 of the gene, which encodes the DNA binding domain of SRF. Cre-mediated recombination of this locus generates the $Srf^{\Delta x}$ deletion allele essentially identical to that in the previously described null allele [10, 11]. Mice heterozygous

for the *Srf*^{flex1} allele and heterozygous for the *Myo-Cre* transgene were bred with homozygous *Srf*$^{flex1/flex1}$ mice to yield mice homozygous for the *Srf*^{flex1} allele and the *Myo-Cre* transgene. Genotyping of litters from these crosses revealed that offspring with the *Myo-Cre*;*Srf*$^{flex1/flex1}$ genotype were born at Mendelian ratios. At birth, the hearts of these mutant mice were beating, but the animals were immobile and died from an inability to breathe. These mutant mice were recognizable by their cyanotic appearance and kyphosis (Fig. 2.1. B).

Efficient deletion of *Srf* from skeletal muscle was confirmed by PCR with genomic DNA (Fig. 2.1. C and D). There was an ~80% reduction in the PCR product from the floxed *Srf* gene in skeletal muscle at E19.5 in the presence of the *Myo-Cre* transgene. Given that cells other than muscle (e.g., neurons and fibroblasts), in which the Cre transgene is not expressed, also are contained in these tissue samples, I estimate that the efficiency of *Srf* gene deletion was at least 90%.

Perinatal Lethality and Skeletal Muscle Hypoplasia Resulting from Skeletal Muscle Deletion of *Srf*

Histological analysis of skeletal muscle from mice at E19.5 or birth lacking skeletal muscle expression of *Srf* showed the presence of multinucleated muscle fibers that were thinner than normal and were separated by prominent interstitial space (Fig. 2.2.A). The diameters of fibers in the mutant were also much more variable than in WT controls. All skeletal muscle groups appeared to be affected comparably in *Srf* mutant animals. Perinatal lethality is likely caused by abnormalities in the diaphragm muscle, which prevent breathing. The mean body weights (±SD) of WT and mutant mice at birth were 1.28 ± 0.05 g (n = 9)

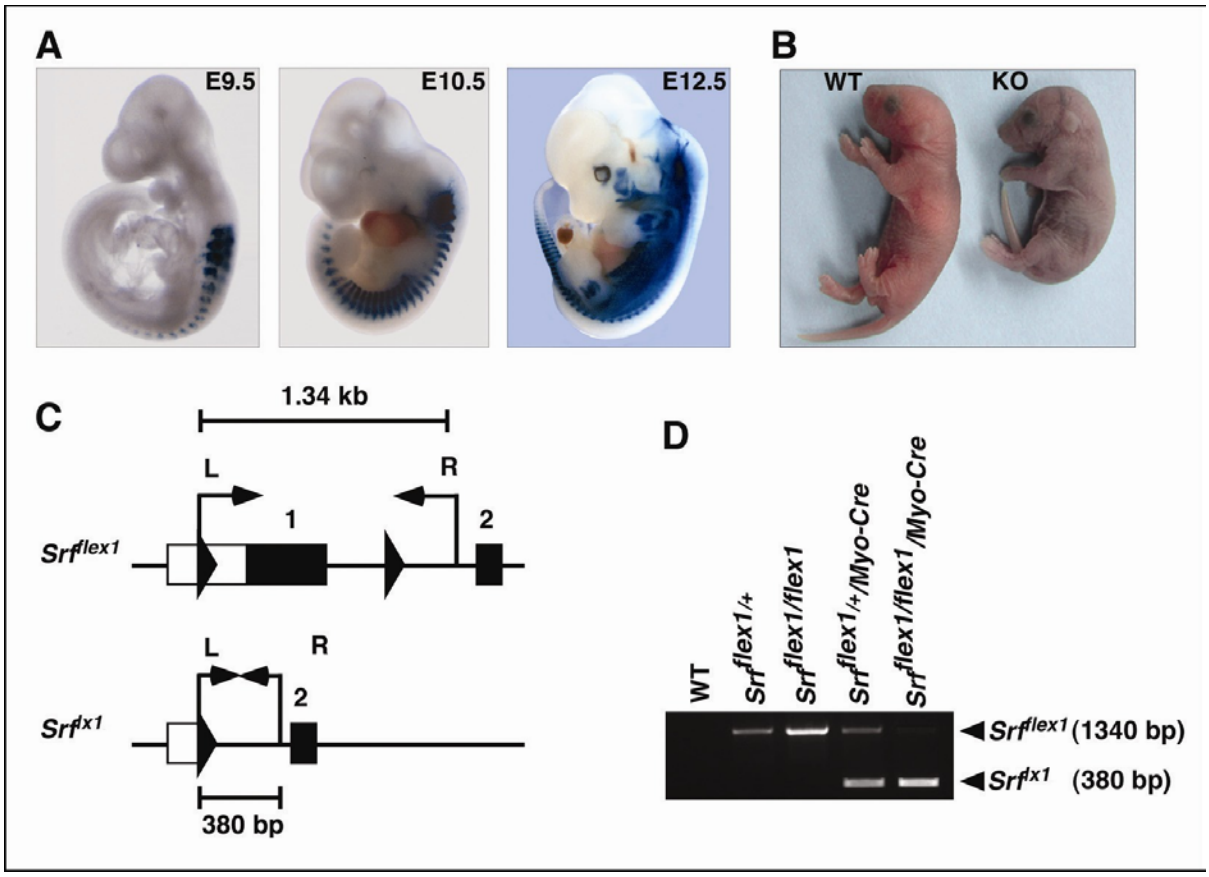

Fig. 2.1. Deletion of *Srf* with a skeletal muscle-specific Cre transgene. (*A*) *Myo-Cre* transgenic mice were bred with ROSA26R indicator mice to determine the temporal and tissue specificity of Cre expression. Whole-mount photographs of β-galactosidase-stained embryos of the indicated embryonic ages are shown. The *lacZ* reporter gene is activated specifically in the skeletal muscle lineage. (*B*) WT and $Srf^{flex1/flex1}$/*Myo-Cre* (KO) mice immediately after birth are shown. The mutant is cyanotic and displays curvature of the spine. (*C*) The structure of the Srf^{flex1} allele before (*Upper*) and after (*Lower*) Cre-mediated recombination is shown. Triangles represent loxP sites. Exons 1 and 2 are shown in black boxes with the 5' UTR as a white box. Primers used by PCR are designated L and R, and sizes of PCR fragments are indicated. (*D*) PCR of genomic DNA from skeletal muscle of mice of the indicated genotypes. Primers L and R yield a product of 1,340 bp with the Srf^{flex1} allele and 380 bp with the $Srf^{\Delta x1}$ allele in the presence of the *Myo-Cre* transgene.

versus 1.06 ± 0.11 g (n = 6) ($P < 0.005$). Because the deletion of *Srf* is specific for skeletal muscle, this difference in body weight reflects the lack of muscle mass in the mutants.

Given that the *MEF2C* enhancer and *myogenin* promoter are activated at the onset of myogenesis, We considered it unlikely that the deficiency of skeletal muscle fibers in *Myo-Cre*;*Srf*$^{flex1/flex1}$ mutants reflected a deficiency in myoblasts. Indeed, the number of nuclei in muscle fibers of WT and mutant mice was comparable, suggesting that the skeletal muscle hypoplasia of the mutant was caused by a failure in growth of muscle fibers rather than a deficiency of muscle cells or a partial block of myoblast fusion.

Ultrastructural analysis of skeletal muscle fibers by electron microscopy showed the presence of sarcomeres in *Myo-Cre*;*Srf*$^{flex1/flex1}$ mutants (Fig. 2.2.B). However, the sarcomere units were smaller, and the fibers were narrowed and disorganized. Electron-dense material, likely glycogen, filled the interstitial spaces between the hypoplastic muscle fibers in the mutant.

Analysis of representative skeletal muscle transcripts at birth by semiquantitative RT-PCR showed a 70% decrease in SRF mRNA in *Myo-Cre*;*Srf*$^{flex1/flex1}$ mutants compared with WT littermates (Fig. 2.3). Skeletal α-actin and cardiac α-actin transcripts also were down-regulated ~30% in the mutants, whereas other transcripts for smooth muscle α-actin, neonatal skeletal myosin heavy chain, MCK, and myogenic basic helix–loop–helix and MEF2 factors were unaffected (Fig. 2.3).

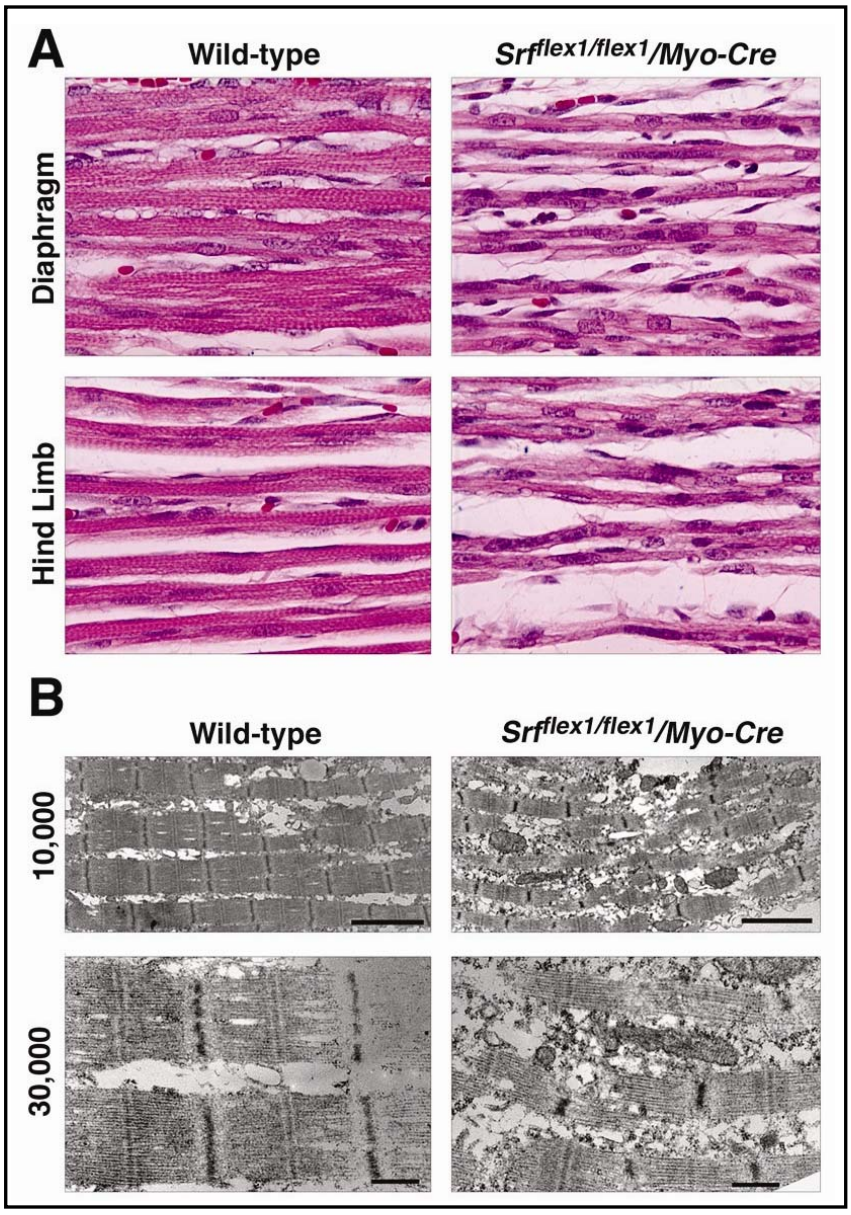

Fig. 2.2. Histology of skeletal muscle of *Srf*$^{flex1/flex1}$*/Myo-Cre* mice. (*A*) Histological sections of representative muscle groups of WT and *Srf*$^{flex1/flex1}$*/Myo-Cre* mice at E19.5 were stained with H&E. The muscle fibers in the mutant are thinner than those of WT. (Bar: 20 μm.) (*B*) Hindlimb muscle of WT and *Srf*$^{flex1/flex1}$*/Myo-Cre* mice was analyzed by electron microscopy at E19.5. The muscle fibers in the mutant are disorganized and less developed than those of WT. Magnifications are shown at left. (Bar: 2 μm, *Upper*; 0.5 μm *Lower*.)

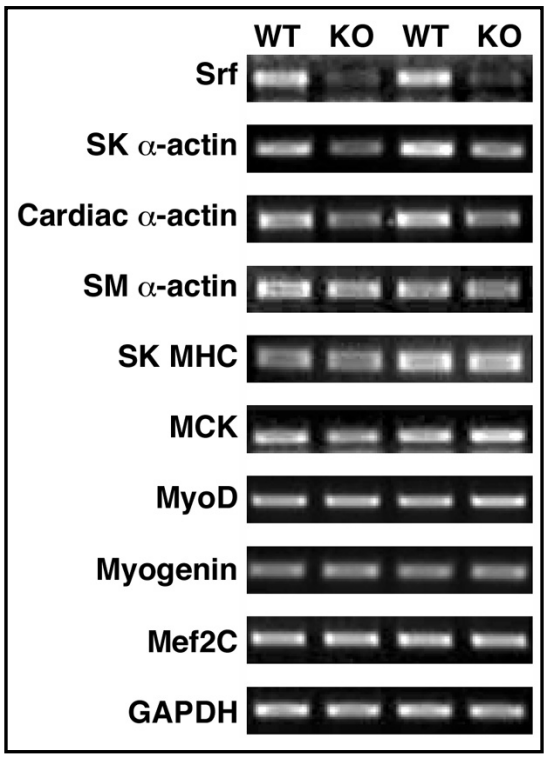

Fig. 2.3. Analysis of muscle markers in *Srf*$^{flex1/flex1}$/*Myo-Cre* mice. RNA was isolated from hindlimb muscles of WT and *Srf*$^{flex1/flex1}$/*Myo-Cre* (KO) mice at birth and analyzed by semiquantitative RT-PCR for the indicated transcripts. Samples from two animals of each genotype are shown.

Skeletal Muscle Deletion of Srf Using MCK-Cre

Because the myogenin and *MEF2C* regulatory elements used to direct Cre recombinase are active early in the pathway of skeletal muscle development, I wondered whether deletion of *Srf* from skeletal muscle at a later time might result in a different phenotype. To explore this possibility, I additionally used a *MCK-Cre* transgene to delete the *Srf*^{flex1} allele. Prior studies have shown that this transgene is activated in skeletal muscle cells during late embryonic development [17]. This transgene also is expressed in the developing heart and smooth muscle cells of the large arteries [17, 18]. Deletion of *Srf* with the *MCK-Cre* transgene resulted in perinatal lethality with complete penetrance. These mutant mice were mobile, nursed, and appeared normal at birth (data not shown). However, by postnatal

day (P) 3, these animals were lethargic and began to display growth retardation (Fig. 2. 4.A). No viable offspring with skeletal muscle deletion were observed beyond P7.

Based on PCR of genomic DNA of mutant mice at P3, I estimate that the *MCK-Cre* transgene directed at least 90% deletion of *Srf* in skeletal muscle (Fig. 2.4.B). Histological analysis of skeletal muscle from *MCK-Cre*;$Srf^{flex1/flex1}$ mutants at P3 showed thinner myofibers than normal, although the phenotype appeared less severe than that of *Myo-Cre*;$Srf^{flex1/flex1}$ mutants (Fig. 2.4.C). The delayed phenotype of these animals compared with those using the *Myo-Cre* transgene for *Srf* deletion is likely to reflect the later activation of the *MCK-Cre* transgene. I detected no abnormalities in the hearts of *MCK-Cre*;$Srf^{flex1/flex1}$ mutants, leading us to conclude that skeletal muscle abnormalities were the cause of death. Expression of *Srf* transcripts in skeletal muscle from *MCK-Cre*;$Srf^{flex1/flex1}$ mutants at P3 was reduced by 80% compared with controls. However, I detected only a modest (<50%) decrease in expression of α-skeletal and α-cardiac actin and no decrease in other muscle genes in these animals (Fig. 2.4.D).

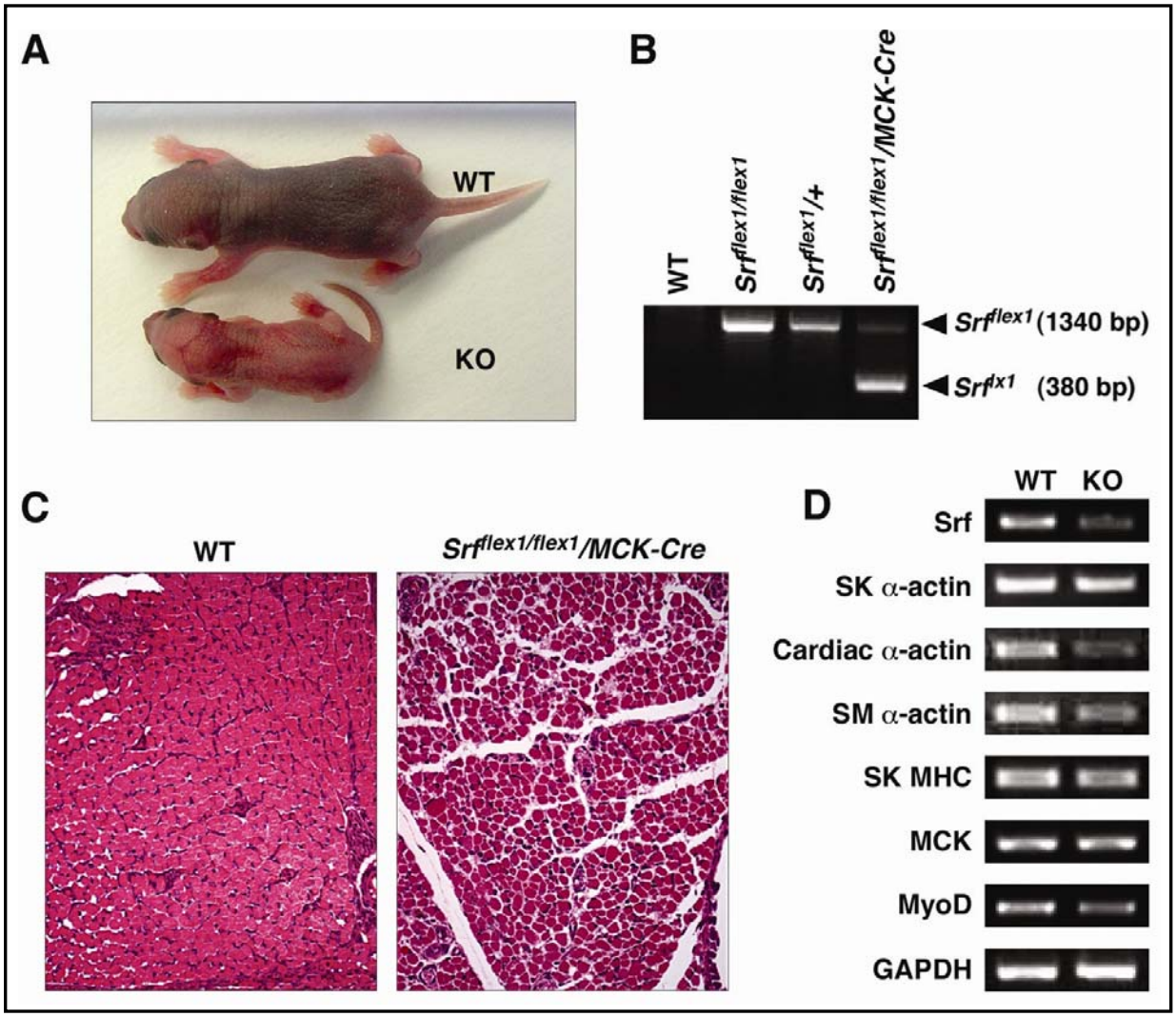

Fig. 2.4. Deletion of *Srf* with a *MCK-Cre* transgene. (*A*) WT and $Srf^{flex1/flex1}$/*MCK-Cre* (KO) mice at P3 are shown. The mutant is severely runted. (*B*) PCR of genomic DNA from mice of the indicated genotypes. Primers L and R yield a product of 1,340 bp with the floxed Srf^{flex1} allele and 380 bp with the deleted $Srf^{\Delta x}$ allele, generated in the presence of the *MCK-Cre* transgene. (*C*) Histological sections of representative muscle groups of WT and $Srf^{flex1/flex1}$/*MCK-Cre* mice were stained with H&E. The cross-sectional area of the muscle fibers in the mutant is smaller than that of WT. (Bar: 20 μm.) (*D*) RNA was isolated from hindlimb muscles of WT and $Srf^{flex1/flex1}$/*MCK-Cre* (KO) mice at P3 and analyzed by semiquantitative RT-PCR for the indicated transcripts.

Discussion:

To determine the role of SRF in skeletal muscle development, I deleted a conditional *Srf* gene specifically in the skeletal muscle lineage by using two Cre transgenes with different temporal patterns of expression in the muscle developmental pathway. The phenotypes of these mutant mice reveal an essential role for SRF in the control of skeletal muscle growth and maturation *in vivo*.

Control of Myofiber Growth and Maturation by SRF

We believe the failure of skeletal muscle to grow and mature properly in mice lacking skeletal muscle expression of *Srf* results in lethality caused by skeletal muscle weakness, which disrupts breathing and/or nursing. The early onset of lethal muscle deficits in these mutant mice is distinct from most myopathic phenotypes in mice, which do not manifest until adulthood reflecting, at least in part, the regenerative capacity of skeletal muscle. The myopathic phenotype resulting from skeletal muscle-specific deletion of *Srf* is also distinct from those of mice lacking myogenic basic helix–loop–helix genes. *MyoD* and *Myf5* play redundant roles in specification of the skeletal muscle cell lineage such that deletion of one gene or the other does not substantially affect muscle development, whereas deletion of both genes eliminates all traces of the skeletal muscle lineage [19, 20]. In contrast, deletion of the *myogenin* gene results in perinatal lethality from a block in myoblast fusion and differentiation [21, 22].

The skeletal muscle phenotype of *Srf* mutant mice could, in principle, reflect an early or late developmental function of SRF. The *Myo-Cre* transgene is activated by E9.5 before

the first round of myoblast fusion, whereas the *MCK-Cre* transgene is activated later during muscle fiber differentiation. The finding that two skeletal muscle Cre transgenes activated at different times in development lead to similar phenotypes, albeit with differing severity, suggests that the *Srf* mutant phenotype reflects a late function of SRF in hypertrophic growth rather than an early developmental role, for example, in myoblast fusion.

Sarcomeric actin genes, as well as other contractile protein genes, require CArG boxes for expression [4]. Thus, it is intriguing that some CArG box-dependent genes were expressed normally in *Srf*-deficient skeletal muscle. We suggest two possible explanations for this finding. (*i*) Residual SRF caused by incomplete or delayed gene deletion might be adequate to activate certain SRF-dependent genes that are more sensitive to SRF levels than others. (*ii*) SRF-independent mechanisms might bypass a requirement of SRF for activation of some CArG box-dependent genes.

Given the evidence for the involvement of SRF in myoblast differentiation *in vitro* [7-9], why does myogenesis appear to proceed normally in mice lacking skeletal muscle expression of SRF? We suggest three possibilities, which are not mutually exclusive. (*i*) Myogenesis *in vitro* might have a more stringent dependency on SRF. (*ii*) The kinetics of *Srf* gene deletion *in vivo* might be delayed such that an initial requirement for SRF in activation of the differentiation program is bypassed. (*iii*) Residual, low-level expression of SRF in the Srf^{lx1} animals might be sufficient to support the initial steps in myogenesis, whereas later steps in muscle growth and maturation might require higher SRF levels.

The apparent block to myofiber growth after skeletal muscle deletion of *Srf* is reminiscent of the cardiac phenotype resulting from cardiac expression of dominant negative

SRF [23] or cardiac deletion of *Srf*, which results in embryonic lethality from a defect in ventricular growth and maturation [12, 13]. Conversely, overexpression of SRF results in lethal cardiomyopathy with associated myocyte hypertrophy in adult cardiac muscle [24]. Thus, SRF might play comparable roles in regulating growth of skeletal and cardiac muscle *in vivo*.

Mechanisms for Skeletal Muscle Hypertrophy

Growth of skeletal muscle during late fetal and postnatal development involves the assembly of sarcomeres and an increase in volume of individual myofibers. Several signaling pathways have been shown to control skeletal muscle hypertrophy [25]. Signaling by insulin-like growth factor-1 to Akt and its downstream effectors promotes hypertrophy, and SRF has been shown to be a target of Akt signaling [26]. The secreted bone morphogenetic protein myostatin also suppresses muscle hypertrophy, and follistatin, its antagonist, promotes hypertrophy [27, 28]. SRF could be a critical component of these hypertrophic signaling pathways. Alternatively, the reduction in expression of one or more SRF target genes, α-*actin*, for example, could perturb myofiber growth through secondary mechanisms. In this regard, α-skeletal actin knockout mice die during the perinatal period from abnormalities in skeletal muscle growth and force generation [29].

Consistent with the notion that SRF plays a role in hypertrophic growth of skeletal muscle, SRF expression is up-regulated during load-induced hypertrophy of skeletal muscle [30], and the CArG box in the α-*skeletal actin* promoter is a target for hypertrophic signaling [31]. The recognition that SRF plays a role in skeletal muscle growth and maturation suggests

strategies for enhancing SRF activity in the settings of muscle-wasting disorders, possibly by modulating the signaling pathways that stimulate the activity of SRF or its cofactors.

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Chapter III

The Serum Response Factor Coactivator Myocardin is Required for Vascular Smooth Muscle Development

Abstract

Formation of the vascular system requires differentiation and patterning of endothelial and smooth muscle cells (SMCs). Although much attention has focused on development of the vascular endothelial network, the mechanisms that control vascular SMC development are largely unknown. Myocardin is a smooth and cardiac muscle-specific transcriptional coactivator of serum response factor, a ubiquitous transcription factor implicated in smooth muscle gene expression. When expressed ectopically in nonmuscle cells, myocardin can induce smooth muscle differentiation by its association with serum response factor. Mouse embryos homozygous for a myocardin loss-of-function mutation die by embryonic day 10.5 and show no evidence of vascular SMC differentiation. Myocardin is the only transcription factor known to be necessary and sufficient for vascular SMC differentiation.

Introduction

The cardiovascular system is the first organ system to form and function during embryogenesis. Vascular development begins with the organization of endothelial cells into a primitive vascular plexus that becomes progressively remodeled to ultimately form a complex vascular network [1]. Smooth muscle cells (SMCs) are recruited to the endothelial vasculature and ensheathe it, providing support and contractility to the vascular system. Several peptide growth factors and their tyrosine kinase receptors have been shown to play key roles in assembly and patterning of the endothelial vasculature and recruitment of SMCs. In contrast, little is known of the transcriptional events responsible for development of vascular SMCs *in vivo*.

Vascular SMCs are derived from a variety of embryonic progenitors, including lateral mesoderm, cranial mesenchyme, and the neural crest [2, 3]. Differentiation of SMCs is triggered by extracellular cues and is accompanied by the transcriptional activation of an array of smooth muscle (SM) genes whose products confer the unique contractile, morphological, and structural properties that distinguish them from other muscle cell types. The MADS box transcription factor SRF plays a critical role in SM gene activation. SRF binds to a DNA sequence known as a CArG box, which is required for the expression of virtually every SM gene analyzed to date [4-8]. The importance of SRF for SM gene expression has also been suggested by the finding that a dominant-negative SRF mutant can block SM differentiation in epicardial explant cultures [9]. However, the role of SRF in SM development *in vivo* has been clouded by the fact that SRF knockout mice die during gastrulation from a lack of mesoderm well before vascular development is initiated [10].

Moreover, because SRF is expressed throughout the embryo, it alone cannot account for the specificity of SM gene transcription. Thus, it has been proposed that SRF controls SM genes by recruiting cell type-specific cofactors. Indeed, the transcriptional activity of SRF in cultured cells can be modulated by its association with positive and negative cofactors and by extracellular signaling, but there is little or no direct evidence for the involvement of either of these types of mechanisms in the control of SM genes by SRF *in vivo* [11].

Recently a SRF coactivator, myocardin, which is expressed specifically in cardiac and smooth muscle cells, was identified by Da-zhi Wang in our lab. Myocardin associates with the MADS box of SRF through a basic and glutamine-rich domain; this interaction brings the powerful transactivation domain (TAD) of myocardin to CArG box-containing target genes with resulting transcriptional activation [12]. Myocardin contains an SAF-A/B, Acinus, PIAS (SAP) domain, found in a variety of nuclear proteins involved in chromatin remodeling and gene expression [13]. The SAP domain of myocardin is not required for association with SRF, but it provides specificity to target gene activation and is required for the activation of some genes but not others. Expression in *Xenopus* embryos of dominant negative mutants of myocardin that associate with SRF but lack transcriptional activity prevents heart formation, revealing an essential early role for myocardin in cardiac gene expression [12].

To determine the function of myocardin during embryogenesis, we targeted the mouse *myocardin* gene by homologous recombination. Mice lacking myocardin die by embryonic day (E) 10.5 from a complete absence of vascular SMCs. In contrast, cardiac development occurs normally in myocardin mutant embryos. The avascular phenotype of myocardin mutant mice, combined with studies demonstrating that myocardin can activate

expression of SM genes in nonmuscle cells, demonstrates that myocardin is a master regulator of SM development both sufficient and necessary for SMC differentiation.

Materials and Methods

Generation of Myocardin Mutant Mice

The gene structure of *myocardin* has been described [14]. A *myocardin*-targeting vector was constructed to delete exons 8 and 9 by using a pN-Z-TK2 vector, which contains a nuclear *LacZ* (*nLacZ*) cassette and a *neomycin-resistance* gene under the control of the RNA polymerase II promoter and two herpes simplex virus *thymidine kinase* (*TK*) gene cassettes (a generous gift of R. Palmiter, University of Washington, Seattle). The targeting vector was electroporated into 129 SvEv-derived ES cells, and selection was performed with G-418 and FIAU, respectively. Four hundred ES cell clones were isolated and analyzed by Southern blotting for homologous recombination. Three clones with a disrupted *myocardin* gene were injected into 3.5-day mouse C57BL/6 blastocysts, and the resulting chimeric male mice were bred to C57BL/6 females to achieve germline transmission of the mutant allele.

RT-PCR

Total RNA was purified from tissues with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For RT-PCR, total RNA was used as a template for reverse transcriptase and random hexamer primers. The details were described in Chapter II. The sequences of primers used for PCR were listed in Table 2.1.

Immunostaining and Histology

Whole-mount immunostaining was performed as described [15]. Briefly, embryos were fixed in 4% paraformaldehyde at 4^{o}C overnight, Rinsed with PBS for 10 min twice at

RT, dehydrated through 25%, 50%, 75%, 100% methanol in PBS, bleached with 5% H_2O_2 in methanol for 5 hours at RT, rehydrated through 75%, 50% then 25% methanol in PBS, and finally wash twice with PBS. The embryos were blocked in PBSMT (3% skim milk, 0.5% Triton X-100 in PBS) at RT for 1 hour twice. Then they were incubated in 10 ug/ml either purified anti-mouse CD31 (PECAM antibody, MEC13.3, anti-mouse platelet-derived endothelial adhesion molecule, Pharmingen, San Diego, CA) or anti-SM α-actin (Clone 1A4, Sigma) in PBSMT at 4^{o}C overnight. The embryos were rinsed with PBSMT 5 times for 1 hour each, and then incubated in 1:100 dilution of horseradish peroxidase-conjugated goat anti-rat IgG (H+L) (Kirkegaad and Perry Laboratories Inc., Gaithersburg, MD / Roche) in PBSMT at 4^{o}C overnight. They were rinsed with PBSMT 4 times 1 hours each at 4^{o}C, rinsed with PBST (0.2% BSA, 0.1% Triton X-100, PBS) for 30 min and with PBS for 10 min. The embryos were then stained with the DAB kit (Vector labs). Embryos used for histology were fixed in 4% paraformaldehyde, sectioned, and processed for hematoxylin and eosin (H&E) staining or *in situ* hybridization by using standard procedures [16], 35S labeled RNA probes were generated using Maxiscript kit (Amersham).

SM22-lacZ Transgenic Mice

Staining of transgenic embryos for lacZ expression was described in Chapter II. The SM22-lacZ transgene contained the 1,343-bp SM22 promoter linked to lacZ [17]. The transgene was introduced into the myocardin mutant background by interbreeding the appropriate strains of mice.

Results

Generation of Myocardin Knockout Mice

To determine the function of myocardin during mouse development, Dazhi Wang, a former postdoctoral fellow in the lab, generated myocardin-deficient mice by targeted disruption of the *myocardin* gene. The protein-coding region of the mouse *myocardin* gene encompasses 13 exons and spans ~93 kb of genomic DNA (Fig. 3.1.A). Exons 8 and 9, which encode the basic, glutamine-rich domain, and part of the SAP domain [14], were replaced with a lacZ gene and a neomycin-resistance gene (Fig. 3.1.B). The basic and glutamine-rich domains are required for interaction with SRF. Deletion of these domains abolishes all myogenic activity of myocardin [18, 19]. Thus, this mutation inactivates the gene.

I found that mice heterozygous for the mutant *myocardin* allele were viable, fertile, and phenotypically normal. Genotyping of off-spring from heterozygous intercrosses in the isogenic 129 background or in a 129/C57BL/6 mixed genetic background yielded WT and myocardin$^{+/-}$ mice in an approximate 1:2 ratio but no myocardin$^{-/-}$ mice, indicating that the homozygous mutation resulted in embryonic lethality. Analysis of the genotypes of embryos from timed matings showed Mendelian ratios up to E10.5 but no live homozygous mutants at later developmental time points.

To confirm the gene-targeting event, I performed RT-PCR analysis of mRNA from the hearts of WT and mutant embryos at E9.5, using primers representing exon sequences within and surrounding the deleted region of the gene (Fig. 3.1.C). These assays revealed that exons 8 and 9 of *myocardin* were replaced with the *LacZ-Neo* cassette, as expected, and that this mutation resulted in alternative splicing of the targeted allele, such that exon 7 was

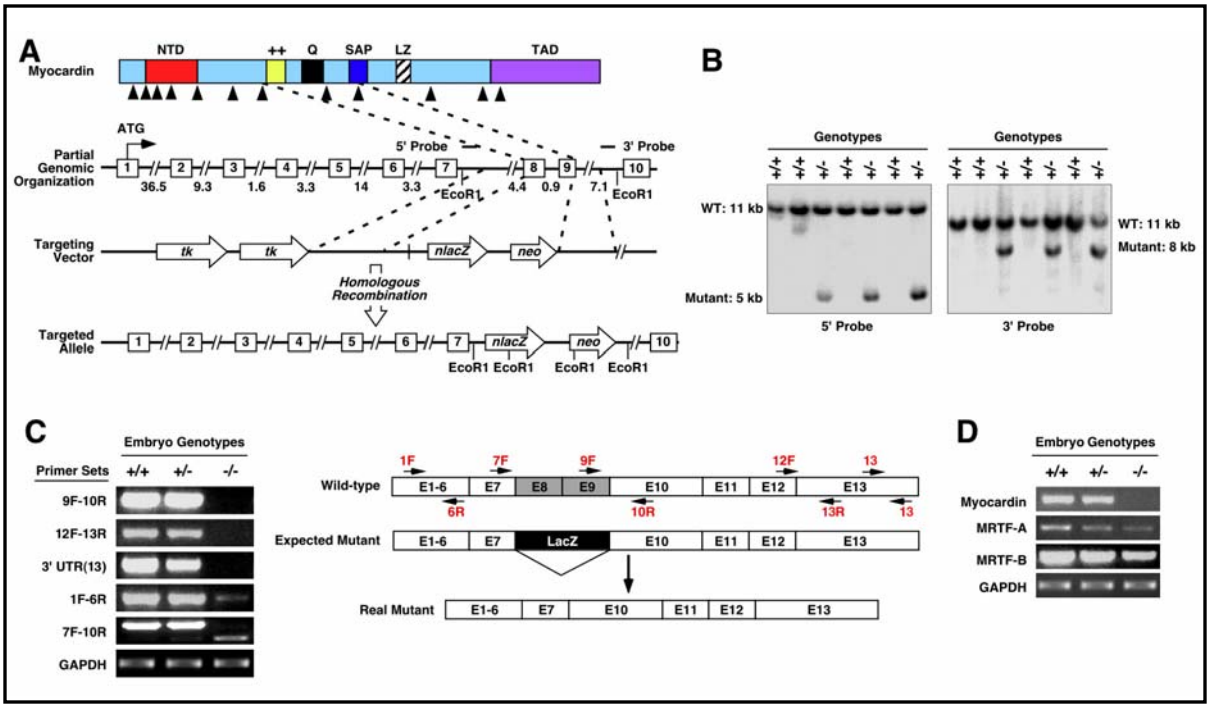

Fig. 3.1. Generation of myocardin mutant mice. (*A*) Targeting strategy. Homologous recombination resulted in deletion of exons 8 and 9 and insertion of lacZ and neomyocin-resistance cassettes. The positions of 5' and 3' probes used for Southern analysis in *B* are shown. Intron junctions within the coding region are shown by arrowheads beneath the schematized protein. Exons are shown in boxes, and sizes of introns are indicated. (*B*) Southern blot analysis. Genomic DNA from ES cell clones was isolated from tail biopsies and analyzed by Southern blot with 5' and 3' probes after digestion with *Eco*RI. The positions of WT and mutant bands are shown. (*C*) Analysis of myocardin transcripts by RT-PCR. RNA was isolated from hearts of WT and myocardin mutant embryos at E9.5 and analyzed by RT-PCR by using different pairs of primers, as shown to the left of each panel. Genotypes are shown at the top. A schematic of exons (E) with positions of primers is shown at the right. Transcripts for GAPDH were detected as a control for RNA loading and integrity. In the targeted allele, exon 7 is spliced to exon 10. (*D*) RNA was isolated from hearts of WT and myocardin mutant embryos at E9.5 and analyzed for myocardin, MRTF-A, and MRTF-B transcripts by RT-PCR.

spliced to exon 10. This was further confirmed by sequence analysis of RT-PCR products. Because of such alternative splicing, the lacZ gene was not expressed in mutant mice (data not shown). Notably, the truncated transcript generated from the mutant allele was expressed at a much lower level than the WT myocardin transcript, presumably because of instability of the mutant transcript (Fig. 3.1.C).

Because myocardin shares extensive amino acid homology with MRTF-A and MRTF-B, which can also act as SRF cofactors as I will describe later, I assayed their expression by RT-PCR of RNA from hearts of E9.5 embryos. As shown in Fig. 3.1.D, both transcripts were readily detectable in WT and mutant hearts, and neither was up-regulated in the absence of myocardin.

Lethal Vascular Abnormalities in Myocardin Mutant Embryos

Analysis of myocardin$^{-/-}$ embryos obtained from timed matings revealed no abnormalities before E8.0, and most embryos appeared to develop normally up to E8.5 (data not shown). However, homozygous mutant embryos could be readily identified at E9.5 by their pale yolk sacs, which lacked blood vessels (Fig. 3.2.A. a–d). Homozygous mutant embryos also showed growth retardation and delayed development at E9.5. The gross morphology of the hearts in mutant embryos appeared normal with completed rightward looping and normal chamber formation.

Histological analysis of transverse sections of mutant embryos at E9.5 confirmed the normal appearance of the atrial and ventricular chambers but revealed severe vascular defects in which the dorsal aortae were clearly underdeveloped (Fig. 3.2.B). However, the anterior

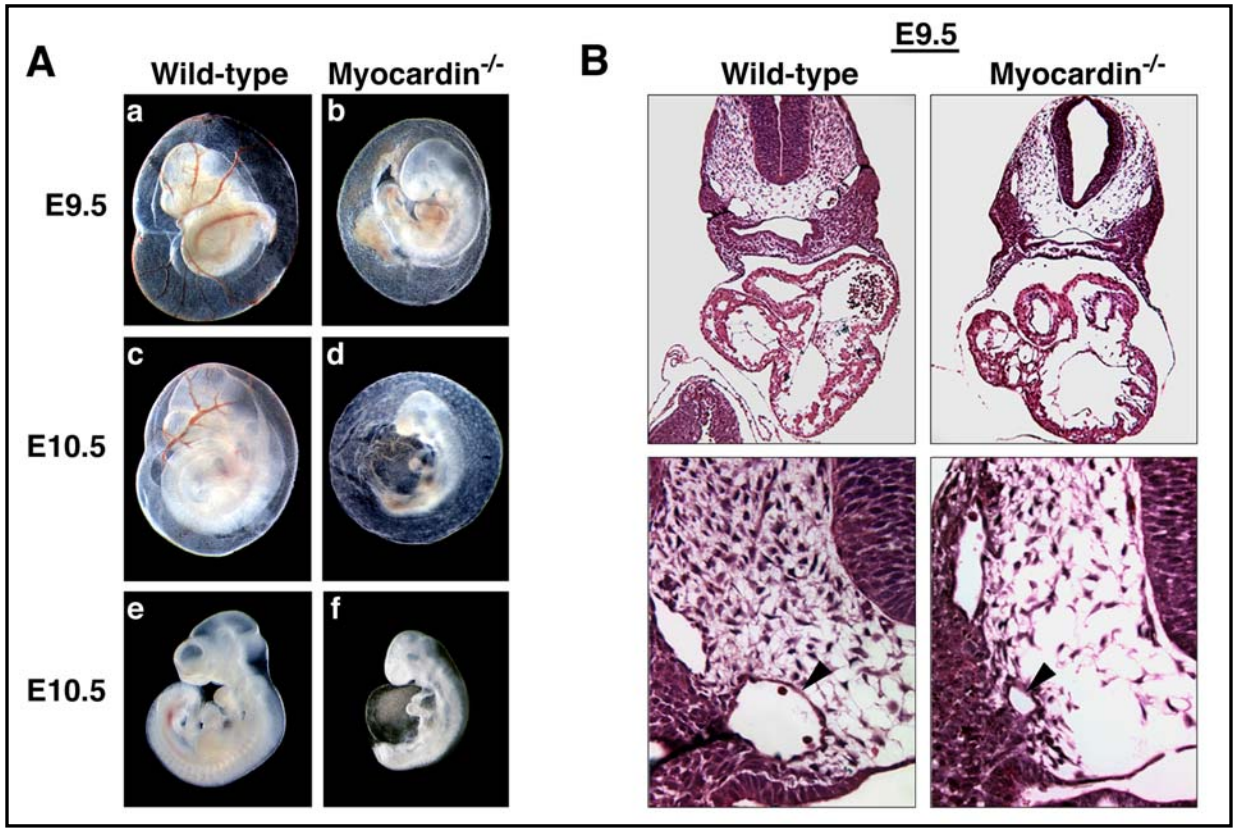

Fig. 3.2. Vascular abnormalities in myocardin mutant embryos. (*A*) Shown are WT and mutant yolk sacs and embryos at E9.5 and E10.5. In *e* and *f*, the yolk sacs were removed from the embryos shown in *c* and *d*. (*B*) Hematoxylin/eosin sections of WT and mutant embryos at E9.5. (*Lower*) High magnifications of the region with the dorsal aorta, indicated by an arrowhead.

cardinal veins appeared normal in mutant embryos at this stage. It is noteworthy that SM marker genes are not yet activated in the cardinal veins of WT embryos, whereas differentiated SMCs are present in the dorsal aortae at this stage. By E10.5, mutant embryos were severely delayed developmentally and pericardial effusion was often observed, indicative of cardiovascular insufficiency (Fig. 3.2.A. e and f).

Normal Endothelial Cell Differentiation and Organization in Myocardin$^{-/-}$ Embryos.

Vascular development initiates around E7.5 in the mouse with the differentiation and migration of endothelial progenitor cells [1-3]. To visualize the embryonic vasculature, I performed whole-mount antibody staining for PECAM-1, an endothelial marker [20]. At E8.5, PECAM staining of the newly formed vasculature was indistinguishable in WT and myocardin mutant embryos (data not shown). At E9.5, differentiated endothelial cells were properly positioned in mutant embryos, even though mutant embryos began to show growth retardation by this stage (Fig. 3.3. A and B). Notably, the complexity and patterning of the cranial vasculature, the intersomitic vasculature, and the dorsal aorta as revealed by PECAM-1 staining appeared similar in WT and myocardin mutant embryos. Transverse sections clearly showed the presence of PECAM-1-positive endothelial cells in the dorsal aorta and cardinal veins of both WT and mutant embryos, although the dorsal aorta in mutant embryos was smaller than normal (Fig. 3.3. C–F). Similarly, PECAM staining was detected in the endocardial layer of the heart of WT and myocardin mutant embryos. These findings indicated that vascular endothelial cell differentiation and organization were unaffected by the *myocardin* mutation.

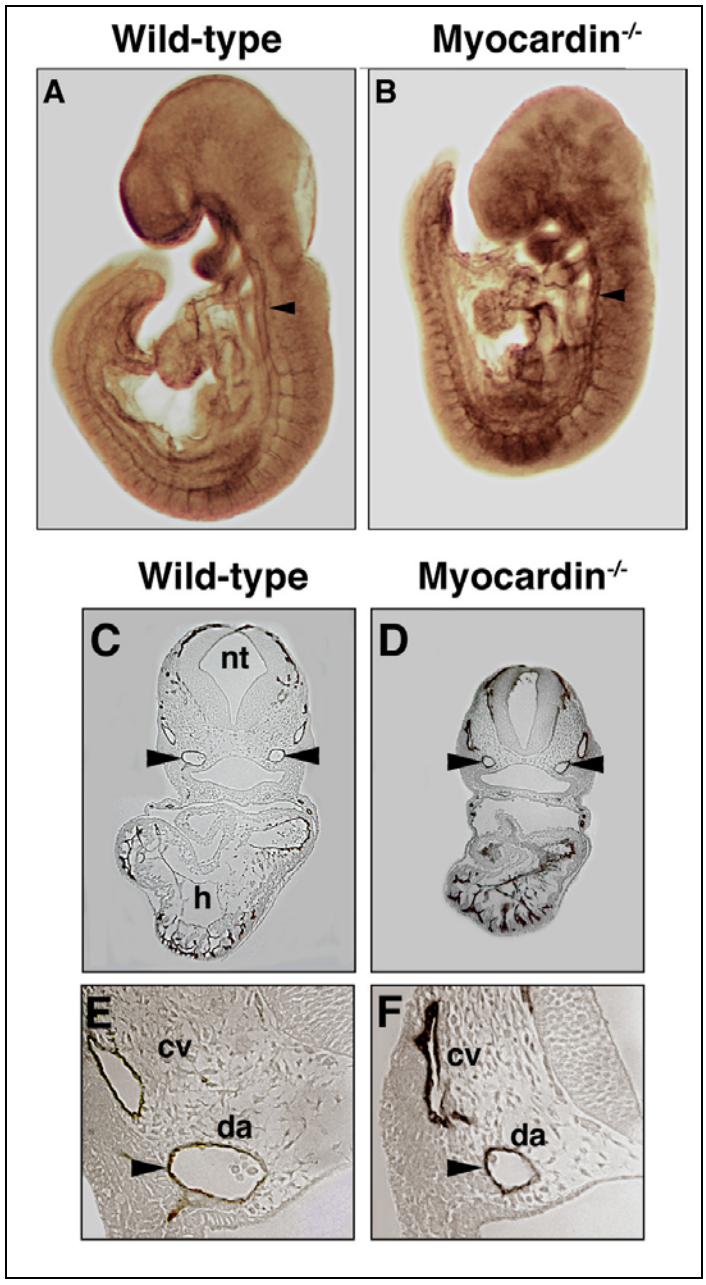

Fig. 3.3. Endothelial cell patterning detected by PECAM staining is unperturbed in myocardin mutant embryos. (*A* and *B*) WT and myocardin mutant embryos at E9.5 stained for PECAM. The arrowhead points to the dorsal aorta. (*C–F*) Histological sections of embryos stained for PECAM (*C* and *E*, WT; *D* and *F*, myocardin$^{-/-}$). *E* and *F* show high magnifications of the region of the dorsal aorta, indicated by arrowheads. cv, cardinal vein; da, dorsal aorta; h, heart; nt, neural tube.

Defects in Vascular SMC Differentiation in Myocardin$^{-/-}$ Embryos

To determine whether the vascular abnormalities in myocardin mutant embryos resulted from a defect in SMC differentiation, I stained E9.5 embryos with an Antibody against SM α-actin. As shown in Fig. 4A, SM α-actin-positive SMCs were present in the dorsal aorta and cardinal veins of WT embryos. However, no such SM α-actin-positive cells were detected in the vasculature of myocardin$^{-/-}$ embryos. Comparison of transverse sections of SM α-actin-stained embryos revealed that SMCs were missing from the dorsal aortae of myocardin$^{-/-}$ embryos (Fig. 3.4. A). Unexpectedly, however, there was no decrease in the expression of SM α-actin in myocardin$^{-/-}$ hearts.

I further examined the expression of SM genes by *in situ* hybridization to embryo sections at E9.5 (Fig. 3.4. B). The *SM22* and *SM α-actin* genes are direct target genes of SRF and are induced by myocardin in transfected fibroblasts [8, 12, 19, 21, 22]. Transcripts for both genes were expressed in the heart and developing vasculature of WT embryos (Fig. 3.4. B). In contrast, neither transcript was detected in the vasculature of myocardin$^{-/-}$ embryos, although normal expression was detected in the hearts of mutant embryos. The atrial natriuretic factor (*ANF*) gene, a cardiac-specific target of myocardin, was also expressed normally in mutant embryos. These findings demonstrated that vascular SMC differentiation was specifically disrupted in myocardin$^{-/-}$ embryos, which is the likely cause of vascular abnormalities and embryonic lethality.

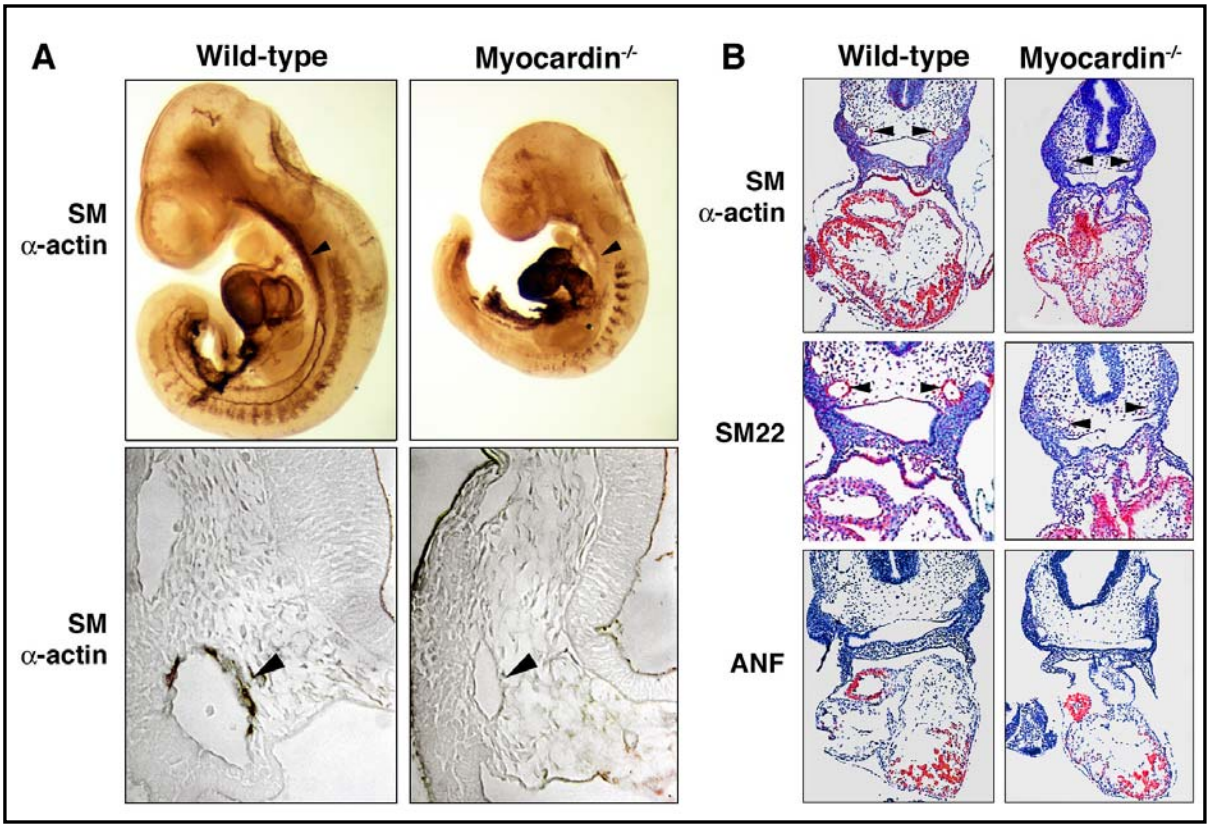

Fig. 3.4. Lack of expression of SM markers in myocardin mutant embryos. (*A*) WT and myocardin mutant embryos at E9.5 stained for SM α-actin. The arrowhead points to the dorsal aorta. (*B*) Detection of smooth and cardiac muscle transcripts by *in situ* hybridization to E9.5 embryo sections. Silver grains are shown pseudocolored red. Arrowheads point to the dorsal aortae.

Expression of SM22-lacZ in Myocardin$^{-/-}$ Embryos

The *SM22* promoter contains two CArG boxes that are required for expression in smooth, cardiac, and skeletal muscle cells at E9.5 and for transactivation of the promoter by myocardin *in vitro* [12, 22, 23]. The finding that the endogenous *SM22* gene was expressed in the heart of myocardin$^{-/-}$ embryos raised the question whether this promoter region was regulated by myocardin/SRF *in vivo* or whether another regulatory region was able to sustain the expression of *SM22* in the heart of mutant embryos. I therefore introduced an SM22-lacZ transgene into the myocardin mutant background by breeding to the corresponding transgenic line. As shown in Fig. 3.5, SM22-lacZ expression was specifically ablated in the dorsal aortae of homozygous mutant embryos, but not in the heart or somites, confirming that myocardin is required specifically for vascular SM gene expression.

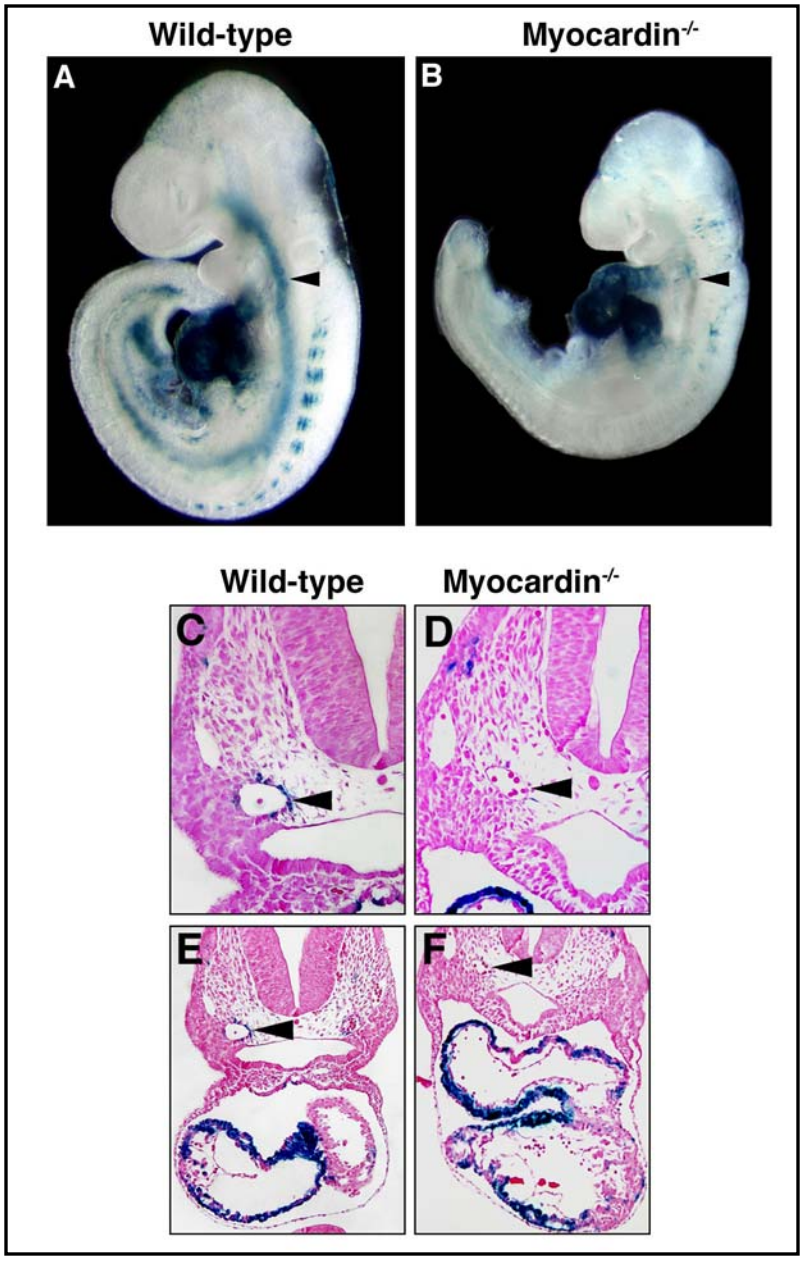

Fig. 3.5. Ablation of SM expression of SM22-lacZ in myocardin mutant embryos. (*A* and *B*) WT and myocardin$^{-/-}$ mutant embryos harboring an SM22-lacZ transgene were stained for lacZ expression at E9.5. Arrowheads point to the dorsal aortae. (*C–F*) Sections of WT and mutant embryos from *A* and *B* counterstained with light eosin (*C* and *E*, WT; *D* and *F*, myocardin$^{-/-}$). *E* and *F* show low magnifications to include the heart. Arrowheads point to the dorsal aortae.

Discussion:

The phenotype of myocardin mutant mice reveals an essential role of myocardin in differentiation of vascular SMCs. These findings coupled with studies demonstrating the ability of myocardin to induce SM gene expression in nonmuscle cells [19, 24, 25] establish myocardin as the only transcription factor known to be both necessary and sufficient for SM differentiation.

The Role of Myocardin in SM Development

SRF has been reported to activate SM genes in transfection assays by recruiting several other cofactors. For example, the homeodomain protein Mhox and two A/T-rich DNA-binding proteins referred to as MRF α and β have been proposed as SM-restricted activators of SRF target genes [26, 27]. The combination of GATA6 and LIM domain proteins of the cysteine-rich lim-only protein family has also been reported to be sufficient and necessary for SM gene expression in transfected cells, apparently by enhancing SRF DNA binding [28]. However, it remains unclear which, if any, of these purported SRF cofactors are essential for SM gene expression *in vivo* or whether there is so much redundancy among SRF cofactors that no single cofactor is indispensable for SM gene activation.

The abnormalities in vascular development in myocardin mutant embryos are highly specific to SMCs and occur in the absence of associated cardiac abnormalities, which contrasts with numerous other mouse mutants in which vascular demise is secondary to cardiac dysfunction. The complete block of SM development in myocardin$^{-/-}$ embryos

demonstrates that myocardin is an essential activator of the SM differentiation program *in vivo* and that no other factor can substitute for this promyogenic function, despite numerous reports that other transcription factors can cooperate with SRF to stimulate SM gene expression *in vitro*. These findings also demonstrate that SRF alone is incapable of activating SM target genes *in vivo* without recruiting myocardin.

Myocardin and Cardiac Gene Expression

Based on the absence of cardiac gene expression in *Xenopus* embryos expressing a dominant-negative myocardin mutant, and the ability of myocardin to activate cardiac gene promoters in transfection assays [12], we anticipated that myocardin would be required for heart development in the mouse. Nevertheless, we detected no abnormalities in cardiac morphogenesis or gene expression in myocardin mutant embryos. Even direct target genes of myocardin, such as *SM22, SM* α*-actin*, and *ANF* were expressed normally in the heart.

How can these findings be explained? We favor the possibility that MRTF-A or -B, which are expressed in the developing heart [14], or other cardiac transcription factors, may substitute for myocardin at this early stage, and such redundancy is lacking in the SM lineage. It is interesting to note that the early heart tube resembles a vessel and expresses many SM genes, which are later down-regulated. We propose that myocardin controls an early muscle regulatory program shared by the smooth and cardiac muscle lineages and that cardiac muscle cells possess additional myogenic regulators that modify this program. Combining the myocardin mutation with mutations in MRTF genes or other cardiac transcription factors should further illuminate the potential role of myocardin in the developing heart.

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Chapter IV

Potentiation of Serum Response Factor Activity by a Family of Myocardin-related Transcription Factors

Abstract

Myocardin is a SAP (SAF-A/B, Acinus, PIAS) domain transcription factor that associates with serum response factor (SRF) to potently enhance SRF-dependent transcription. Two myocardin-related transcription factors (MRTFs), A and B, which also interact with SRF and stimulate its transcriptional activity, were identified and cloned. Whereas myocardin is expressed specifically in cardiac and smooth muscle cells, MRTF-A and -B are expressed in numerous embryonic and adult tissues. In SRF-deficient embryonic stem cells, myocardin and MRTFs are unable to activate SRF-dependent reporter genes, confirming their dependence on SRF. Myocardin and MRTFs comprise a previously uncharacterized family of SRF cofactors with the potential to modulate SRF target genes in a wide range of tissues. Mice expressing a dominant negative mutant of MRTF-A in skeletal muscle showed a myopathic phenotype similar of mutant mice lacking SRF gene in skeletal muscle, which reveal an essential role for the partnership of SRF and myocardin-related transcription factors in the control of skeletal muscle growth and maturation *in vivo*.

Introduction

As described earlier, Serum Response Factor (SRF) regulates both muscle-specific and growth factor-inducible genes by binding the DNA consensus sequence CArG box [1]. The spectrum of genes activated by SRF is dictated by its differential affinity for different CArG-box sequences [2] and its association with a variety of positive and negative cofactors, many of which are cell type-specific and signal-responsive [3].

In addition to its role in proliferation and myogenesis, targeted inactivation of the mouse *Srf* gene has revealed a requirement of SRF in early embryogenesis and mesoderm formation [4, 5]. SRF-deficient [*Srf*(–/–)] embryonic stem (ES) cells retain their ability to proliferate [4, 6] and provide a powerful system for identifying transcriptional programs that depend on SRF as well as for analyzing the potential requirement of SRF for the activities of its cofactors.

The SAP domain transcriptional coactivator, myocardin, is an extraordinarily powerful SRF cofactor expressed specifically in smooth and cardiac muscle cells [7]. Myocardin selectively activates smooth and cardiac muscle promoters by its interaction with SRF. Expression of a dominant-negative myocardin mutant in *Xenopus* embryos blocks heart formation, suggesting that myocardin cooperates with SRF to activate cardiac gene expression. However, as I have shown earlier, myocardin mutant mice showed normal heart development but no evidence of vascular smooth muscle differentiation during embryogenesis [8]. When expressed in nonmuscle cells *in vitro*, myocardin activates smooth muscle gene expression [9].

Because SRF regulates numerous growth factor-inducible genes that are expressed in cells in which myocardin is not expressed, we investigated whether myocardin-related proteins might modulate SRF activity outside the cardiovascular system. Two myocardin-related transcription factors (MRTFs), referred to as MRTF-A and MRTF-B, that differentially stimulate SRF-dependent transcription were identified. In contrast to myocardin, MRTF-A and -B are expressed in a wide range of embryonic and adult tissues. In S*rf*(–/–) ES cells, myocardin and MRTFs are unable to transactivate SRF-dependent promoters, confirming the obligate role of SRF as a mediator of transcriptional activities of these factors.

Materials and Methods

Bioinformatics and cDNA Cloning

The mouse myocardin cDNA sequence was used to search NCBI databases to identify related genes by Da-Zhi Wang. Several human, mouse, and *Xenopus* cDNA clones and ESTs with homology to myocardin were identified. These sequences were used as probes to screen cDNA libraries for full-length cDNAs. The gene structures of *myocardin*, *MRTF-A*, and *MRTF-B* were deduced from available mouse genomic sequences.

RNA Analysis

Adult mouse multiple-tissue Northern blots (CLONTECH) were hybridized with cDNA probes encompassing the complete ORFs of MRTF-A and -B as described [7]. The cDNA probes for Northern blotting were labeled with ^{32}P-dCTP. For *in situ* hybridization, 3'-untranslated regions of MRTF-A and -B were transcribed *in vitro* in the presence of [^{35}S]UTP to make antisense and sense (as a control) riboprobes. *In situ* hybridization was performed as described [10].

Transfection Assays

SRF and myocardin expression constructs have been described [11]. MRTF-A and -B cDNAs encoding full-length proteins or different deletion mutants were subcloned into the pcDNA3.1 expression vector (Invitrogen) in frame with a C-terminal Myc epitope tag. For GAL4 transfection experiments, full-length proteins or the TADs (residues 692–929 and 784–1080 of MRTF-A and -B, respectively) were fused in frame to the GAL4-(1–147) DNA-

binding domain. Unless otherwise indicated, 100 ng of luciferase reporter and 100 ng of each activator plasmid were used. The total amount of DNA per well was kept constant by adding expression vector without a cDNA insert. Cytomegalovirus-lacZ was used as an internal control to normalize for variations in transfection efficiency. A retroviral SRF expression construct (pHeinz; D. Boos, O. Heidenreich, and A.N., unpublished data) was also used in some experiments using *Srf*(–/–) ES cells as indicated.

The SM22-luciferase construct contains the 1,434-bp promoter [12]. The atrial natriuretic factor (ANF)-luciferase construct contains the 638-bp promoter [13]. The 4xSM22 CArG-near-luciferase construct has been described [11]. ES cell transfections used lipofectamine (Invitrogen), and associated luciferase assays were performed as described in [14]. The generation and maintenance of the 100 *Srf*(–/–) ES cell line has been described [4].

DNA-Binding Assays

Gel-mobility shift assays were performed as described [2]. Myocardin and MRTF-A and -B proteins were transcribed and translated *in vitro* with a TNT T7-coupled reticulocyte lysate system (Promega). The DNA probe was labeled with ^{32}P-dCTP using a Klenow-fill in method and purified using a G25 DNA purification column from Roche. The 20 ul binding reaction contains 1 ug of poly (dI-dC) (Roche), 50,000 cpm of probe and 2ul of TNT for each protein in gel shift buffer (15 mM Hepes pH7.9, 40 mM KCl, 1 mM EDTA, 0.5 uM DTT and 5% glycerol). After incubation at room temperature for 15 minutes, the reactions were separated on a 5% PAGE gel containing 0.5 X TBE. The sequence for the SM22-CArG far probe is CTAGGTTTCAGGGTCCT GCCATAAAGGTTTTTCCCGGCCGCC [12].

GST Protein-Binding Assays

A cDNA encoding human SRF was cloned in frame to GST in the pGEX-KG vector (Amersham Pharmacia). GST-SRF fusion protein was expressed and purified as described [15]. Briefly, the constructs were transformed into BL21-CodonPlus^{TM}-RIL cells (Stratgene). A single colony was inoculated in 10 ml LB with ampicillin and shaken overnight at 37 ^{o}C. The next day, the culture was transferred into 500 ml of LB with ampicillin, and shaken at 37 ^{o}C until an O.D. 0.6 or so. Then IPTG was added to a final concentration of 50 uM. The culture was shaken at room temperature for another 4 hours. The bacteria were collected by spinning at 2,000g for 10 minutes at 4 ^{o}C. (The pellet can be stored at -80 ^{o}C.) The cells were lysed in 10 ml cold PBS containing 0.5 mM EDTA, 0.5 mg/ml Lysozyme (Sigma), 1 mM PMSF and protease inhibitor cocktail (Roche). After spinning at 10,000 rpm at 4 ^{o}C for 30 minutes, the lysate was incubated with 1 ml of 50% slurry glutathione beads (Amersham). After rotation at 4 ^{o}C for 90 minutes, the mixture was then washed with cold PBS containing 0.5% Triton X-100 for 3 times. ^{35}S-labeled myocardin and MRTF-A and -B were translated in a T7-coupled reticulocyte lysate system. For GST protein-binding assays, equal amounts of either GST-SRF or GST protein alone (as negative control) were incubated with myocardin, MRTF-A, or MRTF-B in GST binding buffer (20 mM Tris, pH 7.3/150 mM NaCl/0.5% Nonidet P-40/protease inhibitors) for 1 h at 4°C. After washing three times with GST binding buffer, proteins associated with GST-agarose beads were analyzed by 10% SDS/PAGE.

Western Blot Analysis

Skeletal muscle extracts were prepared and used for Western blotting with anti-FLAG antibodies and horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biosciences). Signal was detected with Western blotting Luminol Reagent (Santa Cruz Biotechnology), followed by exposure of blots to BioMax film (Kodak).

Histology

Skeletal muscle was dissected from the hind limbs of WT and mutant mice and fixed with 4% paraformaldehyde. Embedding of tissues, histological sectioning, and staining with hematoxylin and eosin (H&E) were performed by standard procedures.

Results:

Identification of a Family of MRTFs

Da-Zhi Wang found ESTs that share significant homology with myocardin from the NCBI database. I cloned Full-length cDNAs encoding two myocardin-related proteins, MRTF-A and -B by screening mouse embryo cDNA libraries or applying a PCR-based cloning strategy using these EST sequences. The protein structures of mouse myocardin and MRTF-A and -B are schematized in Fig. 4.1.A. The overall amino acid identity between the three proteins is ~35%, whereas they share >60% amino acid identity within the basic, glutamine (Q)-rich, and SAP domains (Fig. 4.1. A and B). Outside of these regions, homology among these three proteins is restricted to the N-terminal region and the C-terminal region, which functions as a TAD. It is also notable that the amino acid identity between MRTF-A and -B (42%) is greater than that between MRTFs and myocardin.

Expression Patterns of MRTFs

The Northern blotting analysis that I performed showed that MRTF transcripts are present in a wide range of adult tissues (Fig. 4.2. A and B). MRTF-A has two major transcripts (~4.5 and ~2.5 kb) present in all tissues examined, with the most abundant expression in heart and liver. MRTF-B apparently has one major transcript of ~9 kb with dominant expression in heart and brain. A transcript of ~3 kb, which apparently represents an alternatively spliced form of MRTF-B, is also detected in testis.

In contrast to the expression of myocardin in heart and a subset of smooth muscle cells [7], MRTF-A and -B transcripts were detected throughout the embryo at embryonic day

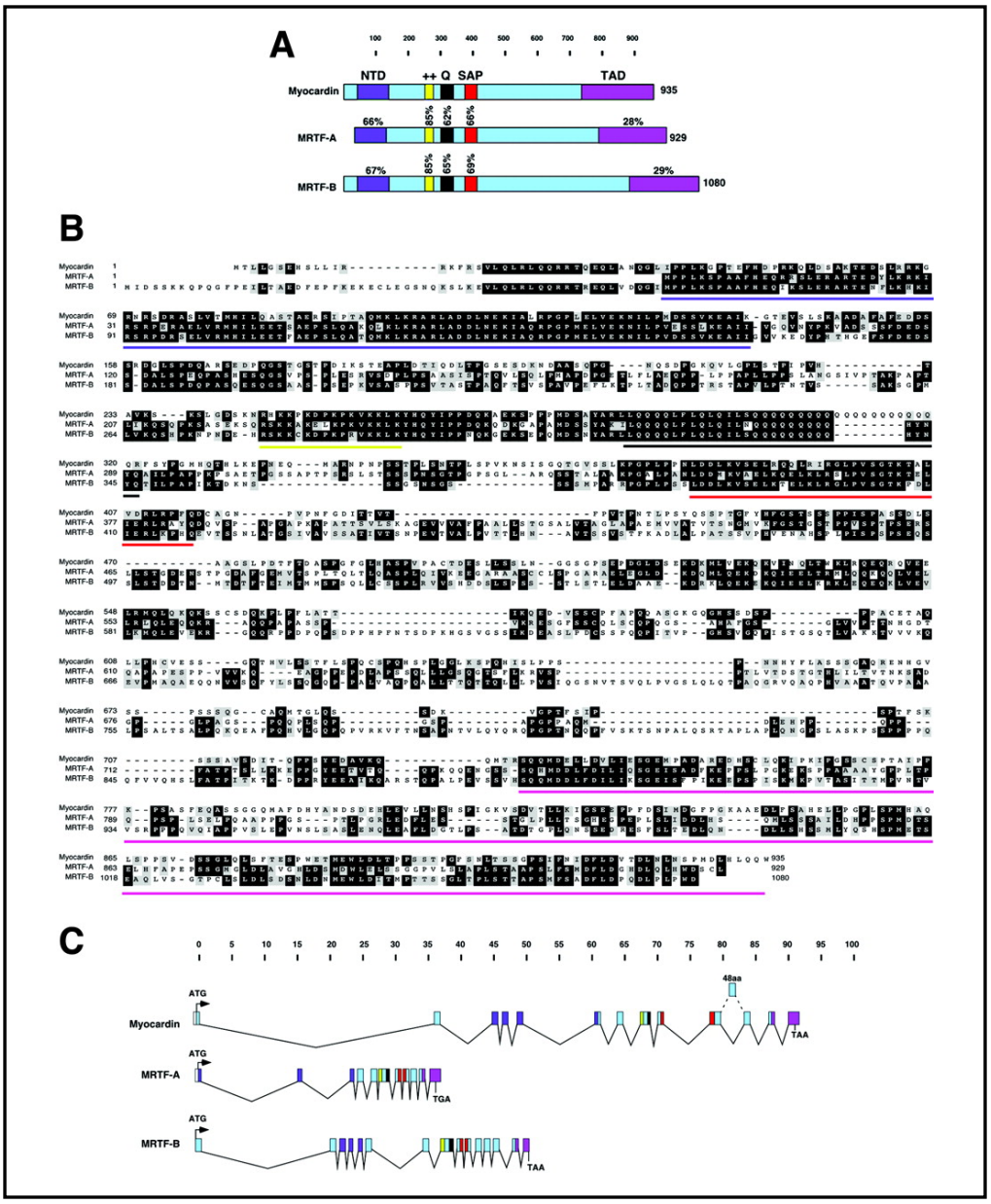


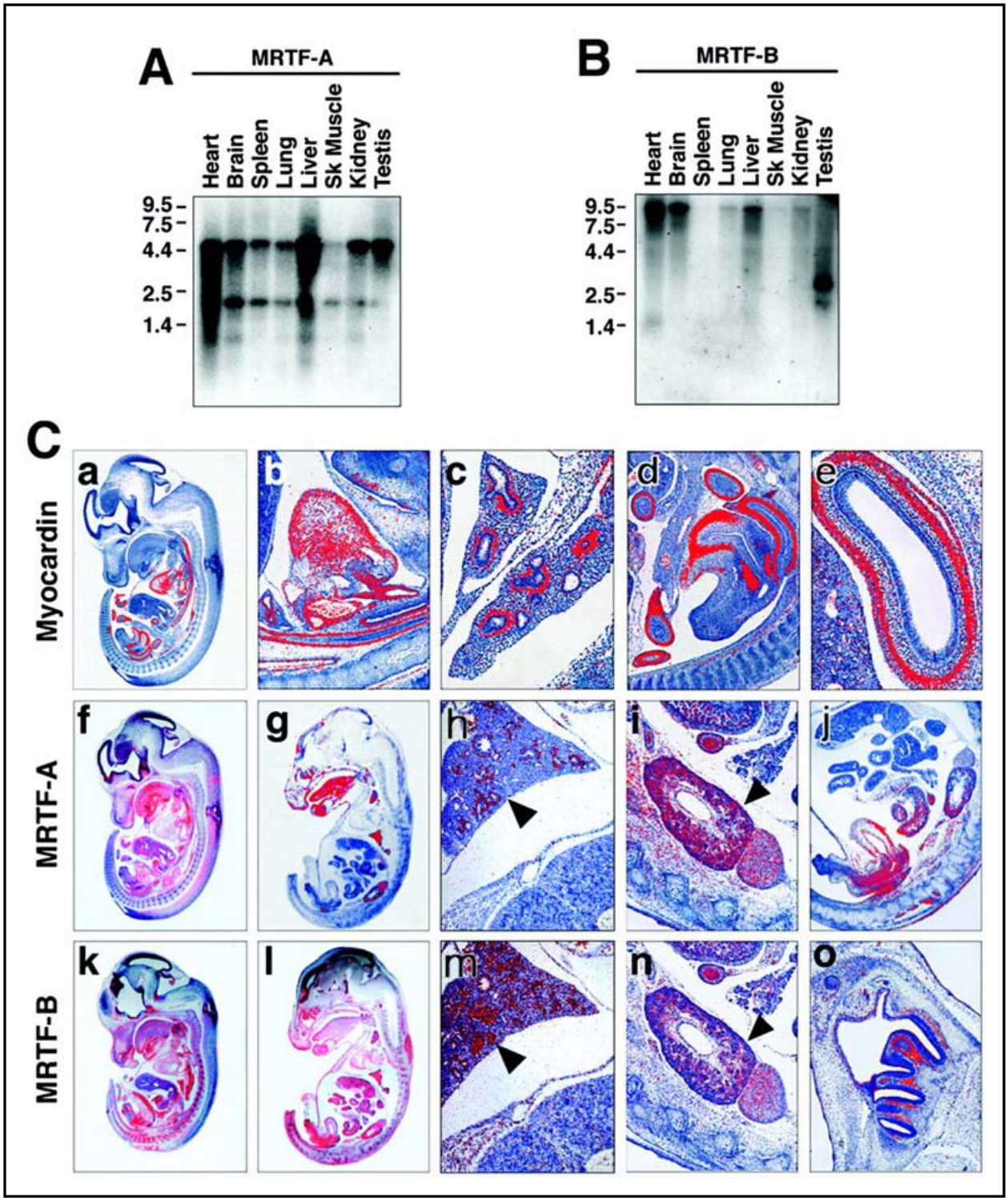
Fig. 4.1. Structure of the myocardin family of transcription factors. (*A*) Schematic diagrams of myocardin, MRTF-A, and MRTF-B proteins. ++, basic region; NTD, N-terminal domain; Q, glutamine-rich region. The number of amino acids in each protein is shown to the right, and percent identity between the indicated domains of each MRTF and myocardin is shown. (*B*) Amino acid sequence homology between myocardin and MRTFs. Colored bars correspond to the conserved regions shown in *A*. (*C*) Gene organization of mouse *myocardin*, *MRTF-A*, and *MRTF-B*. The colors of exons correspond to the regions shown in *A*. Dashed lines designate an alternative exon. Kilobases of genomic DNA are shown above the gene structures. Translation initiation (ATG) and termination codons (TAA and TGA) are indicated.

(E)10.5 (data not shown). By E13.5, MRTF-A continued to be expressed at a low level in most tissues, but higher expression was detected in a subset of neural mesenchymal cells, skeletal muscle of the tongue, and epithelial cells of the colon and small intestines (Fig. 4.2.C.f). At E15.5, the expression of MRTF-A in the above tissues became more obvious (Fig. 4.2.C.g). MRTF-A expression was detected also in epithelial cells of lung, kidney, bladder, and colon at this stage (Fig. 4.2.C. h–j).

Like MRTF-A, MRTF-B is expressed in epithelial cells of the lung, kidney, colon, and testis (Fig. 4.2.C. k–n). However, unlike MRTF-A, MRTF-B is expressed in the smooth muscle of the colon and small intestines. MRTF-B expression is also pronounced in mesenchymal cells adjacent to the olfactory epithelium (Fig. 4.2.C. o). Expression of MRTF-B in the developing lung differs from that of MRTF-A. Whereas the expression of MRTF-A is restricted to epithelial cells, MRTF-B seems to be expressed in both epithelial and mesenchymal cells (compare Fig. 4.2. C. h with m).

Stimulation of SRF-Dependent Transcription by MRTFs

To assess the potential transcriptional activity of MRTF-A and -B, I fused their complete coding regions to the GAL4 DNA-binding domain and assayed their abilities to activate a GAL4-dependent luciferase reporter in transfected COS cells. In this assay, myocardin and both MRTFs showed an increase in transcriptional activity (Fig. 4.3.A). The C-terminal regions of MRTF-A and -B were much more potent as transactivators than the full-length proteins (Fig. 4.3.B), as observed with myocardin (Fig. 4.3.B; ref. 8).


A
MRTF-A
Heart
Brain
Spleen
Lung
Liver
Sk Muscle
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Testis
9.5
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B
MRTF-B
Heart
Brain
Spleen
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Liver
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Fig. 4.2. Expression patterns of myocardin and MRTFs in adult and embryonic tissues. (*A* and *B*) Northern blot analyses of MRTF-A and -B, respectively, in adult mouse tissues. (*C*) Expression of myocardin (*a–e*), MRTF-A (*f–j*), and MRTF-B (*k–o*) in mouse embryos as detected by *in situ* hybridization. Myocardin is expressed in cardiac and smooth muscle cells of an E13.5 embryo (*a*). A higher magnification of the same embryo showing myocardin expression in heart, smooth muscles of esophagus, and dorsal aorta (*b*), lung (*c*), bladder and small intestine (*d*), and stomach (*e*) is shown. MRTF-A is expressed in the tongue (*f* and *g*), lung and diaphragm (*h*), kidney (*i*), bladder (*j*), and colon of E13.5 (*f*) and E15.5 (*g–j*) mouse embryos. Expression of MRTF-B in E13.5 (*k*) and E15.5 (*l–o*) mouse embryos. Note higher-level expression present in the lung (*m*), kidney (*n*), and olfactory epithelium (*o*). Arrowheads in *h* and *m* point to the lung and in *i* and *n* to the kidney.

Because MRTF-A and -B share homology with the basic and Q-rich regions of myocardin, which interact with SRF [7], I tested whether they also could potentiate the activity of SRF using luciferase reporters linked to the *SM22* and *ANF* promoters, both containing a pair of CArG boxes. MRTF-A and myocardin activated these reporters to similar levels (Fig. 4.3.C). In contrast, MRTF-B was less effective in activating the *SM22* reporter and showed almost no transcriptional activity with the *ANF* reporter despite the fact that MRTF-B was as potent as myocardin and MRTF-A when fused to the GAL4 DNA-binding domain. Similar to myocardin, MRTF-A and -B required the CArG boxes in the *SM22* and *ANF* promoters for transcriptional activation, because these factors were unable to transactivate promoters with CArG box mutations (Fig. 4.3.C). MRTF-A also activated a luciferase reporter containing the Elb minimal promoter and four tandem copies of an *SM22* CArG-near to a level comparable to that of myocardin, whereas MRTF-B activated this reporter only to a minimal level (Fig. 4.3.C). Similar to myocardin, MRTFs did not activate the c-*fos* promoter efficiently, which contains a single CArG box (data not shown). Myocardin, MRTF-A, and MRTF-B were expressed at comparable levels as determined by Western blot analysis of transfected cells (data not shown).

Myocardin and MRTFs Fail to Activate SRF-Dependent Transcription in *Srf* Null ES Cells.

To further investigate the potential dependence of myocardin and MRTFs on SRF for transcriptional activity, I examined their abilities to transactivate SRF-dependent promoters in *Srf*(–/–) ES cells. Myocardin activated expression of the *SM22* promoter in wild-type ES

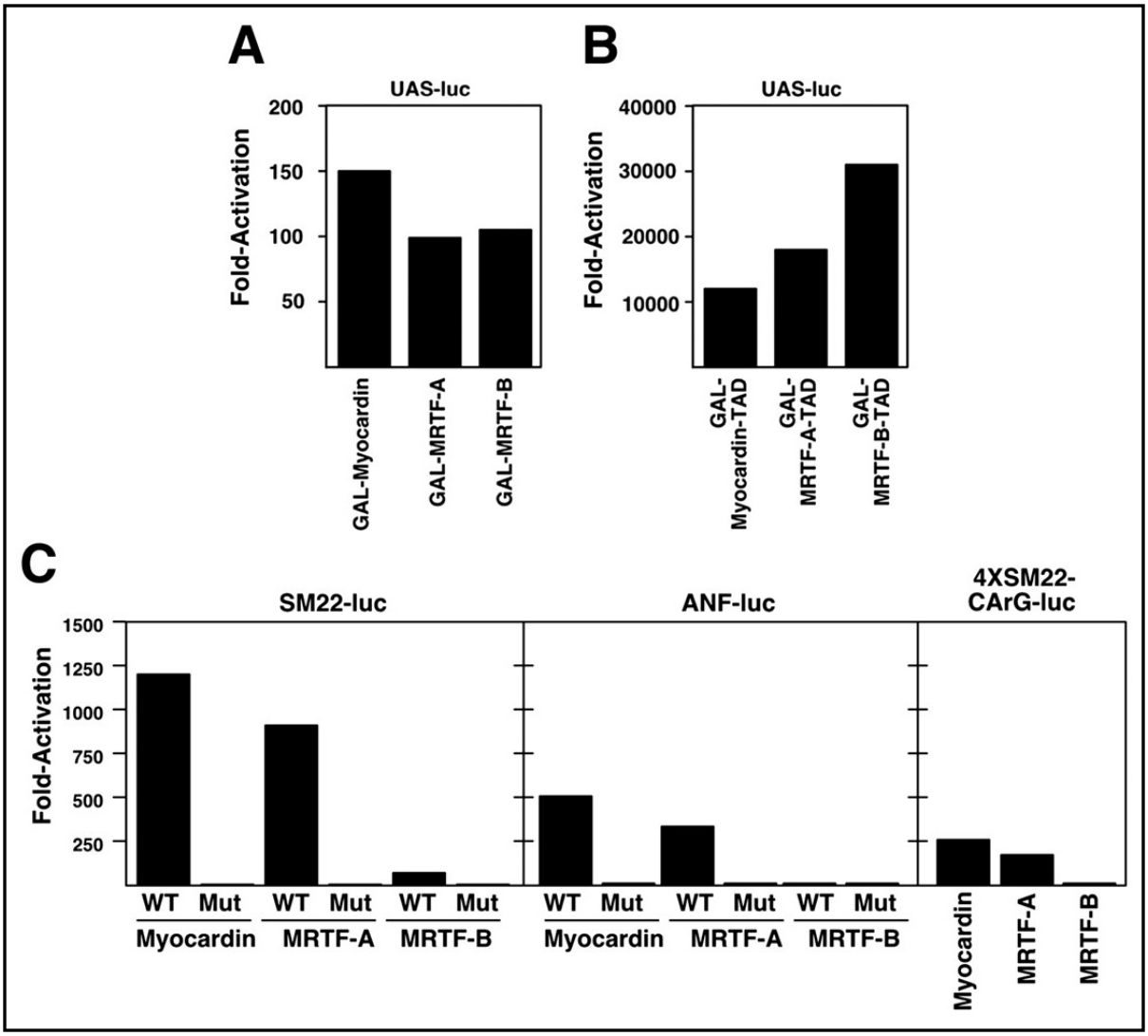

Fig. 4.3. Transcriptional activity of MRTFs. The complete ORFs (*A*) or TADs (*B*) of myocardin and MRTFs were fused to the GAL4 DNA-binding domain and tested for transcriptional activity by using a GAL4-dependent luciferase reporter (UAS-luc) in transfected COS cells. (*C*) Transactivation of luciferase reporters linked to *SM22* or *ANF* promoters or four tandem copies of CArG-near from the *SM22* promoter by myocardin and MRTFs, as indicated. WT refers to the wild-type promoter, and Mut refers to the promoters with mutations in the two CArG boxes. Values are presented as the fold activation of expression above the background level of expression of vector alone. All transfection assays were performed at least three times, and representative data are shown.

cells (Fig. 4.4.A) but not in *Srf*(–/–) ES cells (Fig. 4.4.B and data not shown). The MRTFs also were incapable of activating this reporter in *Srf*(–/–) ES cells, whereas introduction of SRF into *Srf*(–/–) ES cells with an SRF-expressing murine retroviral construct restored transcriptional activity to myocardin and MRTFs. These findings demonstrate that myocardin and MRTFs can potently activate CArG-box-dependent gene expression in a strictly SRF-dependent fashion.

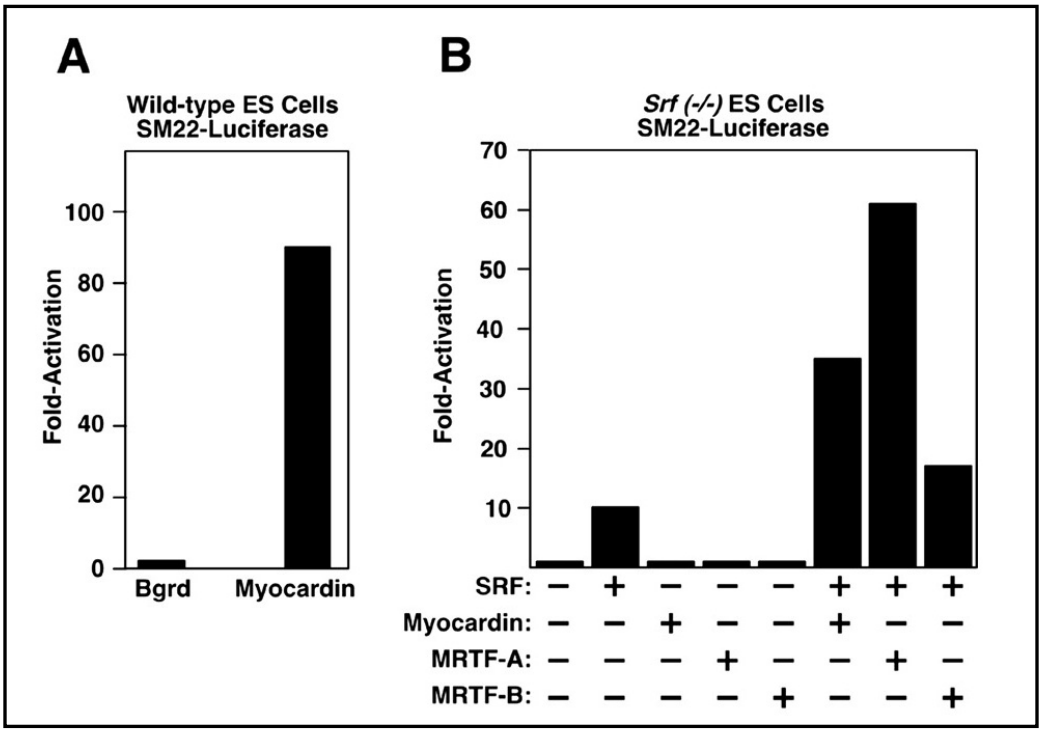

Fig. 4.4. Lack of transcriptional activity of myocardin and MRTFs in *Srf*(–/–) ES cells. Wild-type (*A*) and *Srf*(–/–) (*B*) ES cells were transiently transfected with the SM22-luciferase reporter and expression plasmids encoding myocardin, MRTFs, and SRF. Values are presented as the fold activation of expression above the background level of expression of vector alone. All transfection assays were performed at least three times, and representative data are shown.

Physical Interaction of SRF and MRTFs.

To determine whether MRTF-A and -B formed ternary complexes with SRF on DNA, we performed gel-mobility shift assays using *in vitro*-translated proteins and labeled probes corresponding to the SM22 CArG box. Ternary complex formation between myocardin and SRF was readily observed, whereas unexpectedly, ternary complex formation between MRTF-A and SRF was barely detectable, and complex formation between MRTF-B and SRF was undetectable under our assay conditions (Fig. 4.5.A).

Using a GST protein-binding assay, we found that GST-SRF interacts with myocardin, MRTF-A, and MRTF-B (Fig. 4.5.B). At present we cannot reconcile the difference between the gel-mobility shift assay and the GST protein-binding assay. A likely explanation for this finding is that the gel-mobility shift assay is more stringent and requires a higher affinity complex than does the GST protein-binding assay. The fact that myocardin and MRTFs have different affinities for SRF may explain (at least partially) why they transactivate *SM22* and *ANF* reporter genes differentially despite their similar transcriptional activities when fused to the GAL4 DNA-binding domain.

Skeletal Muscle Hypoplasia Resulted from Expression of dnMRTF-A.

As I have shown in Chapter II, SRF is critical for skeletal muscle growth and maturation. Since MRTFs are strong SRF co-activators and they are expressed in skeletal muscle, I wondered if MRTFs are required by SRF to exert its function during skeletal muscle development. To answer this question, I overexpressed a dominant negative mutant of MRTF-A, which contains the leucine zipper domain and the SRF-binding region, but lacks

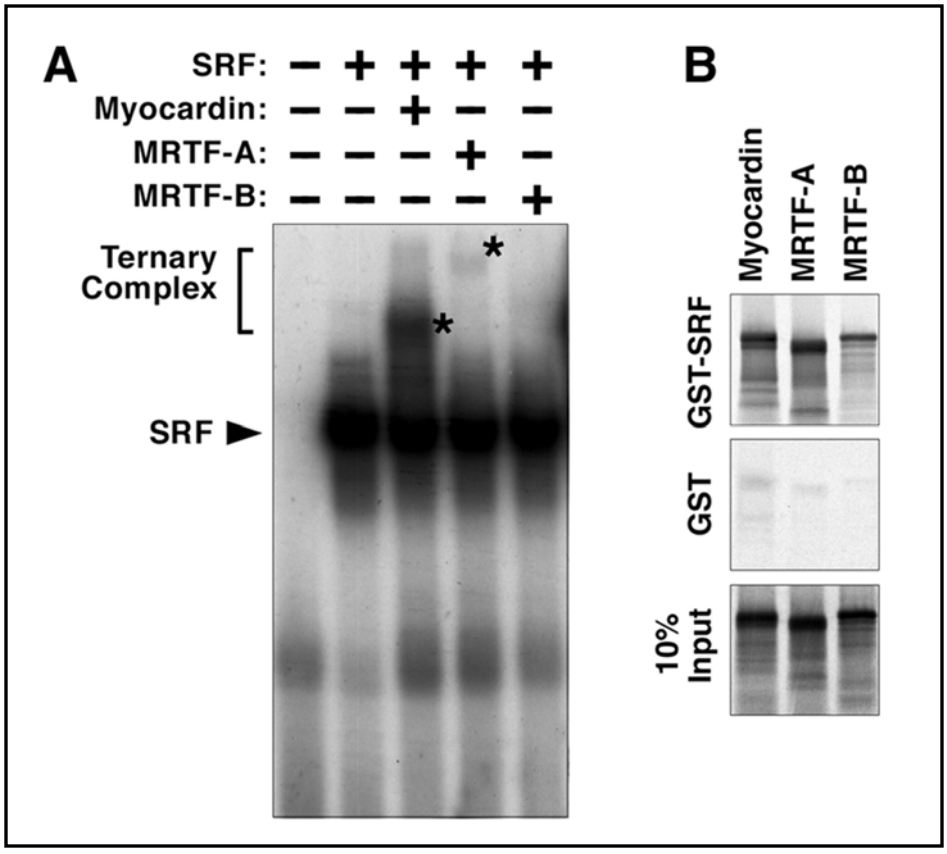

Fig. 4.5. Interaction of SRF and MRTFs. (*A*) Gel-mobility shift assays were performed with a ^{32}P-labeled oligonucleotide probe for *SM22* CArG-far and *in vitro* translation products of myocardin, MRTF-A, or MRTF-B in the presence and absence of SRF. Asterisks designate the position of the ternary complexes formed between SRF and myocardin or MRTF-A. The ternary complex between SRF and MRTF-A was very weak and between SRF and MRTF-B was undetectable. (*B*) GST-SRF protein interaction. Myocardin, MRTF-A and -B, translated *in vitro* with [^{35}S]methionine, were incubated with either GST-SRF-agarose beads or GST-agarose beads as indicated. After washing, proteins associated with beads were separated on 10% SDS/PAGE and analyzed by autoradiography. One-tenth of the *in vitro*-translated proteins were also separated directly on the gel as a loading control.

the transcription activation domain, in mouse skeletal muscle using a MCK promoter. This mutant can compete with WT MRTFs for association with SRF and can form heterodimers with WT MRTFs, and suppress SRF activity *in vitro* (data not shown). Expression of FLAG-tagged dnMRTF-A in skeletal muscle of transgenic mice was confirmed by Western blot analysis (Fig. 4.5.A).

Mice expressing dnMRTF-A were viable, but failed to thrive and showed skeletal myopathy and hypoplasia reminiscent of, although less severe than, the phenotype resulting from skeletal muscle-specific *Srf* deletion (Fig. 4.5. B and C). The severity of the muscle phenotype depended on the level of dnMRTF-A expression. Transgenic line 1, which expressed FLAG-dnMRTF-A at a level ~4-fold higher than line 2, showed a more severe myopathic phenotype (Fig. 4.5. A and C and data not shown). In contrast with mice lacking skeletal muscle expression of *Srf*, these transgenic mice survived to adulthood, likely because dnMRTF-A is unable to completely silence SRF activity. Myofibers from MCK-dnMRTF-A transgenic mice also showed extensive fibrosis and centrally located nuclei, indicative of muscle damage and regeneration (Fig. 4.5.C). Transgenic animals also were runted, reflecting the failure in skeletal muscle growth. The mean body weights (±SD) of WT and transgenic mice at 8 weeks of age were 21.4 ± 1.6 g ($n = 12$) versus 18.9 ± 1.0 g ($n = 9$) ($P < 0.005$). We detected no abnormalities in cardiac structure in MCK-dnMRTF-A transgenic mice. RNA analysis showed a decline in expression of skeletal and cardiac α-actin genes, as well as the *MCK* gene, in these transgenic mice (Fig. 4.5.D). Based on the intensity of MRTF-A transcripts in WT and transgenic mice (line 1), we estimate the transcript encoding dnMRTF-A to be expressed at a level ~4-fold higher than the endogenous MRTF-A transcript.

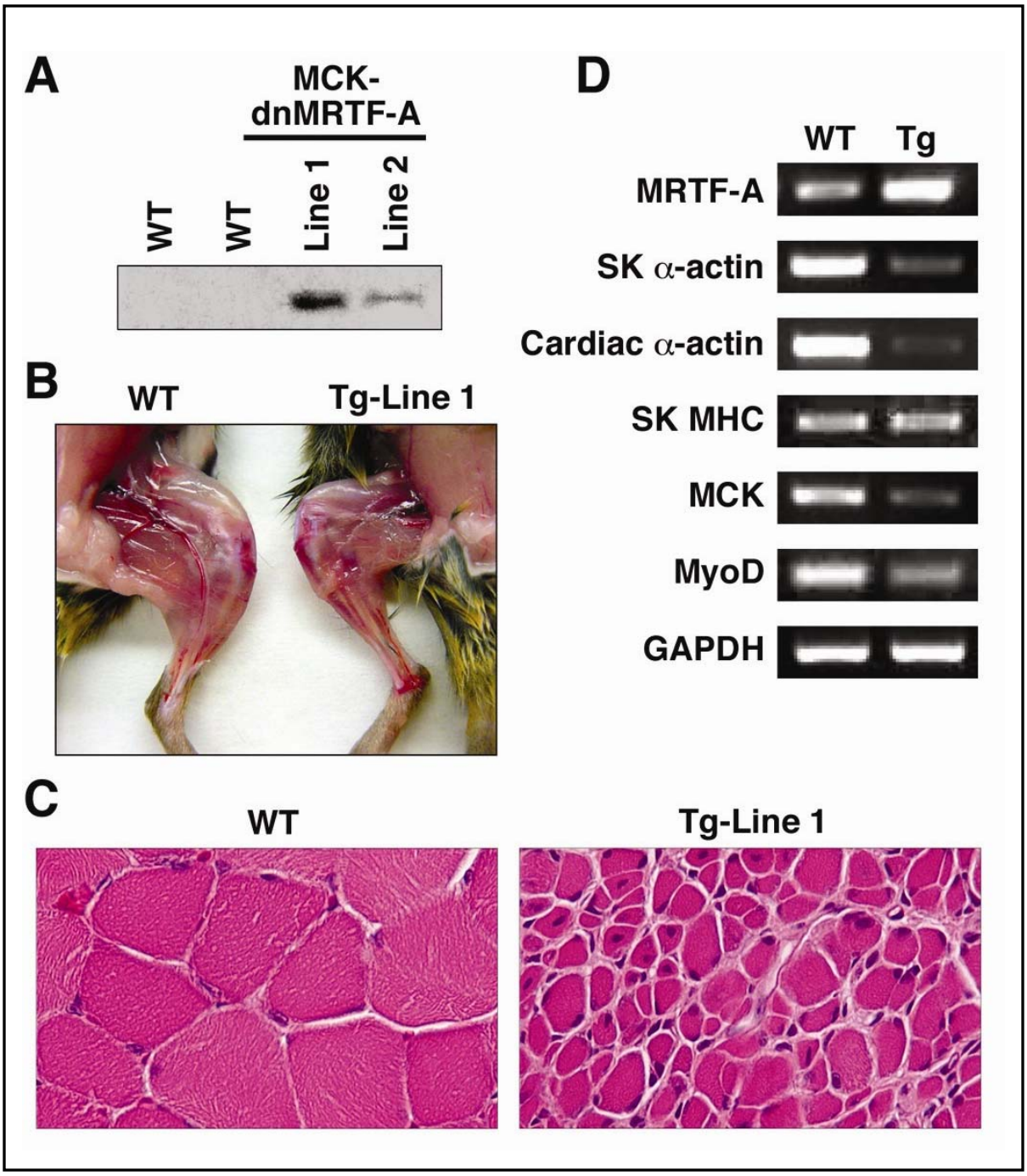
A
MCK-dnMRTF-A
WT
WT
Line 1
Line 2
B
WT
Tg-Line 1
C
WT
Tg-Line 1
D
WT
Tg
MRTF-A
SK α-actin
Cardiac α-actin
SK MHC
MCK
MyoD
GAPDH

Fig. 4.6.. Skeletal muscle abnormalities resulting from expression of dnMRTF-A. (*A*) Western blot analysis of skeletal muscle from *MCK-dnMRTF-A* transgenic mice. Extracts from skeletal muscle of WT and *MCK-dnMRTF-A* transgenic mice were analyzed by Western blot with anti-FLAG antibody to detect FLAG-tagged dnMRTF-A. Two transgenic lines are shown. (*B*) Hindlimb muscles of WT and *MCK-dnMRTF-A* transgenic (line 1) mice at 4 weeks of age are shown. The transgenic animal shows severe skeletal myopathy. (*C*) Histological sections of hindlimb muscles of WT and *MCK-dnMRTF-A* transgenic mice at 4 weeks of age were stained with H&E. The muscle fibers in the transgenic animals are thinner than those of WT. Transgenic line 1 shows the most severe phenotype with extensive fibrosis and centrally located nuclei. (Bar: 20 μm.) (*D*) RNA was isolated from hindlimb muscles of WT and *MCK-dnMRTF-A* transgenic (Tg) mice line 1 at 4 weeks of age and analyzed by semiquantitative RT-PCR for the indicated transcripts.

Discussion:

Myocardin and MRTFs comprise a previously uncharacterized family of SRF cofactors with extraordinary transcriptional potency. Whereas myocardin is expressed in a cardiac- and smooth muscle-specific manner, MRTF-A and -B are widely expressed. Their different expression patterns and differential effects on SRF activity suggest that myocardin and MRTFs participate in distinct SRF-dependent programs of gene expression.

The Myocardin Family.

Myocardin and MRTFs share a common structural organization with conserved N-terminal, basic, Q-rich, and SAP domains. These proteins also contain TADs near their C termini, which are less conserved than these other domains (see Fig. 6). Myocardin and the MRTFs constitute a subclass of SAP domain transcription factors. The SAP domain is a conserved 35-aa motif that contains two amphipathic α-helices that resemble helices 1 and 2 of the homeodomain [16]. SAP domains are found in a variety of nuclear proteins including the nuclear matrix attachment factors SAF-A and -B [17, 18], Acinus, which is a target for caspase cleavage that participates in chromatin degradation during apoptosis [19], and PIAS (protein inhibitor of activated STAT), a transcriptional repressor that associates with a variety of transcription factors [20].

The SAP domains of SAF-A and PIAS interact with matrix attachment regions, which has been proposed to stimulate transcription by forming active domains of chromatin [21]. Myocardin also can bind matrix attachment regions through its SAP domain (Wang DZ, Wang Z, and Olson EN, unpublished data). A myocardin mutant lacking the SAP domain

retains the ability to transactivate the *SM22* promoter but is unable to activate the *ANF* promoter [7]. Whether this differential requirement of the SAP domain of myocardin for transcriptional specificity reflects a role of matrix attachment region binding or other cofactor associations remains to be determined.

The Q-rich domain of myocardin is required for association with SRF [7], and the transcriptional potency of myocardin and MRTFs correlates with the length of the Q-rich domain. Q-rich domains have been identified in a variety of other transcription factors and are presumed to mediate interactions with other components of the transcriptional machinery [22, 23].

The human *MRTF-A* gene was reported to be translocated to chromosome 1 in the recurrent and specific t (1, 22) translocation in acute megakaryocytic leukemia [24, 25]. This translocation creates a fusion protein with the human protein One-Twenty-Two (OTT)/RBM15 (RNA-binding motif protein-15). OTT belongs to a family of nuclear proteins that share homology in a putative RNA-binding motif [26, 27]. Our finding that MRTF-A is a potent transcriptional coactivator of SRF raises the possibility that OTT-MAL may induce aberrant growth via SRF.

Potentiation of SRF Activity by Myocardin and MRTFs.

Our results show that myocardin and MRTFs are unable to activate SRF-dependent promoters in *Srf*(–/–) ES cells, confirming that SRF is an obligatory partner for these factors, at least on the SRF-dependent promoters tested, and probably others. Whether myocardin and MRTFs can cooperate with other transcription factors is an interesting question for the future.

In contrast to the high-affinity association between myocardin and SRF, the interaction of MRTFs and SRF is relatively weak and was detectable in a GST protein-binding assay but not in a DNA-binding assay. Because both MRTFs can transactivate CArG-box-dependent promoters and require SRF for this activity, we suggest that, similar to myocardin, MRTFs act through SRF to activate transcription. Consistent with this notion, dominant negative mutants of MRTFs can interfere with the activity of myocardin and vise versa (Wang D, Li S, and Olson EN, unpublished results), suggesting they may compete for the association with SRF.

Regulation of SRF Activity by Cofactor Interactions.

The activity of SRF is modulated through its interactions with a plethora of transcriptional cofactors. Among them, a family of Ets domain-containing proteins forms ternary complexes with SRF on the serum response element of the c-*fos* promoter [3]. This family of TCFs includes the ubiquitously expressed proteins SAP-1, SAP-2/Net [28, 29], and Elk-1 [30, 31]. It is worth noting that despite their names, the latter proteins do not contain SAP domains. In addition to binding to the MADS domain of SRF, the TCF proteins can also bind directly to the Ets domain-binding consensus core motif GGA(A/T), which is adjacent to the CArG box in the c-*fos* promoter. Whether the association of SRF with myocardin/MRTFs and TCFs is mutually exclusive remains to be determined.

Given the tissue distribution of MRTF-A and –B, as well as their differing affinities for SRF, it is reasonable to speculate that MRTF-A and -B may play different roles from that of myocardin. Considering that the function of TCF and SRF is signal-dependent [32, 33], it

will be interesting to determine how cellular and/or extracellular stimuli regulate the functions of the myocardin family of transcriptional cofactors.

A Role for MRTFs in Muscle Development

Members of the myocardin family stimulate SRF activity and have been implicated in differentiation of cardiac and smooth muscle [7, 9, 34-39]. Consistant with the results from Chapter II with the deletion of SRF specifically in skeletal muscle, I found a dominant negative mutant of MRTF-A inhibits muscle growth and cause skeletal muscle myopathy. Similarly, a dominant negative mutant of MRTF-B/MKL2 inhibits differentiation of skeletal muscle cells in vitro [40]. MRTFs form homodimers and heterodimers through a leucine zipper [9]. The dominant negative mutant used in these studies contains the leucine zipper domain and the SRF-binding region, but lacks the transcription activation domain. This mutant can compete with WT MRTFs for association with SRF and can form heterodimers with WT MRTFs with diminished transcriptional activity. Although we favor the interpretation that dnMRTF-A blocks muscle growth by disrupting the functions of MRTF-A or MRTF-B, it is also possible that it displaces other transcription factors from SRF or even interferes with the activities of transcriptional partners that function independently of SRF.

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Chapter V

Requirement of Myocardin-Related Transcription Factor-A for Development of Mammary Myoepithelial Cells

Abstract

The mammary gland consists of a branched ductal system that acquires increasing complexity during pregnancy and lactation. The mammary network is comprised of milk-producing epithelial cells that form the ductile tubules surrounded by a myoepithelial cell layer that provides contractility required for milk ejection. Myoepithelial cells bear a striking resemblance to smooth muscle cells, but they are derived from a different embryonic cell lineage and little is known of the mechanisms that control their differentiation. Members of the myocardin family of transcriptional coactivators cooperate with serum response factor to activate smooth muscle gene expression. I show that mice homozygous for a loss-of-function mutation of the myocardin-related transcription factor MRTF-A are viable, but females are unable to effectively nurse their offspring due to a failure in differentiation of mammary myoepithelial cells that prevents milk secretion. The phenotype of MRTF-A mutant mice reveals a highly specific and essential role for MRTF-A in mammary myoepithelial cell differentiation and points to commonalities in the transcriptional mechanisms that control differentiation of smooth muscle and myoepithelial cells.

Introduction

In contrast to most organs, the mammary gland develops primarily after birth in response to endocrine signals. During embryogenesis, the nascent mammary gland forms by budding of embryonic ectoderm and invasion of adjacent mesenchyme to give rise to a primitive ductal tree, which increases in size and branching pattern in response to hormonal signaling during puberty. During pregnancy, mammary ductal branching further increases and a secretory lobulo-alveolar compartment forms at the termini of the ductal branches, allowing for production and secretion of milk. After weaning of their offspring, the mother's lobulo-alveolar compartment remodels to the virgin-like state. Thus, the mammary gland undergoes a cyclical process of hormone-dependent differentiation and de-differentiation [1-3].

The mammary tree in adult females is composed of a luminal epithelial layer of milk-producing cells surrounded by a basal layer of myoepithelial cells that provides structural support and contractility required for milk release [4]. Myoepithelial cells possess characteristics of both epithelial cells and smooth muscle cells (SMCs). They are true epithelial cells since they are derived from ectoderm, they express cytokeratins as the major component of the intermediate filament system, they form desmosomes, hemidesmosomes and cadherin-mediated junctions, and they are permanently separated from surrounding stroma by a basement membrane. On the other hand, like SMCs, myoepithelial cells contain numerous fine filaments in their cytoplasm, express several smooth muscle structural proteins, and possess contractile activity [5, 6]. Contraction of myoepithelial cells is triggered by oxytocin stimulation, resulting in release of milk [2, 7, 8].

Although numerous studies have focused on the differentiation and functions of luminal epithelial cells, little is known of the mechanisms that control the development of myoepithelial cells, and no transcription factors that control their differentiation have yet been identified. However, the striking resemblance of myoepithelial cells to SMCs suggests possible shared developmental regulatory mechanisms.

As described in Chapter III, differentiation of SMCs is dependent on serum response factor SRF [9-15]. Members of the myocardin family of transcriptional coactivators interact with SRF and potently enhance the expression of SRF-dependent genes [16-23]. Myocardin is expressed specifically in cardiac and smooth muscle cells, whereas the myocardin-related transcription factors (MRTFs) MRTF-A/MAL/MKL1 and MRTF-B/MKL-2 are expressed in a wide range of cell types [24-29].

Conditional deletion of the *Srf* gene in the smooth muscle lineage results in early embryonic lethality due to a failure in SMC differentiation, precluding an analysis of possible functions of *Srf* after birth [30]. Similarly, as described in Chapter III, *myocardin* knockout mice die at embryonic day (E) 10.5 from an apparent failure in differentiation of SMCs [19], and *MRTF-B* null mice die at about E14.5 from abnormalities in a subset of SMCs and cardiac abnormalities (Oh J and Olson EN, unpublished).

Here I describe the phenotype of mice lacking *MRTF-A*. In contrast to mice lacking *myocardin* or *MRTF-B*, mice homozygous for a null mutation in the *MRTF-A* gene are viable. However, post-partum MRTF-A$^{-/-}$ females are unable to productively nurse their offspring. Analysis of the molecular basis of this maternal abnormality reveals an essential role of MRTF-A in differentiation of mammary myoepithelial cells, which are required for

ejection of milk from the mammary gland. We conclude that MRTF-A is a highly specific regulator of myoepithelial cell differentiation and that members of the myocardin family act in a wide range of cell types to control smooth muscle genes during embryogenesis and adulthood.

Materials and Methods

Generation of *MRTF-A* Knock-out Mice

The gene structure of *MRTF-A* has been described in chapter IV. A *MRTF-A*-targeting vector was constructed to delete exons 9 and 10 by using a pN-Z-TK2 vector, which contains a nuclear *LacZ* (*nLacZ*) cassette and a *neomycin-resistance* gene under the control of the RNA polymerase II promoter and two herpes simplex virus *thymidine kinase* (*TK*) gene cassettes (a generous gift of R. Palmiter, University of Washington, Seattle). Genomic DNA flanking *MRTF-A* exons 9 and 10 was PCR amplified from a mouse 129SvEv genomic DNA library (Stratagene) and inserted into the targeting vector as short and long arms, respectively. The targeting vector was electroporated into 129 SvEv-derived ES cells, and selection was performed with G-418 and FIAU, respectively. Five hundred ES cell clones were isolated and analyzed by Southern blotting for homologous recombination. Three clones with a disrupted *MRTF-A* gene were injected into 3.5-day mouse C57BL/6 blastocysts, and the resulting chimeric male mice were bred to C57BL/6 females to achieve germline transmission of the mutant allele.

RT-PCR Analysis

Total RNA was purified from tissues with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For RT-PCR, total RNA was used as a template for reverse transcriptase and random hexamer primers as described in Chapter II. Primer sequences are listed in Table 2.1.

Immunostaining and Histology

As described previously [31], the fourth pair of mammary glands was surgically dissected, fixed with carnyon fixative for 1 hour, washed with 70% ethanol and then distilled water, then stained with carmine alum staining solution (0.2% carmine and 0.5% aluminum potassium sulfate), washed again and cleared with xylenes for visualization of the stained lobular-alveolar structure.

Histological sectioning and staining with hematoxylin/eosin were performed according to standard techniques. For immunostaining, sections were deparaffinized in xylenes, rehydrated through graded ethanol to PBS, and permeabilized in 0.3% Triton X-100 in PBS. Nonspecific binding was blocked by 1.5% normal horse serum in PBS and primary antibodies were applied at a 1:200 dilution in 0.1% BSA in PBS overnight at 4°C. Sections were washed in PBS and fluorescein or Texas red-conjugated secondary antibodies (Vector Laboratories) were applied at a 1:200 dilution in 1% normal horse serum for 1 hr. Antibodies used were mouse SM α-actin antibody (clone 1A4, Sigma), rabbit cytokeratin 14 antibody (Zymed) and mouse CD10 (anti-CALLA) antibody (56C6, Labvision).

TUNEL Staining

Dead-end fluorometric TUNEL system was purchased from Promega (Madison, WI) and staining was performed according to user's manual. Briefly, sections were deparaffinized in xylenes, rehydrated through graded ethanol to PBS, and permeabilized by proteinase K treatment for 8 min at RT. Sections were fixed with 4% formaldehyde again, and equilibrated

with reaction buffer, then incubated with nucleotide mix and TdT enzyme in reaction buffer at 37°C for 1 hr.

RNA *in situ* Hybridization

In situ hybridization of paraffin sections was performed as described in Chapter III [18]. Identical bright and dark field images were captured and silver grains were pseudo-colored red using Adobe Photoshop, after which images were superimposed.

Cell Culture

The two cell lines originally established from human breast tissue, Hs578T and Hs578Bst, were obtained from American Type Culture Collection (Rockville, MD). These cells were cultured according to ATCC's culture conditions.

Results

Generation of *MRTF-A* Mutant Mice

The mouse *MRTF-A* gene, located on chromosome 15, contains 14 exons distributed across ~37 kb. To introduce a loss-of-function mutation in the gene, I deleted a 1.7 kb region encompassing a portion of exon 9 and all of exon 10, which encode the basic, glutamine-rich, and SAP domains (Fig. 5.1.A). The basic and glutamine-rich domains are required for the interaction of myocardin and MRTFs with SRF, and the SAP domain confers target gene specificity [16-18, 32]. Deletion of these domains results in functional inactivation of MRTF-A. The deleted genomic region was replaced with a lacZ expression cassette fused in-frame with exon 9 and a neomycin resistance gene. The targeted *MRTF-A* locus was identified by Southern blot analysis of genomic DNA (Fig. 5.1.B).

MRTF-A null offspring were produced at predicted Mendelian ratios from intercrosses of *MRTF-A* heterozygous mutant mice, indicating that *MRTF-A* is not required for embryonic or post-natal development. Homozygous *MRTF-A* mutant mice were viable and fertile, and intercrosses of null mice yielded normal-sized litters.

To confirm the gene-targeting event and determine whether the mutant allele might encode truncated *MRTF-A* transcripts, I performed RT-PCR of mRNA isolated from hearts of adult mice of the different genotypes, using primers representing exon sequences within and surrounding the deleted region of the gene (Fig. 5.1.C and D). These assays confirmed that the predicted exons were deleted and also showed that exon 8 was spliced to exon 11, thereby deleting the *lacZ-neo* cassette (Fig. 5.1.C). Sequencing of the RT-PCR product from the mutant allele showed that this aberrant splicing event caused a frame-shift in the *MRTF-A*

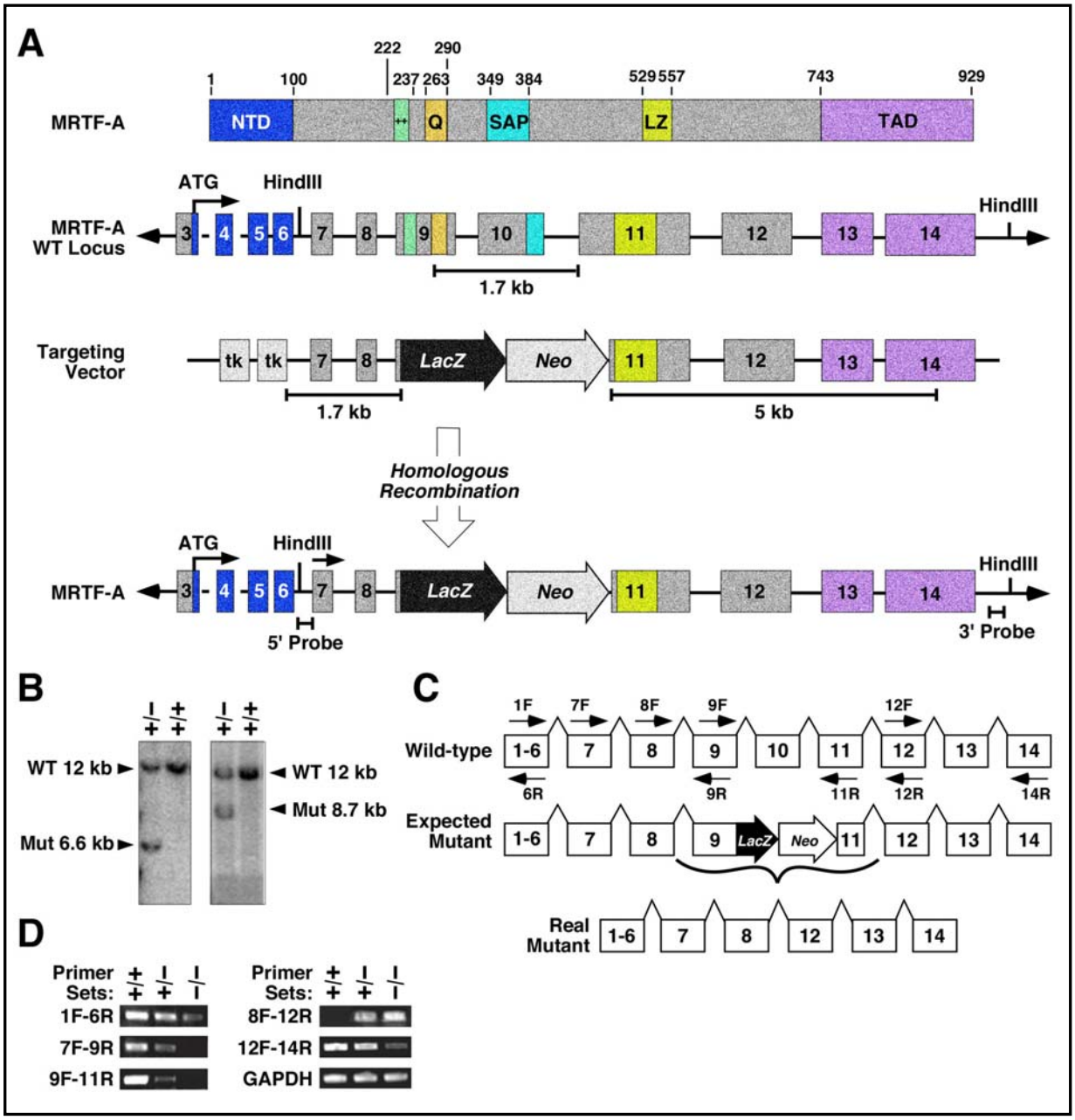
A
MRTF-A
NTD
Q
SAP
LZ
TAD
MRTF-A WT Locus
ATG
HindIII
1.7 kb
Targeting Vector
tk
LacZ
Neo
5 kb
Homologous Recombination
5' Probe
3' Probe
B
WT 12 kb
Mut 6.6 kb
Mut 8.7 kb
C
Wild-type
Expected Mutant
Real Mutant
D
Primer Sets:
1F-6R
7F-9R
9F-11R
8F-12R
12F-14R
GAPDH

Fig. 5.1. Generation and analysis of *MRTF-A* knockout mice. *(A)* Gene targeting strategy. The mouse MRTF-A protein is schematized at the top. Amino acid positions are indicated and functional domains are shown in color on the corresponding exons. The targeting vector, which contained a 2.4 kb 5' arm and a 5 kb 3' arm, replaced a 1.7 kb region of the gene with a LacZ-neo cassette. Positions of 5' and 3' probes are indicated. Positions of PCR primers used for genotyping are shown at the bottom by horizontal arrows. *(B)* Southern blot analysis. Genomic DNA from ES cell clones was isolated from tail biopsies and analyzed by Southern blot with 5' and 3' probes after digestion with HindIII. *(C)* Positions of primers used for RT-PCR. A schematic of the exons of the *MRTF-A* gene and positions of primers used for RT-PCR is shown. The expected mutation would contain the LacZ-neo cassette between exons 9 and 11. However, RT-PCR from mRNA isolated from heart tissue of mutant mice revealed that exon 8 was spliced to exon 12, as shown at the bottom. *(D)* RT-PCR was performed with RNA from heart tissue using primers shown in panel C. Genotypes of mice are shown at the top.

open reading frame such that the mutant transcript would create a truncated protein with residues 1-209 fused to 687-929 and lacking the SRF-interaction domain.

MRTF-A Mutant Females are Unable to Productively Nurse Their Offspring

Although *MRTF-A* null mice showed no obvious abnormalities, I noticed that the offspring of *MRTF-A* null females failed to thrive and none survived beyond 14 days of age (Fig. 5.2 and Table 1). The growth retardation of offspring of *MRTF-A* null females was independent of their genotype, suggesting an abnormality in the mutant mothers rather than the offspring. Indeed, wild-type pups fostered to *MRTF-A* null females also failed to thrive, whereas *MRTF-A* null pups fostered to wild-type mothers grew normally (Fig. 5.2). *MRTF-A* deficient females nursed and attended to their young, and they did not exhibit abnormal maternal nurturing behaviors. Offspring also suckled from the mothers' nipples, but little or no milk was present in their stomachs. These findings suggested that *MRTF-A* is required specifically for females to productively nurse their young.

Abnormal Mammary Development in *MRTF-A* Mutant Mice

Consistent with the notion that *MRTF-A* mutant females displayed a defect in nursing, the mammary glands dissected from mutant lactating females were pale compared to those of wild type lactating females (Fig. 5.3.A). To visualize the ductal and alveolar structures in the mammary glands of females at different maternal stages, I performed whole-mount immunostaining. In the mutant females, the large club-shaped terminal end bud (TEB) formed normally after puberty and elongation and branching of the mammary tree showed no

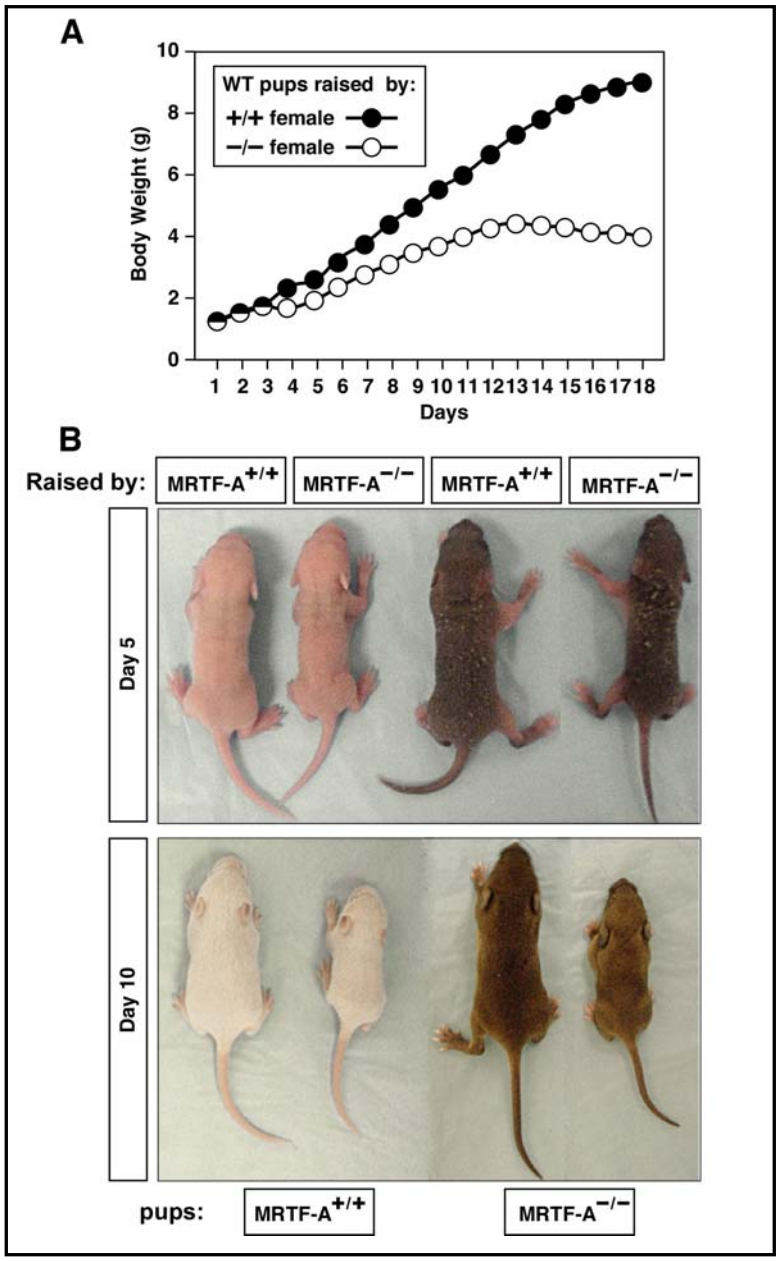

Fig. 5.2. Growth retardation of neonates nursed by MRTF-A$^{-/-}$ mothers. *(A)* Postnatal growth curves. Body weights of wild-type pups raised by wild-type and MRTF-A$^{-/-}$ mothers were determined on successive days after birth. Pups raised by MRTF-A$^{-/-}$ mothers fail to thrive. *(B)* Pups on day 5 (top) and day 10 (bottom) after birth are shown. The genotypes of the mothers and pups are shown at the top and bottom, respectively. Wild type and MRTF-A$^{-/-}$ pups thrive with wild type mothers, whereas wild type and MRTF-A$^{-/-}$ pups fail to thrive with MRTF-A$^{-/-}$ mothers.

apparent abnormalities (Fig. 5.3.B, a and b). During pregnancy, additional ductal branching occurred and terminal alveoli formed in the mutants, just as well as in the wild type females (Fig. 5.3.B, c and d). On day 1 after delivery, the mutant females also completed ductal-alveolar development and the mammary trees of wild-type and mutant females were indistinguishable (Fig. 5.3.B, e-j). However, beginning at day four of lactation, the density of alveoli became substantially reduced relative to wild type mammary gland (Fig. 5.3.B, g-j). Some large ducts, which were all surrounded by extensive alveoli in the wild type mammary gland, were still visible in the mutant (Fig. 5.3.B, h). On day 12, the wild type mammary gland was filled with alveoli, while there were spaces between the alveoli in the mutant mammary gland. Notably, at this stage, the alveoli of the mutant female appeared larger and less organized than those of wild type females (Fig. 5.3.B, j). After weaning, the mutant mammary gland underwent a remodeling process, known as involution, in the same fashion as the wild type, and the gland regressed to the resting phase (Fig. 5.3.B, k and l).

Histological analysis confirmed normal ductal-alveolar development of the mutant female during resting stage and pregnancy (Fig. 5.3.C). For both wild type and mutant 8 week old virgins, the mammary glands were filled with fat tissue (asterisk), and the ducts were lined by a single layer of epithelial cells surrounded by myoepithelial cells and dense stroma. During pregnancy, both ducts and alveoli were visible (large and small arrow heads), and the epithelial cells began to secrete milk protein and lipid in the mutant as well as in the wild type mammary gland (arrow). Histological sections also revealed that the mammary gland of wild type lactating female was filled with alveoli, and that their lumens were defined by highly organized thick alveolar walls. In contrast, adipocytes were still present between

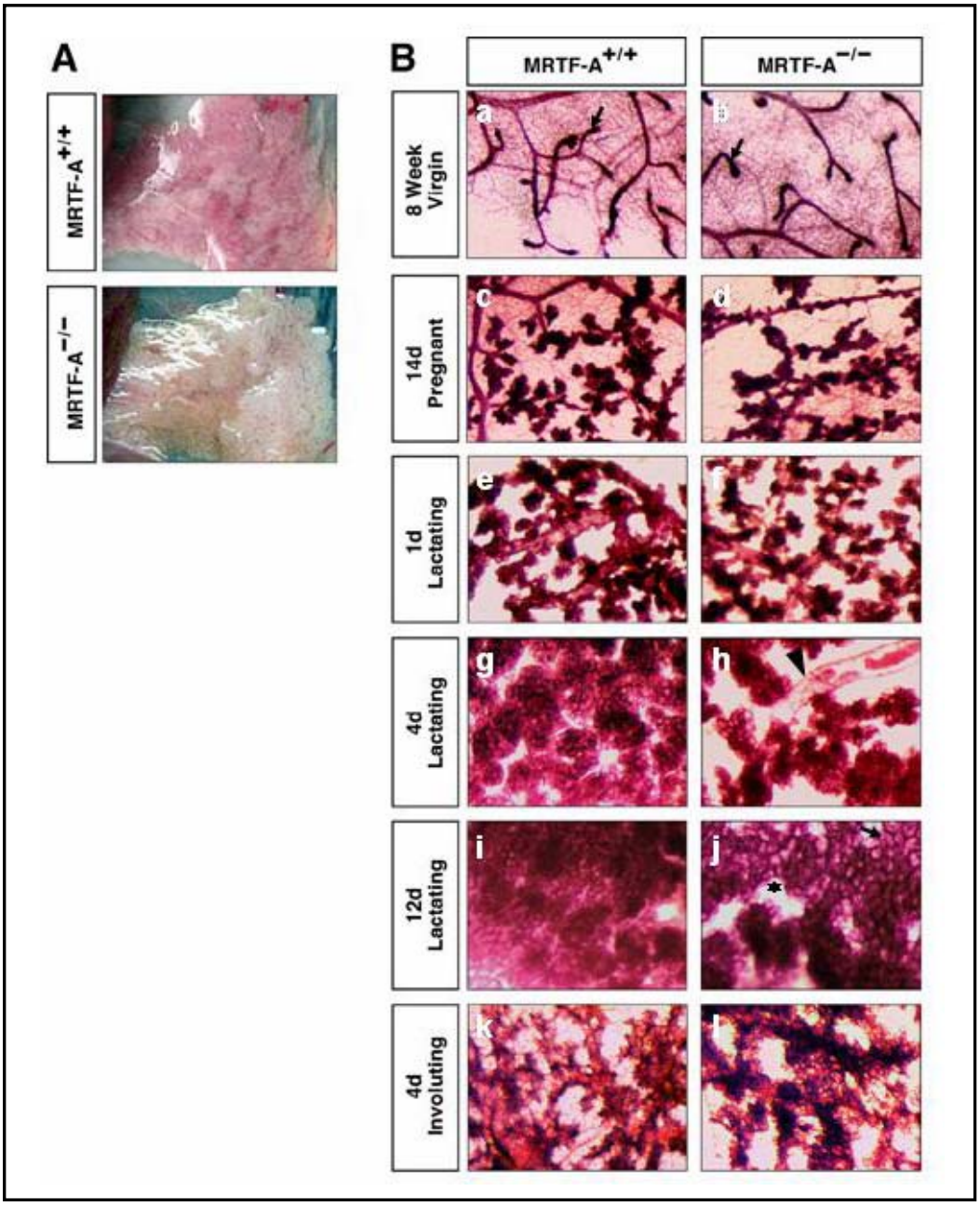

Fig. 5.3. Histology of *MRTF-A* mutant mammary glands. *(A)* Gross appearance of mammary glands at day 12 of lactation. mammary gland from mutant lactating female is pale compared that of wild type lactating female. *(B)* Whole-mount staining of luboloal-alveolar network. (a-f) normal mammary development from virgin to pregnancy to initiation of lactation. (g-j) luboloal-alveolar structures of *MRTF-A* mutant females are underdeveloped during middle and late lactation. Large tubular structure is still visible at lactating day 4 in the mutant (arrow head, h). In the mutant mammary gland at lactation day 12, the alveoli are enlarged compared to the wild type (arrow, j) and there is space among alveoli (asterisk, j).

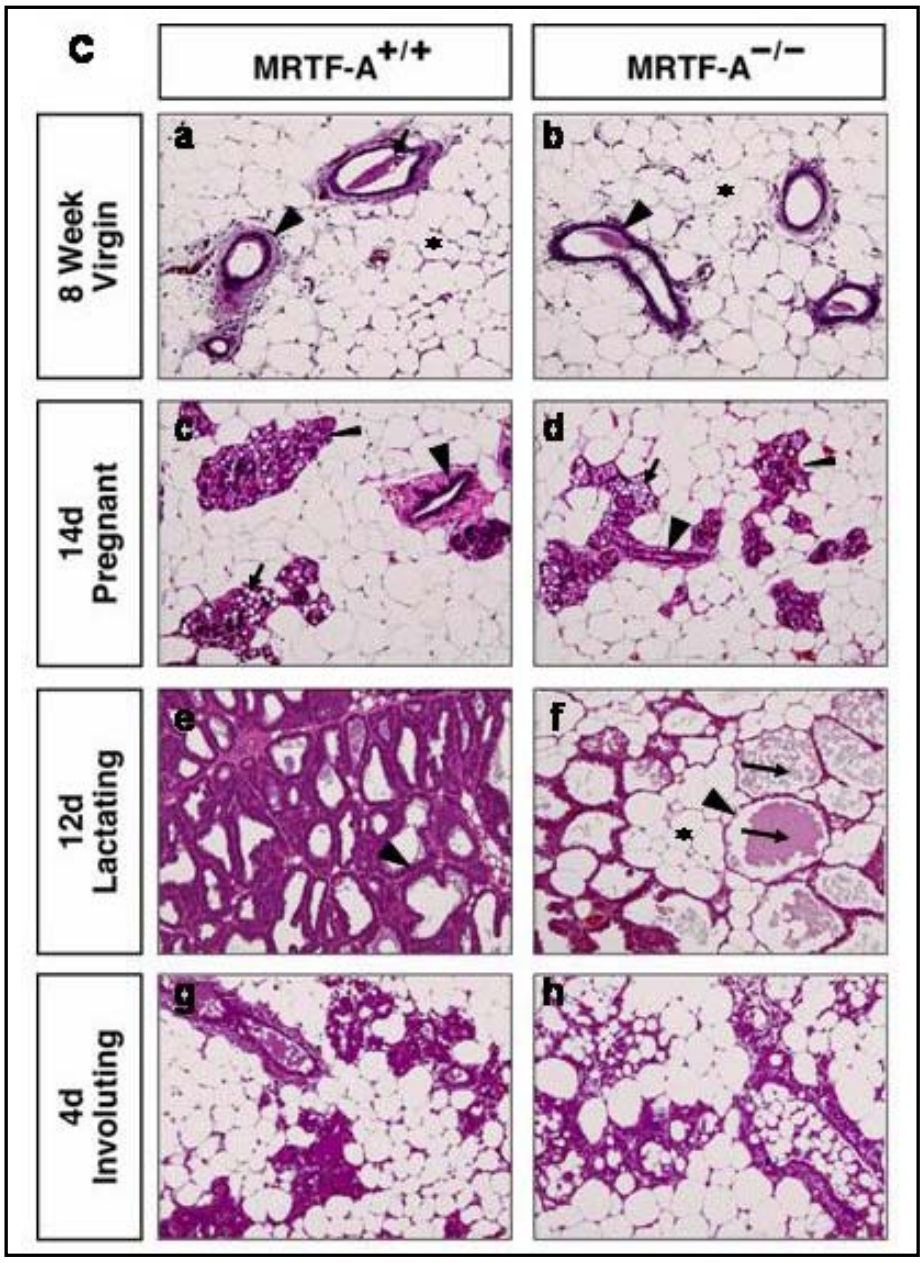

Fig. 5.3. Histology of *MRTF-A* mutant mammary glands. *(C)* Histological sections of mammary gland from different developmental stages. (a-d) normal ductal-alveolar development during resting and pregnant stages of *MRTF-A* mutant females. Arrow head, milk ducts; thin arrow head, alveoli; arrow, milk droplets; asterisk, adipocytes. (e and f) during lactation, the alveolar lumens of *MRTF-A* mutant female were enlarged with thin walls (arrow head), milk protein and lipids were trapped in the alveoli (arrow). Fat tissue is present between alveoli (asterisk).

alveoli of the lactating mammary glands of the mutant mice (asterisk), and the alveoli were dilated with much thinner walls compared to the wild type glands (arrow head). Milk was trapped in the lumens of mutant mammary glands, indicated by the purple protein staining and the lipid droplets (arrow).

Myoepithelial Cell Defects in *MRTF-A* Mutant Mice

To pinpoint the cell type responsible for the nursing defects of *MRTF-A* mutant mothers, I examined markers of the different mammary cell types. RT-PCR analysis indicated that *MRTF-A* and *MRTF-B* were expressed at constant levels in mammary tissue of 8-wk virgins, 14-day pregnant, 3-day lactating, 12-day lactating, and 4-day involuting mammary glands (Fig. 5.4.A). Myocardin expression was not detected by RT-PCR analysis. Transcripts encoding milk proteins, α-lactalbumin, β-casein and WAP (whey acidic protein) were expressed normally at all stages in *MRTF-A* mutant mammary glands [33, 34]. The luminal epithelial cell-specific cytoskeletal protein cytokeratin 18 was also expressed at a normal level in the mutants (Fig. 5.4.A) [35]. Thus, luminal epithelial cell differentiation and function appeared unperturbed in the mutant mammary glands.

Since myoepithelial cells express both smooth muscle genes and certain epithelial genes, I examined the expression of the myoepithelial-specific epithelial genes, cytokeratin 14 (ck14) and common acute lymphoblastic leukemia antigen (CALLA, CD10) in the mammary gland [35, 36]. The RT-PCR analysis of CK14 and CALLA indicated that expression of these genes was reduced at the early lactating stage (L4) and almost abolished at the late lactating stage (L12).

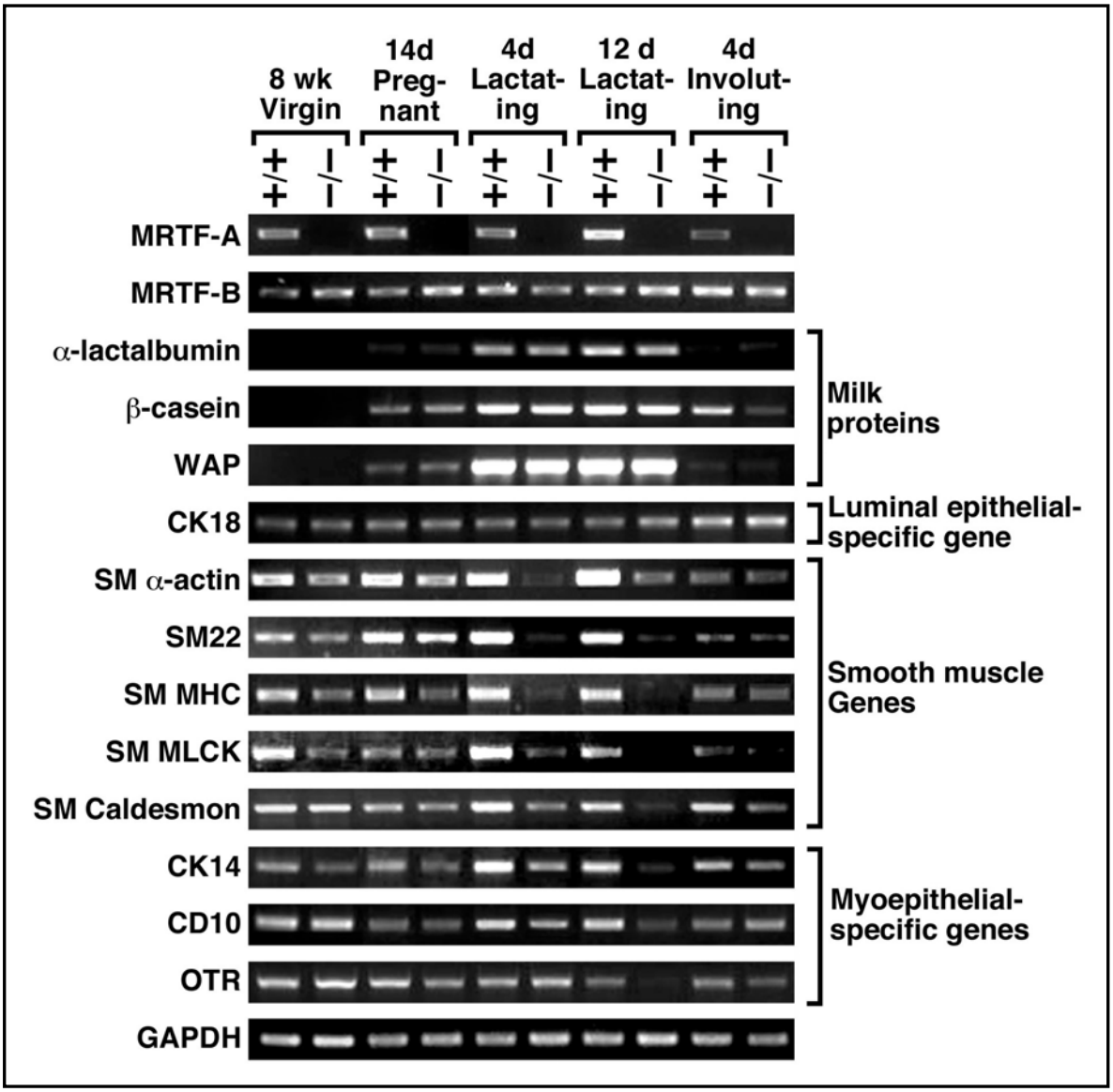

Fig. 5.4. Analysis of mammary gene transcription in *MRTF-A* mutant mice. *(A)* RT-PCR analysis of mammary genes. MRTF-A transcript is absent in the mutant, while MRTF-B is constantly expressed during the mammary cycle in both wild-type and MRTF-B mutant. Milk proteins (α-lactalbumin, β-casein and WAP) are expressed at the same level in wild-type and mutant mammary gland during lactation. Secretory epithelial-specific cytokeratin18 (CK18) is not change in the mutant either. Smooth muscle proteins (SM α-actin, SM22, SM-MHC, SM-MLCK and SM caldesmon) are down-regulated specifically during lactation. Other myoepithelial-specific proteins (cytokeratin 14, CD10 and oxytocin receptor) are down-regulated specifically at late lactation. GAPDH is the loading control.

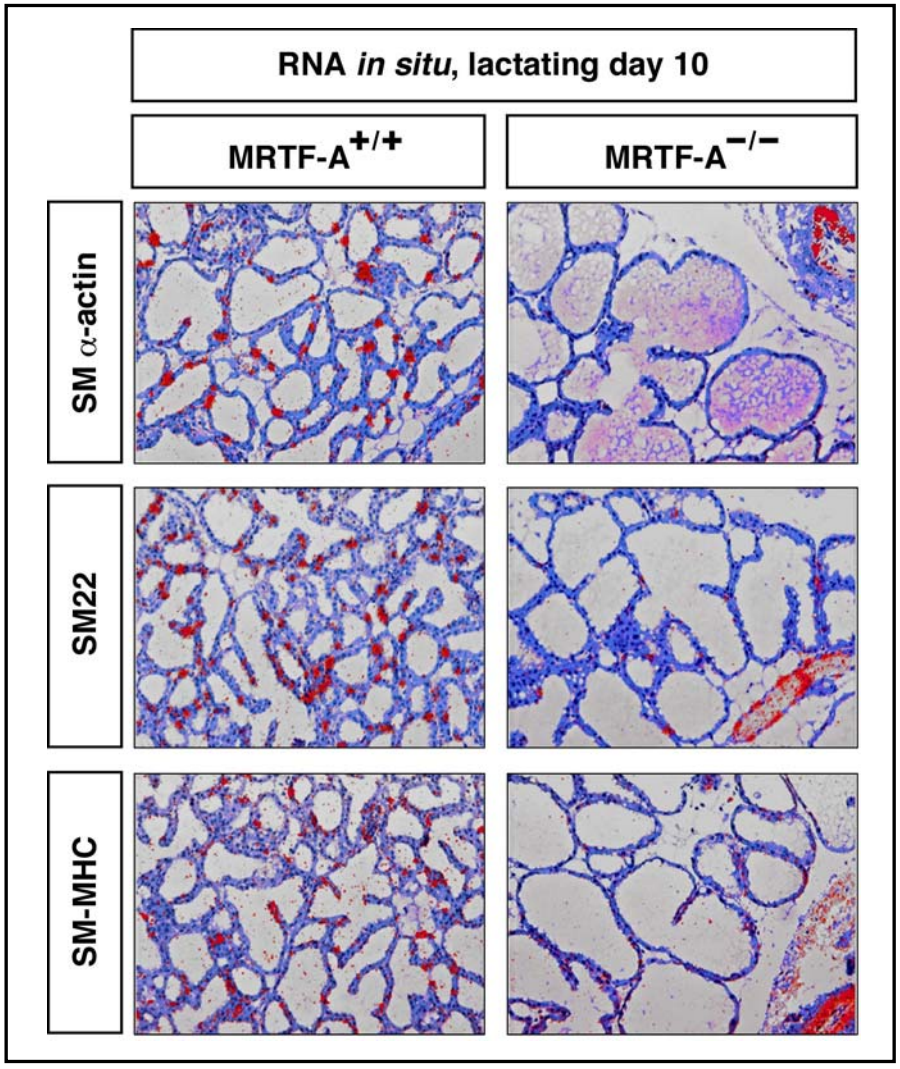

Fig. 5.4. Analysis of mammary gene transcription in *MRTF-A* mutant mice. (B) Transcripts for the indicated smooth muscle genes were detected by in situ hybridization to sections of mammary gland from wild type and MRTF-A$^{-/-}$ females at 10-days of lactation. Silver grains are pseudocolored red.

During lactation, smooth muscle genes are up-regulated in the mammary gland of wild-type females [37]. In mammary tissue of *MRTF-A* mutant virgins or 14-day pregnant mice, there was a slight decrease in expression of smooth muscle marker genes compared to wild-type. However, in the mammary myoepithelial cells of lactating *MRTF-A* mutant females, I observed a pronounced loss of smooth muscle markers (Fig. 5.4.A). This dramatic down-regulation of smooth muscle gene expression in myoepithelial cells from mutant mammary gland was confirmed by in situ hybridization (Fig. 5.4.B).

Abnormalities in Myoepithelial Cell Differentiation in MRTF-A Mutant Females

To further examine the differentiation of myoepithelial cells, I performed immunohistochemistry using an antibody against smooth muscle α-actin (Fig. 5.5) [38]. SM α-actin positive myoepithelial cells form a single layer around the ducts in mammary glands. In wild type and *MRTF-A* mutant 8 week-old virgin females, SM α-actin was expressed at a comparable level. However, at 20 weeks of age and during pregnancy, the wild type myoepithelial cells around the mammary ducts showed a stronger and thicker staining pattern with a stellate shape, while the mutant myoepithelial cells maintained a staining pattern similar to that of 8 week-old virgin and showed no enhancement of staining. Strikingly, during late lactation, the mutant myoepithelial cells showed almost no SM α-actin expression, while the wild type myoepithelial cells formed a discontinuous, basket-like single layer around the alveolar lumens. However, upon involution (4 days after weaning), the expression of SM α-actin in *MRTF-A* mutants returned to a level comparable to that of wild-type females. Immunohistochemistry with other smooth muscle proteins, such as SM MHC

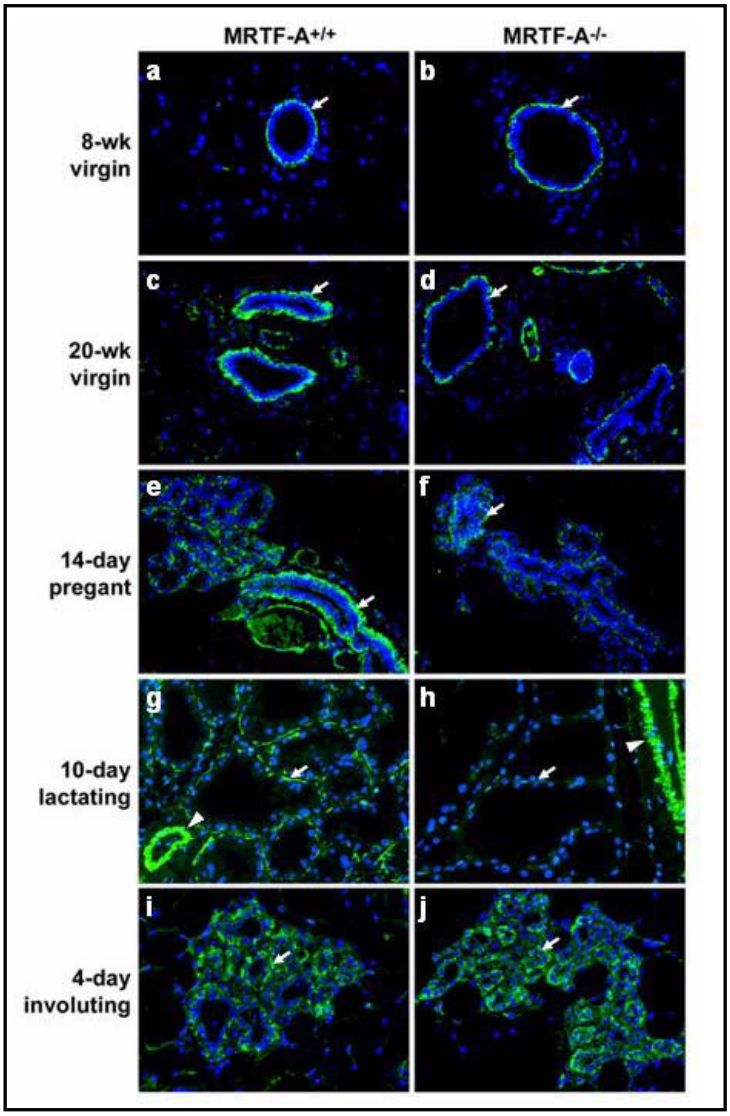

Fig. 5.5. Detection of smooth muscle markers in mammary glands of wild type and *MRTF-A* mutant females. SM-α-actin expression (green) was detected in histological sections of mammary glands from wild type and MRTF-$A^{-/-}$ females at the indicated stages. Blue staining is DAPI staining of nuclei. (a and b) similar pattern of SM α-actin positive myoepithelial cells in wild-type and MRTF-$A^{-/-}$ mutant virgin females. (c-f) in mature and pregnant wild type female, SM α-actin staining around milk ducks became thicker and stronger, while the mutant mammary glands maintained a thin, single layered pattern the same as young virgin females (arrow). (g and h) at day 12 of lactation, SM α-actin was absent around the alveoli in the mutant mammary gland (arrow), while the vascular smooth muscle strongly expressed SM α-actin (arrow head). (i and j) SM α-actin expression in the mutant mammary gland was similar with that of the wild-type during involution.

and SM calponin showed similar expression pattern in *MRTF-A* mutant mammary glands (data not shown), which is consistent with the mRNA expressions of these genes, as seen with RT-PCR and RNA in situ hybridization.

Lack of Myoepithelial Cells in *MRTF-A* Mutant Mammary during Late Lactation

Immunostaining using antibodies against CALLA (CD10) and cytokeratin 14 (CK14) confirmed that at lactating day 12, expression of CALLA and cytokeratin 14 was abolished (Fig. 5.6.A and B). Double immunostaining with antibodies against SM α-actin and CK14 indicated that at lactating day 4, both of these two proteins were still expressed in most of the myoepithelial cells of the *MRTF-A* mutant, although at a decreased level compared to the wild-type, while at lactating day 12, both SM α-actin and CK14 were ablated. Noticeably, at lactating day 4, cell number around alveoli lumens in mutant mammary gland was similar to that of wild-type. In contrast, at lactating days 10 and 14, the number of cells surrounding the lumens was greatly reduced in the mutant mammary gland. The wild-type alveolar lumens were surrounded by three layers of cells, two layers of epithelial cells separated by one layer of myoepithelial cells. However, in MRTF-A mutant mammary gland, the walls between two adjacent alveoli lumens were only composed of one or two layers of cells, which resulted in a thin appearance of these walls. Taken together, these results imply that the myoepithelial cells were ablated at late lactating states.

To elucidate the mechanism of loss of myoepithelial cells, I performed TUNEL assay to evaluate apoptosis. TUNEL assay showed no increase in apoptosis in the mutant mammary gland at the resting stage or at pregnant stages, lactating day 2, 4, 7 and 12 (data

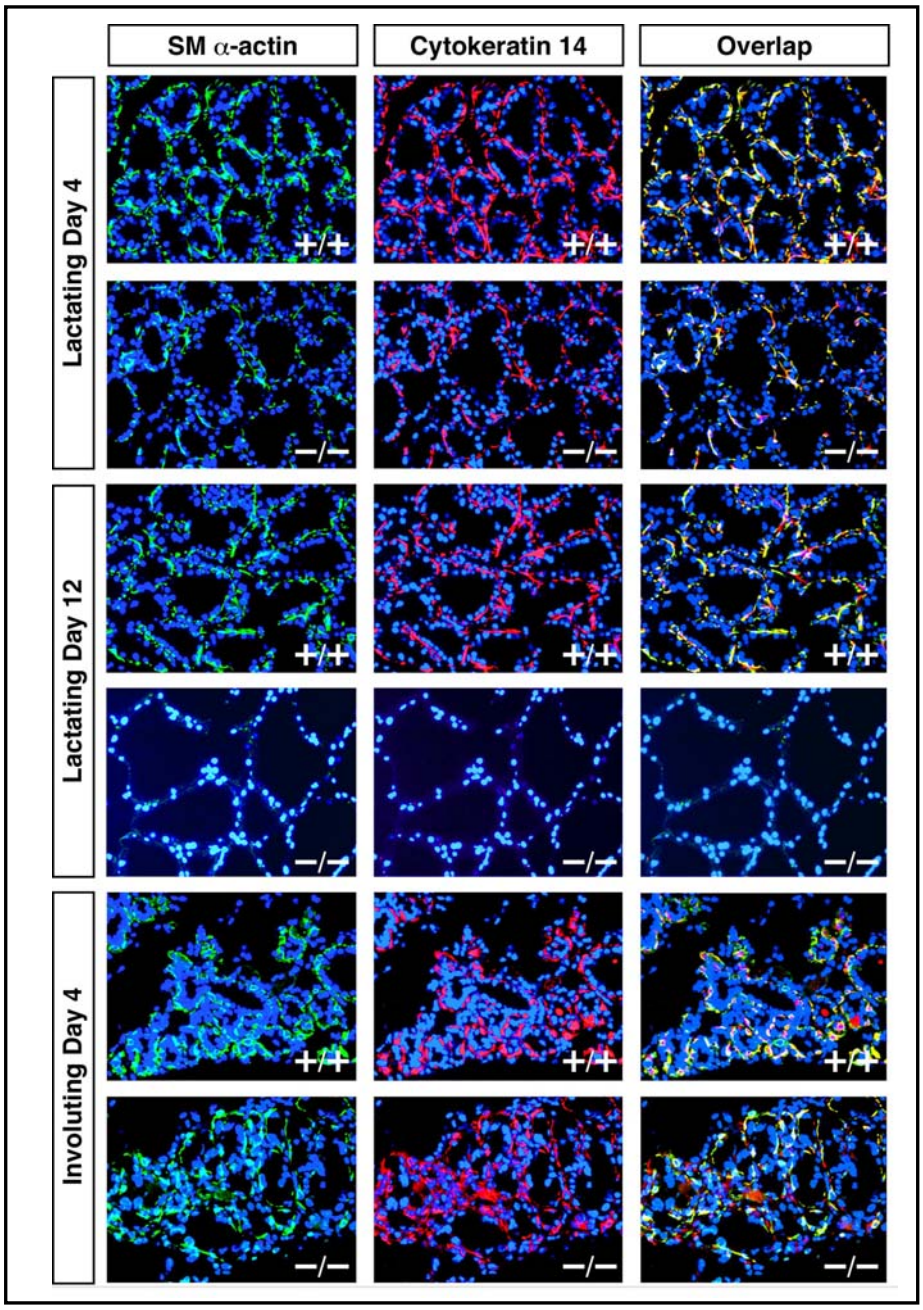

Fig. 5.6. Lack of myoepithelial cells in *MRTF-A* mutant mammary gland during late lactation. *(A)* Immunostaining with antibodies against SM α-actin and cytokeratin 14 with lactating mammary tissue. The mutant myoepithelial cells eventually lost expressions of both these two proteins in a similar fashion, indicated by the fact that these two proteins co-localized with each other. At lactating day 12, expressions of both proteins were abolished, and cell numbers around alveoli were decreased in *MRTF-A* mutant mammary gland. During involution, the expressions of these two proteins came back to a comparable level as the wild-type. Green, SM a-actin; red, CK14; Blue, DAPI staining of nuclei

not shown), while massive apoptosis was detected at lactating day 10 in the mutant mammary gland (Fig 5.6.C). The percentage of apoptotic cells was 20 fold higher in the mutant mammary gland than in the wild-type at this time point. These data indicate that failure of the smooth muscle program leads to failure of expression of other cytoskeletal proteins of the myoepithelial cells, disruption of normal functions of these cells and ultimately to cell death of these myoepithelial cells. The remnant smooth muscle structure proteins during early lactation probably could maintain a low level of milk ejection that sustains the survival of the offspring of the MRTF-A mutant females prior to lactating day 10. However, at late lactation phase, with most myoepithelial cells ablated in the mutant mammary network, milk could not be ejected and the offspring would die due to starvation.

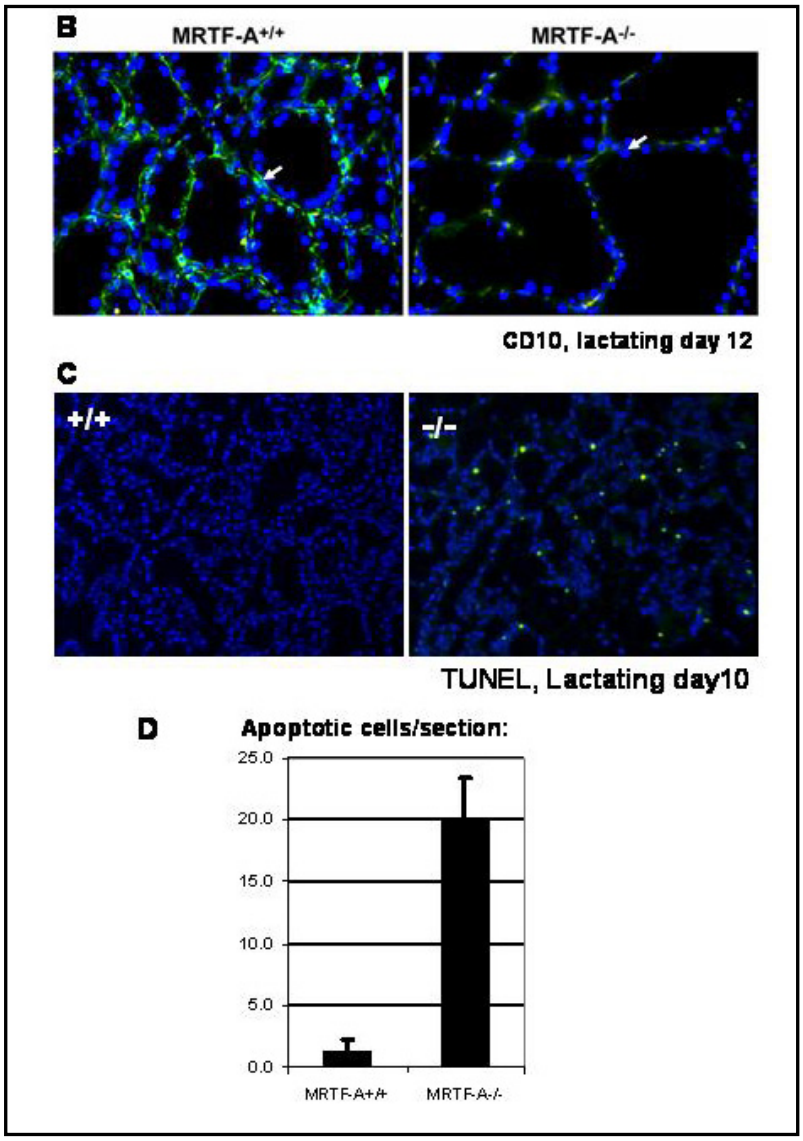

Fig. 5.6. Lack of myoepithelial cells in *MRTF-A* mutant mammary gland during late lactation. *(B)* Expression of CD10, which is also specifically expressed in myoepithelial cells, is diminished at late lactating stage in *MRTF-A* mutant. *(C)* Excessive apoptotic cell death in *MRTF-A* mutant mammary gland at lactating day 10, detected by TUNEL assay. *(D)* Quantification of apoptotic cell death in the mammary glands of wild-type and *MRTF-A* mutant females at lactating day 10.

Discussion

The results of this study reveal an essential role for *MRTF-A* in the differentiation of mammary myoepithelial cells, a specialized smooth muscle-like cell type required for milk ejection from the mammary gland. As a consequence of the failure in myoepithelial cell differentiation, *MRTF-A* mutant mothers are unable to productively nurse their offspring. These findings reveal a commonality in the molecular mechanisms that control differentiation of smooth muscle and myoepithelial cells, both of which depend on activation of contractile protein genes by members of the myocardin family of SRF coactivators.

Myoepithelial development

Myoepithelial cells are found within the secretory and ductal portions of most glands. Their contractile function, which is controlled by hormonal and neural signals, is essential for ductal secretion. Myoepithelial cells also transport metabolites to secretory cells and provide structural integrity to glandular tissues through their association with the basement membrane [39]. The ultrastructure, gene expression pattern, and contractile properties of myoepithelial cells are strikingly similar to those of SMCs. However, in contrast to SMCs, which are derived from mesodermal precursors and neural crest cells, myoepithelial cells of the mammary gland are derived from ectoderm. To our knowledge, the molecular mechanisms responsible for myoepithelial cell differentiation have not been previously defined.

The mammary ductal tree forms normally in *MRTF-A* mutant mice, but myoepithelial cells fail to differentiate, as shown by the lack of expression of smooth muscle contractile

protein genes such as those encoding SM α-actin, SM MHC and SM caldesmon. As a result, mutant mothers are unable to release their milk.

Given the accumulation of excess milk in the lumens of the mutant mammary gland, it appeared that the nursing defect was caused by a failure of milk ejection. Oxytocin, a neurohypophyseal hormone, is essential for stimulating myoepithelial cell contraction and milk ejection. However, RT-PCR analysis showed normal expression level of this hormone in the brains of *MRTF-A* mutants (data not shown). Oxytocin receptor is exclusively expressed in the myoepithelial cells within the mammary gland [40, 41]. The oxytocin mRNA level is not changes in the *MRTF-A* mutant mammary tissue at early lactation (day 4), when the myoepithelial-specific smooth muscle proteins are already down-regulated. At lactating day 12, oxytocin receptor is diminished since the myoepithelial cells were ablated due to apoptosis. Taking into consideration the overt myoepithelial cellular abnormalities, it is unlikely that the nursing deficiency of *MRTF-A* mutant females is caused by defective oxytocin circulation and signaling.

Myoepithelial cells line the secretory and ductal areas of most glands, so it is curious that the phenotype of *MRTF-A* mutant mice is so restricted to mammary myoepithelial cells. Myoepithelial cells are also associated with salivary, lacrimal, and sweat glands, but we did not detect abnormalities in these glandular tissues, raising the possibility that other members of the *myocardin* family may substitute for *MRTF-A* function in those tissues. In this regard, it is interesting to note that *MRTF-B* are expressed at normal levels in mammary glands of *MRTF-A* mutant females. Expressional analysis using established mammary epithelial and myoepithelial cell lines Hs578T and Hs578Bst [42] indicated that MRTF-A and B are

expressed in both cell lines (data not shown). This could suggest that *MRTF-A* is uniquely required for differentiation of mammary myoepithelial cells or that the loss of *MRTF-A* reduces the level of *myocardin* family members below a critical threshold required for myoepithelial cell differentiation. Another interesting finding with the myoepithelial defects is that the phenotype is only prominent during lactation, while the smooth muscle gene expression is normal in resting mammary gland. It is possible that during resting stage, MRTF-B is able to compensate the loss of MRTF-A and sustain the smooth muscle program, while during pregnancy and lactation, when extensive proliferation and differentiation of myoepithelial is necessary, MRTF-B cannot offset the loss of MRTF-A anymore. It is also intriguing to determine if female hormones, which play important roles during mammary development regulate MRTF-A and B's activity differentially.

MRTF-A is required for myoepithelial differentiation and survival

In the lactating mammary gland of *MRTF-A* mutant female, not only smooth muscle genes but also other myoepithelial specific genes, such as cytokeratin 14 and CALLA, are down-regulated. Notably, There are no conserved SRF binding sites at least within 40kb upstream of transcription initiation site of these genes and there are no previous reports that expression of these genes might be regulated by SRF. Since the smooth muscle structure genes and the cytokeratins together compose the cytoskeletal structure of the myoepithelial cells, it is likely that the lack of smooth muscle proteins probably leads to the dispensability of cytokeratins, and in turn leads to degradation of these proteins or repression of their gene

expression. Although we cannot exclude the possibility that MRTF-A has other partners, other than SRF, that control the expression of these myoepithelial-specific genes.

It is reported that failure of milk ejection promotes mammary involution [43, 44]. Milk stuck within the alveoli was observed in *MRTF-A* mutant mammary gland from early lactation (day 4). However, excessive apoptosis was not detected until lactation day 10. Moreover, the lubola-alveolar structure of the mutant mammary gland maintained the lactating appearance throughout lactation, and milk protein expression was sustained at a level comparable to wild-type mice. In *MRTF-A* mutant mice, there was no indication of premature involution. However, there was dramatic increase of apoptotic cells specifically at lactating day 10, and by day 12 the apoptosis was not detected. The alveolar structures were maintained, although the alveolar walls were much thinner and composed of fewer cells in the mutant mice compared to wild-type mice. Noticeably, the decrease of smooth muscle and other myoepithelial-specific genes proceeded apoptotic cell death. In nervous system, it has been suggested that failure to retain synaptic connectivity is linked to neuronal dysfunction and this is often followed by neuronal cell death [45]. In addition, it has been shown that Nkx2.5, which is critical for the expression of cardiac-specific genes, is also required for survival of cardiac myocyte under stress [46]. In the *MRTF-A* mutant mammary gland, the absence of smooth muscle genes and other myoepithelial genes should impair the normal function of these myoepithelial cells, and it is reasonable to speculate that failure of normal differentiation and function would lead to programmed cell death of myoepithelial cells.

Regulation of cell migration and cytoskeletal development by the myocardin family and SRF

We showed previously that *myocardin* null mice die at E10.5 from an apparent lack of differentiated SMCs [19]. However, the interpretation of this mutant phenotype was complicated by the fact that *myocardin* is only expressed in a small subset of SMCs at this stage of development. In addition, *myocardin* mutant embryos displayed abnormalities in the yolk sac development, making it difficult to distinguish whether the effects of myocardin gene deletion on embryonic vascular development are primary or secondary to yolk sac abnormalities.

A null mutation in the *MRTF-B* gene also results in embryonic lethality at ~E12.5 due to a spectrum of cardiovascular defects (J. Oh and E. Olson, unpublished). Thus, each member of the *myocardin* family is required for the activation of smooth muscle gene expression, but each is uniquely required in a different cell type at a different developmental stage. It will eventually be interesting to generate mice lacking different combinations of the *myocardin* family genes in order to determine if there are cell types in which the myocardin family members are functionally redundant and whether there might be alternative pathways leading to smooth muscle gene expression in a subset of cell types.

MRTF-A has been shown to mediate the effects of Rho signaling and changes in the actin cytoskeleton to SRF-dependent transcription [32, 47]. Mice overexpressing a dominant -negative form of MTRTF-A in skeletal muscle showed skeletal myopathy and hypoplasia [48]. Similarly, a dominant negative mutant of MRTF-B/MKL2 inhibits differentiation of skeletal muscle cells *in vitro* [49]. Remarkably, however, *MRTF-A* mutant mice display no

obvious abnormalities in skeletal, cardiac or smooth muscle, presumably due to functional redundancy between MRTF-A and MRTF-B.

Implications: Potential Roles of MRTF-A in Breat Cancer

In addition to their role in milk secretion, myoepithelial cells have been suggested to possess tumor suppression activities [50-52]. Myoepithelial cells produce anti-invasive protease inhibitors and anti-angiogenic molecules, such as protease nexin II, α1-antitrysin, tissue inhibitor of metalloproteinase 1, thrombospondin-1 and soluble basic fibroblast growth factor receptor [53-55]. Thus, myoepithelial cells can induce growth arrest and apoptosis of breast carcinoma cells by interfering with the invasive behavior of tumor cells and inhibiting angiogenesis. Taking into consideration the role of SRF and MRTFs in controlling expression of growth responsive genes, such as c-fos and egr-1 [28, 56], it will be of interest to determine whether MRTF-A plays a role in breast cancer.

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Chapter VI

VITAE

Shijie Li was born in Wulian, Shangdong Province, China on September 11th, 1978, the only son of Yigui Wang and Xinliang Li. After completing his work at No. 1 High School of Wulian in 1996, he entered College of Life Sciences, Peking University at Beijing, China. He received the degree of Bachelor of Science with a major in Biochemistry and Molecular Biology from Peking University in June 2000. In August 2000, he entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. He joined the laboratory of Dr. Eric Olson in May 2001. He married Shurong Chang in July 2003. He graduated with a Ph.D. degree in August 2005.

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