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Serum Response Factor Micro-Managing Cardiogenesis

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Abstract

Serum response factor (SRF), a cardiac enriched transcription factor, is required for the appearance of beating sarcomeres in the heart. SRF may also direct the expression of microRNAs (miRs) that inhibit the expression of cardiac regulatory factors. The recent knockout of miR-1-2, an SRF gene target, showed defective heart development, caused in part by the induction of GATA6, *Irx4/5*, and *Hand2*, that may alter cardiac morphogenesis, channel activity and cell cycling. SRF and co-factors play an obligatory role in cardiogenesis, as major determinants of myocyte differentiation not only by regulating the biogenesis of muscle contractile proteins but also by driving the expression of silencer miRNA.

Introduction

The heart is the first organ to form in mammals controlled by an exquisite developmental program that ultimately assembles sarcomeres that rhythmically beat. Cardiac progenitors receiving the appropriate set of signaling inputs upregulate several cardiac-restricted transcription factors such as *Nkx2*, *GATA-4*, and *Tbx5* that are among the earliest markers of the cardiac lineage. Despite their apparent importance, deletion of these genes in knockout mice failed to prevent cardiomyocyte terminal differentiation (31). However, recent *Srf* conditional mutants blocked the appearance of SRF in nascent cardiomyocytes, completely prevented the appearance of organized sarcomeres that beat. Moreover, SRF null mutants in worms have placed SRF at the highest point in the regulatory hierarchy as a transcription factor governing myogenesis (14). Thus, SRF is recast here, as the obligatory Sarcomeric Regulatory Factor required for cardiogenesis.

SRF belongs to an ancient DNA binding protein family, which shares a highly conserved DNA-binding/dimerization domain termed the MADS box (29;Fig.1). SRF target genes are characterized by the presence of single or multiple copies of the SRF binding consensus element known as the CArG box and the full spectrum of known and novel CArG elements in the genome was recently named the CArGome (42). CArG boxes are found primarily in promoters of genes involved with contractility, cell movement, and cell growth signaling (42,50) and some of the recently discovered miRNAs, that exert important silencing activity required for normal heart development (49).

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A central problem in cardiovascular development is to understand how SRF activity can be differentially controlled according to muscle cell types and or signaling pathways. The discovery that SRF activity is controlled to a large extent by its interaction with signal-regulated or tissue-specific regulatory cofactors provided the mechanistic insights into the problem. In fibroblasts, SRF controls transcription of many cellular “immediate-early” genes, whose expression is activated by growth factor or mitogenic stimuli (41). The SRF gene is an important target for the ERK signaling pathways that converge on ternary complex factors (TCF), some of which are a subfamily of ETS domain transcription factors such as Elk-1 (38, Fig. 2). Phosphorylation of the Elk-1 B-box enhanced co-association with SRF and stimulated growth factor driven SRF gene activity. Thus, SRF expression is controlled by itself during cellular growth, leading to a positive feedback loop.

Combinatorial Interactions of SRF and co-factors

SRF activity is controlled to a large extent by its interaction with tissue-specific regulatory cofactors. In myogenic cells, as shown in Figures 1 and 2, a large number of positive-acting SRF cofactors, which include GATA4 and Nkx2.5, can form complexes with each other and with SRF also have their own DNA binding sites (36). The cysteine-rich LIM-only proteins may act as bridging factors to facilitate formation of GATA-SRF complexes, thus allowing muscle differentiation (7,8). The LIM zinc fingers in CRP2 were found to collaborate with Brg1 of the SNF/SWI complexes recruited SRF and remodeled silent cardiac myocyte chromatin and directed SRF-dependent smooth muscle gene activity (8). Myocardin is another important co-factor which drives smooth muscle gene activity by association with SRF (33). Myocardin sumoylation in the presence of coexpressed SUMO-1/PIAS1, acted as a molecular switch to promote myocardin's role in cardiogenic gene expression (44). In addition, the myocardin-related transcription factors (MRTF-A/-B) are a second family of signal-regulated SRF cofactors that have been well characterized (33). Activity of the two MRTFs, is regulated by a novel signaling pathway controlled by Rho-family GTPases and monomeric actin (34). YY1, a competitor of SRF's DNA binding activity (24) and the heart-enriched homeodomain-only cofactor HOP (9) block myogenic gene activity.

SRF MADS box serine-162 phosphorylation, a switch for preventing muscle differentiation gene programs

A long-standing conundrum in myogenesis concerns the mechanisms whereby SRF could regulate both replication- and differentiation-dependent genes for apparent antagonistic processes. Growth factor-stimulated PKC pathways converge on the c-fos SRE to promote myoblast proliferation blocking differentiation. Recently, Iyer et al (18) showed that phosphorylation of serine-162 in the SRF MADS box $\alpha 1$ coil is a key determinant for switching SRF-dependent gene expression between replication and muscle differentiation (Fig.2). Serine 162 was shown to be a highly efficient PKC- α phosphorylation site, that's well conserved across evolution. An SRF phosphorylation mimetic mutant generated by substitution of serine 162 with aspartate (SRF-S162D) completely abolished SRF binding to the CArG boxes, blocked expression of myogenic genes in differentiated embryoid bodies, but allowed for robust expression of immediate early genes through a TCF stabilizing complex. Specific antibodies generated against phosphorylated SRF serine 162 peptides showed highly phosphorylated SRF in growing myocytes that was greatly reduced following the withdrawal from the cell cycle, acting as a myogenic regulatory switch.

Identification of SRF gene targets

Several years ago there were a limited number of known SRF gene targets. In a genome wide search for SRF targets, we estimate that there are approximately 1200 genes that contain

conserved SRF binding sites, of these about 250 genes have been validated, as bonafide targets. It will be important to determine how SRF selects among these myriad of potential targets to primarily express genes that direct tissue restricted expression. In a recent study, Zhang et al (50) used Protein A-TEV-tagged chromatin immunoprecipitation technology to collect direct SRF-bound gene targets from P19 cells, induced by Me2SO treatment into an enriched cardiac cell population. In identifying SRF DNA binding targets sequencing revealed a great enrichment for CArG boxes many of which were located within 5 Kb of the transcription start sites (50). In addition, several transfactor binding sites, represented by NKEs, E boxes, HNF1/4, STATs, Smad, mTEF, Ets, NF- κ B and YY1 sites appeared at a much greater frequency than by chance in the mouse genome (Table I). Previous studies showed that SRF cofactors Nkx2.5 and GATA4, strongly enhanced SRF-DNA binding affinity, permitting the formation of higher order DNA binding complexes at relatively low SRF levels (36). Similarly, CRP2 LIM protein, which bridges SRF and GATA factors (7) and myocardin, which form ternary complexes with SRF (33), greatly enhanced SRF DNA binding. Thus, the combination of SRF co-factors that are enriched in cardiac myocytes increase SRF binding affinity to DNA and enriched cognate DNA binding sites closely embedded near CArG boxes may help guide SRF to cardiogenic genes but not others. In fact, Cooper et al (12), using chromatin immunoprecipitation with SRF antibodies and genomic tile arrays concluded that differential occupation of validated SRF gene targets is likely influenced by the SRF co-factors found in differentiated cell types.

Embryonic SRF Expression Is Largely Restricted to Cardiac and Skeletal Muscle Tissues

SRF is expressed in a highly restrictive pattern throughout mouse development (2). As development proceeds, SRF transcripts becomes restricted to the cardiac crescent and greatly increases in the heart tube and presomitic mesenchyme in the tail and somites (Fig.3). These areas are of significance to cardiac and skeletal muscle, because pre-cardiac mesoderm cells migrate through the streak to take up residence in the anterior lateral plate, whereas skeletal muscle originates in myotomes of the paraxial mesoderm. SRF β -Galactosidase “knock-in” reporter mice faithfully reproduced the endogenous SRF gene expression pattern, reinforced the notion of restricted developmental expression of SRF primarily in committed myogenic precursors. SRF through its 3' enhancer was shown to direct transgene activity in the heart and a downstream gene target of the mutual interactive cofactors Tbx2 and Tbx5, and the histone acetyltransferase, TIP60 (2).

SRF orchestrates cardiac sarcomerogenesis

Recent SRF inactivation studies in the developing mouse heart were performed through a conditional knock-out strategy using Cre recombinase driven by late expressing transgenic promoters such as, SM22, and or α/β myosin-heavy chains (25,28). These studies revealed a role for SRF in reducing transcription of smooth muscle and myofibrillar proteins. Unfortunately, SRF protein does not rapidly turn over, and even though the SRF locus was ablated the appearance of beating cardiac myocytes was not prevented from appearing (28); thus, this obfuscated SRF's primary role in controlling sarcomerogenesis.

To knock out SRF before it accumulates in the heart, we engineered a mouse that carried both *Srf^{lacZ}* and *Nkx2.5^{Cre}* on chromosome 17 which was then bred to *SRF^{lox/lox}* mice to generate a conditional SRF knockout (*Srf^{cko}*) in the heart forming region. This time, SRF protein failed to accumulate in the embryonic myocytes. The initiation of contractile actins' expression at late cardiac crescent stage (7.75-8.0 dpc) was scrutinized for the induction of cardiac and smooth muscle α -actin gene activities which were thoroughly blocked in *Srf^{cko}* mutant. At this developmental stage, no morphological defects were detected in the *Srf^{cko}* mutant embryos,

yet by ~8.0-8.5 dpc, *Srf^{cko}* embryonic mutants failed to initiate cardiac beating, revealed the earliest cardiac defect due to the conditional ablation of a cardiac transcription factor. Microarray analysis of RNA isolated from the *Srf^{cko}* mutant embryo in comparison to haploid *SRF^{LoxP/LacZ}* mice showed blocked expression of many sarcomeric thin filaments, thick filaments and M-bands including *Myl2* and *Myom1* (Fig.3) Excitation-contraction coupling pathway proteins *Gja5*, *Jph,2* and *KCNMB1* also failed to appear in the *Srf^{cko}* mutant hearts. In addition, the appearance of *Smyd1*, a histone methyltransferase required for chromatin remodeling and sarcomere formation in Zebrafish was blocked in the *Srf^{cko}* mutant myocytes. Also, expression of the “left sided” *Hand1* gene was reduced in *Srf^{cko}* mutant. Thus, SRF orchestrates cardiac myogenesis through multiple levels of regulation from the appearance of cardiogenic chromatin remodeling factors, myofibrillar proteins and excitation contraction coupling proteins.

The Gene Ontology (GO) project allowed us to analyze the global biological impact of SRF on the expression of functionally related groups of genes (Fig. 3). Ten GO terms fulfilled the Benjamini test (conservative correct p-values), and they included *muscle contraction*, *actin cytoskeleton*, *myosin*, *contractile fiber*, *sarcomere*, *myofibril*, *cytoskeleton*, *A band*, *motor activity*, *muscle development*, *cytoskeleton organization and biogenesis*, thus, correlating well with SRF primary role as the sarcomeric regulatory factor. Surprisingly, we also observed strong upregulation of genes associated with the 4 GO terms *biomineral formation*, *ossification*, *bone remodeling* and *extracellular matrix*. Microarrays revealed genes that were strongly induced in the absence of SRF which included GATA-6, BMP4, endothelin and periostin, key factors involved with endocardial specification (Fig. 3). GATA6 contributes to septal and valvular development via its direct transcription target, BMP-4 through the induction of periostin (27). Transformation of atrioventricular (AV) canal endocardium into invasive mesenchyme correlates spatially and temporally with the expression of BMPs in the AV myocardium critical for the induction of periostin (30). Also, periostin induces proliferation of differentiated cardiomyocytes and may promote cardiac repair (22).

SRF directs the expression of miR-1-2

Although, SRF is not thought of as an inhibitory transfactor there is a greater likelihood that SRF indirectly exerts gene silencing activities through its regulation over miRNAs. Based on sequence conservation and the ability to fold into a hairpin structure, the human genome is predicted to encode as many as a thousand miRNAs, which are estimated to regulate as many as 30% of mRNA transcripts (3). Informatics helped us to determine that approximately 169 miRNA genes in mammalian genomes contain at least one CArG element in their promoter regions, while at least 40 miRNA genes contain 3 or more CArGs (Table 1). Obviously, ablation of SRF may also block the expression of many miRNAs, causing rampant and complex dysregulation in cardiac development. In support of this idea, Srivastava and colleagues (48) showed that SRF regulates the expression of miR-1-2, through its 3 CArG boxes in the miR-1-2 promoter.

miRNAs control cellular regular pathways through posttranscriptional regulation of protein expression (1,29). miRNA may modify cellular events, function as molecular switches and even silence superfluous mRNAs in specific cell lineages (11,17). miRNAs are transcribed in the nucleus and processed by Drosha and Dicer to generate miRNAs of 20–22 nucleotides (4). Mature miRNA in the RNA-induced silencing complex (RISC) are guided to the 3' UTR of target mRNAs and bind target mRNAs by partial sequence matching in the seed region of 2-8 nucleotides causing degradation of the mRNA transcript and or translational inhibition. Disruption of miRNAs in *Caenorhabditis elegans* and *Drosophila* suggest several ways by which miRNAs may control cellular events. In some cases, they function to “fine-tune” physiologic events, but in others they function as molecular “switches” (5,19,23,37). miRNAs

can also function in a “fail-safe” mechanism to silence mRNAs that are unwanted in specific cell lineages (11,17). In mice, interference with miRNA biogenesis by tissue-specific deletion of Dicer revealed a requirement of miRNA function during limb outgrowth (16) and in development of skin progenitors (46). Clearly, miRNAs powerful effects on cellular phenotypes, effect on such a substantial fraction of the genome, and evolutionary conservation across divergent species underscore their importance as key regulators of physiological and pathological processes that are only beginning to be appreciated. To date, the *in vivo* functions of only a handful of miRNAs have been determined in mammals and until recently their *in vivo* requirement of specific miRNAs in mammals through targeted deletion was known.

Disruption of the Dicer Allele in Cardiac Progenitors

Interference with miRNA biogenesis by tissue-specific deletion of Dicer revealed a requirement of miRNA function during cardiogenesis. To understand the role of miRNAs in the developing heart, Zhao et al (2007) deleted a floxed Dicer allele (16), using Cre recombinase under control of the endogenous Nkx2.5 regulatory region, which directs expression in cardiac progenitors (26). Dicer, which is essential for processing of premiRNAs into the mature form, was efficiently deleted in the heart, and the embryos died from cardiac failure by E12.5 (Fig. 4). Embryos lacking Dicer showed cardiac failure due to a variety of developmental defects, including pericardial edema and underdevelopment of the ventricular myocardium. Most markers of initial cardiac differentiation and patterning, such as Tbx5, Hand1, Hand2, and Mlc2v, were expressed normally. Microarray analysis of E11.5 hearts revealed upregulation of several genes, such as the endoderm marker a fetoprotein and the skeletal muscle-specific gene, fast skeletal troponin; numerous genes were also downregulated, including those encoding the homeodomain only protein (HOP), myoglobin, and the potassium channel Kcnd2. These phenotypes are consistent with the defects during heart development observed in zebrafish embryos devoid of Dicer function (15). The early lethality in the Dicer mutant revealed an essential requirement for miRNA function in the developing heart.

SRF driven bicistronic miRNA 1-1 and miRNA-133 have important regulatory roles in heart and skeletal muscle

Srivastava and colleagues previously described muscle-specific miRNAs, such as the bicistronic miR-1 and miR-133 cluster and miR-206. Two members of the miR-1 class of RNAs miR-1-1 and miR-1-2 are specifically expressed in cardiac tissue and skeletal muscle. The regulation of muscle miRNA expression appears to be largely controlled by myogenic transcriptional networks involving SRF, MyoD, MEF2, and myocardin (8,35,37,48). For example, mouse miR-1 and miR-133 are expressed in both skeletal and cardiac muscle lineages; however, they originate from different polycistronic loci (e.g., miR-1-1 and miR-133a-2 are clustered on mouse chromosome 2, while miR-1-2 and miR-133a-1 are clustered on mouse chromosome 18 (9). Promoter analyses demonstrate that both the chromosome 2 and 18 clusters contain upstream enhancers with CArG elements, and the activity of those enhancers is increased by myocardin co-expression (9,48 Fig. 5). Cardiac muscle-specific and skeletal muscle-specific expressions of those miRNAs are controlled by SRF/myocardin and MyoD/MEF2, respectively, suggesting that the tissue-specific expression of miRNAs are likely regulated at the transcriptional level. In addition, we confirmed that greater than 90% of miR-1 RNA transcripts were blocked in the SRF^{cko} hearts. These studies also suggest that tight temporal and spatial regulation of miRNA expression is important for their function.

Dysregulation of cardiogenesis, miRNA-1-2 knockout

Mice lacking miR-1-2 have a spectrum of abnormalities, including ventricular septal defects in a subset that suffer early lethality, cardiac rhythm disturbances in those that survive, and a striking myocyte cell-cycle abnormality that leads to hyperplasia of the heart with nuclear division persisting postnatally. Zhao et al (49) determined in vivo miR-1-2 targets, including the cardiac transcription factor, *Irx5*, which represses the potassium channel, *Kcnd2* (13). Their study also suggested that the combined loss of *Irx5* and *Irx4* disrupted ventricular repolarization with a predisposition to arrhythmias. The increase in *Irx5* and *Irx4* protein levels in miR-1-2 mutants corresponded well with a decrease in *Kcnd2*, expression. Clearly, both gain- and loss-of-function of miR-1 and Dicer mutant embryos, affect conductivity through potassium channels supports a central role for miR-1 for fine tuning the regulation of cardiac electrophysiology in pathological and normal conditions.

Consistent with the role for miR-1 in muscle differentiation, overexpression of miR-1 in the developing mouse heart resulted in reduced ventricular myocyte expansion and decreased the number of proliferating myocytes (48). This phenotype was explained in part by the presence of an miR-1 target site in the 3' UTR of the *Hand2* cardiac transcription factor (48), whose genetic ablation in the mouse produced a similar failure in ventricular myocyte expansion (39). In agreement with this notion, overexpression of miR-1 in the mouse heart decreased *Hand2* protein levels (48). Similarly, introduction of miR-1 and miR-133 in *Xenopus* embryos interferes with the heart development (9). Additional genetic studies of *Drosophila* support the view that miR-1 is an important regulator of cardiogenesis and cardiac gene expression (23). Collectively, miRNAs are an emerging class of molecules important for cardiac and skeletal muscle development. Figure 5 summarizes miR-1 activities in the heart.

miRNA-208 knockout revealed a critical role in stress-dependent cardiac growth

miRNAs also have important roles in regulating the cardiac response to stress by hypertrophic growth that eventually diminishes contractility, from the down-regulation of α -myosin heavy chain (α MHC) and up-regulation of β MHC, the primary contractile proteins of the heart. In an elegant study from Eric Olson's laboratory, van Rooij et al. (43) deleted the cardiac-specific miR-208 that is encoded by an intron in the α -MHC gene and found miR-208 mutant mice failed to undergo stress-induced cardiac remodeling, hypertrophic growth, and β -MHC upregulation. Importantly, transgenic expression of miR-208 was sufficient to induce β -MHC. miR-208-deficient hearts also resembled hyperthyroid hearts and miR-208 regulates β -MHC by repressing the thyroid hormone receptor associated protein 1 (THRAP1), a cofactor of the thyroid hormone receptor (TR) and a predicted miR-208 target mRNA. Thus, miR-208 is important for cardiac growth and gene expression in response to stress and hypothyroidism.

The studies by Zhao et al. (49) and van Rooij et al (43) also indicates that miRNA dosage, is important during heart development and hypertrophy and could constitute potential risk factors for adult cardiovascular disease and heart failure. In addition, Chen and colleagues showed that expression of miR-133 repressed myoblast differentiation by repressing SRF expression (9). Thus, miR-133 is controlled by SRF, yet directs a negative regulatory loop through inhibiting SRF translation (Fig. 5).

It is not surprising that SRF activity, must also be tightly regulated through a miR-133 dependent negative feedback loop, because enhanced levels of SRF have led to dilated cardiomyopathy, while too little SRF showed that SRF activity was required for the maintenance of normal sarcomeric organization and contractility (32). Possibly, the delicate balance of miR-133 directed silencing of SRF activity in cardiac hypertrophy may be more

profound in human heart disease. Chang et al (6) found SRF was cleaved by caspase 3 in human heart failure to become a dominant-negative transfactor. This SRF fragment contains an intact MADS box but not the C-terminal transactivation domain. Therefore, cleaved SRF competes with full-length SRF for binding to CArG boxes, interfering with SRF's global role in controlling genes required for sarcomerogenesis but may also hinder the miRNAs that silence gene activities needed for steady heart's basic functions.

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cardiogenesis. The mouse knockout of the muscle-specific miRNA, miR-1-2, revealed numerous functions in the heart, including regulation of cardiac morphogenesis, electrical conduction, and cell cycle control. miR-1-2 deletion revealed an enrichment of miR-1 ‘‘seed matches’’ and a strong tendency for potential miR-1 binding sites to be located in physically accessible regions.

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Figure 1. SRF contains an evolutionary conserved MADS box domain that allows for dimerization of SRF monomers, binding to a CArG element and association with co-factors

A Schematic diagram of human SRF with a transcription activation domain (TAD) and the DNA binding and dimerization domain between 141aa to 223aa. **B** Diagram of the SRF-DNA crystal structure was adapted from Pellegrini et al. (1995) **C** Sequence of SRF targets showed a high level of similarity for preferred base composition across the CArG elements named CArGome, as reported by Sun et al. (2006). **D** Cardiogenic enriched transcription factors Nkx2-5, GATA4, GATA6, CRP2 and myocardin support SRF myogenic activity and associate with the SRF MADS box. The Homeodomain factor, HOP and the Ets factor, ELK-1, are competitive inhibitors of Nkx2.5 and myocardin, respectively.

Figure 2. SRF gene activity directed by growth factor signaling and by cardiac specific transactors

A The first diagram corresponds to growth factor signaling in which the SRF gene is a downstream target for the ERK signaling pathways that converge on ternary complex factors (TCF), such as the ETS domain transcription factor, Elk1. SREs are dependent upon TCF Elk-1 and drive a positive feedback loop. In addition, growth factor stimulation lead to PKC- α phosphorylation of S162 in the MADS box in a manner that suppressed the expression of muscle differentiation genes, while permitting and or increasing the association between Ets factors and SRF to up-regulate c-fos transcription. The MADS box served as a regulatory platform that mediates interactions between SRF and a myriad of transcription cofactors, hence differential phosphorylation of MADS box residues directed specific SRF transcriptional complexes toward the expression of proliferation dependent genes in myoblasts. **B** The second diagram shows cardiogenic specific regulation of the SRF gene. Tbx2, Tbx5, and TIP60, were shown to strongly activate SRF activity (2). Also shown is the competitive inhibition of SRF – cardiogenic cofactor interactions by ETS factors. In the heart SRF and cardiogenic co-factors facilitate SRF binding to promoters that drive the expression of other cardiogenic transcription factors, sarcomeric contractile proteins, ion channels and miRNAs.

Figure 3. Analysis of *Srf*^{cko} mutant on global gene transcription revealed downregulation of contractile proteins and ion channels and upregulation of bone related proteins in embryonic hearts Top panel shows SRF *lacZ* expression in E7.5-E9.5 mouse embryos containing a β -Galactosidase reporter gene knock-in into the SRF genomic locus. LacZ stain revealed highly restricted expression in embryonic cardiac, skeletal, and smooth muscle tissues. To dissect out SRF's role during cardiac myocyte commitment and differentiation, we generated lineage specific deletion of *Srf* with Cre/loxP system. *Srf* and *Nkx2.5* reside on the same chromosome in murine genome, at 17B3 and 17A3.3 respectively. To solve this technical problem, we first selected a chromosome carrying both *Srf*^{lacZ} and *Nkx2.5*^{cre}. This double positive allele, *Srf*^{lacZ::Nkx2.5}^{cre}, was then bred with *Srf*^{fllox/+} or *Srf*^{fllox/fllox} to generated early cardiac specific *Srf* knockout. This double allele, *Srf*^{lacZ::Nkx2.5}^{cre}, was transmitted normally in C57/BL6

background. (Adapted from Zhiyv Niu, Ph.D., Thesis, Program in Cardiovascular Sciences, Baylor College of Medicine). RNA samples isolated from embryonic hearts were pooled (~30, somite 6-9) hybridized against Affymetrix array 430a2 chip. Microarray raw data analysis was done with dCHIP software (www.dchip.org). Genes affected by the SRF knockout were treated for functional classification using DAVID 2007 (<http://david.abcc.ncifcrf.gov/>). The GO term classification uses a program that analyzes for functional associations. The ablation of SRF in the heart revealed 10 GO terms that were associated with downregulated genes and fulfill the Benjamini test (conservative correct p-values) including *muscle contraction*, *actin cytoskeleton*, *myosin*, *contractile fiber*, *sarcomere*, *myofibril*, *cytoskeleton*, *A band*, *motor activity*, *muscle development*, *cytoskeleton organization and biogenesis*. Conversely, upregulated genes were associated with the 4 GO terms *biomineral formation*, *ossification*, *bone remodeling* and *extracellular matrix*.

Figure 4. miRNA Biogenesis Is Necessary for Cardiogenesis

A Quantitative RT-PCR for relative mRNA levels of Dicer's RNase III domain. **B** Protein level of Dicer in wild-type (wt) or *Nkx2.5-Cre; Dicer^{flox/flox}/Dicer^{flox/flox}* embryos measured by western blot using an antibody that recognizes the RNase III domain. **C** and **D** *Nkx2.5-Cre; Dicer^{flox/flox}/Dicer^{flox/flox}* (mutant) embryos showed developmental delay and pericardial edema (arrow) at E12.5. **E** and **F** Transverse sections of E11.5 embryos showing thin-walled myocardium in mutant. **G**–**N** Whole-mount in situ hybridization with indicated cardiac markers in wild-type and mutant E9.5 embryos. **O** RT-PCR using primers specific for miR-1-1 or miR-1-2 in wild-type hearts at E8.5, E10.5, E12.5, and P10 with or without reverse transcriptase (RT). ra, right atrium; la, left atrium; rv, right ventricle; lv, left ventricle; h, head; ht, heart; pa, pharyngeal arch; lm, lateral mesoderm; lb, limb bud. Figure was adapted from Zhao et al. (49).

Figure 5. miR1-2 regulates cardiac morphogenesis, cardiac conduction and cell cycle

Schematic diagram shows the two miR-1 bicistronic genes on chromosome 2 and 18 and summarizes some of the roles for miR-1-2 and miR133 in regulating *Irx4/5*, *GATA-6* and *Hand2* and *SRF* expression that were described in recent studies. Hypothetical regulation of the endocardial cell lineage specification via *GATA-6*, *BMP4* and *periostin* was shown, but not examined for in Zhao et al (49).

Table I

Enrichment of DNA-binding motifs from SRF and co-accessory factors from SRF-PATChIP pulldown

	Expected	Observed	Observed/expected
CArg box	8	89	11
CArg like box	12	66	5
NKE	12	65	6
Gata family	102	118	1
mTATA box	17	46	3
MyoD family	8	34	4
HNF1/4	7	35	5
STAT family	11	26	2
mTEF motif	1	10	10
Ets box	28	34	1

A total of 16,650 bp were sequenced from cloned 234 fragments pulled out by SRF-PATChIP from P19 converted to beating myocytes. Within these sequences we predicted the random appearance of the transcription DNA-binding sites shown above by MatInspector, observed to expectation indicated enrichment for most of the co-accessory transcription-binding sites. An exception was GATA-binding sites that are so numerous to begin with as compared with other transactors

Table II
MicroRNA genes containing 3 or more SRF binding sites

microRNA	CArGs	microRNA	CArGs
MIRN7-1	6	MIRN488	3
MIRN451	6	MIRN450-1	3
MIRN144	6	MIRN377	3
MIRN133A1	6	MIRN365-1	3
MIRN497	5	MIRN324	3
MIRN21	5	MIRN30C2	3
MIRN195	5	MIRN30A	3
MIRN125B2	5	MIRN214	3
MIRN93	4	MIRN206	3
MIRN9-2	4	MIRN205	3
MIRN365-2	4	MIRN196A2	3
MIRN25	4	MIRN196A1	3
MIRN211	4	MIRN181B1	3
MIRN199A2	4	MIRN181A-1	3
MIRN182	4	MIRN148A	3
MIRN154	4	MIRN146B	3
MIRN122A	4	MIRN10B	3
MIRN106B	4	MIRN1-1	3
MIRN1-2	4	MIRNLET7C	3
MIRNLET7A2	3	MIRN551B	3
MIRN99A	3	MIRN568	3

Approximately one third of the 500 or more miRNA genes in mammalian genomes contain at least one CArG element in or near their promoter regions. At least 43 miRNA genes contain 3 or more CArG elements including miR-1-1 and miR1-2.