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Lrrc10 is a novel cardiac-specific target gene of Nkx2-5 and GATA4

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Abstract

Cardiac gene expression is precisely regulated and its perturbation causes developmental defects and heart disease. Leucine-rich repeat containing 10 (*Lrrc10*) is a cardiac-specific factor that is crucial for proper cardiac development and deletion of *Lrrc10* in mice results in dilated cardiomyopathy. However, the mechanisms regulating *Lrrc10* expression in cardiomyocytes remain unknown. Therefore, we set out to determine trans-acting factors and cis-elements critical for mediating *Lrrc10* expression. We identify *Lrrc10* as a transcriptional target of Nkx2-5 and GATA4. The *Lrrc10* promoter region contains two highly conserved cardiac regulatory elements, which are functional in cardiomyocytes but not in fibroblasts. In vivo, Nkx2-5 and GATA4 endogenously occupy the proximal and distal cardiac regulatory elements of *Lrrc10* in the heart. Moreover, embryonic hearts of *Nkx2-5* knockout mice have dramatically reduced expression of *Lrrc10*. These data demonstrate the importance of Nkx2-5 and GATA4 in regulation of *Lrrc10* expression in vivo. The proximal cardiac regulatory element located at around −200 bp is synergistically activated by Nkx2-5 and GATA4 while the distal cardiac regulatory element present around −3 Kb requires SRF in addition to Nkx2-5 and GATA4 for synergistic activation. Mutational analyses identify a pair of adjacent Nkx2-5 and GATA binding sites within the proximal cardiac regulatory element that are necessary to induce expression of *Lrrc10*. In contrast, only the GATA site is functional in the distal regulatory element. Taken together, our data

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Disclosure Statement
None.

demonstrate that the transcription factors Nkx2-5 and GATA4 cooperatively regulate cardiac-specific expression of *Lrrc10*.

Keywords

LRRC10; Nkx2-5; GATA4; transcriptional regulation; cardiac gene expression

1. Introduction

Leucine-rich repeat containing 10 (LRRC10) is a cardiac-specific factor in mice, zebrafish and humans [1–4], which exhibits robust expression in the developing and adult heart [1, 4]. Lrrc10 plays critical roles in cardiac development and function in zebrafish [3] and LRRC10 interacts with actin and actinin in the heart [5], suggesting a prominent function for LRRC10 in cardiac physiology and a potential role in human heart disease.

Tissue-specific expression of genes requires precise coordinated molecular controls to confer specificity. Given the remarkable cardiac-specific expression of *Lrrc10* from the precardiac region to the adult heart and its important roles in cardiac function [1, 3–5], it is critical to investigate the molecular mechanisms that regulate expression of *Lrrc10*. *Lrrc10* expression is dynamically regulated during development, with an approximately four-fold increase in mRNA levels at birth [1]. *Lrrc10* homozygous knockout mice exhibit prenatal cardiac functional deficits and progressive dilated cardiomyopathy in postnatal life [5], indicating that proper regulation of *Lrrc10* expression is essential for normal cardiac function. The promoter region of *Lrrc10* contains multiple DNA binding motifs for key cardiac transcription factors [4, 6] and *LacZ* transgenic reporter mice containing 7 Kb upstream of the *Lrrc10* sequence recapitulate its cardiac-specific expression in vivo [4]. However, the critical transacting factors and cis-elements that regulate cardiac *Lrrc10* expression remain to be elucidated.

Nkx2-5 is a cardiac-restricted transcription factor essential for proper cardiac development [7–9] and conduction system function [10–13]. Mutations of *Nkx2-5* result in congenital heart disease, electrophysiological abnormalities, and sudden death in animal models [7, 8, 10–13] and humans [14]. Nkx2-5 regulates cardiac transcription often in conjunction with other transcriptional cofactors, including GATA4 [15–19], SRF (serum response factor) [17, 20, 21], Tbx5 [12, 22], and Jarid2 [23]. GATA4 is a zinc finger transcription factor that is required for early cardiac development and adult cardiac function [24–27]. GATA4 regulates cardiac gene expression by forming complexes with transcriptional factors, including Nkx2-5 [15–19], NFAT (nuclear factor of activated T cells) [28], Tbx5 [25, 29], SRF [17, 30, 31], Smad1/4 [32] and Jarid2 [23]. Furthermore, mutations of GATA4 have been shown to cause cardiac septal defects in humans [25]. Cardiac-specific deletion of *Gata4* [33] or perinatal knockout of *Nkx2-5* [10] in mice results in compromised cardiac function and dilated cardiomyopathy, suggesting a prominent role for Nkx2-5- and GATA4-mediated transcription in adult cardiac function and disease.

Nkx2-5 is expressed very early in the precardiac region when *Lrrc10* expression is first detected [1] and regulates the expression of a number of transcriptional targets in the heart, including endothelin-converting enzyme-1 [34], Jarid2 [35], and β-catenin [36]. GATA4 also controls the transcription of several important cardiac genes, such as carnitine palmitoyltransferase Iβ [31], troponin I [37], troponin C [38], brain natriuretic peptide [39–41], and α-myosin heavy chain [42]. Moreover, Nkx2-5 and GATA4 physically interact [15, 16, 18] and have been shown to cooperatively regulate the expression of essential cardiac

target genes, including ANF [15, 18, 19, 43], T- and L-type Ca^{2+} channels [44], connexin 40 [22], α-actin [16, 17, 20, 21], and Id2 [45].

Here, we provide evidence that *Lrrc10* is a novel transcriptional target of Nkx2-5 and GATA4. In vivo, Nkx2-5 and GATA4 occupy highly conserved cardiac regulatory regions of the *Lrrc10* genomic locus in the heart and deletion of *Nkx2-5* in mice results in dramatically reduced *Lrrc10* expression. In vitro, we identify proximal and distal cardiac regulatory elements (PCE and DCE, respectively) near the *Lrrc10* promoter by reporter gene assays. Nkx2-5 and GATA4 synergistically activate the *Lrrc10* promoter containing the PCE and respective binding sites are required for Nkx2-5- and GATA4-mediated activation. The DCE contains a highly conserved GATA site critical for GATA4-mediated activation and is cooperatively activated by SRF, Nkx2-5, and GATA4.

2. Materials and Methods

2.1. Animals

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH) and the University of Wisconsin Research Animal Resource Center policies. Procedures were approved by a University of Wisconsin-Madison Institutional Animal Care and Use Committee. Genotyping of *Nkx2-5*$^{-/-}$ embryos was described elsewhere [9].

2.2. Cell Culture and Luciferase Reporter Gene Assays

Mouse neonatal cardiomyocytes were cultured as described previously [1]. *Lrrc10* reporter plasmids were constructed by cloning various regions of the *Lrrc10* genomic locus by PCR and ligating the PCR product into the pGL3 basic vector carrying a firefly luciferase gene (Promega). Transient transfection assays were performed as described [23, 46]. Briefly, cardiomyocytes were cotransfected with *Lrrc10* reporter constructs and a β-galactosidase-CMV vector using Lipofectamine LTX (Invitrogen). Luciferase assays were performed 48 hours post transfection using the Luciferase Assay System (Promega) and normalized to β-galactosidase activity. Assays were repeated at least three times in duplicate. Reporter gene assays were also performed in which 10T1/2 cells were cotransfected with plasmids encoding the cardiac transcription factors Nkx2-5, GATA4, and/or MEF2A in the pcDNA3.1-myc vector (Promega) [23, 47] and/or pCGN-SRF [48]. Nkx2-5 binding elements (NKEs) and/or GATA binding sites within the −178 bp *Lrrc10* promoter-enhancer reporter were mutated as depicted in Figure 4B using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). All new constructs were subjected to diagnostic digestion and confirmed by DNA sequencing. The oligonucleotides used for constructing *Lrrc10* mutant reporter genes are as follows with mutated nucleotides in bold and NKEs and GATA factor sites underlined:

Proximal mNKE/GATA forward (F)

5′GCCTGTCACCAGACACTCAAGCTGC**C**A**C**CAGGAG**T**A**T**T**CG**ATTCTGTCAA AGTGACTGGCTTT′

Distal mNKE

5′GGAGAGTAGATAAGAGATCAGGGCTT**TT**G**CC**ATTAAAGACAAATATGTG AGTTGGAC′

Distal mGATA F

5′GCCCTTGTCGAGGAGAGTA**AC**TAAGAGATCAGGGCTTAAG′

2.3. Quantitative Real Time PCR

(qRT-PCR) qRT-PCR was performed as described previously [5]. Data are normalized to 18S expression. The following primers were designed using Primer Express 1.0 (ABI Prism):

Lrrc10 F 5′GCCACCTCCTCCCCTTCTT′; *Lrrc10* R 5′GCCTTCAACCTCAGCTGTTCA′;

18S F 5′CGCCGCTAGAGGTGAAATTCT′; *18S* R 5′CGAACCTCCGACTTTCGTTCT′

2.4. Quantitative Chromatin Immunoprecipitation (qChIP)

qChIP experiments were performed as described previously [49, 50] on two pooled wild type mouse hearts at postnatal day three with Nkx2-5 (Supplemental Fig 1), GATA4 (Santa Cruz), or SRF (Santa Cruz) specific antibodies. Preimmune serum or nonspecific IgG (Sigma) was used as a negative control. Primers were designed to amplify 50- to 150-bp amplicons. Products were measured using SYBR Green fluorescence (FastStart SYBR Green Master, Roche; iCycler, BioRad) and normalized to a standard curve of input chromatin. The following primers were used for amplification of the conserved regions of the *Lrrc10* locus:

- 5.5 Kb F 5′ACAAACAATTTGTTCCGCTGAA′; -5.5 Kb R 5′CAAGGTTGAGGAGCCCTTAGC′;
- 3 Kb F 5′CCATTTGTGGTCACCACTGG′; -3 Kb R 5′TCGCGCGGTCTTACCTCTAT′;
- 2.3 Kb F 5′TTTACTCTCACACAAGGATGTATGCA′
- 2.3 Kb R 5′ CCAAGCAATGTTCAGACAATTCTATAAT′
- 1.7 Kb F 5′TGGCAGCACTTTGGGTCAT′; -1.7 Kb R 5′GGAAGCGTCTGTCCCAGAGTT′
- 200 bp F 5′AGTGATTAAAGACAAATATGTGAGTTGGA′;
- 200 bp R 5′AAAGCCAGTCACTTTGACAGAATAAA′

2.5. Anti-Nkx2-5 Antibody Generation, Western Blotting, and In Situ Hybridization

To generate anti-Nkx2-5 antibody, mouse Nkx2-5 was purified from *E. coli* as a Maltose Binding Protein (MBP) fusion protein and the MBP tag was removed by TEV cleavage leaving a 6 His N-terminal tag on Nkx2-5. The protein was confirmed by SDS-PAGE followed by Coomassie blue staining and was used to immunize rabbit to generate polyclonal anti-Nkx2-5 antibodies (Proteintech Group). Affinity of the antiserum was tested using an enzyme-linked immunosorbent assay and specificities of anti-Nkx2-5 antibodies were confirmed by Western blotting and immunostaining as described previously [5] (Supplemental Fig 1). The LRRC10 antibody was previously described [1]. Whole-mount in situ hybridization of mouse embryos using antisense cRNA probes against *Lrrc10* was previously described [1].

2.6. Statistical Analysis

All statistical analysis was performed by a student’s t-test unless otherwise stated. Results are expressed as mean ± standard error of the mean (SEM).

3. Results

Lrrc10 exhibits robust, cardiac-specific expression in the developing and postnatal heart [1] and plays critical roles in cardiac development and function [3, 5]. We have previously shown that embryonic hearts express abundant *Lrrc10* mRNA, which is increased significantly at birth and maintained at an elevated expression level in the adult heart [1]. To determine if this expression pattern is recapitulated at the protein level, Western blotting was performed on mouse heart extracts at various ages. LRRC10 protein expression is also abundant in the embryonic heart, significantly increased in the newborn heart, and maintains elevated abundance in the adult (Supplemental Fig 2). These results suggest that a transcriptional mechanism dynamically regulates LRRC10 protein levels during cardiac development and in the postnatal heart.

Given the intricate transcriptional regulation of *Lrrc10* expression in development, we set out to identify the enhancer/promoter regions of the *Lrrc10* genomic locus that confer cardiac-specific expression of *Lrrc10* and the corresponding trans-acting factors. A VISTA alignment was performed to identify highly conserved and potential functionally important regions of the *Lrrc10* genomic locus (Fig 1A). VISTA alignment of the genomic sequences of mouse, rat, chimpanzee, and human revealed highly conserved regions of 70% or greater sequence homology located at approximately −3 Kb, −2.3 Kb, −1.7 Kb, and −200 bp of the transcription initiation site (TIS). No highly conserved regions were found within 10 Kb downstream of the stop codon (data not shown). Because the *Lrrc10* genomic locus contains highly conserved regions within −4 Kb of the TIS (Fig 1A) [4], we analyzed this region for potential transcription factor binding sites using the TFSEARCH program [51] and rVista [52]. Results revealed the presence of multiple DNA binding sites for critical cardiac transcription factors such as Nkx2-5, GATA4, MEF2, and SRF (Fig 1B). Notably, a cluster of three Nkx2-5 binding sites (NKEs) is located around −3.1 Kb of the TIS of *Lrrc10*, which is flanked by GATA binding sites. Additionally, two NKEs that each lie adjacent to GATA binding sites are found within −200 bp of the TIS (Fig 1B), suggesting a potential role for Nkx2-5 and GATA4 in regulation of *Lrrc10* expression.

To dissect the cis-elements that mediate cardiac expression, we performed transient transfection assays using reporter genes containing the *Lrrc10* promoter region linked to a luciferase reporter. Various serial deletion reporters were constructed (Fig 1C) and luciferase activity was evaluated in primary cardiomyocytes or a fibroblast cell line. The −3984 bp *Lrrc10* reporter showed a marked 100-fold activation in cardiomyocytes (Fig 1D), but no activation in fibroblasts (Fig 1E), suggesting that cardiac regulatory elements are located within the −4 Kb region. A marked decrease in luciferase activity was observed from the −3984 bp to −2909 bp reporter constructs in cardiomyocytes but not in fibroblasts (Fig 1D, E). However, further deletion from −2909 bp to −1807 bp constructs did not further decrease cardiac-specific activation, indicating that there is a functionally important cardiac regulatory element present between the −3984 and −2909 bp region. This regulatory region located around −3 Kb (referred to hereafter as the distal cardiac regulatory element, DCE) contains three putative NKEs flanked by GATA binding sites around −3.1 Kb (Fig 1B), which are deleted in the −2909 bp reporter. In contrast, deletion of the −3984 bp to −2909 bp region markedly increased reporter activity in fibroblasts (Fig 1E), suggesting a repressive element functions in fibroblasts between the −3984 bp and −2909 bp region. Therefore, the region between −3984 bp and −2909 bp likely ensures cardiac-specific expression of *Lrrc10* by repressing ectopic expression in non-cardiomyocytes.

Further serial deletion of reporters did not appear to affect cardiac-specific expression of *Lrrc10* (Fig 1D). Although there is a Tbx binding site present in the *Lrrc10* promoter (Fig 1B), it is in a region that is not highly conserved (Fig 1A) and deletion of the region from

−1807 bp to −999 bp containing the Tbx binding site does not affect cardiac-specific activity (Fig 1D). The −178 bp region was sufficient to mediate cardiac-specific activity (Fig 1D), and therefore, this region is hereafter referred to as the proximal cardiac regulatory element, PCE. The PCE contains a pair of adjacent NKEs and GATA binding sites but no SRF DNA binding site (Fig 1B). Because binding sites for the cardiac transcription factors SRF, MEF2, GATA4, and Nkx2-5 are located within 4Kb of the TIS, the ability of these factors to activate the *Lrrc10* promoter was evaluated in transient transfection assays. Results indicate that Nkx2-5, SRF, or GATA4 alone activates the −3984 bp *Lrrc10* reporter, however, no activation is observed in response to MEF2A (Fig. 1F).

Given the significant activation of the *Lrrc10* promoter by Nkx2-5 and GATA4, we hypothesized that Nkx2-5 and GATA4 are key mediators of the cardiac-specific expression pattern of *Lrrc10*. To determine if Nkx2-5 endogenously occupies conserved regions of the *Lrrc10* locus (Fig 1A) in the heart, qChIP analyses were performed on postnatal mouse heart tissue using primers specific for these conserved regions and an Nkx2-5 antibody. Significant occupancy of Nkx2-5 was observed at the highly conserved regions −3 Kb, −2.3 Kb, and −200 bp relative to the TIS of *Lrrc10* (Fig 2A), which all contain NKEs (Fig 1B, data not shown). The most substantial accumulation of Nkx2-5 occurs at two conserved regions at −3 Kb and −200 bp (Fig 2A), corresponding to the DCE and PCE, respectively (Fig 2A, 1B). Minor accumulation of Nkx2-5 occurs at the -2.3 Kb region (Fig 2A). However, truncation of the NKE within this region (at −2285/−2279 bp) does not reduce transcriptional activities in cardiomyocytes (Fig 1D, −2909 bp vs. −1807 bp reporters), suggesting this NKE is not functional. qChIP assays using a GATA4 antibody revealed that the most significant occupancy of GATA4 also occurred at −3 Kb and −200 bp of the TIS of *Lrrc10* (Fig 2B), regions that contain GATA binding sites. GATA4 additionally occupies regions containing putative GATA binding sites at −5.5 Kb and −1.7 Kb, but to a much lesser extent (Fig 2B). These results indicate that high levels of occupancy by Nkx2-5 and GATA4 occur at conserved regions −3 Kb and −200 bp of the *Lrrc10* locus in the heart, which contain cardiac regulatory elements (Fig 1D) and NKEs and GATA binding sites (Fig 1B).

To assess whether expression of *Lrrc10* is regulated by Nkx2-5 in vivo, *Lrrc10* expression was evaluated in *Nkx2*-5 knockout (*Nkx2-5*$^{-/-}$) mice. *Nkx2-5*$^{-/-}$ mice die around embryonic day (E) 10 with arrested cardiogenesis after partial looping [9]. Evaluation of *Lrrc10* transcript levels by qRT-PCR reveals a 57% reduction in *Lrrc10* mRNA expression in *Nkx2-5*$^{-/-}$ hearts (Fig 2C), providing crucial evidence that Nkx2-5 is a key regulator of *Lrrc10* expression in vivo. *Lrrc10* mRNA level was normalized to a house keeping gene and other genes are expressed at normal levels in *Nkx2-5*$^{-/-}$ hearts despite severe morphological defects [9, 53], suggesting downregulation of *Lrrc10* is specifically due to the absence of Nkx2-5. However, it cannot be ruled out that *Lrrc10* expression is reduced in *Nkx2-5*$^{-/-}$ hearts as a consequence of gross morphogenetic abnormalities. Furthermore, whole mount in situ hybridization of E9.5 embryos demonstrates dramatically reduced expression of *Lrrc10* in the *Nkx2-5*$^{-/-}$ heart as compared to the control heart (WT) (Fig 2D, E). Transverse sections of in situ labeled embryos show cardiomyocyte-specific expression of *Lrrc10* in WT (Fig 2F) that is significantly reduced in *Nkx2-5*$^{-/-}$ hearts (Fig 2G). Notably, *Lrrc10* is not expressed in the developing endocardium or epicardium (Fig 2F) [1]. *Lrrc10* expression was not evaluated in *Gata4* knockout embryos due to early embryonic lethality prior to the formation of the heart tube and the well-established genetic and functional redundancy of GATA factors such as cardiac expression of GATA5 and 6 [54, 55].

Because the *Lrrc10* promoter is activated by SRF, Nkx2-5, and GATA4 (Fig 1F) and Nkx2-5 and GATA4 endogenously occupy the highly conserved DCE containing putative binding sites (Fig 2A, B), we tested whether these cardiac factors regulate the expression of

Lrrc10 via this DCE. Cells were cotransfected with SRF, Nkx2-5, or GATA4 and a −3984 bp or a −2909 bp *Lrrc10* reporter lacking the DCE harboring binding sites for these factors. A dramatic reduction in GATA4 mediated activation of the *Lrrc10* promoter was observed when the conserved GATA site (−3044/−3041 bp) within the DCE was removed (Fig 3A). However, truncation of NKEs and SRF binding sites within this DCE failed to alter Nkx2-5- or SRF-mediated activation, respectively (Fig 3A). To investigate whether SRF, Nkx2-5, and GATA4 cooperatively regulate expression of *Lrrc10*, cells were cotransfected with the −3984 bp *Lrrc10* reporter and SRF, Nkx2-5, and/or GATA4. Interestingly, although no one factor independently alters the transcriptional activity of any other factor, cotransfection of SRF, Nkx2-5, and GATA4 synergistically activates *Lrrc10* expression (Fig 3B). Cotransfection of MEF2A did not affect the transcriptional activity of SRF, Nkx2-5, or GATA4 (data not shown), suggesting that MEF2 is not involved in regulation of *Lrrc10* expression.

To determine if SRF, GATA4, or Nkx2-5 alone or in combination regulates *Lrrc10* expression via the DCE (−3984/−2909 bp), a reporter gene containing only the DCE with the *Lrrc10* promoter (−50/+56 bp) was constructed (Fig 3C, *top*). SRF, GATA4, or Nkx2-5 alone activates this DCE-containing reporter independent of the PCE (Fig 3C, *left*). Cotransfection of any two factors results in synergistic activation and cotransfection of SRF, GATA4, and Nkx2-5 results in robust activation (greater than 120-fold) of the DCE reporter (Fig 3C, *left*). In contrast, negligible activation is observed by the *Lrrc10* promoter (−50/+56 bp) alone in response to SRF, GATA4, and/or Nkx2-5 (Fig 3C, *right*).

To investigate whether Nkx2-5 and GATA4 cooperatively regulate *Lrrc10* transcription via the PCE, the −178 bp *Lrrc10* reporter was cotransfected with Nkx2-5 and GATA4 alone or together. Results indicate that Nkx2-5 or GATA4 alone induce *Lrrc10* reporter activity 18- and 54-fold, respectively, while together Nkx2-5 and GATA4 synergistically activate *Lrrc10* reporter activity 184-fold (Fig 4A). Interestingly, there are two NKEs that are each adjacent to GATA binding sites (Fig 1B, 4B) within the PCE. To identify the functional binding sites that are responsible for the Nkx2-5- and GATA4-mediated activity of the PCE, we mutated the NKEs and GATA sites within this region (Fig 4B). A −178 bp *Lrrc10* reporter or a mutant construct containing mutations in one or both of the NKEs or GATA sites was cotransfected with Nkx2-5 or GATA4 (Fig 4C, D). Mutation of either one of the NKEs in the PCE (pmN/G or dmN) diminished Nkx2-5-mediated activation. When both NKEs were mutated (pmN/G/dmN), Nkx2-5-mediated reporter activity was reduced further (Fig 4C). Mutation of either GATA site (pmN/G or dmG) drastically reduced GATA4-mediated activation and mutation of both GATA binding sites (pmN/G/dmG) almost abrogated activation by GATA4 (Fig 4D). Taken together, reporter assay data indicate that NKEs in the PCE, but not in the DCE, play important roles in activating *Lrrc10* transcription, whereas GATA sites in both the DCE and PCE are crucial.

To investigate whether SRF contributes to activation of the PCE, the −178 bp *Lrrc10* reporter was cotransfected with Nkx2-5, GATA4, and/or SRF. Nkx2-5 and GATA4 synergistically activate the reporter but SRF alone does not activate the PCE. When cotransfected, SRF augments synergistic activation of the −178 bp reporter by Nkx2-5 and GATA4 (Fig 5A). To determine if SRF endogenously occupies regulatory regions of *Lrrc10* in the heart, qChIP assays were performed with an SRF antibody or IgG control (Fig 5B). There is robust accumulation of SRF at the conserved region at −3 Kb of the *Lrrc10* TIS (Fig 5B), supporting cooperative regulation of the DCE by Nkx2-5, GATA4, and SRF (Fig 3B, C). Although a low level of accumulation of SRF is observed at the −1.7 Kb region, there are no cardiac regulatory elements (Fig 1D, E, −1807 bp vs. −999 bp reporters) or SRF binding sites (SREs) located within this region. In contrast, no accumulation of SRF was

observed at the PCE. Based on our findings, our working model of transcriptional regulation of *Lrrc10* is depicted in Fig 5C (See Discussion).

4. Discussion

Cardiac-specific expression of *Lrrc10* begins very early in the precardiac region [1]. *Lrrc10* expression at the mRNA [56] and protein levels is significantly increased in the heart soon after birth, suggesting a transcriptional regulatory mechanism mediates the cardiac-specific upregulation of *Lrrc10*. Since *Lrrc10* expression is remarkably cardiac-specific [4, 56] and is dynamically regulated during development, it is critical to determine the molecular mechanisms that regulate expression of *Lrrc10*. Here, we provide in vivo evidence that *Lrrc10* is a novel target gene of the cardiac transcription factors Nkx2-5 and GATA4 by showing that these factors occupy the cardiac regulatory regions of *Lrrc10* in the heart and that deletion of *Nkx2-5* dramatically reduces *Lrrc10* expression. We identify two cardiac regulatory elements located around −3 Kb (DCE) and −200 bp (PCE) of the *Lrrc10* TIS, which confer *Lrrc10* reporter activity specifically in cardiomyocytes but are inactive in fibroblasts.

The DCE at −3 Kb from the TIS of *Lrrc10* is endogenously occupied and cooperatively activated by SRF, Nkx2-5, and GATA4. Deletion of the DCE markedly reduces GATA4-mediated, but not SRF- or Nkx2-5-mediated activation of the *Lrrc10* promoter, suggesting that the conserved GATA site (−3044/−3041) in the DCE plays a major functional role (Fig 5C). Importantly, GATA4, SRF, and Nkx2-5 cooperatively activate transcription via this DCE independent of the PCE. GATA4 may be involved in recruiting SRF and/or Nkx2-5 or perhaps other yet unidentified cardiac transcription factors to the DCE to regulate *Lrrc10* expression. Although there are other GATA sites within the DCE, these sites are not conserved in human, suggesting that the conserved GATA site at −3044/−3041 bp, which is in closest proximity to the SRE and NKEs in this region, is likely the GATA4-responsive DNA sequence.

Indeed, formation of higher order transcriptional complexes among cardiac transcription factors including SRF, Nkx2-5, and GATA4 is often needed for cooperative regulation of transcription. For example, Nkx2-5 autoregulates its own transcription in the second heart field via conserved enhancers by combinatorial but not individual expression of Nkx2-5, SRF, GATA4, GATA6, myocardin, and p300 [57]. The *Xenopus* MLC2 gene contains GATA4 binding sites that flank a YY1/CarG-like site that mediate its myocardial expression [58] and the zebrafish ventricular myosin heavy chain (*vmhc*) promoter contains a proximal cardiac enhancer element with multiple NKEs and GATA factor binding sites upstream of an SRF binding site [59], both similar to the configuration observed in the DCE of *Lrrc10*. In the case of *Lrrc10*, the SRF binding site within the DCE is not critical for SRF-mediated activation, but SRF cooperates with Nkx2-5 and GATA4 (Fig 5B).

We also identify a PCE within the −178 bp region of the *Lrrc10* promoter that is sufficient to confer cardiac-specific expression and is synergistically activated by Nkx2-5 and GATA4. The PCE is endogenously occupied by Nkx2-5 and GATA4. The PCE contains two NKEs (−155/−149 and −62/−56 bp) with adjacent upstream GATA binding motifs that are critical for Nkx2-5- and GATA4-mediated activation, respectively. Although SRF augments synergistic activation of the PCE by Nkx2-5 and GATA4, SRF does not significantly accumulate at the PCE in the heart when assayed by qChIP. Cooperation of SRF with Nkx2-5 and GATA4 at the PCE may be observed as a consequence of overexpression in transfection assays. Alternatively, accumulation of SRF at the PCE may not be detectable under the experimental ChIP conditions employed. Therefore, SRF may regulate *Lrrc10* expression primarily via the DCE in vivo.

Although a previous study attempted to characterize the cis elements mediating cardiac-specific expression of *Lrrc10*, experiments were performed only in vitro using NIH3T3 fibroblast cells [6], which are not suitable cells for identifying regulatory elements that confer cardiac-specific expression. Moreover, the functional significance of the cis elements was not investigated in cardiomyocytes nor was regulation of *Lrrc10* expression evaluated in vivo. In contrast, we clearly demonstrate that fibroblasts do not activate reporter genes containing the *Lrrc10* promoter regions (Fig 1E). Nonetheless, Fan et al. reported that a different GATA binding site (−2894/−2889 bp) downstream of the DCE is dispensable while the GATA site at −71/−68 bp is important for GATA4-mediated activation [6], supporting our finding that the GATA sites within the DCE (−3044/−3041 bp) and PCE (−171/−168 and −71/−68 bp) are the GATA4 responsive DNA sequences. In addition, the MEF2 site in the DCE was not functional, consistent with the previous study [6].

Nkx2-5 and GATA4 physically interact via the homeobox domain of Nkx2-5 and a C-terminal zinc finger of GATA4 to synergistically activate cardiac target gene expression [16–19]. The promoters of essential cardiac genes such as α-cardiac actin [16], ANF [15, 18], CARP [60], Id2 [45] and the A1 adenosine receptor [61] are synergistically activated by Nkx2-5 and GATA4. Binding to adjacent NKE and GATA sites by Nkx2-5 and GATA4, respectively, is required to cooperatively activate the minimal (−150 bp) cardiac enhancer of connexin40 [22] and to synergistically activate the Id2 [45] and A1 adenosine receptor [61] promoters. In contrast, regulation of CARP requires GATA4, but not Nkx2-5, binding to DNA [60], and neither Nkx2-5 nor GATA4 binds DNA to activate cardiac α-actin expression but are instead recruited to its promoter by SRF [16, 17, 20, 21]. Conversely, in the case of *Lrrc10*, SRF is likely recruited to the DCE by GATA4 and to the PCE by GATA4 and/or Nkx2-5 to induce full activation of *Lrrc10* transcription.

Most notably, the ANF enhancer element within −270 bp of the TIS contains two adjacent NKEs with upstream GATA binding sites in very close proximity (within 25 bp), an arrangement very similar to the PCE of *Lrrc10*, which contains two adjacent NKEs with GATA sites within 15 bp upstream. The transcriptional activity of GATA4 and Nkx2-5 and corresponding DNA binding sites are required for synergistic activation of ANF [15, 18]. Due to the close proximity of GATA binding sites and NKEs in the PCE of *Lrrc10* and dramatic reduction in activation when either GATA site or either NKE is mutated, we speculate that GATA4 and Nkx2-5 bind their respective DNA binding sites and physically interact with each other to potentiate their ability to activate *Lrrc10* expression. However, further investigation is required to determine the critical endogenous cardiac regulatory sequences and exact molecular mechanism of Nkx2-5 and GATA4-mediated activation of *Lrrc10*. Regardless of the mechanism, our data taken together demonstrate that *Lrrc10* is a bona fide cardiac target gene of Nkx2-5 and GATA4.

The PCE of mouse *Lrrc10* maps to the 5′UTR of the human *LRRC10* genomic locus, suggesting that regulatory elements may reside within the 5′UTR of human *LRRC10*, which has been reported in other genes. The expression of rat carnitine palmitoyltransferase Iβ [62] and human 5-hydroxytryptamine receptor 4 [63] is regulated by an enhancer element in the 5′UTR that contains putative overlapping NKE and GATA binding sites or a putative NKE, respectively. Zebrafish ventricular myosin heavy chain (*vmhc*) contains overlapping NKEs in the second intron [59, 64] that are functionally important in the regulation of ventricular specific expression [64]. Additionally, splice variants 1A and 1C of the mouse cardiac sodium channel (*Scn5a*) contain cardiac enhancer elements within the 5′UTR and within the first exon, respectively [65]. Importantly, the human integrin β3 gene contains a functional regulatory element in the 5′UTR that maps to the 5′ flanking promoter region in avians [66]. Therefore, 5′UTRs in other genes have been shown to be critical in regulation of cardiac gene expression, as is likely the case for human *LRRC10*.

5. Conclusions

We identify the cardiac-specific factor *Lrrc10* as a novel target gene of Nkx2-5 and GATA4. Distal (around −3 Kb) and proximal (within−200 bp) cardiac regulatory elements upstream of *Lrrc10* are critical for mediating its expression and are occupied by Nkx2-5 and GATA4 in the heart. We characterize how *Lrrc10* is transcriptionally regulated by Nkx2-5 and GATA4 in cooperation with SRF, providing fundamental insight into gene regulatory mechanisms necessary for proper cardiac development and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We characterize mechanisms mediating the cardiac-specific expression of *Lrrc10*.
- Distal and proximal cardiac cis- regulatory elements mediate expression of *Lrrc10*.
- Nkx2-5, GATA4, and SRF cooperatively regulate transcription of *Lrrc10*.

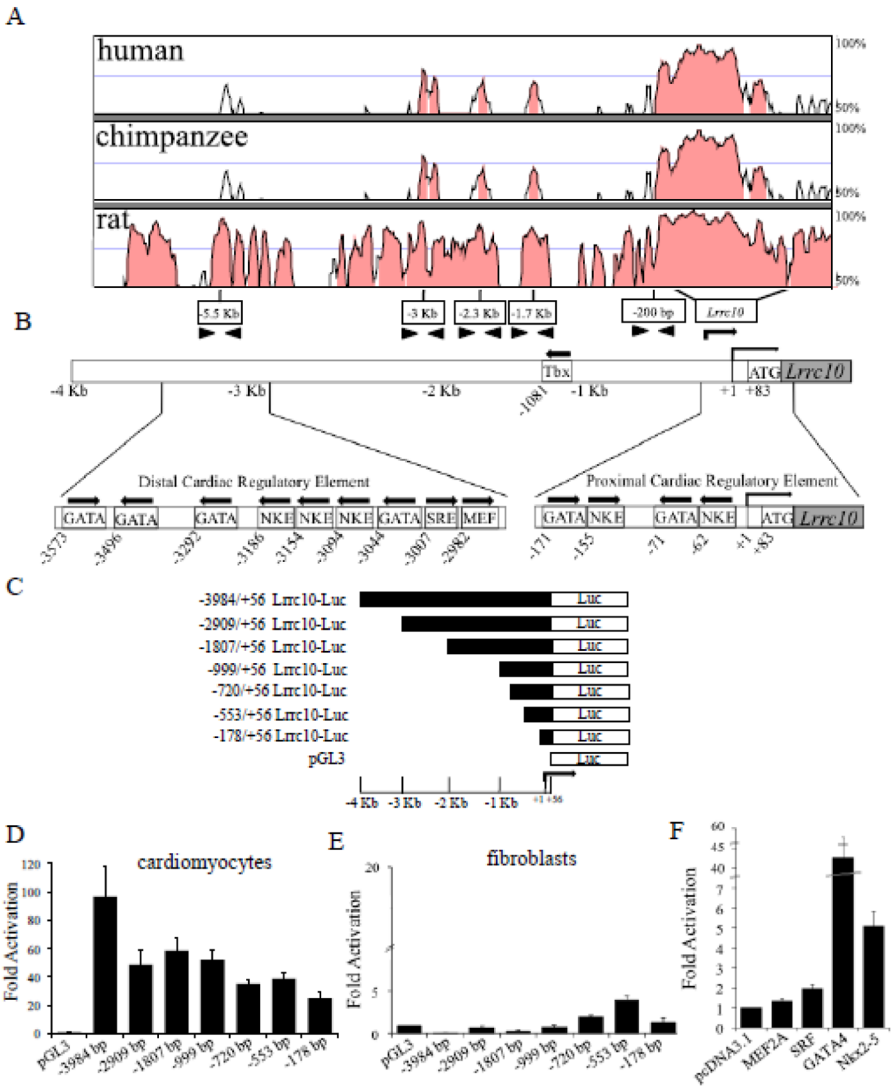

Figure 1.

The highly conserved mouse *Lrrc10* promoter regions exhibit cardiomyocyte-specific activity. (A) A VISTA alignment was performed on the *Lrrc10* locus for mouse, rat, chimpanzee, and human to identify conserved regions. Arrowheads indicate primer sites designed in conserved regions. Rightward arrow indicates transcription initiation site (TIS, +1). Shading denotes regions of greater than 70% conservation. (B) Schematic representation of transcription factor binding sites within conserved regions located approximately −3 Kb and −200 bp of the TIS of *Lrrc10*. GATA, NKE, MEF, and SRE are putative binding sites for GATA4, Nkx2-5, MEF2, and SRF, respectively. Arrows indicate orientation of putative binding sites. (C–E) Determination of cardiac regulatory elements by reporter gene analyses. Deletion analyses of the *Lrrc10* promoter regions were performed in cultured mouse neonatal cardiomyocytes and 10T1/2 cells. (C) Schematic diagram of the 5′ deletions of the *Lrrc10* promoter region used for transient transfections. *Lrrc10* reporter genes are (D) robustly activated in cardiomyocytes but (E) inactive in 10T1/2 cells. (F) A −3984 bp *Lrrc10* luciferase reporter construct is activated in 10T1/2 cells when cotransfected with the cardiac transcription factors Nkx2-5, GATA4, or SRF, but not MEF2A. Fold activation is expressed relative to pcDNA3.1 vector control.

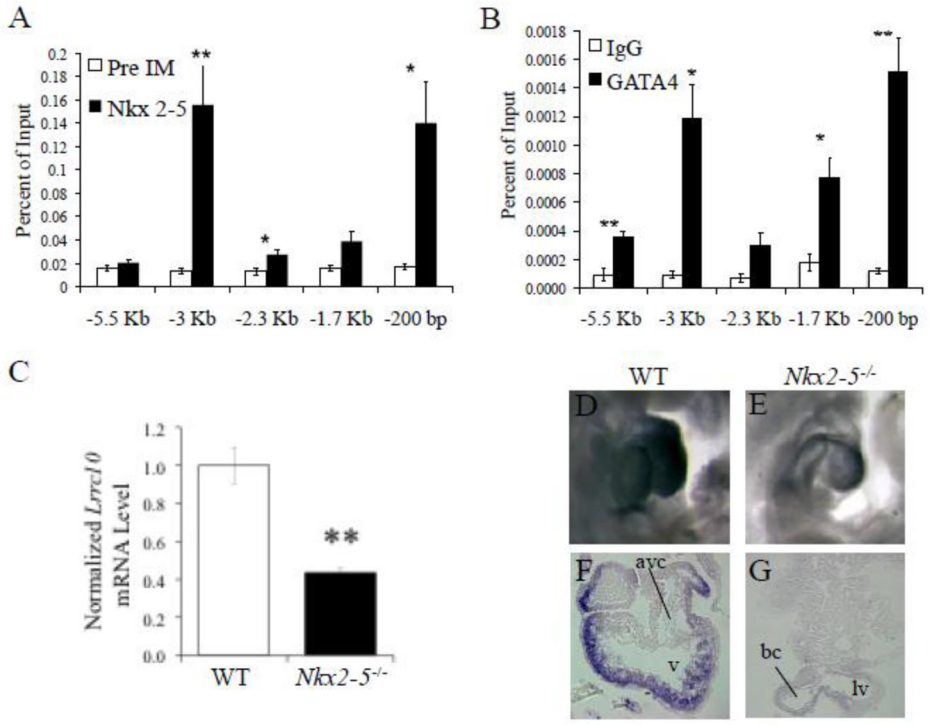

Figure 2.

Lrrc10 is a target gene of Nkx2-5 and GATA4. (A, B) Nkx2-5 and GATA4 endogenously occupy specific regions of the *Lrrc10* promoter. qChIP was performed on wildtype (WT) hearts at postnatal day 3 using (A) Nkx2-5 or (B) GATA4 specific antibodies and primers for conserved regions of the *Lrrc10* promoter as indicated in Fig 1A (n=3). Preimmune serum (Pre IM) or nonspecific rabbit IgG was used as a control. (C–G) *Lrrc10* expression is reduced in *Nkx2-5*$^{-/-}$ hearts. (C) qRT-PCR shows markedly reduced expression of *Lrrc10* in *Nkx2-5*$^{-/-}$ embryos at E9.5 (n=3). Expression was normalized to *18S* expression. (D) WT and (E) *Nkx2-5*$^{-/-}$ embryos at E9.5 were subjected to whole-mount in situ hybridization using digoxigenin-labeled *Lrrc10* antisense cRNA probes. Sense cRNA probes did not give any signal (data not shown). Transverse sections of labeled (F) WT and (G) *Nkx2-5*$^{-/-}$ hearts reveal dramatically reduced expression of *Lrrc10* in the *Nkx2-5*$^{-/-}$ heart. *avc*, atrioventricular canal; *v*, ventricle; *lv*, left ventricle; *bc*, bulbous cordis. *p<0.05, **p<0.01 compared to control.

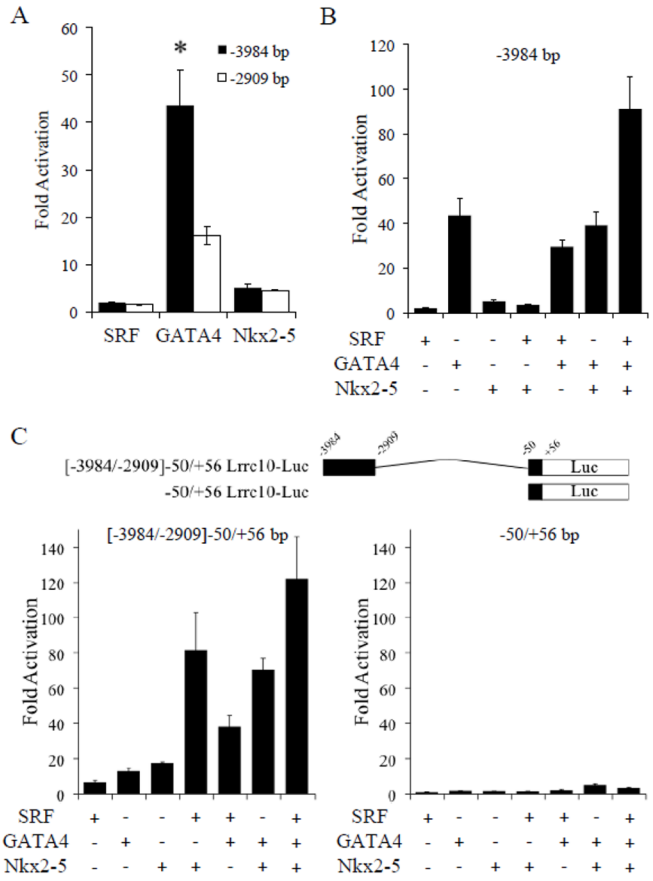

Figure 3.

The distal cardiac regulatory element (DCE) of *Lrrc10* is regulated by SRF, GATA4, and Nkx2-5. (A) There is a conserved functional GATA4 binding site located in the DCE. 10T1/2 cells were cotransfectd with SRF, GATA4, or Nkx2-5 and a −3984 bp *Lrrc10* reporter or a −2909 bp *Lrrc10* reporter lacking the DCE containing three NKEs and conserved SRF and GATA factor binding sites. Deletion of the DCE significantly reduces GATA4-mediated activation of the *Lrrc10* promoter while Nkx2-5 and SRF-mediated activation is unaffected. Nkx2-5, GATA4, and SRF regulate the DCE of *Lrrc10*. (B) 10T1/2 cells were cotransfected with the −3984 bp reporter and Nkx2-5, GATA4, and/or SRF. GATA4, Nkx2-5, and SRF together synergistically activate the *Lrrc10* promoter. C) A reporter gene containing the DCE (−3984/−2909 bp) and the *Lrrc10* promoter (−50/+56) or a reporter gene only containing the promoter was cotransfected with Nkx2-5, GATA4, and/or SRF. Nkx2-5, GATA4, and SRF cooperatively regulate the DCE of *Lrrc10* (*left*). Negligible activation is observed on the *Lrrc10* promoter alone (*right*). Activation is expressed relative to pcDNA3.1 vector control. *p<0.05 compared to −2909 bp reporter.

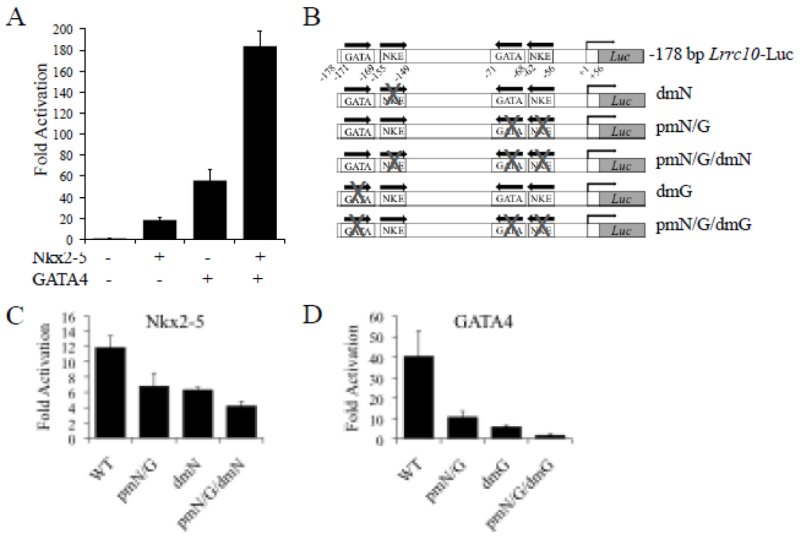

Figure 4.

The proximal cardiac regulatory element (PCE) of *Lrrc10* contains functional Nkx2-5 and GATA4 binding sites. (A) A −178 bp *Lrrc10* reporter was cotransfected with Nkx2-5 and/or GATA4, indicating that Nkx2-5 and GATA4 synergistically induce *Lrrc10* expression. (B) Schematic representation of −178 bp *Lrrc10* reporter genes containing mutations in NKEs and GATA binding sites. Mutation of (C) NKEs or (D) GATA binding sites within the PCE of *Lrrc10* diminishes Nkx2-5- and GATA4-mediated activation, respectively. 10T1/2 cells were cotransfected with a −178 bp *Lrrc10* WT reporter or a mutant reporter containing one or both of the NKEs or GATA sites mutated and Nkx2-5 or GATA4. Activation is expressed relative to pcDNA3.1 vector control.

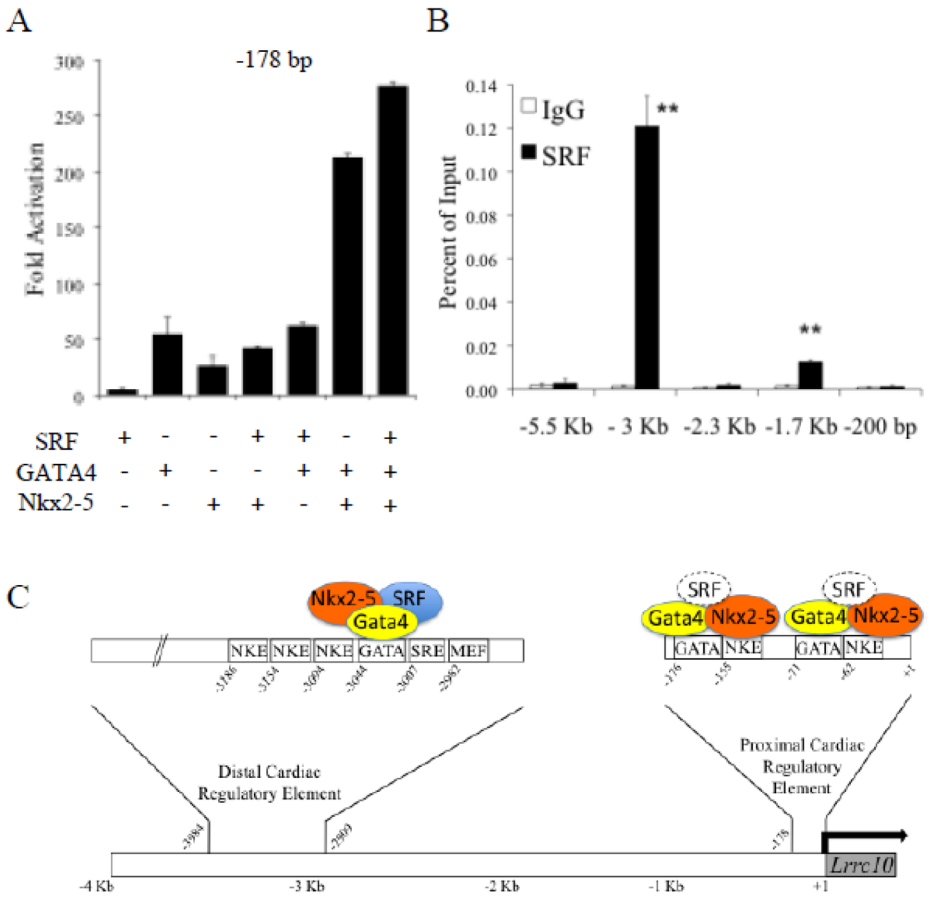

Figure 5.

Roles of SRF in regulation of *Lrrc10* expression and working model. (A) A −178 bp *Lrrc10* reporter was cotransfected with Nkx2-5, GATA4, and/or SRF. (B) SRF endogenously occupies the DCE of *Lrrc10*. qChIP was performed on WT hearts at postnatal day 3 using an SRF antibody or IgG as a control (n=3). (C) Working model of *Lrrc10* transcriptional regulation. The DCE located at around −3Kb is endogenously occupied in the heart and cooperatively activated by SRF, Nkx2-5, and GATA4, and contains a highly conserved functional GATA4 binding site. The PCE of *Lrrc10* located within 200 bp of the transcription initiation site (TIS) is endogenously occupied and synergistically activated by Nkx2-5 and GATA4 via their respective binding sites and SRF may augment activation by Nkx2-5 and GATA4. Dotted outline of SRF denotes lack of endogenous accumulation in the heart as evidenced by qChIP as shown in (B). **p<0.01 compared to IgG.