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Cloning and characterization of the cardiac-specific Lrrc10 promoter

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Leucine-rich repeat containing protein 10 (LRRC10) is characterized as a cardiac-specific gene, suggesting a role in heart development and disease. A severe cardiac morphogenic defect in zebrafish morphants was recently reported but a contradictory result was found in mice, suggesting a more complicated molecular mechanism exists during mouse embryonic development. To elucidate how LRRC10 is regulated, we analyzed the 5′enhancer region approximately 3 kilo bases (kb) upstream of the Lrrc10 start site using luciferase reporter gene assays. Our characterization of the Lrrc10 promoter indicates it possesses complicated cis-and trans-acting elements. We show that GATA4 and MEF2C could both increase transcriptional activity of Lrrc10 promoter individually but that they do not act synergistically, suggesting that there exists a more complex regulation pattern. Surprisingly, knockout of Gata4 and Mef2c binding sites in the 5′enhancer region (−2,894/−2,889) didn't change the transcriptional activity of the Lrrc10 promoter and the likely GATA4 binding site identified was located in a region only 100 base pair (bp) upstream of the promoter. Our data provides insight into the molecular regulation of Lrrc10 expression, which probably also contributes to its tissue-specific expression. [BMB reports 2011; 44(2): 123-128]

INTRODUCTION

The leucine-rich repeat containing protein 10 (LRRC10, also named HRLRRP or SERDIN1) is a cardiac-specific factor that contains 7 Leucine-rich repeat (LRR) motifs (1, 2). LRR motifs are present in a number of proteins and thought to provide a structural framework for protein-protein interactions. These motifs are thought to contribute to diverse functions including enzyme inhibition, cell growth, cell adhesion, signal transduction, regulation of gene expression, apoptosis signaling, and development (3). Expression profile analyses in mice indicate that LRRC10 is exclusively expressed in the precardiac region (E7.5) starting in early embryos and continuing through all developmental stages to the adult heart with a marked elevation in expression upon birth (4). The function of LRRC10 has been characterized by other laboratories, producing some controversy concerning its developmental roles in different species. Knockdown of Lrrc10 in zebrafish embryos using morpholinos caused severe cardiac morphogenic defects including a cardiac looping failure accompanied by significant pericardial edema, and embryonic lethality between day 6 and 7 post fertilization. These developmental defects were accompanied by increases in atrial natriuretic factor (ANF), a hallmark for cardiac hypertrophy and failure, and decreases in cardiac myosin light chain 2 (MLC2), an essential protein for cardiac contractility (5). In striking contrast to these results, Lrrc10 knockout mice developed normally and exhibited no discernable phenotype with no alteration of the expression of ANF and MLC2 (6). These seemingly contradictory results may imply the existence of more complicated molecular regulatory mechanisms and/or that parallel pathways operate during mouse embryonic development that compensate for the loss of Lrrc10 function.

The cardiac-specific expression profile of LRRC10 suggests that this gene is involved in heart development and/or function. Indeed a developmental role of Lrrc10 with a tight spatiotemporal expression pattern has been previously reported (1, 2, 4), however the molecular mechanisms involved in the temporal and spatial regulation of Lrrc10 remain completely unknown. It has been reported that 7 kb of the upstream regulatory sequence is sufficient to recapitulate the cardiac-specific expression pattern of Lrrc10 (2). Thus, we predicted that the tissue-specific expression might be regulated by the upstream cis- and trans-acting elements in this region. Bioinformatic analysis with rVista revealed there are a number of transcriptional binding sites dispersed throughout this 7 kb upstream region including sites for the cardiac-specific transcription factors GATA and MEF located in the region (−2,894/−2,889

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and −2,983/2,973), respectively. Because of their known roles in cardiac development (7-10) these two binding sites are likely contributors to the tissue-specific expression of Lrrc10.

GATA factors contain a highly conserved DNA binding domain consisting of two zinc fingers that directly bind to the nucleotide sequence element (A/T)GATA(A/G) which is contained in the transcriptional regulatory regions of a plethora of cardiac-specific genes (11). Of the six GATA transcription factors (12), GATA4 has been considered to be one of the earliest marker of cardiac myocyte commitment and has been shown to be an essential regulator of cardiac development and differentiation, as well as a regulator of survival and hypertrophic growth of the adult heart (12, 13). Knockout of Gata4 in the entire mouse resulted in early embryonic lethality between embryonic (E) day 7.0 to 9.5 due to defects in endoderm and ventral morphogenesis (7, 8), and Gata4 conditional knockout mice die between E8.5 and E10.5 with abnormalities in heart tube formation (8).

The myocyte enhancer factor 2 (MEF2) family is also considered to include essential regulators of cardiac development. MEF2 dimers, consisting of homo- and heterodimers of four separate gene products in vertebrates (14, 15), bind to the consensus sequence CTA(A/T)4TAG present in the 5' transcriptional regulatory regions of most cardiac muscle structural genes (14, 15). A member of the MEF2 family, MEF2C, is expressed in heart precursor cells prior to linear heart tube formation (9). Targeted disruption of Mef2c in the mouse leads to early embryonic lethality associated with a cardiac looping defect, a general absence of the right ventricle, and down regulation of a subset of cardiac-specific genes (10). MEF2C functions as a co-activator, making a physical interaction with other transcription factors that leads to a synergistic activation of the targeted gene (16, 17). For example, MEF2C can cooperate with GATA4 to activate ANF expression (18). Recently, it was reported that combinations of Gata4, Mef2c, and Tbx5 rapidly and efficiently reprogrammed postnatal cardiac or dermal fibroblasts into differentiated cardiomyocyte-like cells (19). In the Lrrc10 promoter, the MEF2 binding site is adjacent to a GATA site, suggesting that Mef2c and Gata4 act synergistically to regulate the expression of Lrrc10.

Here, we isolated the region approximately 3 kb upstream of the Lrrc10 transcriptional start site and constructed a series of reporter plasmids that we used to assess the transcriptional activity. Using luciferase reporter gene assays, we have characterized the Lrrc10 promoter, and show that GATA4 and MEF2C can both increase transcriptional activity of Lrrc10 promoter but that they do not work together in a synergistic fashion. We have now identified the potential GATA4 binding site, which is located in the 100 bp upstream of the promoter. Our data therefore provide insights into the molecular mechanisms that regulate Lrrc10, which may also contribute to the tissue-specific expression of the Lrrc10.

RESULTS AND DISCUSSION

Characteristics of the 5′-upstream region of Lrrc10 promotor

It has previously been shown that a 7 kilo base region upstream of the Lrrc10 transcriptional start site is sufficient for its cardiac-specific expression pattern (2). Therefore we analyzed this region of the Lrrc10 gene in both mouse and human genomic DNA (rVistar, http://zpicture.dcode.org). A diagram of the Lrrc10 promoter is shown in Fig. 1D. A typical promoter harbours additional DNA sequences: cis- and trans-acting elements that inhibit or activate gene transcription by binding transcription factors (20). Lrrc10 appears to contain a typical promoter region as we found a conserved putative CAAT-box (ACTGGC) in the region −44/−33 and TATA-box (TTATAAA) (−16/−10) upstream the transcription start site (TSS) (Fig. 1B and D). Two highly conserved regions were found in the 10 kb upstream of the Lrrc10 gene, located between approximately −3200 bp and −2800 bp, and −300 bp and +11 bp (Fig. 1A). Both regions contained a number of transcription binding sites (Supplementary Table 1). Consistent with the heart-specific expression of Lrrc10 (1, 2), binding motifs for cardiac-specific transcription factors, including GATA (−2,894/−2,889) and MEF (−2,983/2,973), were found (Fig. 1B, Supplementary Table 1). Unexpectedly, we also found a high conserved GATA binding site in the region between −72 bp and −67 bp in the anti-sense strand of Lrrc10 DNA (Fig. 1C). We predicted these two conserved transcription factors might contribute to the tissue-specific expression profile of Lrrc10.

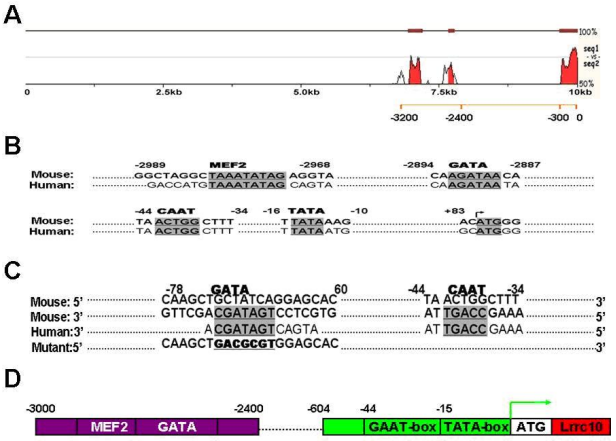

Fig. 1. Bioinformatics analysis of Lrrc10 Promotor. (A) The 10 kb mouse and human sequence of the Lrrc10 gene was aligned with rVistar. The peaks indicate degree of sequence homology. (B) Sequence of the Lrrc10 gene depicting the position of the transcriptional binding sites and promoter elements: GATA, (A/T)GATA(A/G)64); MEF, GGGCGG or CCGCCC59); CAAT-box ACTGGC; TATA-box, TTATAAA. (C) A high conserved GATA binding site in the region between −72 bp and −67 bp in the anti-sense strand of Lrrc10 DNA. (D) Cartoon map of Lrrc10 promoter.

Promoter activity of the 5'-upstream sequence

To confirm the identity of cis- and trans-acting DNA sequences that regulate Lrrc10 transcription, we examined Lrrc10 promoter activity in NIH3T3 cells since both Gata4 and Mef2c are expressed in this cell line. We used a luciferase reporter assay to assess activity of different segments of the prometer (Fig. 2). First, we generated three luciferase reporter constructs containing deletions of the Lrrc10 upstream region (Fig. 2A). Of them, the longest fragment (≈3.2 kb) extends from −3,190 bp upstream of the Lrrc10 to +11 bp downstream of the transcription start site. We also generated two smaller approximately 2.4 and 0.6 kb fragments (Fig. 2A). When compared to the promoter activity of the longer 3.2 kb construct, the 2.4 and 0.6 kb constructs exhibited increased luciferase activity, suggesting the existence of negative regulatory elements in the region from −3190 to −604.

In order to determine the position of this negative transcriptional regulatory activity, we generated four fragments from the 3.2 kb fragment as shown in Fig. 2B. Of these constructs all showed enhanced transcriptional activity compared to the full length 3.2 kb fragment, except for the fragment −2,409 to −604 upstream of the Lrrc10 transcription start site, suggesting that the negative regulatory element resides in this region.

The most proximal 0.6 kb region was further characterized by the generation of five additional constructs, with progressive deletions of approximately 0.1 kb (Fig. 2C). Of these, three constructs occupying the region −494 to −307 showed similar promoter activity as the original 0.6 kb construct.

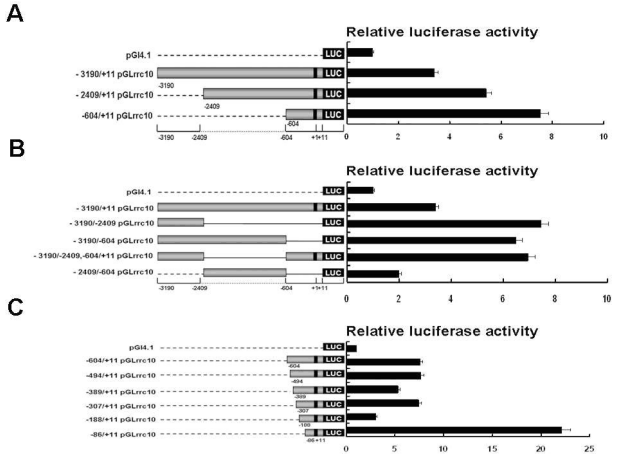

Fig. 2. Analysis of the 5'-upstream Sequence of the Lrrc10 Gene by Means of the Reporter Gene Assay in NIH3T3 Cell Lines. (A, B and C) Schematic representation of the series Lrrc10 promoter constructs. The luciferase activities of various lengths of the reporter plasmid (100 ng) were examined by reporter gene assay. The activity was normalized as to that of renila (5 ng). The relative activity in response to a mock transfection (Pgl4.1) is shown (data is the mean +/− Standard Deviation of duplicate experiments).

Surprisingly, the construct between −188/+11 displayed significantly lower promoter activity, suggesting the presence of a "repressor", a notion further supported by the presence of a high GC content in this region (21). On the other hand, the construct very near the start site (−86/+11) unexpectedly produced the highest luciferase activity (about six times higher than the full length 3.2 kb construct), suggesting the region (−86/+11), which contains the CAAT-box and TATA-box, also contains essential elements required for optimal activity of the Lrrc10 gene.

Collectively analyses of the basal promoter activities using luciferase-reporter assay in a series of 5'-deleted mutant constructs revealed the existence of both positive and negative regulatory elements, suggesting a complicated regulation of Lrrc10 by cis regulatory elements and trans-acting factors. Overall there appear to be a number of negative regulatory elements within the upstream promoter region, which may explain why Lrrc10 is not expressed in tissues other than the heart. This result also suggests that it is the presence of heart-specific transcription factors that are responsible for the tissue specific expression profile of Lrrc10.

Lrrc10 expression is regulated by Gata4 and Mef2c

Gata4 and Mef2c are highly expressed in the developing heart somewhat before the Lrrc10 gene first turns on (4, 7, 9). Our data above suggest that there are several potential conserved GATA4 and MEF2C transcription factors binding sites in the Lrrc10 promoter. We first examined whether Gata4 and Mef2c could exert any effect on the transcriptional activity of the Lrrc10 promoter. Therefore, we tested whether overexpression of Gata4 or Mef2c could increase mRNA expression of the Lrrc10 gene. The results showed that Gata4 and Mef2c both increased the mRNA expression of Lrrc10 individually in a P19CL6 cell line (Fig. 3A), suggesting that the Lrrc10 expression is positively regulated by these two genes.

We then tested whether Gata4 and Mef2c could both affect the promoter activity of Lrrc10 using a luciferase reporter assay. In this case, the three original Lrrc10 reporter constructs, with lengths of the 3.2, 2.4 and 0.6 kb fragments, were used. Transfection with a Gata4 construct significantly increased the transcriptional activity of all three Lrrc10 reporter genes, with shorter promoter fragments exhibiting higher activity (Fig. 3B). Transferction with Mef2c also significantly increased the transcriptional activity of the 2.6 kb Lrrc10 reporter gene but only slightly increased the transcriptional activity of the 3.2 and 0.6 kb Lrrc10 reporter genes (Fig. 3B). These results indicate that Lrrc10 is positively regulated by Gata4 and Mef2c.

Gata4 and Mef2c were described previously as acting synergistically (18) and there are two potential binding sites located in the Lrrc10 promoter (Fig. 1A). We next tested whether Lrrc10 is synergistically regulated by Gata4 and Mef2c. We found that with increasing level of Gata4 expression, the activity of the full length promoter construct (−3,190/+11) was increased (Fig. 3C), however the effect of Mef2c is relativly

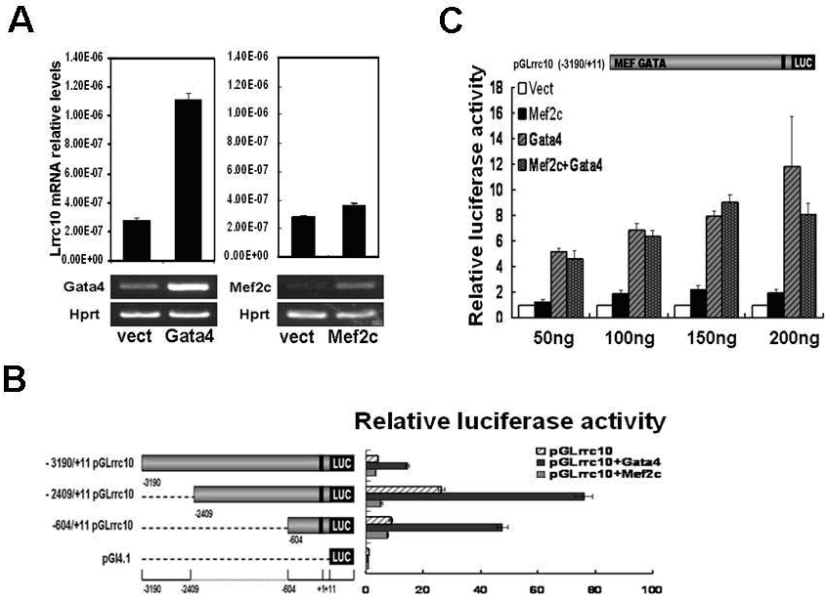

Fig. 3. Gata4 and Mef2c Regulated the Transcriptional Activity of Lrrc10 and Effects of Mutations in GATA and MEF of construct (−3,190/+11) in P19CL6 Cell Line. (A) mRNA of Lrrc10 were detected by means of real-time PCR (top) and RT-PCR (bottom) after transfected with pCMV-Tag-Gata4, pCMV-HA-Mef2c and pCMV-Tag, pCMV-HA in NIH3T3. (B) The effect of Mef2c or GATA4 on luciferase activity was examined on different Lengths of Lrrc10 Promoter. (C) Different quantities of pCMV-Tag-Gata4 and pCMV-HA-Mef2c were co-transfected with construct (−3,190/+11), and then luciferase activity was assayed.

small. In addition, the cotransfection of both Mef2c and Gata4 was not significantly different from that of Gata4 alone indicating that these two transcription factors do not act together to promote Lrrc10 expression (Fig. 3C). The reason for the surprisingly small effect of Mef2c remains to be determined; it may be that Mef2c acts in concert with other, as yet unidentified, factors to regulate Lrrc10.

Indentifying the Gata4 and Mef2c binding sites in Lrrc10 promoter

To determine functional significance of the potential conserved GATA4 and MEF2C transcription factors binding sites in the Lrrc10 promoter region, we examined the region of the Lrrc10 promoter that contains conserved binding sites for GATA4 and MEF2C in the regions (−2,983/2,973) and (−2,894/2,889) of Lrrc10 promoter, respectively (Fig. 1). Unexpectedly, deletion of the GATA, MEF or both putative binding sites from the full length promoter construct had no effect on overall promoter activity (Fig. 4A), suggesting that the region (−2,973/−2,889) is not required for transcriptional regulation by Gata4 and Mef2c, and suggesting that it contains additional regulatory elements that remain to be identified.

Because of the strong effect of Gata4 in regulating the Lrrc10 promoter, we next tried to assess whether or not the Gata4 regulates Lrrc10 directly. There are two possible explanations why the putative GATA binding site (−2,973/−2,889) had no effect on promoter activity of Lrrc10 (Fig. 4A): first, there could be other unidentified binding sites in the region between −2,889 to +11 bp for Gata4; second, the regulation by Gata4 of Lrrc10 could be indirect. We analyzed the response to Gata4 with different truncated fragments from −86/+11 to −3,190/+11, and found that activity of each was elevated (Fig. 4C), indicating that there might be at least a GATA binding site existing in 86 bp upstream of the TSS. Interestingly, when the length of the promoter constructs from −86 to −494 were increased, the higher activity were observed, suggesting that the region −86 to −494 may possess other cis-acting elements to enhance the effect of GATA factor, which remains to be solved. After additional sequence analysis we found a highly conserved GATA binding site in the region between −72 bp and −67 bp in the anti-sense strand of Lrrc10 DNA (Fig. 1C). To determine whether or not the conserved GATA binding site was critical for transcription, reporter vectors with deletion of this GATA binding site were introduced into NIH3T3 cells co- transfected with different quantities of GATA4. As shown in Fig. 4B, deletion of GATA binding site greatly reduced the response to GATA4, suggesting this site is required for GATA4 binding. Therefore, we identified a potential GATA4 binding site located in the 100 bp upstream of the TSS but not in the the predicted region (−2,973/−2,889). Our data also suggest that Lrrc10 is under strong negative regulation. This combined with its strong activation by the cardiac-specific transcription factor GATA4 may account for its tissue specific expression.

MATERIALS AND METHODS

mRNA isolation, real time PCR and RT-PCR

Total RNA was extracted from NIH3T3 cell using Trizol reagent (Invitrogen) according to the manufacturer's recommendations, First stranded cDNA was synthesized by mRNA Selective PCR Kit (TaKaRa). Semi-Quantitative and real-time PCR analyses were performed as described (22). As reference we used GAPDH and Hprt. The related primer sequences used

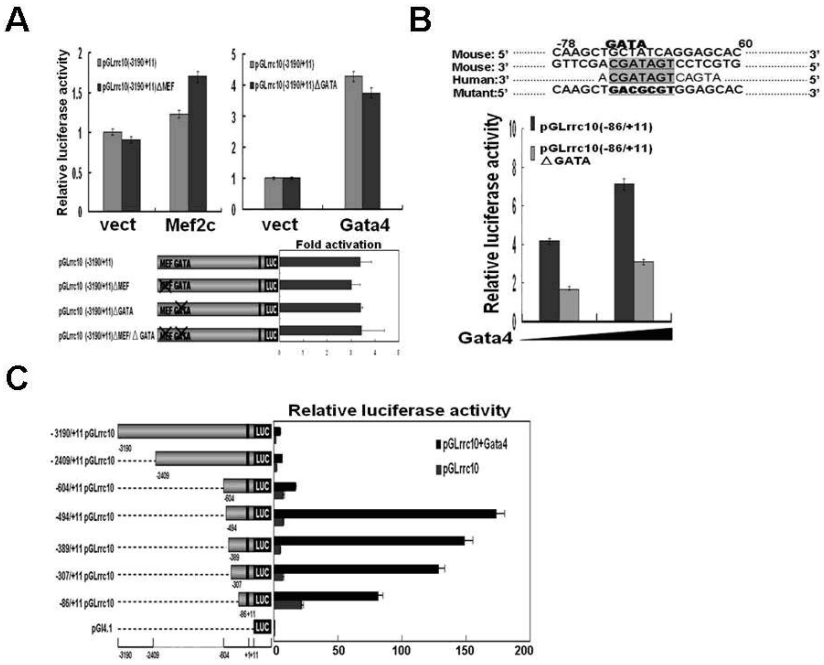

Fig. 4. Indentification of GATA and Mef2c Binding Sites in the Promoter of the Lrrc10 Gene. (A) The effect of Mef2c or GATA4 on luciferase activity was examined by creating plasmids carrying deletions of either Mef2c, or GATA4 binding site. Elimination of one or both of these sties has negligible effect on the reporter gene activity. pGLrrc10 (−3,190/+11) ΔMef2c: deletion region (−2,983/−2,973) in construct (−3,190/+11); pGLrrc10 (−3,190/+11) △Gata4: deletion region (−2,894/−2,889) construct (−3,190/ +11). (B) The effect of GATA4 on luciferase activity was examined by creating plasmids carrying deletions of GATA4 binding site. pGLrrc10 (−86/+11) Gata4: deletion region (−72/-67) construct (−86/+11). (C) The effect of GATA4 on luciferase activity was examinend on different Lengths of Lrrc10 Promoter.

to amplify each gene were listed in Supplementary Table 4. Each experiment was repeated at least three times.

Promoter bioinformatics analysis

The Lrrc10 mouse and human DNA sequence is searched in NCBI (http://www.ncbi.nlm.nih.gov), and submit these two sequence to z-picture (http://zpicture.dcode.org), analysis with rVistar database.

Plasmid construction

Reporter plasmids were constructed by inserting various lengths of the 5'-upstream region of the mouse Lrrc10 gene between the KpnI and HindIII sites of the pGL4.1 Vector (PROMEGA). Plasmids pGLrrc10(−86/+11), pGLrrc10(−188/+11), pGLrrc10(−307/+11), pGLrrc10(−389/+11), pGLrrc10(−494/+11), pGLrrc10(−604/+11), pGLrrc10(-2409/+11), and pGLrrc10 (−3,190/+11) were constructed by inserting the PCR-amplified fragments with primer pairs pGLrrc10s/pGLrrc10A, pGLrrc102s/pGLrrc10A, pGLrrc103s/pGLrrc10A, pGLrrc104s/pGLrrc10A, pGLrrc105s/pGLrrc10A, pGLrrc106s/pGLrrc10A, pGLrrc107s/pGLrrc10A, and pGLrrc108s/pGLrrc10A, respectively. The fragments obtained with primer pairs pGLrrc108s/pGLrrc10a, pGLrrc108s/pGLrrc102a and pGLrrc107s/pGLrrc102a were inserted between the KpnI and HindIII sites of Pgl4.1, resulting in pGLrrc10(−3,190/−2,409), pGLrrc10(−3,190/−604) and pGLrrc10(−2,409/−604), respectively. The fragment amplified by prime pairs pGLrrc108s/ pGLrrc102a inserted between the KpnI sites of pGLrrc10(−604/+11), result in pGLrrc10 (−3,190/−2,409, −604/+11). The primer sequences are listed in Supplementary Table 2. The pCMV-TAG-Gata4 and pCMV-HA-Mef2c constructs were generated by inserting Gata4 and Mef2c cDNA which had cloned into pMD18T vector and sequenced successfully and the cDNA were obtained by prime pairs Mef2c-sense-xhoI/Mef2c-anti-notI. The primer sequences are listed in Supplementary Table 4. The mutant vector were constructed through two segments putting together, segment amplified with primer pairs introduced into sequence motifs by Mut-lp-s-kpnI/ Mut-LPmef2c-a-mluI and Mut-LPmef2c-s-mluI/ Mut-lp-a-kpnI, degested with KpnI/MluI, MluI/KpnI, inserted between KpnI site of construct (−3,190/+11), resulted in pGLrrc10(−3,190/+11) ΔMEF. pGLrrc10(-3190/+11)ΔGATA. and pGLrrc10(−3,190/+11) ΔMEF/ΔGATA were generated as above with Mut-lp-s-kpnI/ Mut-LPGata4-a-mluI and Mut-LPgata4-S-mluI/Mut-lp-a-kpnI, Mut-lpsmg-a-mluI, Mut-LPGata4-a-mluI and Mut-LPgata4-S-mluI/Mut-lp-a-kpnI, respectively. And plasmid pGLrrc10(−86/+11)ΔGATA was generated by insert segment amplified with prime pairs GATA-MUT-S/pGLrrc10A into pGl4.1. The primer sequences were listed in Supplementary Table 3.

Transient expression reporter gene assay and deletion analysis

NIH3T3 cells were transfected using lipofectamine as described above. All of pGLrrc10 reporter vectors were transfected into NIH3T3 cells or pCMV-TAG-Gata4 and pCMV-HA-Mef2c fusion constructs were co-transfected into NIH3T3 cells along with the pGLrrc10 reporter, respectively. The luciferase activity assay was performed 24 h later according to the methods described as the proctcol (Promega).

Acknowledgements
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