Human heart LIM protein activates atrial-natriuretic-factor gene expression by interacting with the cardiac-restricted transcription factor Nkx2.5

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INTRODUCTION

The molecular mechanism underlying the regulation of ANF (atrial natriuretic factor) expression has been studied extensively because of its cardiac-specific pattern of expression during development and its re-expression in response to hypertrophic stimulation [1]. The re-activation of ANF is a hallmark for inducible gene expression in hypertrophied cardiac muscle cells. An understanding of the induction or activation of ANF expression will also provide valuable insights into the mechanism of hypertrophic changes of the ventricle cells in the heart. It has been shown that expression of the ANF gene is activated by either Nkx2.5 (a cardiac-restricted transcription factor) or GATA4 (GATA-binding protein 4, another transcription factor), or by both with synergism [2–4]. The T-box-containing transcription factor Tbx5 (T-box 5) associates with Nkx2.5 and synergistically activates ANF expression in vitro [5,6]. The ANF promoter is also a target of PITX2 (paired-like homeodomain transcription factor 2), and one of the PITX2 isoforms, PITX2C, can synergistically activate the ANF promoter with Nkx2.5.

hhLIM (human heart LIM protein) is a member of the LIM (Lin-11/Isl-1/Mec-3) family of transcription factors that do not have a domain homologous with a DNA-binding domain, but activate transcription by interacting with other transcription factors [7]. The LIM motif is a protein-binding interface found in a diverse group of proteins that include LIM kinases and LIM homeodomain proteins. This motif is frequently found in proteins involved in cell differentiation and cell-fate determination, suggesting that LIM-based protein interactions may mediate specific regulatory processes in the cell [8]. We and others have previously demonstrated that the hhLIM gene plays a critical role in the development of the heart and cardiac hypertrophy [9,10]. A recent report describing structural and functional analyses of hhLIM showed that hhLIM has a role in the transcriptional activation of the ANF gene [11]. These data led us to investigate the molecular mechanism underlying hhLIM regulation of ANF gene expression. The present study demonstrates that hhLIM interacts physically with Nkx2.5 and enhances its ability to activate target gene expression.

EXPERIMENTAL

Plasmid constructs

Full-length and domain-specific regions of human hhLIM were generated in pcDNA3.1, pBIND and pGEX-3X vectors. All new constructs were confirmed by restriction digestion, followed by sequencing. The ANF reporter genes (−638, −288 and −383) and the Nkx2.5 reporter gene NKE-Luc were gifts from Dr Kenneth R. Chien (Massachusetts General Hospital Cardiovascular Research Center, Department of Cell Biology, Harvard Medical School and the Harvard Stem Cell Institute, Boston, MA, U.S.A.), Dr Katherine Yutzey (Division of Molecular Cardiovascular Biology, Department of Pediatrics, Children’s Hospital Research Foundation, Cincinnati, OH, U.S.A.), Professor Andrew F. Russo (Department of Physiology and Biophysics, University of Iowa, Iowa City, IA, U.S.A.), Dr Christopher C. Glensborts (San Diego State University Heart Institute and Department of Biology, San Diego, CA, U.S.A.) and Dr Mona Nemek [Institut de recherches cliniques de Montréal (IRCM), Montréal, Quebec, Canada] respectively. The expression plasmids of human Csx/Nkx2.5 (pEFSA-Nkx2.5), pSSRa-GATA4, and pGEX-3X-Nkx2.5 were gifts from Dr Hiroshi Akazawa (Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan), Dr Youngsook Lee (Department of Anatomy, University of Wisconsin Medical School, Madison, WI, U.S.A.) and Dr David B. Wilson (Department of Pediatrics, Washington University School of Medicine, St. Louis, MO, U.S.A.) respectively.

Abbreviations used: aa, amino acids; ANF, atrial natriuretic factor; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco’s modified Eagle’s medium; FCS, foetal-calf serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GATA4, GATA-binding protein 4; GST, glutathione transferase; HA, haemagglutinin; hhLIM, human heart LIM (Lin-11/Isl-1/Mec-3) protein; NKE, Nkx2.5-binding element; Nkx2.5, a cardiac-restricted transcription factor; NP-40, Nonidet P40; PITX2, paired-like homeodomain transcription factor 2; RT-PCR, reverse transcription PCR; TAD, transcription activation domain; Tbx5, T-box 5.

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Primary cardiomyocyte cultures
Primary cardiomyocytes were cultured as described previously [10]. Briefly, 1-day-old mice were killed by CO2 inhalation and their hearts collected. Atria were removed, and the ventricles were cut into four pieces and digested using 0.05% pancreatin and 0.1% collagenase. After that the cells were plated on gelatinized cell-culture dishes and cultured overnight in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 15% (v/v) FCS (foetal-calf serum) and penicillin/streptomycin (100 units/ml) [10].

Transfection and luciferase assays
Human embryonic kidney A293 cells (A.T.C.C. CRL-1573) and rat cardiomyoblast H9C2 cells (A.T.C.C. CRL-1446) were cultured in DMEM containing 10% (v/v) FBS. The cells were prepared and transfected with Lipofectamine™ (Invitrogen). Briefly, A293 cells or H9C2 cells in 60-mm-diameter dishes were transfected with 1–2 µg of reporter gene and/or various transcription factor (GATA4, Nkx2.5 and/or hhLIM) expression vectors and 0.5 µg of pGL-TK (the minimum promoter region of the herpes-simplex-virus thymidine kinase gene was fused to a luciferase reporter gene of the pGL3 basic vector). At 2 days after transfection, the cells were harvested and firefly (Photinus) luciferase activities as well as Renilla (sea pansy) luciferase activities were measured with the Dual-Luciferase Reporter System (Promega) according to the manufacturer’s protocol. The transfection efficiencies of the A293 and H9C2 cells were 90 and 56% respectively, pGL-TK was included as an internal control for variations in transfection efficiency. A minimum of three independent transfections were performed for each experimental group. The post-test comparison was performed by using a paired t test. Results were accepted as significant when P < 0.05 [12]. To examine whether co-transfection of hhLIM with Nkx2.5 affects the expression levels of Nkx2.5, Western-blot analysis was performed as described previously [10].

RNA isolation and quantitative RT-PCR (reverse transcription PCR)
A293 cells were transfected with pEFSA-Nkx2.5 or pcDNA3-hhLIM as described above. After 48 h, total RNA was isolated with Trizol reagent (Gibco BRL). RT-PCR was performed as described previously [9]. For the ANF gene, oligonucleotides 5′-GGGGGTAGATGTGAGAGGA-3′ and 5′-CTCCAGAGGG-TATTCAACCA-3′ were used as the primers. For the GAPDH (gyceraldehyde-3-phosphate dehydrogenase) gene (used as an internal control), oligonucleotides 5′-ACCAGCTTCA-TGCCATCAC-3′ and 5′-TTCACACCCGTTGTTGTA-3′ were used. PCR conditions were: 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s. The PCR products were subjected to electrophoresis on 1% agarose gel and stained with ethidium bromide [13].

Co-immunoprecipitation assays
To examine the association of hhLIM with Nkx2.5 in vivo, co-immunoprecipitation was performed as described previously [14]. Briefly, A293 cells were transfected with 3 µg of pcDNA-hhLIM and/or 3 µg of pEFSA-Nkx2.5 using Lipofectamine™ reagents (Invitrogen). The cells were briefly sonicated in 1.5 ml of a lysis buffer [50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 0.5% NP-40 (Nonidet P40), 10% glycerol, 10 mM Na2HPO4, and 1 mM dithiothreitol]. The cell extracts were diluted with the lysis buffer containing 0.1% NP-40 and precleared by incubation with 5 µg of rabbit IgG and 50 µl of Protein A–agarose at 4°C for 2 h. The precleared cell extracts were incubated with 3 µg of polyclonal anti-HA (anti-haemagglutinin) antibodies (Santa Cruz Biotechnology) at 4°C for 3 h, followed by incubation with Protein A–agarose at 4°C for 2 h. After washing five times with NETN buffer (100 mM NaCl, 1 mM EDTA, 1 mM Tris/HCl, pH 8.0, 0.5% NP-40 and 1 mM dithiothreitol) at room temperature (25°C) for 5 min, bound proteins were resolved by SDS/PAGE, followed by Western blotting with the anti-Myc antibody (Santa Cruz Biotechnology) [15].

Mammalian two-hybrid assays
The CheckMate Mammalian Two-Hybrid System (Promega) was used for mammalian two-hybrid assays. A293 cells were co-transfected with 1 µg of pBind or pBind-hhLIM and 1 µg of pG5lac, along with 1 µg of pEFSA-NKx2.5, or both. Cells were harvested after 48 h, and firefly and Renilla luciferase activities were measured as described above. Firefly luciferase activities were normalized to Renilla luciferase activities. A minimum of three independent transfections were performed for each experimental group [16,17].

GST (glutathione transferase) pull-down assays
Full-length and domain-specific regions of the hLIM were generated in pGEX-3X using the following oligonucleotides: F7 sense, 5′-ATGCCAAACTGGGGCGAGG-3′; antisense, 5′-TCATATTCT-TCTTTTTCACGT-3′; F5, antisense, 5′-TCAGGCAGCTT-CGGACTCTCCA-3′; F4, antisense, 5′-TCAACAGGGCCTT-CCTGCAAGG-3′; F8, sense, 5′-ACACGCACGAGCTGCGGCT-3′; F9, sense, 5′-TTCGATGTGGCGAAGTCAGT-3′. GST fusion proteins were generated by inducing protein expression with 1 mM isopropyl β-D-thiogalactopyranoside for 4 h and purifying bacterial lysates over glutathione–agarose. For pull-down assays, Nkx2.5 proteins were incubated with equal amounts of GST–hhLIM or each GST–hhLIM deletion mutant protein for 2 h at 4°C in buffer containing 20 mM Tris/HCl, pH 7.5, 300 mM NaCl, 1 mM dithiothreitol, 0.4% NP-40, 0.5 mg/ml BSA and 1 mM PMSF. Protein complexes were washed three times with the above-described buffer and then once with the same buffer containing 150 mM NaCl but lacking BSA. After washing, bound proteins were resolved by SDS/PAGE and detected by Western blotting [18].

Electrophoretic-mobility-shift assays
A double-stranded oligonucleotide probe containing the NKE (Nkx2.5-binding element) corresponding to the −271 to −235 bp region of the ANF promoter was produced by annealing a sense strand (5′-TCTGCTCTTCTCACCTTGAAGTGCGGCCC-TCTTG-3′) and an antisense strand (5′-CAAGAGCCCCCATCAGAGGTGAGAGAGCA-GAGAGCA-3′). Electrophoretic-mobility-shift assays were conducted as described previously [19]. Labelled probes and purified GST–Nkx2.5 (5 ng) and GST–hhLIM (0.5, 10 or 20 ng) were used for each 20 µl reaction mixture in a buffer containing 50 mM KCl, 3 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.5% NP-40, 10 µg/ml BSA, 10% (v/v) glycerol, 20 mM Hepes, pH 7.6, and 5 µg/ml poly(dI·dC) (Sigma). The mixture was incubated at 37°C for 20 min, and then the protein–DNA complex was separated by native PAGE and visualized by autoradiography [20].

ChIP (chromatin immunoprecipitation) assays
A293 cells transfected with expression plasmids encoding hhLIM and Nkx2.5 were cross-linked by formaldehyde, and chromatin was immunoprecipitated with anti-Nkx2.5 polyclonal antibody
hhLIM activates ANF gene expression by interacting with Nkx2.5

Figure 1 Effect of hhLIM on the expression of the ANF gene in cardiomyocytes, H9C2 cells or A293 cells

(A) Primary cardiomyocytes, H9C2 cells and A293 cells were co-transfected with the luciferase reporter construct containing the −638 bp flanking region of the ANF gene, along with hhLIM expression plasmids. After 48 h, cells were harvested and luciferase activities were measured with the Dual-Luciferase Reporter System (Promega). Results are means ± S.E.M. for three separate transfection assays with duplicate plates. *P < 0.05 compared with respective control activation. (B) Primary cardiomyocytes, H9C2 cells and A293 cells were transfected with pcDNA3 or pcDNA3-hhLIM. After 48 h, total RNAs were isolated and ANF mRNA levels were evaluated using semi-quantitative RT-PCR. GAPDH mRNA was used as an internal control. The left panel shows a representative result from three independent experiments. The right panel shows the densities of specific scanned and quantified bands. *P < 0.05 compared with the respective control.

(Santa Cruz Biotechnology). The total input was the supernatant from the no-antibody control. Quantitative PCR of the precipitated chromatin fragments was performed. Oligonucleotides spanning the NKE in the ANF promoter were designed using Primer Express® software (Applied Biosystems). For each amplification, melting curves and gel electrophoresis of the PCR product were used to verify the identities of the PCR products. All real-time PCRs were performed in triplicate [4,21,22].

RESULTS
hhLIM activates ANF gene expression

To determine the effects of hhLIM on ANF promoter activity, we performed a reporter-gene assay. Primary neonatal rat cardiomyocytes, H9C2 cells and A293 cells were transfected with the hhLIM expression plasmids along with the ANF promoter-enhancer (−638 bp) reporter. As shown in Figure 1(A), hhLIM enhanced ANF promoter activity by 3-fold in the cardiomyocytes and H9C2 cells, but not in the A293 cells, indicating that hhLIM activates ANF gene expression in a cell-type-specific manner.

To confirm further that hhLIM promotes the expression of the ANF gene in cardiomyocytes, H9C2 cells and A293 cells were transfected with pcDNA3 or pcDNA3-hhLIM, and ANF mRNA levels were evaluated using semiquantitative RT-PCR. As shown in Figure 1(B), ANF mRNA was expressed in the cardiomyocytes and H9C2 cells, but not in the A293 cells, and there was an evident increase in ANF mRNA levels in these two cell types transfected with pcDNA3-hhLIM. These results further demonstrate that hhLIM promotes the expression of the ANF gene.

hhLIM does not contain a TAD (transcription activation domain), but promotes the expression of the ANF gene in an Nkx2.5-dependent manner

The above findings suggested that hhLIM might be a transcription activator of the ANF gene. To test this idea, expression plasmids (pBind-hhLIM) encoding a Gal4–hhLIM fusion protein were transfected into A293 cells along with the luciferase reporter pG5luc. Cells transfected with pG5luc exhibited no luciferase activity. When the chimaeric genes containing the Gal4-DNA-binding domain and the full-length hhLIM or various truncated hhLIMs were tested, the fusion proteins could not augment the transcriptional activity of the luciferase reporter (results not shown), strongly suggesting that hhLIM lacks a functional TAD.

Since hhLIM does not have a TAD, there are several possible mechanisms by which hhLIM activates the ANF promoter. Previous studies have demonstrated that the cardiac-specific ANF promoter-enhancer is a transcriptional target for both Nkx2.5 and GATA4, and that either Nkx2.5 or GATA4 alone activates, or together they synergistically activate, ANF expression. Given these findings, we hypothesized that hhLIM may activate ANF transcription co-operatively with Nkx2.5 and GATA4. To
determine this, serial mutational analyses of the ANF reporter genes were performed (Figure 2A). The ANF reporter genes were co-transfected with Nkx2.5 and GATA4 in the presence or absence of hhLIM. The 134 ANF-Luc (includes −134 to +65) deleted further upstream of the ANF promoter showed an activation pattern similar to that of 383 ANF-Luc. hhLIM also enhanced activation of 109 ANF-Luc (includes −109 to +65) reporter gene expression co-operatively with Nkx2.5. It should be noted that hhLIM activated 109 ANF-Luc, which does not contain the GATA sequences, but contains an NKE. To further test this, hhLIM, Nkx2.5 or GATA4 expression constructs and 638 ANF-Luc were co-transfected in A293 cells. As shown in Figures 2(B) and 2(C), hhLIM and Nkx2.5, but not hhLIM and GATA4, synergistically activated the ANF gene, suggesting that hhLIM increases the transcriptional activation function of Nkx2.5.

This hypothesis was further tested using ANF promoter mutants in which NKE elements were deleted. As shown in Figure 2(D), the NKE element, but not the GATA element, is essential for synergy between hhLIM and Nkx2.5. The same results were obtained using the reporter of the Nkx2.5-binding consensus sites. As shown in Figure 2(E), the NKE reporter was activated by Nkx2.5, and hhLIM significantly increased the reporter activity by 40% in an Nkx2.5-dependent manner. RT-PCR identified that hhLIM and Nkx2.5 could activate ANF mRNA expression cooperatively (Figure 2F).

**hhLIM interacts with Nkx2.5 in vivo and in vitro via its N-terminal domain**

To examine whether hhLIM can interact with Nkx2.5 in vivo, two approaches, co-immunoprecipitation assays and mammalian two-hybrid assays, were taken. For the co-immunoprecipitation assays, A293 cells were transfected with the expression plasmids encoding HA-tagged Nkx2.5, myc-hhLIM, or both. Following immunoprecipitation with an anti-HA monoclonal antibody, the precipitates were immunoblotted using an anti-myc polyclonal antibody. The results revealed that myc-tagged hhLIM was detected in the anti-HA antibody precipitates, but not in the control IgG precipitates, indicating that hhLIM associates with Nkx2.5 (Figure 3A). A similar in vivo interaction was further confirmed by mammalian two-hybrid assays (Figure 3B). As predicted, the chimera protein pBind-F5 (1–120 aa (amino acids)), which contains the first hhLIM LIM motif fused in-frame with the DNA-binding domain of Gal4, did not activate the pG5Luc reporter gene because it lacks a functional TAD (Figure 3C). When an Nkx2.5 expression plasmid was co-expressed with pBind-F5 (1–120 aa), luciferase expression was enhanced approx. 4-fold, suggesting that Nkx2.5 binds to hhLIM and activates luciferase gene expression via the Nkx2.5 TAD. As predicted from our in vitro protein-binding assays, when pBind-F8 (44–194 aa), containing only the second LIM motif, was co-transfected with Nkx2.5, no significant induction of pG5Luc expression was detected (Figure 3C). We conclude that hhLIM and Nkx2.5 associate with each other in vivo and that their association is mediated through the first LIM motif of hhLIM.

To map further the domain of hhLIM required for its binding to Nkx2.5, full-length hhLIM and various deletion mutants were tested for their ability to interact with Nkx2.5 by GST pull-down assays. A schematic diagram of hhLIM mutants and a summary of the binding results are shown in Figure 4. The full-length hhLIM and the C-terminal deletion mutants, in which aa 120–194 or 44–194 were deleted, interacted well with Nkx2.5 (lanes 1–3 in Figure 4B). When the N-terminal part (aa 1–44 and aa 1–120) of the LIM domain was deleted, the interaction of the mutant with Nkx2.5 was abolished (lanes 4 and 5 in Figure 4B). Co-immunoprecipitation assays further identified that the N-terminal domain of hhLIM mediates the interaction of hhLIM with Nkx2.5 (Figure 4C).

**hhLIM enhances the binding activity of Nkx2.5 with the ANF promoter**

To exclude the possibility that co-transfection of hhLIM with Nkx2.5 increases the expression of Nkx2.5, Western-blot analyses were performed. As shown in Figure 5(A), the co-transfection of hhLIM with Nkx2.5 did not affect the expression level of Nkx2.5. Therefore the increased activation of the ANF reporter gene by hhLIM is not due to an increase in expression of Nkx2.5. To elucidate further the mechanisms whereby hhLIM promotes ANF gene expression activated by Nkx2.5, we next examined the effect of hhLIM on the binding activity of Nkx2.5 with the ANF promoter. We first expressed GST–hhLIM and GST–Nkx2.5 fusion proteins in *Escherichia coli* BL21 and purified them with glutathione–Sepharose 4B. GST–Nkx2.5 and GST–hhLIM fusion proteins were incubated with an [32P]-labelled −271/−235 bp fragment of the ANF promoter, which contains NKE boxes. The protein–DNA complex generated was separated by native PAGE and visualized by autoradiography. As shown in Figure 5(B), purified GST–hhLIM did not bind to the probe. Increasing the amount of purified GST–hhLIM increased the DNA-binding activity of Nkx2.5 in a dose-dependent manner. That is to say, hhLIM enhances Nkx2.5 binding to the NKE-containing region of the ANF promoter. To determine further whether hhLIM could increase the DNA binding of Nkx2.5 to the NKE-containing region of the ANF promoter within intact chromatin, ChIP assays were performed. ChIP assays based on semi-quantitative PCR analysis of DNA precipitated with an anti-Nkx2.5 antibody showed that the NKE-containing DNA fragments increased with an increase in hhLIM, suggesting that hhLIM can enhance the DNA-binding activity of Nkx2.5 (Figure 5C). Addition of increasing amounts of hhLIM using hhLIM expression plasmid increased Nkx2.5 DNA binding in a dose-dependent manner. Compared with the group transfected with 0.25 µg of pcDNA-hhLIM, the group transfected with 1µg of plasmid increased the Nkx2.5 DNA binding by 8.85-fold. To strengthen these findings, we performed a similar ChIP using hhLIM antibody for precipitation. As shown in Figure 5(D), the hhLIM–Nkx2.5 complex bound to the ANF promoter in a chromatin context and validated the existence of this interaction.

**DISCUSSION**

Transcriptional activation plays a critical role in precisely controlling gene expression in a spatial and temporal manner. Many gene-specific activators in bacteria and eukaryotes have been identified. The present study demonstrates that hhLIM, a transcriptional factor, activates ANF gene expression by physical interaction with Nkx2.5.

The precise regulation of temporal and spatial expression of tissue-specific genes may require interactions among transcriptional activators and repressors. Likewise, specification and differentiation of the cardiac muscle lineage appear to require a combinatorial network of many trans-acting factors. For example, cardiac actin expression is activated by a serum response factor, which recruits Nkx2.5 or myocardin for synergistic activation [12,23–26]. hhLIM, which is found exclusively in striated muscle, positively influences cardiac hypertrophy. An interesting feature of hhLIM is its dual subcellular location, as it is present in the nucleus at the beginning of cell hypertrophy and accumulates in the cytoplasm at later stages [10]. How and where hhLIM
Figure 2  hhLIM increases ANF promoter activity in an Nkx2.5-dependent manner

(A) Mutational analyses of the ANF reporter genes. The mutant ANF reporter genes were co-transfected with Nkx2.5 and GATA4 and/or the hhLIM expression vector into A293 cells. Results are means ± S.E.M. for three separate transfection assays with duplicate plates. Above the histogram is a schematic diagram of the ANF enhancer–promoter showing the positions of the Nkx2.5-binding sites (N) and GATA4-binding sites (G). *P < 0.05 compared with the hhLIM-transfected group. (B) A293 cells were transiently co-transfected using expression vectors for hhLIM, Nkx2.5, GATA4 or the backbone vector as control, along with the ANF–luciferase reporter. *P < 0.05 compared with the hhLIM-transfected group. (C) A293 cells were transiently co-transfected using expression vectors for hhLIM, Nkx2.5 or the backbone vector as control, along with the ANF–luciferase reporter. *P < 0.05 compared with the hhLIM-transfected group. (D) The synergy between hhLIM and Nkx2.5 requires an NKE-binding site in the context of the ANF promoter. Transient co-transfections in A293 cells were carried out as described above and the promoter described represents either the −288 bp promoter (WT) or the −288/+59 bp promoter, which removes the NKE element [NKE(mut)] and the −288/+59 bp promoter, which removes the GATA element [GATA(mut)]. *P < 0.05 compared with the hhLIM-transfected group. (E) hhLIM increases transcriptional activation by Nkx2.5. A reporter plasmid (1 µg) containing the Nkx2.5-binding consensus sequence (AGTTAATTG) linked to the pGL3 promoter NKE–Luc was co-transfected with 0.5 µg of Nkx2.5 and/or 0.5 µg of hhLIM expression constructs into A293 cells as indicated. *P < 0.05 compared with the control. (F) H9C2 cells were transfected with the indicated expression vectors. At 3 days after transfection, total RNA was isolated and gene expression was assayed by RT-PCR. GAPDH was measured as an internal control. The left panel shows a representative result from three independent experiments; the right panel shows the densities of specific scanned and quantified bands. *P < 0.05 compared with the control.
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Figure 3 hhLIM physically interacts with Nkx2.5 in vivo

(A) Co-immunoprecipitation assays of hhLIM and Nkx2.5. A293 cells were transfected with expression plasmids encoding Nkx2.5, myc-tagged hhLIM, or both. After incubation for 48 h, cells were harvested, lysed, then subjected, first, to anti-HA antibody immunoprecipitation and then to immunoblotting using anti-Myc polyclonal antibody. GAPDH was measured as an internal control for total lysates prior to the co-immunoprecipitation. (B) Schematic diagram of the mammalian two-hybrid assays for hhLIM with Nkx2.5 in A293 cells. Nkx2.5 intrinsically contains a TAD, so it can promote the transcription of the reporter gene by binding to Gal4–hhLIM. (C) Mammalian two-hybrid analyses of hhLIM and Nkx2.5 interactions in A293 cells. A293 cells were co-transfected with pBind, pBind–F5 (1–120 aa) or pBind–F8 (44–194 aa) and the pGL3 luc reporter gene, along with expression plasmids encoding Nkx2.5. After 48 h, the luciferase activities of the cell extracts were tested with the Dual-Luciferase Reporter System (Promega).

functions to trigger cardiac hypertrophy are important questions that need to be answered in order to characterize fully the intricate regulatory pathways that establish and maintain a normal phenotype. In the present study we found that hhLIM promotes the expression of the ANF gene in H9C2 cells, but not in A293 cells. This finding suggests that hhLIM contributes to cardiac-specific expression of the ANF gene.

The transcription factors involved in activation of ANF expression include Nkx2.5, GATA4, MEF2 (myocyte-specific enhancer factor 2), PITX2, and Tbx5 (T-box 5), which bind to their cis-elements in the ANF enhancer region [27–29]. The Gal-based reporter assays revealed that hhLIM itself had no transcriptional activity, but hhLIM may physically interact with other factors, enhancing their activation function. Although overexpression of hhLIM had no effect on the ANF promoter in A293 cells, co-transfection of hhLIM with Nkx2.5 induced much stronger transactivation than Nkx2.5 alone, suggesting that Nkx2.5 and hhLIM synergistically transactivate the ANF promoter. By contrast, co-transfection of GATA4 with hhLIM did not enhance activation. That is to say, hhLIM mediates the up-regulation of ANF expression by interacting with Nkx2.5, but not GATA4. Nkx2.5 and hhLIM also synergistically activated the luciferase construct containing multimerized Nkx2.5-binding sites. The mutation of the NKE abolished the activation activity of hhLIM. In conclusion, the Nkx2.5-binding element is an absolute requirement for achieving synergy between hhLIM and Nkx2.5.

Our co-immunoprecipitation and mammalian two-hybrid assays revealed that the hhLIM interaction with Nkx2.5 is mediated through the first LIM motif of the hhLIM protein. The interaction of hhLIM with Nkx2.5 enhances the binding of Nkx2.5 to the ANF promoter, suggesting that hhLIM promotes the expression of the ANF gene by facilitating the binding of Nkx2.5 to the NKE boxes in the ANF promoter. The enhancement in the binding of Nkx2.5 to the NKE element induced by hhLIM can be explained by the following possibilities. One is that hhLIM stimulates the expression of Nkx2.5. The other possibility, although the two are not mutually exclusive, is that hhLIM may affect the conformation of the Nkx2.5–NKE complex, thereby facilitating DNA sequence recognition by Nkx2.5. Our observation supports the
hhLIM activates ANF gene expression by interacting with Nkx2.5

Figure 5 hhLIM increases the DNA-binding activity of Nkx2.5 in vivo

(A) Co-transfection of hhLIM with Nkx2.5 did not affect the expression of Nkx2.5. After co-transfection of the Nkx2.5 and hhLIM expression plasmids, the cell extracts were prepared and subjected to immunoblotting with anti-Nkx2.5 or anti-hhLIM antibodies. (B) Electrophoretic mobility-shift assays using purified GST–hhLIM and GST–Nkx2.5. 32P-labelled probes corresponding to the −272/−253 bp region of the ANF promoter were incubated with the purified GST–fusion proteins. The DNA–protein complex was separated by non-denaturing PAGE and then visualized by autoradiography. The arrow and arrowhead indicate the Nkx2.5–hhLIM–NKE complex and Nkx2.5–NKE complex respectively. (C) ChIP assays. Semi-quantitative PCR was used to detect the NKE-containing region of the ANF promoter in chromatin fragments immunoprecipitated with anti-Nkx2.5 antibody. M, size markers. Top panel: representative result from three independent experiments; bottom panel: densities of specific scanned and quantified bands. (D) ChIP assays. Semi-quantitative PCR was used to detect the NKE-containing region of the ANF promoter in chromatin fragments immunoprecipitated with anti-hhLIM antibody. M, size markers. Top panel: representative result from three independent experiments; bottom panel: densities of specific scanned and quantified bands.

latter possibility. Our data suggest that the interaction of hhLIM and Nkx2.5 enhances the DNA-binding activity of Nkx2.5, suggesting that hhLIM promotes ANF expression by recruiting and stabilizing Nkx2.5 to the correct DNA target or by promoting DNA sequence recognition by Nkx2.5. It is possible that a threshold level of activated Nkx2.5 is required to initiate cardiac hypertrophy in these cells. hhLIM would, in effect, lower the threshold of Nkx2.5 that is needed for the activation of the ANF promoter, since the DNA-binding activity of Nkx2.5 is enhanced in the presence of hhLIM. Therefore, cells that express low levels of Nkx2.5 (which normally are not converted into cardiomyocytes) can enter the cardiac-hypertrophy program in the presence of hhLIM. The increase in the DNA binding of Nkx2.5 mediated by hhLIM may be of great importance in cardiac hypertrophy given that the hypertrophy of cardiomyocytes is blocked by the absence of hhLIM protein.

This work was supported by the Program for New Century Excellent Talents in University (grant no. NCET-05-0261), the Key Project of Chinese Ministry of Education (grant no. 206016), the National Natural Science Foundation of People’s Republic of China (grants nos. 30300132 and 30570661) and the Major State Basic Research Development Program of China (grant no. 2005CCA03100).

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