HIF-1α Signaling Upstream of NKX2.5 Is Required for Cardiac Development in Xenopus

HIF-1α is originally identified as a transcription factor that activates gene expression in response to hypoxia. In metazoans, HIF-1α functions as a master regulator of oxygen homeostasis and regulates adaptive responses to change in oxygen tension during embryogenesis, tissue ischemia, and tumorogenesis. Because Hif-1α-deficient mice exhibit a number of developmental defects, the precise role of HIF-1α in early cardiac morphogenesis has been uncertain. Therefore, to clarify the role of HIF-1α in heart development, we investigated the effect of knockdown of HIF-1α in Xenopus embryos using antisense morpholino oligonucleotide microinjection techniques. Knockdown of HIF-1α resulted in defects of cardiogenesis. Whole mount in situ hybridization for cardiac troponin I (cTnI) showed the two separated populations of cardiomyocytes, which is indicative of cardia bifida, in HIF-1α-depleted embryos. Furthermore, the depletion of HIF-1α led to the reduction in cTnI expression, suggesting the correlation between HIF-1α and cardiac differentiation. We further examined the expression of several heart markers, nkx2.5, gata4, tbx5, bmp4, hand1, and hand2 in HIF-1α-depleted embryos. Among them, the expression of nkx2.5 was significantly reduced. Luciferase reporter assay using the Nkx2.5 promoter showed that knockdown of HIF-1α decreased its promoter activity. The cardiac abnormality in the HIF-1α-depleted embryo was restored with co-injection of nkx2.5 mRNA. Collectively, these findings reveal that HIF-1α-regulated nkx2.5 expression is required for heart development in Xenopus.

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HIF-1α is originally identified as a transcription factor that activates gene expression in response to hypoxia. In metazoans, HIF-1α functions as a master regulator of oxygen homeostasis and regulates adaptive responses to change in oxygen tension during embryogenesis, tissue ischemia, and tumorogenesis. Because Hif-1α-deficient mice exhibit a number of developmental defects, the precise role of HIF-1α in early cardiac morphogenesis has been uncertain. Therefore, to clarify the role of HIF-1α in heart development, we investigated the effect of knockdown of HIF-1α in Xenopus embryos using antisense morpholino oligonucleotide microinjection techniques. Knockdown of HIF-1α resulted in defects of cardiogenesis. Whole mount in situ hybridization for cardiac troponin I (cTnI) showed the two separated populations of cardiomyocytes, which is indicative of cardia bifida, in HIF-1α-depleted embryos. Furthermore, the depletion of HIF-1α led to the reduction in cTnI expression, suggesting the correlation between HIF-1α and cardiac differentiation. We further examined the expression of several heart markers, nkx2.5, gata4, tbx5, bmp4, hand1, and hand2 in HIF-1α-depleted embryos. Among them, the expression of nkx2.5 was significantly reduced. Luciferase reporter assay using the Nkx2.5 promoter showed that knockdown of HIF-1α decreased its promoter activity. The cardiac abnormality in the HIF-1α-depleted embryo was restored with co-injection of nkx2.5 mRNA. Collectively, these findings reveal that HIF-1α-regulated nkx2.5 expression is required for heart development in Xenopus.

Hypoxia inducible factor-1 (HIF-1) is a transcriptional factor that has the fundamental function in mammalian develop-
**HIF-1α Regulates Cardiac Development**

**EXPERIMENTAL PROCEDURES**

Construction of Plasmids and Mutagenesis—*Xenopus* cDNAs of cTnl (L25721), nkx2.5 (L25600), gata4 (BC071107), tbx5 (AF133036), hand1 (Z95080), hand2 (AA676713), and bmp4 (AJ005076) were cloned into pCS2+ (11) or pBlueScript SK(–) (Stratagene) vectors. Mouse Hif-1α (BC026139) was obtained from L.M.A.G.E. (Consortium Id clone 4019056). Mouse Gata4 (NM_008092) and Smad4 (NM_008540) cDNAs were cloned into pRES-puro3 (Clontech). *Xenopus* hif-1α (AJ277829) cDNA was subcloned with BamHI and EcoRI into both pBlueScript SK(–) and pCS2+. A genomic clone of mouse Nkx2.5 containing the 3′ flanking sequence was kindly provided by Dr. K. E. Yutzey (Cincinnati Children’s Hospital Medical Center) (12). The sequence of all plasmids was verified by DNA sequencing (Applied Biosystems Inc., Foster City, CA).

A constitutively active form of *Xenopus* hif-1α (P558G) (13) was developed using the QuikChange mutagenesis protocol (Stratagene) with oligos 5′-gtagttgaggattctctc-3′ and its complementary sequence. After sequence verification, the Bsu36I-NheI fragment containing the desired mutation was exchanged with the corresponding region of wild-type hif-1α in pCS2+. A constitutively active form of mouse Hif-1α (P577G) was similarly developed by mutagenesis with oligos 5′-gtagtctg-gtctgcttatcc-3′ and its complementary sequence.

*Synthetic RNA and Antisense Morpholino Oligonucleotides (MOs)—*Synthetic mRNA was made from linearized plasmid DNA using mMessage mMachine *in vitro* transcription kits (Ambion, Austin, TX). MOs were obtained from GeneTools (Philomath, OR). The hif-1α MO1 (hif-1α MO1, hif-1α MO2) were designed to non-overlapping regions of *Xenopus* hif-1α (BC043769) 5′-UTR (MO1, 5-gttcactactgatccctccatg-3′; MO2, 5′-ctccgaagctgtgtcaagcgccgag-3′). *Xenopus* nkx2.5 MO contained the sequence 5′-ggcttgagtggtcaggtgagtc-3′. Control MO (CMO; Gene Tools) was used as a control.

*Embryo Manipulation and Microinjection—*Fertilized *Xenopus laevis* embryos were generated by standard techniques and staged, according to Nieuwkoop and Faber (14). For blastomere injection, regularly cleaving embryos were selected. The synthetic mRNA encoding hif-1α (P558G) (750 pg) was co-injected into one of the two vegetal-dorsal blastomeres of the selected embryos at the 8-cell stage, leaving the other side as control. Knockdown was carried out using MO (40 ng) similarly to mRNA injection. For the double injection to an embryo, MO was microinjected into both of the vegetal-dorsal blastomeres at 40 ng/blastomere. For lineage tracing, mRNA encoding green fluorescent protein (GFP) (500 pg) was co-injected with mRNA or MOs into the blastomere. During and after injection, embryos were maintained in 3% Ficoll in Steinberg’s solution at 20 °C. After incubation overnight, embryos were separated to two groups (right or left side group) according to the position of the lineage tracer, GFP, using fluorescence microscopy. Then embryos were transferred to Steinberg’s solution containing 10 units/ml penicillin and 10 μg/ml streptomycin, and collected at stage 33/34.

*In Vitro Translation and Transcription System—*The DNA consisting of 5′-UTR (102 bp), N-terminal of hif-1α (300 bp) (BC043769), and *venus*, a variant of GFP (DQ092360), was inserted into BamHI and EcoRI sites of the pCS2+ vector (5′-UTR-HIF(N)-Venus). *In vitro* transcription and translation of 5′-UTR-HIF(N)-Venus was performed by using an *in vitro* transcription and translation-coupled system (Tnt™ Coupled Reticulocyte Lysate System; Promega), according to the manufacturer’s protocol with the following modifications: in a 25-μl reaction, 0.5 μg of 5′-UTR-HIF(N)-Venus and various amounts of hif-1α MO (0, 2, and 10 μM final concentration) were added to the Tnt reaction mixture and incubated at 30 °C for 90 min. Five microliters from the reaction mixture were subjected to 10% SDS-PAGE followed by immunoblot analysis with anti-GFP antibody, kindly provided by Dr. N. Mochizuki (National Cardiovascular Center Research Institute).

*Immunoprecipitation of Endogenous HIF-1α—*Total cell lysates were prepared from *Xenopus* embryos microinjected with hif-1α MO in one of two vegetal-dorsal blastomeres at the 8-cell stage. At stage 20, 10 embryos were lysed in 800 μl of immunoprecipitation buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl2, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol and protease inhibitor mixture (Roche)). After incubation with gentle agitation for 30 min at 4 °C, the extracts were centrifuged at 10,000 × g for 15 min. 400 μg of supernatants were used for immunoprecipitation. After pre-treatment with normal mouse serum IgG (Santa Cruz, Santa Cruz, CA) and protein G-Sepharose (GE Healthcare) for 1 h, the supernatants were incubated with shaking at 4 °C in the presence of 2 μg of anti-HIF-1α monoclonal antibody (H1alpha67, Novus Biologicals, Littleton, CO) or normal mouse IgG overnight prior to the addition of 10 μl of protein G-Sepharose for 1 h. Beads were washed in immunoprecipitation buffer three times. Eluted samples with 2× SDS sample buffer were subjected to immunoblot analysis using the same anti-HIF-1α antibody.

*Whole Mount in Situ Hybridization and Histological Analysis—*Whole mount *in situ* hybridization was performed as described previously (15) using digoxigenin-labeled antisense RNA probes transcribed with a DIG RNA Labeling kit (Roche). Positive signals were visualized using BM purple (Roche). For the detection of two probes (tie2 and α-globin) in the same sample, one and then the other was synthesized with DIG-11-UTP and fluorescein-UTP, respectively (Roche). Signals were visualized using BM purple for one (tie2) and with DAB (Vector Laboratories, Burlingame, CA) for the other (α-globin) (16). Embryos were dehydrated and embedded in paraffin after whole mount *in situ* hybridization. Transverse sections were made at 7-μm intervals and counterstained with eosin.

*RT-PCR Analysis—*Total RNA was extracted with Isogen (Nippogone, Tokyo, Japan), followed by DNase I (Takara Bio, Otsu, Japan) treatment. The cDNA was synthesized using a SuperScript™ III First-strand Synthesis System (Invitrogen). PCRs were carried out using the following primers: ornithine decarboxylase, forward, 5′-cagttcatcgctgagtttg-3′, reverse,
5'-caacatgaaacctcaccc-3' (55 °C annealing, 30 cycles); hif-1α, forward, 5'-tggatggtagagtgcgct-3'; reverse, 5'-agattctgccctggcctac-3' (52 °C annealing, 30 cycles); nkx2.5, forward, 5'-gacgtacagtgctggtgtc-3'; reverse, 5'-tgaaggctcagctaggtg-3' (55 °C annealing, 30 cycles).

Reporter Gene Assays—Monkey kidney (CV-1) cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (IRH, Lenexa, KS), 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells grown in 12-well plates were transfected with 0.4 µg of reporter plasmid and 1.6 µg of effector plasmids (pIRES-puro3-Hif-1α (P577G), pIRES-puro3-mGata4, or pIRES-puro3-Smad4), using FuGENE HD, according to the manufacturer’s protocol (Roche). After 48 h, luciferase assay was performed with a Dual Luciferase system according to the manufacturer’s protocol (Promega, Madison, WI). Renilla luciferase plasmid, pRL-null, was co-transfected as a control to standardize the transfection efficiency. All transfection experiments were performed in triplicate.

The pGL4.10–9kb Nkx2.5 promoter (Nkx2.5 promoter–Luc) (100 pg) and pRL-null (2 pg) (an internal control) were injected into one side of the vegetal-dorsal blastomeres at the 8-cell stage. In the same way, the promoter/reporter constructs were co-injected with 40 ng of CMO or hif-1α MO1. Embryos injected with luciferase reporter constructs were collected at stage 19–20 and homogenized in passive lysis buffer using 30 µl of buffer/embryo. Samples were left on ice for 10 min followed by centrifugation (10,000 g) for 5 min at 4 °C to pellet debris. Luciferase assays were performed with 10 µl of clear supernatant and 100 µl of firefly or Renilla luciferase substrate using a Lumat LB 9507 luminometer (Berthold Technology, Bad Wildbad, Germany). For each sample, 15 pools containing 5 embryos each were used. The experiment was repeated three times.

Visualization of Nkx2.5 Promoter Activity in Vivo—cDNA encoding Venus was replaced with that encoding Luciferase in pGL4.10[luc2] (Promega) with the Ncol and Xhol sites (pGL4.10-Venus). The mouse 9kb-Nkx2.5 promoter was cleaved out from the Nkx2.5 promoter–Luc with KpnI and Ncol, and ligated into same site of pGL4.10-Venuses. The Nkx2.5 promoter–Venus (100 pg) was injected into one of the two vegetal-dorsal blastomeres with CMO or hif-1α MO1. The expression pattern and the activity of the Nkx2.5 promoter were analyzed with green fluorescence at stage 20.

Statistical Analysis—Statistical significance was evaluated by one-way analysis of variance followed by Student’s t test for unpaired values. Differences with a p value less than 0.05 were considered significant.

RESULTS

Expression Pattern of Xenopus HIF-1α during Development—We compared the sequence of Xenopus hif-1α cDNA that we cloned with those of human (Fig. 1A). The deduced amino acid sequence of Xenopus hif-1α showed high identity with human. The domains of basic helix-loop-helix, Per-Arnt-Sim (PAS), N-terminal transactivation domain, and the C-terminal transactivation domain were conserved in the predicted Xenopus HIF-1α.

To examine whether hif-1α is expressed in Xenopus embryos, the expression of hif-1α mRNA was analyzed by RT-PCR. The hif-1α mRNA was detected in fertilized eggs and in embryos from stages 1 to 25 (Fig. 1B). Furthermore, to examine the spatial expression of hif-1α mRNA in embryos at later stages, we performed whole mount in situ hybridization analysis. At mid-neurula (stage 23) the transcripts were detected along the dorsal axis in the brain and somites (Fig. 1C, panel a). At approximately stage 28, when the first cardiac differentiation markers are expressed, immediately prior to fusion of the heart tube, hif-1α expression was observed in the heart primordia (Fig. 1C, panel b). In tailbud embryos (stage 33/34), hif-1α continued to be expressed in the branchial arches, somites, and heart (Fig. 1C, panels c, e, and f) as well as in the ventral blood island (VBI) (Fig. 1C, panel d), eyes, and kidneys (Fig. 1C, panel c), similar to mouse and human.

Role of HIF-1α in Vascular Development—HIF-1α is known to activate the transcription of genes encoding angiogenic growth factors such as vascular endothelial growth factor, which is secreted by hypoxic cells and stimulates endothelial cells to promote angiogenesis (17). To understand the role of HIF-1α for vascular formation, we examined whether HIF-1α affects the distribution of primitive blood and endothelial cells in developing embryos. For the gain of function experiment, the
HIF-1α Regulates Cardiac Development

Constitutively active form of hif-1α (P558G) mRNA was injected into one of the two vegetal-dorsal blastomeres of embryos at the 8-cell stage. The other un-injected vegetal-dorsal blastomere of the embryos served as an internal control for the injection. Co-injection of GFP mRNA enabled us to identify the side that was derived from the blastomere injected with mRNAs. The embryos were harvested at tailbud stage 32, and analyzed by whole mount double in situ hybridization for tie-2, a specific marker of differentiating vascular cells (20), and α-globin, a hematopoietic cell marker. Overexpression of HIF-1α (P558G) augmented the formation of the vascular vitelline network (VVN) meshwork with an increase in endothelial cell number, whereas the increased HIF-1α level had little effect on the formation of the VBI (Fig. 2A, panels e–h).

We next investigated the effect of decreased expression of HIF-1α on embryogenesis using antisense morpholino oligonucleotide-mediated knockdown of HIF-1α. Two MOs, hif-1α MO1 and hif-1α MO2, were designed to inhibit HIF-1α expression. hif-1α MO1 was directed against the 5′-UTR spanning the ATG start codon, whereas hif-1α MO2 was against the non-overlapping region of the 5′-UTR with hif-1α MO1 (Fig. 2B).

The potency and specificity of these MOs were tested by an in vitro transcription and translation system. The translation of the HIF(N)-Venus protein was inhibited by hif-1α MO1 and hif-1α MO2, but not CMO (Fig. 2C). We examined the specificity of hif-1α MOs in vivo by co-injecting the 5′-UTR-HIF(N)-Venus DNA with MOs into one of the two vegetal-dorsal blastomeres at the 8-cell stage embryos. Green fluorescence from

assayed at tailbud stage 33/34 with whole mount double in situ hybridization for tie-2 and α-globin, endothelial and hematopoietic markers, respectively. Panels a–d, Xenopus embryos un-injected; panels e–h, embryos injected with hif-1α (P558G) mRNA (750 pg); and panels i and j, embryos injected with hif-1α antisense morpholino 1 (hif-1α MO1) (40 ng). MO or mRNA were injected into one of the two vegetal-dorsal blastomeres at the 8-cell stage. The embryos were

FIGURE 2. The gain- or loss-of-function of HIF-1α. A, effects of injection of hif-1α mRNAs indicated at the top on vascular development. Panels a–d, Xenopus embryos un-injected; panels e–h, embryos injected with hif-1α (P558G) mRNA (750 pg); and panels i and j, embryos injected with hif-1α antisense morpholino 1 (hif-1α MO1) (40 ng). MO or mRNA were injected into one of the two vegetal-dorsal blastomeres at the 8-cell stage. The embryos were
HIF(N)-Venus was dramatically reduced by co-injection of hif-1α MOs compared with CMO (Fig. 2D). The inhibition of HIF(N)-Venus translation in vivo was confirmed by immunoblotting using anti-GFP antibody (Fig. 2E). These results indicate the specificity and efficiency of hif-1α MOs in vivo.

We further examined the effect of hif-1α on endogenous expression of HIF-1α (Fig. 2F). HIF-1α in embryos from those treated with hif-1α MOs was significantly reduced to 75% of that treated with CMO, although it was not completely decreased, because hif-1α MOs were injected into one of the two blastomeres at the 8-cell stage embryos. These results revealed that hif-1α MOs could be capable of knockdown of HIF-1α in embryos.

Injection of hif-1α MO1 caused a marked reduction in the expression of tie-2 and α-globin, specific markers of differentiating vascular cells and hematopoietic cells, respectively, in the VVN meshwork and VBI area (Fig. 2A, panels i–l), indicating the decrease in endothelial cells and hematopoietic cells. These results imply that HIF-1α might be important for the generation of the common precursors of vascular endothelial cells and hematopoietic cells.

**Depletion of HIF-1α Results in Cardia Bifida**—Although hif-1α is expressed during cardiogenesis and in cultured embryonic ventricular myocytes (21), the role of HIF-1α in cardiac differentiation and development has been not well known. We performed knockdown of HIF-1α in *Xenopus* embryos by injecting hif-1α MO into one of the two vegetal-dorsal blastomeres. In this manner, the un-injected side serves as an internal control. Consistent with the previous report (22), these two vegetal-dorsal blastomeres contributed to heart and somitic tissues. Compared with control tadpoles at stage 42, HIF-1α-depleted tadpoles showed cardiac malformations. They had small or undetectable hearts, although wild-type embryos have beating hearts (Fig. 3A). In addition, we could not observe the blood flow in most of the HIF-1α-depleted tadpoles (Table 1).

To further investigate the role of HIF-1α in cardiac development, we examined the expression of a cardiac differentiation marker, cTnl (cardiac troponin I), at the tailbud stage (stage 33/34), at which the myocardium has formed a tube in all but the most anterior region. cTnl transcripts were restricted to two diffuse lateral patches in hif-1α MO-injected embryos (Fig. 3B, panels c and e), whereas it encompassed a large triangular area in the ventral midline in wild-type embryos (Fig. 3B, panel a). The formation of the heart tube was not observed in transverse sections of hif-1α MO-injected embryos (Fig. 3B, panels d and f). This phenotype was attributable to the failure in migration of the bilateral heart precursors into the ventral midline and fusion of these cells, which is called cardia bifida. Cardia bifida was increased by hif-1α MO injection (hif-1α MO1, 82.7%; hif-1α MO2, 52.9%), compared with control MO injection (CMO, 14.7%) (Table 2). The hif-1α MO1 was used for the following experiment as a representative of hif-1α MO. Quantitation of differentiated myocardium (marked by cTnl expression) showed that the area of the HIF-1α-depleted side was smaller (or absent) than the control side. Remarkable reduction of cTnl expression was observed in 92% of embryos (supplementary Fig. S1 and supplementary Table 1). At the later stage (stage 42), the area of cTnl expression was smaller or not detected at the midline in the hif-1α MO1-injected embryos, compared with CMO-injected embryos (supplementary Fig. S2, a–c). The transverse section more clearly showed that the pre-cardiac region was derived from the blastomere, where hif-1α MO injection was small or undetectable and lay asym-

### TABLE 1

**Summary of cardiac defects found in *Xenopus* injected with antisense morpholino against HIF-1α**

*Xenopus* embryos at 8-cell stage were microinjected with hif-1α morpholino oligonucleotides (hif-1α MO1, n = 41; hif-1α MO2, n = 40). Morpholino-injected embryos were subjected to morphological analysis at stage 41.

| Phenotype                  | hif-1α MO1 Count | hif-1α MO1 % | hif-1α MO2 Count | hif-1α MO2 % |
|----------------------------|-----------------|--------------|-----------------|--------------|
| Heart, no beating          | 20/41           | 48.7         | 20/40           | 50.0         |
| Heart, small               | 37/41           | 90.2         | 35/40           | 87.5         |
| Blood, no circulation      | 27/41           | 65.8         | 28/40           | 70.0         |

### TABLE 2

**Frequency of cardia bifida in embryos injected with antisense morpholino against HIF-1α**

*Xenopus* embryos at 8-cell stage were microinjected with morpholino oligonucleotides (CMO, n = 34; hif-1α MO1, n = 75; hif-1α MO2, n = 68). All embryos were assayed for expression of cTnl using whole mount *in situ* hybridization at stage 33/34. The frequency of cardia bifida was increased by hif-1α MOs injection compared with CMO injection (refer to Fig. 3B).

| Heart morphology | CMO Count | CMO % | hif-1α MO1 Count | hif-1α MO1 % | hif-1α MO2 Count | hif-1α MO2 % |
|------------------|-----------|-------|-----------------|--------------|-----------------|--------------|
| Normal heart     | 29/34     | 85.3  | 13/75           | 17.3         | 32/68           | 47.1         |
| Cardia bifida    | 5/34      | 14.7  | 62/75           | 82.7         | 36/68           | 52.9         |
HIF-1α Regulates Cardiac Development

In situ hybridization analyses revealed that the expression of these heart markers except bmp4 was dramatically reduced at the side derived from the blastomere where hif-1α MO1 was injected (Fig. 4). bmp4 expression was reduced in a few embryos (13%) (Table 3). The frequency of the reduced expression of gata4, hand1, hand2, and tbx5 at the region was 66.7, 43.1, 43.9, and 50.0%, respectively. Notably, nkx2.5 was reduced in most of the embryos (93.7%). Reduction of tbx5, hand1, and hand2 expression appeared to be attributable to the inhibition of cardiomyocyte differentiation at an earlier stage, as revealed by the reduced expression of gata4 and nkx2.5. These findings, therefore, prompted us to examine whether HIF-1α regulates the expression of gata4 and nkx2.5 during early heart development.

Regulation of the Nkx2.5 Gene Promoter by HIF-1α—The expression of nkx2.5 was significantly reduced by depletion of HIF-1α, raising the possibility that HIF-1α regulates the expression of nkx2.5. To elucidate the role of HIF-1α on Nkx2.5 expression, we examined the promoter activity of Nkx2.5 in cultured CV-1 cells. Nkx2.5 promoter-Luc, the 9-kb upstream region of the mouse Nkx2.5 gene, was cloned into a promoter-less luciferase reporter gene vector, pGL4.10 vector (Nkx2.5 promoter-Luc). Nkx2.5 promoter-Luc was co-transfected into CV-1 cells with control vector (pIREs puro3), mouse Hif-1α, Gata4, and Smad4. Hif-1α, Gata4, and Smad4 increased the promoter activity of Nkx2.5 (Fig. 5A). These results suggest that HIF-1α controls the expression of Nkx2.5.

Thus, we tried to investigate HIF-1α-regulated nkx2.5 gene expression in Xenopus embryos. The expression profile of nkx2.5 during Xenopus development was examined. The nkx2.5 expression started at early neural plate stage 13 and remained throughout tail-bud stage 25, with a peak in neural tube stage 17–21, as assessed by RT-PCR (Fig. 5B). We considered that the embryos at stage 19–21 were suitable for Nkx2.5 promoter assay according to the result of nkx2.5 expression.

Because it has been reported that several regulatory elements are conserved in the Nkx2.5 promoters of human, mouse, and Xenopus (31) and that structural domains are conserved in HIF-1α among them (Fig. 1A), we assumed that the mouse Nkx2.5 promoter could reflect the endogenous Xenopus HIF-1α function in embryos. To visualize Nkx2.5 promoter activity in embryos, we constructed the plasmid containing Nkx2.5 promoter-Venus. It allowed us to visualize Nkx2.5 promoter activity by yellow fluorescence in developing embryos. Nkx2.5 promoter activation was indeed observed around the cardiac primordial region of embryos at stage 20 (Fig. 5C, panels a and b), consistent with that found in transgenic mice harboring the transgene, −9 kb of Nkx2.5 5′ flanking sequence linked to the reporter gene (12).

### Table 3

| Hybridization probe | Count | % of embryos with reduced expression |
|---------------------|-------|--------------------------------------|
| nkx2.5              | 82/88 | 93.2                                 |
| gata4               | 44/66 | 66.7                                 |
| hand1               | 25/58 | 43.1                                 |
| hand2               | 18/41 | 43.9                                 |
| tbx5                | 17/34 | 50.0                                 |
| bmp4                | 3/23  | 13.0                                 |

K. Nagao, Y. Taniyama, and R. Morishita, unpublished observations.
HIF-1α Regulates Cardiac Development

We furthermore, examined the effect of hif-1α MO on the nkx2.5 transcriptional activity in *Xenopus* embryo. Nkx2.5 promoter-Luc or pGL4.10 vector was injected into one of the two vegetal-dorsal blastomeres of the 8-cell stage *Xenopus* embryos. Significant activation of the luciferase was observed in Nkx2.5 promoter Luc-injected embryos (Fig. 5D). Of importance, luciferase activity was decreased in the presence of hif-1α MO1 (Fig. 5E). To confirm this finding in vivo, we injected Nkx2.5 promoter-Venus with hif-1α MO and found that the fluorescence around the precardiac region was suppressed by depletion of HIF-1α (Fig. 5C, panel d). Consistently, RT-PCR analysis revealed the reduction of Nkx2.5 mRNA expression in hif-1α MO-injected embryos at stage 20 (Fig. 5F).

We supposed that HIF-1α is required for cardiac differentiation via nkx2.5. Upon this hypothesis, we examined whether overexpression of nkx2.5 would preserve the failure of cardiac development caused by depletion of HIF-1α. Both hif-1α MO and nkx2.5 mRNAs were injected into one of the two vegetal-dorsal blastomeres of the embryos at the 8-cell stage. The phenotype caused by depletion of HIF-1α was efficiently rescued by co-injection with the nkx2.5 mRNA (Fig. 6A, 66.3% of embryos showed cardia bifida, n = 217; 125 pg, 23.8%, n = 109; and 250 pg, 21.4%, n = 56). Injection of only nkx2.5 mRNA (250 pg) never affected development of the heart (Fig. 6A, 0%, n = 50).

Because NkX2.5 might be responsible for the phenotype caused by depletion of HIF-1α during cardiac differentiation, we examined the effect of NkX2.5 knockdown on heart development. Similar to HIF-1α-depleted embryos, the embryos injected with nkx2.5 MO (40 ng) showed cardia bifida at stage 33/34 (Fig. 6B, C, and D). However, it is not clear whether GATA4 would have the responsibility for the phenotype caused by depletion of HIF-1α. Although we tried to rescue cardia bifida by injecting gata4 mRNA, injection of gata4 mRNA induced severe gastrulation defects leading to death.3

**DISCUSSION**

We identified HIF-1α as a regulator of cardiac development, as well as endothelial and blood cell differentiation using *Xenopus* embryos. Overexpression of HIF-1α induced the fine structure of the vascular VVN meshwork. In contrast, knockdown of HIF-1α resulted in the decrease in the number of both endothelial cells and blood cells. These results clearly indicate that HIF-1α controls the endothelial cell number and the maturation of blood vessels. In addition, HIF-1α-depleted embryos exhibited the inhibition of cardiac differentiation and showed no heart formation.
HIF-1α Regulates Cardiac Development

**A**

![Bar chart showing the percentage of embryos with cardiobifida](image)

**B**

![Images of embryos](image)

**C**

![Bar chart showing the percentage of embryos with cardiobifida](image)

**FIGURE 6.** **Nkx2.5 functions downstream of HIF-1α.** A, cardiobifida in HIF-1α-depleted embryos was rescued by expression of nkk2.5. Embryos were injected with hif-1α MO1 (40 ng) and nkk2.5 mRNA (0, 125, and 250 pg). The expression pattern of Ctnl was analyzed by in situ hybridization at stage 33/34 to detect the precise migration of cardiomyocytes. Cardiobifida caused by depletion of HIF-1α was restored by co-injection of nkk2.5 mRNA. B, depletion of Nkx2.5 results in cardiobifida in *Xenopus* embryos. CMO-injected embryos (a), nkk2.5 MO-injected embryos (b), and hif-1α MO1-injected embryos (c) were examined for Ctnl by in situ hybridization at stage 33/34. Nkx2.5-depleted embryos showed a similar staining pattern of Ctnl to HIF-1α-depleted embryos. Yellow arrowheads indicate the side derived from the blastomere where hif-1α MO1 was injected. C, the incidence of cardiobifida in nkk2.5 MO-injected embryos, hif-1α MO-injected embryos, or CMO-injected embryos.

cardiobifida. A previous study reports that HIF mediates generation of mesoderm and that blood and vascular progenitor cells are crucial for early embryonic development (17). Several recent studies reveal that cardiomyocytes, endothelial cells, and smooth muscle cells arise from a common progenitor (32–34). We demonstrated that loss of HIF-1α affects the development of endothelial cells/blood cells and cardiomyocytes, suggesting that HIF-1α commonly regulates progenitors originated from the mesoderm.

We reported here that depletion of HIF-1α becomes the cause of cardiobifida in *Xenopus*. During cardiac development, splanchnic mesoderm symmetrically located on either side of the midline is committed to form cardiac tissue (35). The paired cardiogenic tissue migrates ventrally until it fuses in the ventral midline of the embryo (36, 37). Following fusion, the heart tube loops to the right, converting the initial anterior-posterior pattern into left-right symmetry. Subsequently, it remodels into a multichambered heart, which is required for a functional organ (38). Disruption of one of these processes in vertebrates, failure of bilateral symmetrical heart primordial to fuse, has been shown in cardiobifida. This phenotype is known to be caused by defects in cardiac differentiation, endoderm differentiation, extracellular matrix deposition, or migration of the primordial heart. For instance, mice showing cardiobifida are caused by mutations of *Gata4* (39), *Mespl* (40), *furin* (41), *N-cadherin* (42), or *fibronectin* (43). In *Xenopus* embryos, cardiobifida is caused by disruption of BMP signaling using an antagonist (SMAD6) or a dominant negative mutant, truncated BMP receptor (24, 44). It is also caused by the inhibition of *GATA6* (45) or WNT11 (46), non-canonical WNT signaling.

We propose here cardiac defects due to depletion of HIF-1α attributable to incomplete cardiomyocyte differentiation. Early cardiac gene expression is needed for the maintenance and activation of cardiac mesoderm differentiation (47). Depletion of HIF-1α resulted in the reduction of early cardiac gene expression in hif-1α MO-injected embryos (Fig. 4). On the other hand, embryos with the reduced *wnt11*-Related gene (*wnt11-R*) expression exhibited cardiobifida, but not the inhibition of cardiac differentiation (48). In that study, aberrant contact within the myocardial wall leads to defects in fusion of the heart tube. Comparing these results with our present results, we suggested that cardiobifida observed in HIF-1α-depleted embryos may be due to the inhibition of cardiomyocyte differentiation in the one side derived from hif-1α MO-injected blastomere before the migration into midline, because several cardiac genes were down-regulated in the side depleted of HIF-1α.

*Nkx2.5*, one of the earliest genes to be expressed specifically in cardiac precursor cells (49), is necessary for cardiac primordial differentiation and fusing into the single heart tube in *Xenopus*. Mutant embryos *tinman*, the divergent homeodomain homolog of Nkx2.5, fail to develop a heart (50, 51). We noticed the reduced expression of nkk2.5 in HIF-1α-depleted embryos. Interestingly, co-injection of Nkx2.5 mRNA prevented cardiobifida caused by depletion of HIF-1α (Fig. 6A). These results suggest that Nkx2.5 is downstream of HIF-1α signaling. The *Xenopus* embryos injected with the dominant negative form of nkk2.5 exhibited un-fused cardiac primordial with reduced Ctnl staining (52, 53). Our HIF-1α-depleted embryos exhibited a similar Ctnl staining pattern. We further confirmed that Nkx2.5 depletion with nkk2.5 MO inhibited the migration of heart primordial cells into the midline in *Xenopus* embryos (Fig. 6, B and C). We assume that Nkx2.5 is required as transcriptional activators for the earliest stages of heart formation before cardiac primordial migrates into midline. However, in the mouse study, the targeted ablation of Nkx2.5 does not inhibit the formation of the heart tube, although it blocks cardiac development at the stage of looping morphogenesis. This difference might be due to the redundancy in the mouse system, because Nkx2.5 is critical for the initiation of cardiac cell fate decisions.

How does HIF-1α regulate the nkk2.5 gene? Regulation of the *Nkx2.5* gene is highly complex, because there are multiple enhancers/repressor-binding sites in the regulatory regions of the mouse *Nkx2.5* gene. Several consensus binding sites for GATA factors and SMADs have been functional in driving the expression of the mouse *Nkx2.5* (26–30). We suggest that HIF-
1α-regulated nkx2.5 expression is not mediated by BMP4, because the embryos injected with hif-1α MO showed no change in the expression of bmp4 (Fig. 4). In the present study, depletion of HIF-1α affected nkx2.5 expression by suppressing its promoter activity in developing Xenopus embryos. To identify the HIF-1α binding region in the promoter, we constructed a series of deletion constructs of the Nkx2.5 promoter. Deletion from −9.5 to −2.5 kbp did not affect luciferase activity, whereas deletion from −9.5 to −1.2 kbp eliminated the activity, suggesting that the region from −2.5 to −1.2 kbp might be responsible for HIF-1α signaling. Although HIF-1α depletion inhibited gata4 expression (Fig. 4), we could not demonstrate the relationship between HIF-1α and GATA4 acting upstream of Nkx2.5. Further studies are required to explore whether Nkx2.5 is directly or indirectly regulated by HIF-1α.

Understanding the individual modular steps in cardiac morphogenesis is particularly relevant to congenital heart disease, which usually involves defects in specific structural components of the developing heart. The present study would provide new information that regulation of Nkx2.5 by HIF-1α is necessary for normal cardiac development.

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