Molecular Medicine

Essential Role of Developmentally Activated Hypoxia-Inducible Factor 1α for Cardiac Morphogenesis and Function

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Abstract—Development of the mammalian heart is governed by precisely orchestrated interactions between signaling pathways integrating environmental cues and a core cardiac transcriptional network that directs differentiation, growth and morphogenesis. Here we report that in mice, at about embryonic day (E)8.5 to E10.0, cardiac development proceeds in an environment that is hypoxic and characterized by high levels of hypoxia-inducible factor (HIF)1α protein. Mice lacking HIF1α in ventricular cardiomyocytes exhibit aborted development at looping morphogenesis and embryonic lethality between E11.0 to E12.0. Intriguingly, HIF1α-deficient hearts display reduced expression of the core cardiac transcription factors Mef2C and Tbx5 and of titin, a giant protein that serves as a template for the assembly and organization of the sarcomere. Chromatin immunoprecipitation experiments revealed that Mef2C, Tbx5, and titin are direct target genes of HIF1α in vivo. Thus, hypoxia signaling controls cardiac development through HIF1α-mediated transcriptional regulation of key components of myofibrillogenesis and the cardiac transcription factor network, thereby providing a mechanistic basis of how heart development, morphogenesis, and function is coupled to low oxygen tension during early embryogenesis. (Circ Res. 2008;103:1139-1146.)

Key Words: cardiac development  ■ hypoxia  ■ transcription  ■ myofibrillogenesis  ■ HIF1α

Cardiac development is a multi-stage process governed by a network of transcription factors including Nkx2.5, Tbx5, Mef2C, and GATA4 that connect signaling pathways with the regulation of gene expression for the specification of cardiac cell fate, differentiation and morphogenesis. These transcription factors act in an interconnected and coordinated manner at specific stages of cardiogenesis. Although Nkx2.5 and GATA4 activity is predominantly associated with cardiac lineage specification and differentiation, it is also required for induction of downstream cardiogenic transcription factors such as Tbx5 and Mef2C, whose function is primarily linked to looping morphogenesis and chamber formation.1 Thus specific transcription factors induce the expression of genes necessary for progression through the respective stages of cardiogenesis. How the expression and activity of these transcription factors are coordinated spatially and temporally in the course of cardiogenesis remains less clear.

A central feature of mammalian embryogenesis is the development of intraembryonic hypoxia.2 As the embryo increases in size, oxygenation of the avascular early postimplantation embryo by diffusion alone becomes limited, leading to a state of regional embryonic hypoxia. In normal development, regional hypoxia has been implicated to serve as a stimulus for the tissue/region-specific induction of genes required for yolk sac vasculogenesis,3 the establishment of the maternal-fetal circulatory network4 and the initiation of intraembryonic circulation and cardiac function.5,6 Thus, the embryonic hypoxic phase is characterized by the coordinated development of multiple components of the embryonic cardiovascular system with the aim of facilitating intraembryonic oxygenation and relieving the hypoxic stress. Because of the critical role of the heart in oxygen and nutrient conduction, it has been hypothesized that adaptive processes to low oxygen tension occurring early in development could potentially be critical for cardiac development by serving as a stimulus for the induction of transcription factors and structural proteins required for development of the mature heart and the initiation of rhythmic contraction.7

A central mediator of adaptive responses to low oxygen tension is HIF, a heterodimeric transcription factor composed of HIF1α or HIF2α and HIFβ/ARNT subunits. The HIFα subunits are under exquisite oxygen control in that they accumulate rapidly in response to hypoxia to induce an adaptive transcription program. Complete deficiency of

Original received February 20, 2008; revision received September 29, 2008; accepted September 29, 2008.
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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/01.RES.0000338613.89841.c1
HIF1α in mice results in embryonic lethality around embryonic day (E)10.0, characterized by a broad spectrum of abnormalities including striking retarded development, neural tube defects, dysfunctional vasculogenesis and angiogenesis, a reduction in somite numbers and cardiovascular malformations. The main defect observed in HIF1α deficient hearts relates to the absence of a patent ventricular lumen and outflow tract caused by hyperproliferation of myocardial cells. In contrast, mouse embryos lacking HIF2α die between E16.5 and postnatally, and do not display early cardiac developmental or morphological defects. Although one might infer from these studies a possible role specifically for HIF1α in early cardiogenesis, the detailed molecular mechanism underlying a requirement for HIF1α in cardiac developmental processes remains to be defined. Therefore, we generated a ventricular-restricted conditional disruption of HIF1α in the mouse to study its impact on cardiac development. Our results suggest a key role for HIF1α in the regulation of core cardiac transcription factor network and the process of myofibrillogenesis through direct HIF1α-mediated activation of the Mef2C, Tbx5, and titin genes, respectively.

Materials and Methods

Mice and Physiological Measurements

C57/Black6 mice were from Elevage Janvier and the ROSA26 Cre reporter (R26R) line was from The Jackson Laboratory. HIF1α+/ and HIF1α+/− mice were obtained from Randall S. Johnson (University of California, San Diego) and Gregg L. Semenza (Johns Hopkins University School of Medicine, Baltimore, Md), respectively, whereas the MLC2vcre−/− line was from Ju Chen (University of California, San Diego). All mice were maintained at the Institute of Cell Biology, ETH-Zurich specific pathogen-free facility in full compliance with guidelines approved by the Swiss Federal Veterinary Office (SFVO). Embryonic echocardiography measurements were performed on the VisualSonics Vevo 770 machine essentially as recommended by the manufacturer.

Antibodies

Myomesin and EH-myomesin antibodies were generated in-house. The MyBPC antibody was a kind gift from Mathias Gautel (King’s College, London, UK). Antibodies against sarcomeric α-actinin, titin and Ser10-phosphorylated histone H3 were from Sigma, LGC Promochem and Upstate Biotechnology, respectively. Antibodies used in the ChIP assay against HIF1α were from Santa Cruz Biotechnology.

Luciferase Promoter Assays

One kb of the Mef2C and 2kb of the Tbx5 promoter was amplified from mouse genomic DNA and cloned into the pGL3 luciferase reporter vector (Stratagene). The 5.87 kb titin promoter was a kind gift from Gregory A. Cox (The Jackson Laboratory) and was subcloned in its entirety into pGL3. HRE- mutants of the respective promoters were generated by recombinant PCR. Wild-type and mutant CXXCR4 reporters were generated as described, and subcloned into pGL3. The ANF-Luc and the ANF ΔTRE-Luc reporters were generously provided by David Brook (University of Nottingham, UK), whereas the 3xMEF reporter was a kind gift from Eric N. Olson (UT Southwestern Medical Center at Dallas). The HIF1α (P402A/P577A) expression construct was generated as described, whereas the HIF1α (ΔODD) expression construct was kindly provided by H Franklin Bunn (Harvard Medical School).

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Developmental Myocardial Hypoxia Induces HIF1α Accumulation

Pimonidazole, a nitroimidazole-derivative that incorporates in hypoxic cells below a PO2 of 10 mm Hg was used to assess the oxygenation state of E9.5 wild-type mouse embryos. The specificity of pimonidazole incorporation under conditions of hypoxia was confirmed by subjecting primary neonatal mouse cardiomyocytes (NMCs) to 1% oxygen and assessing levels of pimonidazole adduct formation (Figure 1 in the online data supplement). Incorporation of pimonidazole in the whole embryo was restricted to the developing limb buds and the heart, where colocalization was observed with the embryonic cardiomyocyte marker EH-myomesin (Figure 1A). Sections of embryos revealed myocardial pimonidazole incorporation at E9.0 and E10.0 (supplemental Figure II). Negligible incorporation was observed beyond E11.0 (data not shown). Thus the early embryonic heart develops in hypoxia.

To ascertain if myocardial hypoxia was associated with HIF1α accumulation, whole mount immunostaining of wild-type hearts between E8.0 to E11.5 were performed. HIF1α was first detectable in nuclei of cardiomyocytes at E8.0. Its levels increased dramatically at embryonic days E8.5 and E9.5 and diminished thereafter (E10.5) to become undetectable by E11.5 (Figure 1B). Specificity of the HIF1α antibody was confirmed by whole-mount immunostaining of HIF1α+/−/− hearts and by subjecting NMCs to hypoxia (supplemental Figure III, top and bottom).

Although levels of HIF1α mRNA remained relatively constant at the respective time points as analyzed by semi-quantitative (Figure 1C) and quantitative (Figure 1D) RT-PCR, immunoblotting of myocardial extracts revealed a similar pattern of HIF1α protein abundance changes as seen in immunohistochemical sections (Figure 1E). These results suggest a close temporal link between a specific window of time during heart development where the developing myocardium is hypoxic and the consequent accumulation of HIF1α.

HIF1α Is Required for Cardiac Morphogenetic and Functional Development

Next we generated ventricular-restricted HIF1α-deficient mice by mating HIF1α floxed allele mice (HIF1αfl/fl) with the well-characterized myosin light chain-2v (MLC2v) cre recombinase knock-in mouse line (MLC2v-crefl/fl/cre)17,18 with consistent with a previous study20 HIF1αfl/flcre+ embryos were viable and did not exhibit any overt phenotype. Analysis of hearts at E10.0 of HIF1αfl/flcre+ mice however revealed inefficient excision of the HIF1α gene (data not shown) suggesting that although MLC2v-driven cre expression is initiated at E8.251 early expression of cre recombinase is low. To circumvent the low efficiency of cre recombinase activity and achieve efficient deletion of the HIF1α gene by around E8.5 to E10.5, the time during which myocardial hypoxia is apparent, we generated mice that are floxed at 1 HIF1α allele and null on the other (referred to as HIF1α−/−) on
the MLC2v-cre<sup>−/−</sup> background (referred to as HIF1α<sup>−/−</sup>/cre<sup>−/−</sup>). This strategy permitted efficient excision of the HIF1α floxed allele and ablation of HIF1α mRNA (Figure 3A). Specificity and efficiency of MLC2v-cre<sup>−/−</sup>-mediated recombination for the myocardium was confirmed by crossing MLC2v-cre<sup>−/−</sup> mice with the R26R reporter mouse line (Figure III, middle, and supplemental Figure IV).<sup>22</sup> Whereas HIF1α<sup>−/−</sup>/cre<sup>−/−</sup> offspring were phenotypically normal and present at the expected Mendelian frequency, HIF1α<sup>−/−</sup>/cre<sup>−</sup> neonates were not found among 148 offspring.

**Figure 1.** The early embryonic heart is hypoxic. A and B, The tissue hypoxia marker pimonidazole was delivered intraperitoneally to wild-type pregnant C57/Black6 mice 3 hours before euthanasia at the respective time points. A, E9.5 whole embryo mounts were stained for colocalization of pimonidazole and EH-myomesin. Top, Overall pimonidazole incorporation in the embryo. Bottom, Colocalization of pimonidazole incorporation in the myocardium. B, Wild-type embryos were harvested at the respective time points and whole mounts stained for HIF1α, sarcomeric α-actinin, and DAPI. C and D, Ventrices at the respective developmental stage were assayed for relative levels of HIF1α mRNA by semiquantitative RT-PCR (C) and quantitative RT-PCR (D). E, HIF1α protein accumulation at the respective time points was assessed by immunoblotting (top) and quantified (bottom). A representative immunoblot is shown.

**Figure 2.** HIF1α-deficient hearts exhibit aborted development at looping morphogenesis. A, E10.0 HIF1α<sup>−/−</sup>/cre<sup>−</sup> control and HIF1α<sup>−/−</sup>/cre<sup>−</sup> embryos were dissected and photographed from the right (R) and left (L). a-, v-, and ot- indicate atria, ventricle, and outflow tract, respectively. B, E10.0 control HIF1α<sup>−/−</sup>/cre<sup>−</sup> and HIF1α<sup>−/−</sup>/cre<sup>−</sup> hearts are shown and coronal sections of the respective planes of the ventricle (I and II) are shown. C and D, Ventricles of HIF1α<sup>−/−</sup>/cre<sup>−</sup> and HIF1α<sup>−/−</sup>/cre<sup>−</sup> embryos were dissected, dissociated, and stained for sarcomeric α-actinin and Ser10-phosphorylated histone H3 and analyzed by FACS. C, Red circles indicate the sarcomeric α-actinin+Ser10–phosphorylated histone H3<sup>−</sup> double positive population present in hearts of the respective genotype. D, Quantification of the fraction of the double positive population of the respective genotype is shown. E, E10.0 whole mounts of control HIF1α<sup>−/−</sup>/cre<sup>−</sup> and HIF1α<sup>−/−</sup>/cre<sup>−</sup> hearts stained for titin and EH-myomesin expression and imaged by confocal microscopy. All samples were counterstained for DAPI.
cardiac looping occurred normally in HIF1α−/cre− embryos. The decrease in titin expression was restricted to the atria of HIF1α−/cre− embryos. Instead, these hearts developed a single ventricular chamber fused directly to the atria (Figure 2A and 2B), with little evidence of distinct right and left ventricles. Moreover, at E9.5 to E10.5 when the heart of HIF1α−/cre− control embryos exhibited rhythmic contractions, the hearts of HIF1α−/cre− littersmates did not and continued to display peristaltic-like contractions reminiscent of the immature tubular heart (supplemental Movies I and II and expanded Materials and Methods section). HIF1α−/cre− embryos died between E11.5 to E12.0 as a result of cardiac contractile dysfunction. These findings suggest that HIF1α function is essential for normal heart development, in particular for the progression from the looping heart tube stage to ventricular and atrial chamber formation.

Hearts of HIF1α−/cre− embryos also exhibited hyperplasia of the ventricular myocardium resulting in the complete absence of a ventricular cavity (Figure 2B and supplemental Figure V). At E10.0, the ventricular myocardium is normally comprised of 2 to 3 cell layers enveloping the ventricular cavity in which blood is collected and channeled to the atria. Consistent with the above-noted observation, we found a significant higher fraction of actively proliferating ventricular cardiomyocytes in hearts of HIF1α−/cre− embryos at E10.0 than in hearts of HIF1α−/cre− control embryos as indicated by the increased fraction of cells positive for serum10-phosphorylated histone H3, a marker of mitotically active cells, and sarcomeric α-actinin (Figure 2C and 2D). Thus, accumulation of HIF1α during heart development appears to also be critical for constraining uncontrolled ventricular cardiomyocyte proliferation.

As hearts of HIF1α−/cre− embryos lacked the characteristic contractile profile of a functional heart, we asked if the contractile defect occurred as a result of impaired cardiomyocyte myofibrillogenesis. Proper expression and assembly of myofibrillar proteins at the sarcomere is a prerequisite for cardiac contractility and morphological integrity of cardiomyocytes. We assessed the expression of 3 essential sarcomeric components - titin, EH-myomesin and α-actinin. Titin is a giant protein that anchors in the Z-disk and extends to the M-line region of the sarcomere forming a continuous filament along the entire length of the myofibril to provide the structural foundation on which all other myofibrillar proteins including myomesin and α-actinin attach and are assembled in the sarcomere. Histological examination of HIF1α−/cre− embryos indicated a profound decrease in titin protein levels (Figure 2E). No such decrease was seen in HIF1α−/cre− embryos. The decrease in titin expression was restricted to the ventricular myocardium as the atria of both control HIF1α−/cre− and HIF1α−/cre− embryos exhibited normal titin expression and organization (Figure 2E). The expression of 2 other sarcomeric proteins, EH-myomesin (Figure 2E) and α-actinin (supplemental Figure VI), were not dramatically affected in ventricular cardiomyocytes. However, the staining patterns of EH-myomesin and α-actinin unveiled disorganized myofibrils and the complete absence of sarcomere assembly (Figure 2E and supplemental Figure VI). Again, cardiomyocytes of the atria of HIF1α−/cre− embryos displayed properly organized myofibrils as evidenced by staining for EH-myomesin and α-actinin. Together, these results suggest that HIF1α acts to maintain the expression of the sarcomeric protein titin, but not that of the sarcomeric proteins EH-myomesin and α-actinin, during cardiac development. The reduced expression of titin likely contributes to impaired myofibrillogenesis and failure of HIF1α-deficient hearts to complete looping morphogenesis and exhibit normal contractility.
HIF1α Regulates the Expression of Core Cardiogenic Transcription Factors, Cell Cycle Mediators, and Structural Proteins

To identify the molecular basis underlying the effects of HIF1α on myofibrillogenesis, cell proliferation and looping morphogenesis, we compared the expression of a set of genes encoding sarcomeric components, cell cycle regulatory proteins and core cardiac transcription factors in the hearts of HIF1α+/cre− control embryos and HIF1α+/cre+ littermates by semiquantitative RT-PCR (Figure 3A) and quantitative RT-PCR (Figure 3B). Whereas expression of the developmentally predominant cardiac titin N2B isoform24 was present in HIF1α+/cre− control ventricles, it was markedly reduced in HIF1α-deficient ventricles. To rule out the possibility that in HIF1α+/cre− ventricles the lack of titin N2B isoform expression is compensated by that of other titin isoforms, HIF1α+/cre control and HIF1α+/cre− ventricles were analyzed for expression of the titin protein kinase domain, a region ubiquitous to all titin isoforms (Figure 3A and 3B).25

Expression of the titin protein kinase domain was similarly reduced in HIF1α-deficient hearts. Although mRNA levels of α-actinin, myomesin and myosin binding protein C (MyBPC) genes remained unaffected by lack of HIF1α (Figure 3A and 3B), accumulation of myomesin and α-actinin protein was decreased (Figure 3C) which may relate to posttranslational mechanisms regulating the stability of sarcomeric proteins in the absence of its myofibril incorporation26 because of lack of normal titin expression. Genes associated with myocardial cell proliferation such as cyclin D1 and CDK4 were significantly upregulated, whereas genes encoding the cyclin-dependent kinase inhibitors p27 and p21 were downregulated (Figure 3D and 3E). This expression pattern is in accord with the hyperplasia observed in the ventricular myocardiun of HIF1α-deficient embryonic hearts (Figure 2B through 2D), the effect of HIF1α deficiency during chondrogenesis18 and hypoxia-induced growth arrest.27 Intriguingly, transcript levels of the cardiogenic factors Nkx2.5, Tbx5, Met2C were almost absent in HIF1α+/cre− ventricles compared to control HIF1α+/cre− ventricles (Figure 3D and 3E). The expression of GATA4, another member of the core cardiac transcription factor network implicated in cell specification and determination28 was however not changed in the mutant hearts, suggesting that its expression is not dependent on HIF1α (Figure 3D and 3E). These analyses suggest that HIF1α-deficiency affects, directly or indirectly, the expression of at least 1 core structural gene as well as genes orchestrating the core cardiac transcription program.

HIF1α Directly Interacts With the Met2C, Tbx5, and Titin Promoters In Vivo

The dramatic differences in Tbx5, Met2C, and titin gene transcription in HIF1α-deficient and wild-type embryonic hearts, and the fact that HIF1α+/cre− and HIF1α+/cre+ exhibit comparable levels of Tbx5, Met2C, and titin transcription before initiation of MLC2v-driven Cre recombinase expression (supplemental Figure VII) suggests that these genes might be direct targets of HIF1α in vivo. Moreover, Tbx5, Met2C, and titin transcription is dysregulated post-MLC2v-driven Cre recombinase expression but before development of defective cardiac morphogenesis (supplemental Figure VIII), further supporting the notion that downregulation of Tbx5, Met2C, and titin expression observed at E9.5 to 10.0 occurred as a result of Cre recombinase-mediated HIF1α excision and not as a consequence of the morphologically defective state of the HIF1α+/cre− hearts. Analysis of the promoters of mouse Tbx5, Met2C, and titin genes revealed the existence of potential hypoxia response elements (HREs).29 These putative HREs were present at sites −293 and −1514 in Tbx5, 445 in Met2C and −37 and −462 in titin relative to the transcription start site (supplemental Figure IX). The HREs were contained within promoter regions of Tbx5, Met2C, and titin that support transcription of the respective genes in vivo.10−32 HREs were similarly conserved in the respective promoters of the human Tbx5, Met2C, and titin genes (supplemental Figure IX). We performed Chromatin Immunoprecipitation (ChIP) experiments on nuclear extracts of E10.0 control HIF1α+/cre− ventricles using antibodies against HIF1α or its heterodimerizing partner HIFβ/ARNT. Strikingly, ChIP assays showed that both HIF1α and its heterodimerization partner HIFβ associated with certain HREs in the Met2C (Figure 4A), Tbx5 (Figure 4B), and titin promoters (Figure 4C) in native chromatin. Although the expression of Nkx2.5 changed as a function of HIF1α, we failed repeatedly to detect HIF1α binding to the Nkx2.5 promoter (data not shown). These data support the conclusion that the Tbx5, Met2C, and titin genes are direct transcriptional targets of HIF1α in vivo.

To corroborate the above finding for Met2C, Tbx5, and titin, the corresponding regulatory sequences encompassing HRE elements occupied by HIF1α in vivo (as evidenced by ChIP assays) were fused to the luciferase reporter gene and transfected into NMCs and in C2C12 myoblasts (data not shown). When these reporter constructs were cotransfected with an expression plasmid harboring the HIF1α mutant species, HIF1α(P402A/P577A) which contains alanine substitutions at the proline hydroxylation sites Pro402 and Pro577 and thus escapes prolyl-hydroxylation dependent ubiquitin-mediated degradation15) or subjected to hypoxia (1% O2), the promoter activity of Met2C, Tbx5, and titin increased (Figure 4D through 4F, respectively). Mutation of the HRE element in the respective promoters caused significantly reduced responsiveness of the luciferase reporter to HIF1α(P402A/P577A) or hypoxia (Figure 4D through 4F, respectively). Similar results were obtained on transfection with the constitutively active HIF1α(ΔODD) mutant lacking the oxygen-dependent degradation domain (Figure 4F). To further confirm a direct link between HIF1α levels with induction of Met2C, Tbx5, and titin promoter activation, increasing amounts of the HIF1α(ΔODD) expression construct was cotransfected into NMCs in the presence of wild-type Met2C, Tbx5 or titin luciferase reporter constructs, respectively (Figure 4G through 4I). As shown, a dose-dependent increase in Met2C, Tbx5, and titin reporter activity was observed in response to increasing amounts of HIF1α(ΔODD).

In addition, ectopic expression of HIF1α induced the activation a synthetic promoter containing Met2 response
elements (MREs) (Figure 5A). HIF1α(P402A/P577A) also activated the promoter of the ANF gene, an established Tbx5 target in a Tbx5 response element (TRE)-dependent manner (Figure 5B). Wild-type and HRE-mutated luciferase reporters of the respective HIF1α/H9251 constructs and of the hypoxic (Figure 5C). Together, these results strengthen the view that the Tbx5, Mef2C, and titin represent novel direct transcriptional target genes of HIF1α.

Discussion

Here we have demonstrated in the context of heart development how embryonic hypoxia spatially effects cardiac oxygenation and serves as a stimulus for the accumulation and transcriptional activity of the oxygen sensitive factor, HIF1α. Loss of HIF1α in ventricular cardiomyocytes leads to severe cardiac-specific developmental and morphological defects. In contrast to the systemic HIF1α null phenotype where a dramatic reduction in embryonic growth, cardiovascular and neural tube development defects were reported, ventricular-specific deletion of HIF1α did not lead to these defects. Rather, the restricted loss of HIF1α in ventricular cardiomyocytes led specifically to defective cardiac development. Although profound cardiac developmental defects were reported in HIF1α knockout embryos, these studies suffer from the fact that the cardiac phenotype was observed in the context of a systemic lack of HIF1α. The additional defects in embryonic angiogenesis and neural tube morphogenesis in these embryos makes interpretation of the heart phenotype difficult because of the contribution of the vascular and neural cell population to embryonic circulation and development of the secondary heart field, respectively. Thus, by specifically deleting HIF1α in ventricular cardiomyocytes we uncoupled the effects of other components of the embryonic cardiovascular system and specifically addressed the contribution of HIF1α in the development of the primary heart field.

On the basis of our findings, we propose now a model of how the hypoxic environment in the developing heart contributes to cardiac morphogenesis and function. It involves tightly coupled direct interactions between an oxygen-sensitive transcription factor, HIF1α, key genes of the core cardiac transcription factor network including Tbx5 and Mef2C and the gene encoding titin, a sarcomeric protein critical for myofibrillogenesis. According to our model, developmentally triggered hypoxia acts as a physiological signal that induces the accumulation of HIF1α, which directly contributes to maintain the expression of the Tbx5, Mef2C, and titin genes between E8.5 to E10.0 by binding to and activating these genes. This model is supported by the fact that null mutants of Tbx534 and Mef2C35 and partial-deletion mutants of titin26,36 all exhibit cardiac phenotypes similar to those observed in the conditional HIF1α mutant and result in aborted cardiac development during progression to chamber formation, thus being consistent with the fact that HIF1α activity may indeed be critical to integrate hypoxic stress with...
increased Tbx5 expression. Therefore, developmentally regulated HIF1α activation appears to be critical for maintaining the proper operation of the core cardiac gene network, an essential prerequisite for the formation of the 4-chambered heart.

Finally, our data also uncovered an unexpected direct connection between hypoxia-mediated HIF1α induction, titin gene activation, the consequent assembly of a functional sarcomere and development of the capacity for rhythmic cardiac contraction. Thus, HIF1α regulates both, the expression of selected core cardiac regulatory as well as structural genes, which implies that cardiac morphogenesis and contractility are coordinated by a common signaling pathway whose activity is exquisitely regulated by oxygen levels. In summary, the results presented here suggest that the low oxygen environment early in heart development initiates a HIF1α-mediated transcriptional program that facilitates the making of a functional heart, which in turn, is vital for the distribution of oxygen in the embryo and further development of the organism.

Acknowledgments

We thank all members of the laboratory for discussion. We are particularly grateful to R. Johnson (University of California, San Diego) for providing floxed HIF1α and G. Semenza (Johns Hopkins, Baltimore, Md) for HIF1α−/−; J. Chen (University of California, San Diego) and K. Chien (Harvard, Boston, Mass) for MLC2v-cre+ mice; M. Gassmann (University of Zurich) for reagents; T. Pedrazzini (University Lausanne, Lausanne, Switzerland) for providing access to echocardiography; and E. Olson (University of Texas Southwestern Medical Center, Dallas) and R. Eckner (New Jersey Medical School, Newark, NJ) for critical reading of the manuscript. We also thank Tatiana Krebs, Elisabeth Ehler, Stephan Keller, and Alain Hirschy for advice and help.

Sources of Funding

This work was supported by the Swiss Cardiovascular Research & Teaching Network, sponsored by the Swiss University Conference; Swiss National Science Foundation grant 3100-063486 (to J.-C.P.); Gebert-Rüf Foundation grant P038/01 (to J.-C.P.); a Novartis Foundation grant (to J.K.); and the Dr. Josef Steiner Cancer Grant (to W.K.).

Disclosures

None.

References


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_Circ Res._ 2008;103:1139-1146; originally published online October 10, 2008;
doi: 10.1161/01.RES.0000338613.89841.c1

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Online Supplemental Material

Online Supplement

Online Figure I. Pimonidazole specifically incorporates in cells subjected to hypoxia

NMCs were cultured in normoxia (20% O₂) or hypoxia (1% O₂) and stained for pimonidazole-adducts, sarcomeric α-actinin and DAPI.

Online Figure II. The early embryonic heart is hypoxic

The tissue hypoxia marker, pimonidazole, was delivered intraperitoneally to wildtype pregnant C57/Black6 mice 3 hours prior to sacrifice at the respective time points. Sagittal sections of hearts at E9.0 and E10.0 were stained for pimonidazole, EH-myomesin and DAPI.

Online Figure III. Validation of the specificity of the HIF1α antibody

(A) E9.5-10.0 whole mounts of control HIF1α+/+ and HIF1α−/− hearts were stained for HIF1α, sarcomeric α-actinin, and DAPI.

(B) E10.0 whole mounts of control HIF1α+/−/cre− and HIF1α+/−/cre+ hearts were stained for HIF1α, sarcomeric α-actinin, and DAPI.

(C) Wildtype NMCs were cultured in normoxia (20% O₂) or hypoxia (1% O₂) and stained for HIF1α, sarcomeric α-actinin, and DAPI.

Online Figure IV. MLC2v driven Cre recombinase expression is restricted to the ventricular myocardium
E10.0 embryos carrying both MLC2v-cre/+ and the ROSA26 allele were stained for β-galactosidase activity. An overview of the whole embryo (left panel) and a magnified view of the myocardium (right panel) is shown.

Figure V. Cardiac development is aborted at looping morphogenesis in HIF1α/cre+ embryos

E10.0 embryos were stained for sarcomeric α-actinin and DAPI, and sagittal section images of the control HIF1α/cre- and HIF1α/cre+ embryos are shown.

Online Figure VI. HIF1α-deficient hearts exhibit aborted development at looping morphogenesis

E10.0 whole mounts of control HIF1α/cre- and HIF1α/cre+ hearts stained for (F) sarcomeric α-actinin expression and imaged by confocal microscopy. All samples were counterstained for DAPI.

Online Figure VII. HIF1α-deficient (HIF1α/cre+) ventricles exhibit similar levels of Mef2C, Tbx5 and Titin expression compared to control (HIF1α/cre-) ventricles prior to initiation of MLC2v-driven Cre recombinase expression at E8.25

Expression levels of Mef2C, Tbx5 and titin was analyzed by quantitative RT-PCR in HIF1α/cre- and HIF1α/cre+ hearts at E8.0-8.25.

Online Figure VIII. HIF1α-deficient (HIF1α/cre+) ventricles exhibit dysregulated Mef2C, Tbx5 and Titin expression post-MLC2v-driven Cre
recombinase expression, prior to defective cardiac developmental morphogenesis between E8.75-9.25

Expression levels of Mef2C, Tbx5 and titin was analyzed by quantitative RT-PCR in HIF1α^f/-/cre^- and HIF1α^f/-/cre^+ hearts at E8.75-9.25.

Online Figure IX. Localization of HREs on the promoters of Mef2C, Tbx5 and titin

Sequence analysis of Mef2C, Tbx5 and titin promoters showing putative HREs (underlined). The core consensus HRE motif is shown in bold. The HREs are conserved in the promoters of human and mouse Mef2C, Tbx5 and titin.

Online Movie I-II. Mice lacking ventricular HIF1α exhibit dysfunctional peristaltic-like contraction at E10.5

HIF1α^f/-/cre^- (control; Supplemental C embryo) and HIF1α^f/-/cre^+ (heart-specific KO, supplemental cKO embryo) E10.5 embryos were analyzed in situ for cardiac contractility by embryonic echocardiography.
Online Figure I

Online Figure II
Online Figure IV

\(\beta\)-gal: whole embryo  heart

Online Figure V

HIF1\(\alpha^{IR}/\text{cre-}\)  HIF1\(\alpha^{IR}/\text{cre+}\)

Online Figure VI

\(\alpha\)-actinin  DAPI  merge
Online Figure VII

![Graph showing mRNA levels for Me2c, Tbx5, and Titin KD] (Note: actual graph not provided here)

Online Figure VIII

![Graph showing mRNA levels for HIF1α, Me2c, Tbx5, and Titin KD] (Note: actual graph not provided here)
## Online Figure IX

<table>
<thead>
<tr>
<th>Mef2c promoter</th>
<th>Tbx5 promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>-445</td>
<td>-1514</td>
</tr>
<tr>
<td>HRE1</td>
<td>HRE2</td>
</tr>
<tr>
<td>mu gagcgtAGTGct...</td>
<td>mu tggccaaAGTGcg.....</td>
</tr>
<tr>
<td>hu catcggAGTGct...</td>
<td>hu agccaaAGTGac.....</td>
</tr>
</tbody>
</table>

### Titin promoter

| -462           | -37           |
| HRE2           | HRE1          |
| mu ggagaaAGTGag..... | tggtcAGTGtta  |
| hu ggagaaAGTGtg..... | tggtcAGTGtta  |
Online Materials and Methods

Luciferase Promoter assays

1kb of the Mef2C and 2kb of the Tbx5 promoter was amplified from mouse genomic DNA and cloned into the pGL3 luciferase reporter vector (Stratagene). The 5.87kb titin promoter was a kind gift from Gregory A. Cox (The Jackson Laboratory) and was subcloned in its entirety into pGL3. HRE- mutants of the respective promoters were generated by recombinant PCR \(^1,^2\). This was achieved by generating sense and antisense primers bearing HRE mutations which were used to amplify the mutant 5’ and 3’ regions of the promoter, respectively. The 5’ and 3’ products generated from the respective PCR reactions were then pooled at a 1:1 ratio and reamplified using primers targeting the 5’ and 3’ ends of the respective promoters. In doing so, HRE mutants of the respective promoters were generated. Sequence integrity of the respective wildtype and mutant promoters were verified by sequencing and BLAST alignment (http://www.ncbi.nlm.nih.gov/blast). Wildtype and mutant CXCR4 reporters were generated as described\(^3\), and subcloned into pGL3. The ANF-Luc and the ANF ΔTRE-Luc reporters were generously provided by David Brook (University of Nottingham, UK) while the 3xMEF reporter was a kind gift from Eric N. Olson (UT Southwestern Medical Center at Dallas, USA). The HIF1\(\alpha\) (P402A/P577A) expression construct was generated as described\(^3\), while the HIF1\(\alpha\) (ΔODD) expression construct was kindly provided by H Franklin Bunn (Harvard Medical School, USA)\(^4\). NMCs were plated on 35mm plates and transfected with Trogene (Life Technologies) as recommended by the manufacturer, in the presence of 280ng of the respective reporter plasmids, 20ng of the \(\beta\)-galactoside expression construct.
and 1.7 μg of the HIF1α (P402A/P577A) or HIF1α (ΔODD) expression constructs, unless indicated otherwise.

**In vivo pimonidazole delivery and embryonic cardiomyocyte isolation**

Delivery of pimonidazole was performed as described 5 and detection of its incorporation was performed using the Hypoxyprobe-1 kit (Chemicon International) as recommended by the manufacturer. Cardiomyocyte isolation, fixation and staining of whole embryo and heart whole mounts and the subsequent confocal imaging was performed as described 6. FACS and immunoblotting was performed as described 6, 7.

**Semi-quantitative RT-PCR, Quantitative RT-PCR and ChIPs**

RNA was isolated with Trizol (Sigma) and cDNA generated using Superscript II (Invitrogen) from individual hearts as recommended by the manufacturer. The genotypes of the respective hearts were determined retrospectively from embryonic tail biopsies as described 8, 9. Preliminary RT-PCR experiments were performed to determine linear amplification conditions for the different targets. For HIF1α, the final cycle parameters used were 94°C, 15 s and 68°C, 4 min (35 cycles). For HPRT, the cycle parameters were 94°C, 1 min; 62°C, 1.5 min; and 72°C, 3 min (30 cycles). qPCR reactions were setup as recommended by the manufacturer (Roche) and analyzed on the Roche LightCycler® 480. The ChIP assay was performed using material from 7-9 C57/Black6 E10 ventricles and the assay performed using the ChIP-IT kit (Active Motif) as recommended by the manufacturer. In silico promoter analyses and alignments were performed using MatInspector and DiAlignTF (Genomatix Software).

Primer pairs used in RT-PCRs:
(a) HIF1αF (ACCTTCATCGGAAACTCCAAAG) and HIF1αR (ACTGTTAGGCTCAGGGAACCTGA) [HIF1α GenBank accession number Y09085]; and (b) HPRTF (TCAGTCAACGGGGACATAAAA) and HPRTR (GGGGCTGTACTGCTTAAACCAG) [HPRT, GenBank accession number NM013556].

(c) Titin N2BF (ATGTCTGCGAAGCCTCAAAT) and Titin N2BR (CTCTCTGCGCAGCCTTGAACC) [Titin GenBank accession number NM028004];

(d) Titin-KDF (CACTCTGGTCTGCAAGGTGA) and Titin-KDR (GGGTCTGGCTTACCACTGAA) [Titin GenBank accession number NM028004]; (e) α-actininF (TGGCACCCAGATCGAGAAC) and α-actininR (GTGGAACCGCATTTTCCCG) [Actinin2 GenBank accession number NM033268]; (f) MyomesinF (GCACGACCATGAGCGCCACTAC) and MyomesinR (ACCCCTGAGAATGCGGGA) [Myomesin1 GenBank accession number NM010867]; (g) MyBPCF (CAGGGAAGAAACCAGTTCAG) and MyBPCR (GGGTCTGGCTTACCACTGAA) [Mybpc3 GenBank accession number AF097333]; (h) Nkx2.5F (GAACAAAGCCGATGATTCAG) and Nkx2.5R (AACAGCACACTTTTGCCT) [Mef2C GenBank accession number BC026841]; (k) GATA4F (CCCTACCCAGCCTACATGG) and GATA4R (AACAGCACACTTTTGCCT) [Mef2C GenBank accession number BC026841]; (k) GATA4F (CCCTACCCAGCCTACATGG) and GATA4R (AACAGCACACTTTTGCCT) [Mef2C GenBank accession number BC026841]; (l) CyclinD1F (CGTACCCTGACCAATCTC) and CyclinD1R (CTCCTCTCGCAGCCATCTG) [Cyclin D1 GenBank accession number NM008092];
NM007631]; (m) CDK4F (ATGGCTGCCACTCGATATGAA) and CDK4
(TCCTCCATTAGGAACTCTCACAC) [CDK4 GenBank accession number
NM009870]; (n) p21/Cip1F (CCTGGTGATGTCCGACCTG) and
p21/Cip1R(CCATGAGCGCATCGCAATC) [p21/Cip1 GenBank accession number
NM007669]; and (o) p27/Kip1F (TCAAAACGTGAGGTCTAACG) and
p21/Kip1R (CCGGGCCGAAGAGATTTCTG) [p27/Kip1 GenBank accession
number NM009875].
Online References


