Partial rescue of defects in Cited2-deficient embryos by HIF-1α heterozygosity

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Abstract

Hypoxia-inducible factor-1 (HIF-1) initiates key cellular and tissue responses to physiological and pathological hypoxia. Evidence from in vitro and structural analyses supports a critical role for Cited2 in down-regulating HIF-1-mediated transcription by competing for binding with oxygen-sensitive HIF-1α to transcriptional co-activators CBP/p300. We previously detected elevated expression of HIF-1 target genes in Cited2−/− embryonic hearts, indicating that Cited2 inhibits HIF-1 transactivation in vivo. In this study, we show for the first time that highly hypoxic cardiac regions in mouse embryos corresponded to the sites of defects in Cited2−/− embryos and that defects of the outflow tract, interventricular septum, cardiac vasculature, and hyposplenia were largely rescued by HIF-1α haploinsufficiency. The hypoxia of the outflow tract and interventricular septum peaked at E13.5 and dissipated by E15.5 in wild-type hearts, but persisted in E15.5 Cited2−/− hearts. The persistent hypoxia and abnormal vasculature in the myocardium of interventricular septum in E15.5 Cited2−/− hearts were rescued with decreased HIF-1α gene dosage. Accordingly, mRNA levels of HIF-1-responsive genes were reduced in Cited2−/− embryonic hearts by HIF-1α heterozygosity. These findings suggest that a precise level of HIF-1 transcriptional activity critical for normal development is triggered by differential hypoxia and regulated through feedback inhibition by Cited2.

Keywords: Cited2; Hypoxia; Vascular endothelial growth factor-A; Heart development

Introduction

Hypoxia-inducible factor-1 (HIF-1) has fundamental functions in mammalian development and homeostasis and is considered a target for therapy in cancer and cardiac ischemia (Semenza, 2003). It is activated in response to localized tissue hypoxia to regulate transcription of more than 70 hypoxia-inducible genes that mediate angiogenesis, erythropoiesis, vasodilation, and anaerobic metabolism (Iyer et al., 1998). Under normoxic conditions, the HIF-1α subunit is rapidly degraded and transcriptionally repressed. In response to a hypoxic stimulus, HIF-1α protein is stabilized in the cytoplasm and translocates into the nucleus, where it dimerizes with the constitutively expressed HIF-1β subunit (Jiang et al., 1996). The heterodimer HIF-1 then binds hypoxia response elements within the regulatory regions of a variety of target genes to modulate their expression through the recruitment of transcriptional coactivators such as CBP, p300, and SRC-1 (Semenza, 2002). The positive regulation of HIF-1 activity has been the focus of much research. An important but less studied aspect,
however, is the negative regulation of HIF-1 transcriptional activity. Cited2 [cAMP-responsive element-binding protein (CBP)/p300-interacting transactivators with glutamic acid (E) and aspartic acid (D)-rich tail 2], a HIF-1-inducible gene, has been shown to negatively regulate hypoxia-mediated signaling by competing with HIF-1α in binding to the CH1 region of CBP/p300 in vitro (Bhattacharya et al., 1999; Freedman et al., 2003). This is a unique feedback regulatory mechanism to limit excess HIF-1 activation and to maintain normal tissue homeostasis. Cited2 expression can also be regulated by other factors including cytokines, lipopolysaccharide, and shear stress (Bhattacharya et al., 1999; Sun et al., 1998; Yokota et al., 2003), which underscores its involvement in many physiological processes. Cited2 is specifically expressed in the anterior visceral endoderm during early mouse and chicken development (Dunwoodie et al., 1998; Schlange et al., 2000). Its expression persists in the rostral mesoderm as it is translocated caudalwards during the invagination of the foregut and the formation of the heart and is largely restricted to the outflow and inflow regions of the heart, atrioventricular cushion, septum primum, and the crest and apex of the muscular ventricular septum by E13.5 (Dunwoodie et al., 1998; Schlange et al., 2000; Weninger et al., 2005). These expression patterns suggest an important role for Cited2 in tissues that contribute directly and indirectly to early and later steps in cardiac development.

Cited2-deficient embryos on the mixed genetic background exhibit numerous developmental defects, including cardiac malformations, adrenal agenesis, neural crest defects, and exencephaly (Bamforth et al., 2001; Barbera et al., 2002; Yin et al., 2002). Cardiac defects in Cited2−/− embryos included double outlet right ventricle, atrial septal defect (ASD), ventricular septal defect (VSD), overriding aorta, persistent truncus arteriosus, and pulmonary artery stenosis. These defects can be attributed to the loss of at least three Cited2 functions: (1) Cited2 is a co-activator of Tpaf2 that is important for neural crest cell development and in its absence transactivation by Tpaf2 is defective (Bamforth et al., 2001); (2) Cited2 is a negative regulator for HIF-1 and in its absence VEGF-A is overexpressed in the heart (Yin et al., 2002); (3) Cited2 is required for the establishment of the left–right axis in mouse development through a Nodal-Pitx2c pathway and in its absence left–right asymmetry is abnormal (Bamforth et al., 2004; Weninger et al., 2005). Recently, 3 Cited2 mutations, all of which significantly reduced the capacity of Cited2 to transrepress HIF-1 with one also resulting in significantly diminished Tpaf2 co-activation, were found in 392 patients with congenital heart defects (Sperling et al., 2005). The aim of this study was to determine which defects in the Cited2−/− embryos could be attributed to the loss of Cited2 as a negative feedback regulator of HIF-1-mediated transcription.

In this study, we show that malformations of the outflow tract (OFT) and interventricular septum (IVS) and hyposplenia in Cited2−/− embryos could be rescued by HIF-1α haploinsufficiency. We also show that there was a peak of hypoxia and HIF-1α nuclear localization in specific regions of mouse embryonic heart including the OFT and IVS where Cited2 expression was detected in a critical stage of OFT and IVS development. Hypoxia in the myocardium of the OFT and IVS detected by EF5 and HIF-1α immunostaining diminishes by E15.5 after the coronary vasculature has connected to the aortic root. In contrast, in the E15.5 Cited2-deficient hearts, both hypoxic markers continue to intensely stain the OFT and IVS. Furthermore, HIF-1α heterozygosity rescued the excessive hypoxia, VEGF-A overexpression, abnormal coronary vasculature, and defective myocardium at these sites in E15.5 Cited2−/− hearts. These data suggest that Cited2 performs an essential role as a feedback regulator of HIF-1 activity during development of the cardiac OFT and IVS, thus preventing abnormal and ineffective coronary vascularization likely due to excessive VEGF-A production.

Materials and methods

Mouse strains and generation

Cited2−/− and HIF-1α−/− mice were genotyped by Southern blot analyses (Iyer et al., 1998; Yin et al., 2002). The Cited2−/− mice were backcrossed 12 generations into C57BL/6J genetic background before embryonic lethality was investigated. Cited2−/−;HIF-1α−/− mice were generated by crossing the Cited2−/− mice on the C57BL/6j genetic background with HIF-1α−/− mice on the 129Sv×C57BL/6j genetic background. Cited2−/−;HIF-1α−/− and Cited2−/−;HIF-1α−/− embryos were obtained from the intercrosses between the Cited2−/−;HIF-1α−/− mice and Cited2−/− mice.

Embryonic histology and immunohistochemistry and EF5 injection

Embryos were harvested from timed matings, fixed in 4% paraformaldehyde, and embedded in paraffin. Hematoxylin and eosin (HE) stained sections were used for histological examination. Pregnant heterozygotes were injected with 10 nM EF5, 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3-pentafluoropropyl)-acetamide, at 1% of body weight. Three hours later, the embryos were fixed in 4% paraformaldehyde. EF5 staining, which has been described previously (Sugishita et al., 2004a,b), was performed on 10-μm cryosections from 3 each of E11.5, E13.5, and E15.5 wild-type, Cited2−/−;HIF-1α−/−, and Cited2−/−;HIF-1α−/− hearts including at least 1 set of wild-type, Cited2−/−;HIF-1α−/−, and Cited2−/−;HIF-1α−/−;HIF-1α−/− littersmates at each stage by using a mouse monoclonal anti-EF5 Cy3-conjugated antibody (ELK-51 from Dr. Cameron Koch at University of Pennsylvania). Immunohistochemistry was carried out on 10-μm cryosections by using rabbit polyclonal anti-HIF-1α (1:200 dilution), anti-VEGF-A (1:100 dilution, anti-VEGF-A; (1:100 dilution, anti-VEGF-A; BD PharMingen), and anti-myosin heavy chain (MF20, 1:50 dilution, Developmental Studies Hybridoma Bank) antibodies on 10-μm cryosections from 3 each of E11.5, E13.5, and E15.5 wild-type, Cited2−/−;HIF-1α−/−, and Cited2−/−;HIF-1α−/− hearts at 1:100 dilution, anti-VEGF-A; (1:100 dilution, anti-VEGF-A; (1:100 dilution, anti-VEGF-A; BD PharMingen, San Diego, CA), anti-CD31 (1:200 dilution, BD PharMingen), and anti-myosin heavy chain (MF20, 1:50 dilution, Developmental Studies Hybridoma Bank) antibodies on 10-μm cryosections. Anti-CD31 reaction was detected by the Labeled Streptavidin Biotin Kit (Dako, Carpinteria, CA). Developmental Studies Hybridoma Bank) antibodies on 10-μm cryosections. Anti-CD31 reaction was detected by the Labeled Streptavidin Biotin Kit (Dako, Carpinteria, CA). The others were detected with appropriate secondary antibodies conjugated with biotin (1:200 dilution, Vector, Burlingame, CA), and the signal was amplified with fluorescein tyramide signal (Perkin Elmer, Boston, MA).

Real-time RT-PCR

Ventricles from embryos at E15.5 were dissected, frozen, and stored until embryonic DNA was genotyped. Ventricles from 5 each of E15.5 wild-type, Cited2−/−;HIF-1α−/−, and Cited2−/−;HIF-1α−/− hearts, including 3 sets of wild-type, Cited2−/−;HIF-1α−/−; and Cited2−/−;HIF-1α−/− littersmated hearts, were used for RNA extraction. Total RNA was isolated using Trizol reagent and 5 μg total RNA was used for cDNA synthesis using the SuperScript® First Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR reactions were carried out with diluted RT reaction products using iQ™ SYBR Green Supermix PCR kit and iCycler machine (Bio-Rad). β-actin transcript was used as an internal control for normalization.
Coronary vascular permeability measured by Evans blue dye

Extravasation of Evans blue dye (EBD) identifies increased vascular permeability and/or disruption of endothelial integrity. Embryos at E15.5 were injected with 10 mg/ml EBD (Sigma, St Louis, MO) in umbilical veins immediately after dissection (Supplemental Fig. 1). To further examine which specific regions in the embryonic hearts have accumulated EBD, embryos were fixed in 4% paraformaldehyde and cryosections were observed by fluorescence microscope (Shai et al., 2002).

Statistical analysis

Statistical difference between two independent groups was assessed by Fisher’s Exact test or Independent-samples t test; p \leq 0.05 was considered statistically significant.

Results

Embryonic lethality and defects in Cited2−/− embryos on the C57BL/6J genetic background

Previous studies showed that Cited2-null embryos die at late gestation and display numerous developmental defects on both mixed 129SV × C57BL/6J and pure C57BL/6J genetic backgrounds (Bamforth et al., 2001, 2004; Barbera et al., 2002; Yin et al., 2002; Weninger et al., 2005). In this study, Cited2-null embryos on the C57BL/6J genetic background died from E15.5 (Table 1). Twelve Cited2−/− embryos between E14.5 and E17.5 examined by HE-stained transverse sections had cardiac malformations, adrenal agenesis, hyposplenia, exencephaly as well as left–right patterning defects (Supplemental Fig. 2). All of the 12 Cited2−/− embryos had adrenal agenesis, hyposplenia, and cardiac defects including ASD, VSD, overriding aorta, double-outlet right ventricle, and persistent truncus arteriosus in different combinations. The left–right patterning defects including abnormal heart looping, right-sided aortic arches, ASDs with right atrial and pulmonary isomerism, and stomachs lying in the midline were observed in 7 of 12 Cited2−/− embryos. These results suggest that the role of Cited2 in establishing embryonic laterality may not be the only mechanism to account for the cardiovascular malformations in the Cited2-null embryos.

**HIF-1α heterozygosity rescued the cardiac OFT and ventricular septum defects and hyposplenia in Cited2−/− embryos**

In vitro data and gene-targeting results support that the cardiac defects in Cited2−/− embryos may be mediated by the dysregulated expression of HIF-1 target genes (Bhattacharya et al., 1999; Yin et al., 2002). To test the consequences to morphogenesis of varying gene dosages of Cited2 and HIF-1α, mice doubly heterozygous for Cited2 and HIF-1α were generated. These double heterozygotes and Cited2+/−;HIF-1α−/− mice survived with the expected Mendelian ratio. From the intercrosses between the Cited2−/−;HIF-1α−/− mice and Cited2+/−, no Cited2−/−;HIF-1α−/− mice survived to birth (Table 1). The ratio of Cited2−/−;HIF-1α+/− embryos to Cited2−/−;HIF-1α−/− embryos was almost 1 to 1 at E13.5 and E15.5 (E13.5: 6/6; E15.5: 4/6), but 1 to 2.7 (6/16) at E17.5–18.5 (Table 1), indicating that Cited2−/−;HIF-1α−/− embryos survived longer than Cited2−/−;HIF-1α−/− embryos. All of the six Cited2−/−;HIF-1α+/− and 8 Cited2−/−;HIF-1α+/− embryos at 18.5 d.p.c., including 2 pairs of Cited2−/−;HIF-1α+/− and Cited2+/−;HIF-1α−/− littersmates, were examined by HE-stained transverse sections and their phenotypes were summarized in Table 2. Notably, the VSD (Figs. 1a: A–C), overriding aorta or smaller diameter aorta compared with pulmonary artery (Figs. 1a: D–I), and double-outlet right ventricle (Figs. 1a: D–F) observed in Cited2−/−;HIF-1α−/− embryos were absent in 6 out of 8 Cited2−/−;HIF-1α−/− embryos (p = 0.009). Small membranous VSDs were identified in the other 2 Cited2−/−;HIF-1α+/− embryos (data not shown). ASD was observed in 5 Cited2−/−;HIF-1α+/− embryos, 2 Cited2−/−;HIF-1α−/− embryos that also had right atrial and pulmonary isomerism, and 1 Cited2−/−;HIF-1α+/− embryo that did not have right atrial and pulmonary isomerism (Figs. 1a: J–L).

**Table 2**

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<tr>
<th>Phenotypes of Cited2−/−;HIF-1α−/− and Cited2−/−;HIF-1α−/− embryos at E18.5 on the mixed background</th>
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<td>Cited2−/−;HIF-1α−/−</td>
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<td>Ventricular septal defect</td>
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<td>Overriding aorta or smaller diameter aorta</td>
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<td>Double-outlet right ventricle</td>
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<td>Atrial septal defect</td>
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<td>Right atrial isomerism</td>
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<td>Right pulmonary isomerism</td>
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<td>Adrenal agenesis</td>
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**Table 1**

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<th>Cited2−/− genotype of embryos from Cited2−/− intercrosses and Cited2−/−;HIF-1α−/− intercrosses</th>
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| Cited2−/− × Cited2−/− | E11.5 | 9 | 14 | 7 |
| Cited2−/− × Cited2−/− | E12.5 | 11 | 24 | 9 |
| Cited2−/− × Cited2−/− | E13.5 | 6 | 12 | 5 |
| Cited2−/− × Cited2−/− | E14.5 | 13 | 45 | 10 |
| Cited2−/− × Cited2−/− | E15.5 | 9 | 21 | 6 |
| Cited2−/− × Cited2−/− | E16.5 | 13 | 16 | 7 |
| Cited2−/− × Cited2−/− | E17.5–18 | 28 | 69 | 9 |
| Cited2−/− × Cited2−/−;HIF-1α−/− | E13.5 | 10 | 22 | 12 (6 + 6) |
| Cited2−/− × Cited2−/−;HIF-1α−/− | E15.5 | 12 | 26 | 10 (4 + 6) |
| Cited2−/− × Cited2−/−;HIF-1α−/− | E17.5–18.5 | 55 | 1121 | 22 (6 + 16) |
| Cited2−/− × Cited2−/−;HIF-1α−/− | PD10 | 10 | 26 | 0 |

PD: postnatal day.

a 6 Cited2−/−;HIF-1α−/− and 6 Cited2−/−;HIF-1α−/− embryos.

b 4 Cited2−/−;HIF-1α−/− and 6 Cited2−/−;HIF-1α−/− embryos.

c 6 Cited2−/−;HIF-1α−/− and 16 Cited2−/−;HIF-1α−/− embryos.
In contrast, HIF-1α heterozygosity did not eliminate adrenal agenesis, exencephaly, and left–right patterning defects in Cited2−/− embryos. These histological results suggest that reducing the gene dosage of HIF-1α can rescue the OFT and ventricular septum defects in Cited2-deficient embryos irrespective of the presence of the left–right asymmetry defects. HIF-1 mediates changes in gene expression in response to lowered oxygen levels. The accumulation of EF5, a metabolic indicator of hypoxia (Koch, 2002), was used to identify hypoxic regions in each of wild-type, Cited2−/−;HIF-1α+/+, and Cited2−/−;HIF-1α+/− mouse embryonic hearts at E11.5, E13.5, and E15.5 and to determine whether these hypoxic regions corresponded to sites where defects arise in Cited2−/− embryonic hearts.

**Hypoxic regions and relative hypoxic levels in wild-type, Cited2+/−;HIF-1α+/+, and Cited2−/−;HIF-1α+/− mouse embryonic hearts**

HIF-1 mediates changes in gene expression in response to lowered oxygen levels. The accumulation of EF5, a metabolic indicator of hypoxia (Koch, 2002), was used to identify hypoxic regions in each of wild-type, Cited2−/−;HIF-1α+/+, and Cited2−/−;HIF-1α+/− mouse hearts at E11.5, E13.5, and E15.5 and to determine whether these hypoxic regions corresponded to sites where defects arise in Cited2−/− embryonic hearts. Examination of EF5 binding in the E11.5 hearts demonstrated weak immunoreactivity in the myocardium at the right atrioventricular junction of both wild-type and Cited2−/− embryonic hearts (data not shown). At E13.5, before coronary vasculature connects to aortic root (E14.5), the myocardium of the OFT and IVS was hypoxic in both Cited2−/−;HIF-1α+/+ (Fig. 2F) and Cited2−/−;HIF-1α+/− (Fig. 2I) hearts. The atrioventricular junction of the wild-type (Fig. 2J) and Cited2−/−;HIF-1α+/− (Fig. 2L) hearts at E15.5 were weakly but distinctly immunopositive for EF5. No EF5 immunopositivity was observed in the myocardium of the interatrial...
septum was noted in any of the embryos at stages between E11.5 and E15.5 (Figs. 2D–F; data not shown). Taken together, these results show that the myocardium was hypoxic at the stages of ventricular septum formation and hypoxia was most severe in the OFT and IVS at E13.5 and dissipated by E15.5 in the wild-type embryos. In contrast, Cited2-deficient hearts remained highly hypoxic at E15.5 in these cardiac regions and these cardiac regions were also consistently defective. Both the severe hypoxia and cardiovascular defects were absent when the gene dosage of HIF-1α was reduced in these Cited2-deficient embryos (Fig. 2I).

HIF-1α nuclear localization in Wild-type, Cited2−/−;HIF-1α+/−, and Cited2−/−;HIF-1α−/− mouse embryonic hearts

To demonstrate the physiological significance of the hypoxic regions detected by EF5 during development, we examined the expression of HIF-1α in wild-type, Cited2−/−;HIF-1α+/−, and Cited2−/−;HIF-1α−/− embryonic hearts from the pregnant mice that were not injected with EF5. Interestingly, HIF-1α nuclear localization was noted in almost the same regions that were intensely EF5-immunopositive. At E13.5, the myocardium of the OFT, IVS, atria, and ventricles of wild-type (Figs. 3A, D), Cited2−/−;HIF-1α+/− (Figs. 3B, E) as well as Cited2−/−;HIF-1α−/− (Figs. 3C, F) hearts was immunopositive for HIF-1α nuclear reactivity in a similar pattern. By E15.5, high HIF-1α immunoreactivity in the OFT, IVS, and atrial and ventricular wall was still observed in Cited2−/−;HIF-1α+/− hearts (Figs. 3H, K), but it was significantly reduced in both wild-type (Figs. 3G, J) and Cited2−/−;HIF-1α+/− (Figs. 3I, L) hearts. The ventricular wall of Cited2−/−;HIF-1α−/− heart (Fig. 3I) had relatively high HIF-1α immunoreactivity in comparison to wild-type (Fig. 3G). Only low levels of HIF-1α immunopositivity in the atrial walls of both wild-type (Fig. 3G) and Cited2−/−;HIF-1α+/− (Fig. 3I) hearts and in the IVS of Cited2−/−;HIF-1α+/− (Fig. 3L) hearts were detected at this stage. No HIF-1α nuclear localization could be detected in spleen sections from 2 of each wild-type and Cited2−/− embryos at stages between E13.5 and E16.5 (data not shown).
HIF-1α heterozygosity reduced VEGF-A expression level in Cited2−/− embryonic hearts

One of the most potent responses to tissue hypoxia is the induction of an angiogenic response in part mediated by the expression of VEGF (Carmeliet and Collen, 2000). As shown in Fig. 4a, HIF-1α mRNA levels in the E15.5 Cited2−/−;HIF-1α+/− hearts were approximately half those of the wild-type and Cited2−/−;HIF-1α+/− hearts detected by real-time RT-PCR (range, 0.4–0.6). VEGF-A and PGK1 mRNA levels in the E15.5 Cited2−/−;HIF-1α+/− hearts were 2.95- and 2.28-fold higher than those in the wild-type hearts. Interestingly, VEGF-A and PGK1 mRNA levels in the E15.5 Cited2−/−;HIF-1α+/− hearts were reduced to 1.85-fold (1.32–2.39) and 1.19-fold (1.01–1.38), respectively, compared with those in the wild-type hearts, and were significantly lower than those in the E15.5 Cited2−/−;HIF-1α+/− hearts (p=0.005 and p<0.001). VEGF-A immunoreactivity in the IVS of E15.5 Cited2−/−;HIF-1α+/− (Fig. 4b: H) hearts was also stronger than in wild-type (Fig. 4b: D) and Cited2−/−;HIF-1α+/− (Fig. 4b: L) hearts, suggesting that VEGF-A up-regulation occurs selectively in regions of relatively high levels of hypoxia in the ventricle and that HIF-1α heterozygosity reduced VEGF-A expression level in Cited2−/− embryonic hearts.

HIF-1α heterozygosity rescued the defective myocardium and abnormal coronary vasculature in Cited2−/− embryonic hearts

VEGF is essential for vasculogenesis and angiogenesis in the embryo and fetus as well as for neovascularization of ischemic tissues and tumors in the adult organism (Ferrara and Davis-Smyth, 1997). CD31 immunohistochemistry, EBD staining, measurement of the extravasation of EBD, and MF20 immunostaining were performed in 3 of each E15.5 wild-type, Cited2−/−;HIF-1α+/−, and Cited2−/−;HIF-1α+/− littermate hearts. CD31 immunohistochemistry demonstrated that capillary-sized vessels were replaced by dilated sinusoidal vessels in E15.5 Cited2−/−;HIF-1α+/− hearts (Figs. 5a: B, E), compared with normal capillary-sized coronary vessels in E15.5 wild-type (Figs. 5a: A, D) and Cited2−/−;HIF-1α+/− (Figs. 5a: C, F) hearts. In addition, as shown in Fig. 5b, the coronary vascular permeability revealed by...
the extravasation of EBD in E15.5 Cited2−/−;HIF-1α+/+ hearts was 1.9-fold higher than that in wild-type embryonic hearts and HIF-1α heterozygosity significantly decreased the coronary vascular permeability in E15.5 Cited2−/− hearts (p = 0.0083). EBD staining revealed that large regions with EBD accumulation were present in the IVS of E15.5 Cited2−/−;HIF-1α+/+ (Figs. 5c: B, C, F, G) hearts, compared to small regions in the IVS of E15.5 wild-type (Figs. 5c: A, E) and Cited2−/−;HIF-1α+/− (Figs. 5c: D, H) hearts. MF20 immunostaining showed that the myocardium of the IVS and ventricular wall in E15.5 Cited2−/−;HIF-1α+/− (Figs. 4b: I–K) hearts including the one with right atrial and pulmonary isomerism. These results indicate that HIF-1α...
heterozygosity rescued the abnormal vasculature in the myocardium of the OFT and IVS and the defective myocardium in Cited2−/− embryonic hearts.

Discussion

Here we tested the hypothesis that some of the defects in Cited2−/− embryos could be explained by abnormally high levels of HIF-1 activity in the absence of this negative regulator of HIF-1. By reducing the gene dosage of HIF-1α, we found that malformations of the OFT, IVS, and spleen in Cited2−/− embryos were largely rescued. In addition, we demonstrated that Cited2-null hearts at E15.5 had abnormal coronary vessels, persistence of myocardial hypoxia and HIF-1 nuclear localization, and overexpression of HIF-1 regulated genes, which were reduced or normalized by HIF-1α heterozygosity. These results strongly suggest that an appropriate level of HIF-1 transcriptional activity induced by differential microenvironmental tissue hypoxia is critical for the normal development of the myocardium and coronary vessels of the OFT and IVS of mouse embryonic heart, and support a key role for Cited2 as a negative regulator of HIF-1 activity in these regions.

Previous studies have shown that the myocardium of the OFT and IVS is the most hypoxic area in the developing chicken heart and that regional myocardial hypoxia is critical for triggering the remodeling of the embryonic avian OFT (Sugishita et al., 2004a,b; Wikenheiser et al., 2006). Disruption of this hypoxia by exposure to hyperoxic conditions resulted in OFT defects. These studies also revealed that the hypoxic regions in the chicken embryo heart have HIF-1α accumulation in the nuclei. The present study demonstrated that the OFT and IVS of the mouse embryo have relatively high levels of
myocardial hypoxia and HIF-1α nuclear localization transiently during the normal course of cardiac septation and coronary vascular development. HIF-1α is activated in response to localized tissue hypoxia and global HIF-1α deficiency resulted in developmental arrest and lethality by E11 of HIF-1α−/− embryos that manifested neural tube defects, a single abnormal pharyngeal arch, abnormal neural crest migration, and cardiovascular malformations (Iyer et al., 1998; Ryan et al., 1998; Compernolle et al., 2003). Reducing HIF-1α transactivation specifically in endothelial cells using the Flk-1 promoter to induce endothelial cell-specific expression of the dominant negative HIF-2α showed that HIF function is required in endothelial cells for normal heart development (Licht et al., 2005). Conditional knockout of HIF-1α from ventricular cardiomyocytes using the mcl2v-cre resulted in a milder phenotype but with a reduction in the coronary vasculature and changes in heart function (Huang et al., 2004). These studies point to an important role for specific regions of tissue hypoxia and subsequent HIF-1α activation in the normal course of cardiac development and coronary vessel formation.

As in most pathways, HIF-1 is negatively as well as positively regulated. Cited2 has been shown to function as a negative regulator of HIF-1 in vitro through its competitive binding with HIF-1α to the transcriptional co-factors CBP/p300 (Bhattacharya et al., 1999). NMR structure analyses further support the competition hypothesis by demonstrating that the Cited2 transactivation domain disrupts the interaction between the C-terminal transactivation domain of HIF-1α and the CH1 domain of CBP/p300 by binding to an overlapping binding site on CH1 with higher affinity than the HIF-1α C-terminal transactivation domain (Freedman et al., 2003). Furthermore, transcript levels of HIF-1α-responsive genes, Vegf, Glut1, and Pdgk1, in E14.5 Cited2−/− hearts (Yin et al., 2002) and mRNA levels of VEGF-A and Pdgk1 in E15.5 Cited2−/− hearts in this study (Fig. 4a) were 2- to 3-fold higher than those in wild-type hearts. These data supported the role for Cited2 in modulating HIF-1α activity in vitro and suggested that Cited2 plays this role during heart development.

VEGF-A expression induced by HIF-1α in many cell types is a potent component of the complex angiogenic response to localized tissue hypoxia (Carmeliet and Collen, 2000). Overexpression of VEGF-A in Cited2-null embryonic hearts may be a major determinant for OFT and ventricular septum defects for the following reasons. First, the most hypoxic regions of the heart during development include the OFT and IVS (Fig. 2) that are therefore likely to have a higher level of HIF-1α expression and HIF-1α activity and in turn would increase downstream VEGF-A expression. Second, 2- to 3-fold overexpression of VEGF-A from the endogenous locus resulted in lethality of the VEGF-A overexpressing embryos at E12.5 to E14 with severe VSDs and abnormalities in the remodeling of the OFT (Miquerol et al., 2000), which are similar to cardiac malformations in Cited2-deficient embryos (Supplemental Fig. 2). Similar defects were observed in the avian embryos by the injection of VEGF or by the overexpression of VEGF in the OFT myocardium using adenoviral vectors (Feucht et al., 1997; Sugishita et al., 2004b). Third, the most intense expression of VEGF-A was within the myocardium of the OFT, IVS, and atroventricular junction in the VEGF-A overexpressing mice (Miquerol et al., 2000), which is remarkably similar to EF5 and HIF-1α immunostaining patterns. In the present study, the VEGF-A protein level in the myocardium of the IVS in the E15.5 Cited2−/−;HIF-1α+/+ hearts was higher than that of wild-type and Cited2−/−;HIF-1α−/− hearts (Fig. 4b). Finally, in the Cited2-null embryos, OFT and ventricular septum defects were largely rescued when VEGF-A mRNA and protein levels were decreased in heart tissues by HIF-1α haploinsufficiency (Fig. 1a).

Overexpression of VEGF-A in tumors led to formation of structurally and functionally abnormal vessels that are leaky, tortuous, dilated, and saccular and had a haphazard pattern of interconnection while application of VEGF-A inhibitors normalized tumor vasculature and improved perfusion (Jain, 2005). Injection of an adenoviral vector overexpressing vascular permeability factor/VEGF164 also results in abnormal vascularization with the formation of vascular glomeruloid bodies in the ear skin of nude mice (Sundberg et al., 2001). Similarly abnormal vasculature was observed within the myocardium of the OFT and IVS in the Cited2−/−/− embryonic hearts (Figs. 5a: B, E) and may be the consequence of overexpression of VEGF-A in the myocardium. The rescue of the coronary vasculature in the myocardium of the Cited2−/−; HIF-1α−/−/− embryonic hearts where VEGF-A expression is reduced compared to the Cited2−/+; HIF-1α−/+ supports this hypothesis (Figs. 5a: C, F). In addition, the abnormal persistence of the myocardial hypoxia levels and defects of the OFT and IVS in the E15.5 Cited2−/− hearts were also rescued by HIF-1α heterozygosity. Thus, we propose that the increased hypoxia of the OFT and IVS in Cited2−/−; HIF-1α−/− versus Cited2−/+; HIF-1α−/+ hearts at E15.5, as indicated by more intense EF5 and HIF-1α immunostaining, may be due to the abnormally high VEGF-A expression levels in the Cited2−; HIF-1α−/+ embryonic hearts resulting in higher permeability of the coronary vessels and ineffective tissue perfusion (Figs. 5b, c). In addition, the relatively high HIF-1α immunoreactivity in the ventricular wall in Cited2−/−; HIF-1α−/+ versus wild-type hearts at E15.5 may be explained by the relatively high level of VEGF-A mRNA in Cited2−/−; HIF-1α−/+ hearts. Our results strongly suggest that differential myocardial hypoxia and the proper level of HIF-1 activity are critical for normal development of the OFT, IVS, and coronary vessels in the mouse embryo. However, uncontrolled HIF-1 activity in the absence of Cited2 is detrimental and results in abnormal vasculature that can lead to persistent elevated hypoxia that might contribute to further dysregulated HIF-1 activity.

In addition to the role for Cited2 as a negative regulator of HIF-1 activity, Cited2 serves other functions and the defects of the Cited2 null may be the result of the misregulation of several pathways that influence heart development. Tpap2, a transcription factor necessary for neural tube and neural crest cell development, interacts with Cited2 for its transcription (Bamforth et al., 2001). In view of the multiple and profound effects of the cardiac neural crest on cardiac development (reviewed in Stoller and Epstein, 2005; Hutson and Kirby,
2003), it would be important to identify whether the absence of Cited2 either directly or indirectly affects steps in neural crest cell development. The typical cardiac defects associated with neural crest cell defects such as persistent truncus arteriosus and overriding aorta were observed in some of the Cited2−/− embryos. However, muscular VSDs detected in Cited2-null embryos are not typically associated with any models of neural crest cell deficiency described so far (Hutson and Kirby, 2003). In addition, there is little or no difference in the expression patterns of neural crest markers, such as Crabp-1, Wnt-1, Wnt-3, RXRα, and Pax3, between wild-type and Cited2-null embryos (Barbera et al., 2002; Yin et al., 2002). Thus, a deficiency in Tfp2 function and subsequent abnormalities in neural crest cells does not appear to be the sole mechanism to explain the full spectrum of cardiovascular defects in Cited2-null embryos.

Laterality defects were reported in a proportion of Cited2-null embryos with evidence that Cited2 is acting upstream of Nodal, Lefty2, and Pitx2 in the lateral mesoderm and of Lefty1 in the presumptive floor plate (Bamforth et al., 2004; Weninger et al., 2005). A major function of the Pitx2c-mediated left–right asymmetry pathway is to pattern the aortic arches, OFT, and atrioventricular valves and cushions (Liu et al., 2002; Franco and Campione, 2003). Since the Cited2–Tfp2a/c complex regulates Pitx2c transcription and embryos null for either Cited2 or Pitx2c develop similar cardiac defects (Liu et al., 2002; Franco and Campione, 2003; Bamforth et al., 2004), the role of Cited2 in establishing embryonic laterality was suggested to explain the diverse cardiovascular malformations in embryos lacking Cited2 (Bamforth et al., 2004).

However, 4 of 13 Cited2-deficient hearts displayed normal Pitx2c expression in dorsal ventricular cells irrespective of their laterality phenotype; 9 of 28 Cited2-null embryos did not exhibit isomerism, but still developed a complex array of cardiovascular defects (Weninger et al., 2005). In our study, all 12-cosogenic C57BL/6J Cited2−/− embryos had a wide spectrum of cardiac defects, while only 7 of 12 Cited2−/− embryos had left–right patterning defects. Therefore, some of the cardiac malformations in Cited2−/− embryos may be independent of left–right patterning defects.

Bamforth et al. (2004) further showed that the laterality defects were significantly more frequent in Cited2−/− embryos on the C57BL/6J background (7/11, 64%) than on the mixed background (3/15, 20%), indicating that background-specific genetic modifiers have an important role in determining the left–right patterning defects. In their report, both the incidence of the OFT (7/15, 47%) and ventricular septal (10/15, 67%) defects in Cited2-null embryos on the mixed background and on the C57BL/6J background (10/11, 91%) were much higher than the incidence of the laterality defects, respectively, suggesting that the OFT and ventricular septal defects in Cited2−/− embryos, at least in part, are independent of the left–right patterning defects. Moreover, Yin et al. (2002) reported that the majority of Cited2−/− embryos (16/19, 84%) on the mixed background had the OFT and ventricular septal defects and Weninger et al. (2005) demonstrated that the incidence of the OFT (21/24, 87%) and ventricular septal defects (22/24, 92%) in Cited2-null embryos on the mixed background was extremely high. These results support that, unlike the laterality defects, the incidence of OFT and ventricular septal defects in Cited2−/− embryos is not significantly modified by genetic background.

In conclusion, our results establish an essential role for Cited2 in the development of the OFT and IVS of mouse embryonic heart by down-regulating the HIF-1 regulated hypoxia response. The absence of this regulatory function provides a mechanism to explain malformations of the OFT, IVS, and spleen in Cited2-deficient embryos in the absence of laterality defects. Interestingly, a recent study by Sperling et al. (2005) reported three functionally relevant mutations of Cited2 in 392 patients with congenital heart defects. All of these mutations significantly reduced the capacity of Cited2 to transrepress HIF-1α with one also resulting in significantly diminished Tfp2c co-activation. The importance of embryonic hypoxia has long been recognized as an environmental risk factor of congenital heart defects. To date, however, the mechanism by which prenatal hypoxia contributes to congenital heart defects has not been established. The Cited2 knockout mouse in combination with other mouse lines may serve as useful models to investigate the molecular mechanism by which hypoxia regulates critical events in normal and abnormal embryonic heart development. These studies may also provide potential therapeutic strategies in treating patients with congenital heart defects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.072.

References


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