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Review

The Role of Hypoxia in Development of the Mammalian Embryo

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Hypoxia inducible factor (HIF) is a transcription factor that acts in low-oxygen conditions. The cellular response to HIF activation is transcriptional upregulation of a large group of genes. Some target genes promote anaerobic metabolism to reduce oxygen consumption, while others "alleviate" hypoxia by acting non-cell-autonomously to extend and modify the surrounding vasculature. Although hypoxia is often thought of as being a pathological phenomenon, the mammalian embryo in fact develops in a low-oxygen environment, and in this context HIF has additional responsibilities. This review describes how low oxygen and HIF affect gene expression, cell behavior, and ultimately morphogenesis of the embryo and placenta.

Introduction

Some 2.4 billion years ago, photosynthesis lead to the accumulation of oxygen to levels that were likely toxic to many obligate anaerobes. Organisms that could defend themselves against oxidative stress, and at the same time utilize oxygen for energy, survived and evolved. As time went on, a cellular requirement for oxygen became critical, and animals developed a biochemical response to low levels of oxygen. There are a number of oxygen-sensing pathways that promote hypoxia tolerance by activating transcription and inhibiting translation: the energy and nutrient sensor mTOR, the unfolded protein response that activates the endoplasmic stress response, and the nuclear factor (NF)-κB transcriptional response (Perkins, 2007; Wouters and Koritzinsky, 2008). In addition, the transcriptional response mediated by hypoxia inducible factor (HIF) is a key feature of the cellular response to hypoxia, and its role in mammalian embryo development is the focus of this review.

HIF is a heterodimeric transcription factor consisting of two subunits, Hifα and aryl hydrocarbon receptor nuclear translocator (Arnt; also called Hif1β) (Figure 1A); these factors interact via two Per-Arnt-Sim (PAS) domains, bind DNA via N-terminal basic helix-loop-helix (bHLH) domains, and activate transcription with C-terminal transcriptional transactivation domains (TADs). In mammals, Arnt is constitutively expressed, but the expression and activity of Hifα are regulated by cellular oxygen concentration. In the absence of hypoxic stress—that is, at oxygen concentrations above 5%—prolyl hydroxlase domain proteins (Phd1–3) hydroxylate two proline residues of Hifα, enabling the binding of the von Hippel-Lindau tumor suppressor protein (Vhl). Vhl is the recognition component of an E3 ubiquitin-protein ligase that targets Hifα for ubiquitylation and proteasomal degradation (Ruas and Poellinger, 2005; Schofield and Ratcliffe, 2005). In addition, the factor inhibiting HIF (Fih) hydroxylates an asparagine residue in the TAD, blocking binding of the transcriptional coactivator CBP/p300 (Lisy and Peet, 2008). Under low-oxygen conditions (less than 5%), the rate of prolyl and asparaginyl hydroxylation is reduced, and as a result Hifα accumulates, dimerizes with Arnt, and translocates to the nucleus, where it binds DNA and associates with transcriptional coactivators.

There are three Hifα genes (Hif1α, Hif2α, and Hif3α) (Figure 1B). Hif1α dimerizes with Arnt forming the HIF1 transcription factor, and HIF2 is formed similarly via the association of Hif2α and Arnt: both HIF1 and HIF2 activate transcription. Three Hif3α isoforms (Hif3α, neonatal and embryonic PAS [NEPAS], and inhibitory PAS protein [IPAS]) have been identified; Hif3α and NEPAS have a single TAD, unlike Hif1α and Hif2α (which have two), whereas IPAS lacks a TAD (Yamashita et al., 2008). Hif3α and NEPAS negatively regulate HIF1 and HIF2 activity indirectly by competing for available Arnt, and then only weakly activating transcription. IPAS inhibits HIF1 and HIF2 activity through a different mechanism by binding Hif1α and Hif2α and preventing their heterodimerization with Arnt (Hara et al., 2001; Yamashita et al., 2008). HIFs bind to the hypoxia responsive element (HRE) (Ruas and Poellinger, 2005) to regulate the transcription of some 200 genes in response to hypoxia (Elvidge et al., 2006; Manalo et al., 2005). Currently, experimental evidence of direct transcriptional activation by HIF activity exists for around 70 of these (Wenger et al., 2005), and some target genes specifically activated by HIF1 or HIF2 have been identified (Aprelikova et al., 2006; Elvidge et al., 2006; Hu et al., 2003).

The functional repertoire of the HIF transcription system expanded with the increase in complexity and size that occurred during animal speciation. In the nematode worm *Caenorhabditis elegans*, where oxygen delivery occurs by diffusion, hypoxia is resisted through reduced energy expenditure and a HIF-dependent switch to anaerobic metabolism, achieved via upregulated expression of glucose transporters and glycolytic enzymes. Simple diffusion of oxygen is inadequate in larger organisms, where an oxygen delivery system is required (Fisher and Burggren, 2007). The fruit fly *Drosophila melanogaster*, for example, has co-opted the HIF transcription network to facilitate the formation of the oxygen-delivering tracheal system (Gorr et al., 2006). Interestingly, the hypoxia-responsive *Drosophila* Hif1α homolog (sima) is not required for formation of the tracheal system. Instead trachealess, which is not induced by hypoxia, dimerizes with the Arnt homolog (tango) and specifies tracheal cells. The further increase in complexity of vertebrates, and the evolution of endotherms (birds and mammals), necessitated an efficient solution to the mounting demands for oxygen and

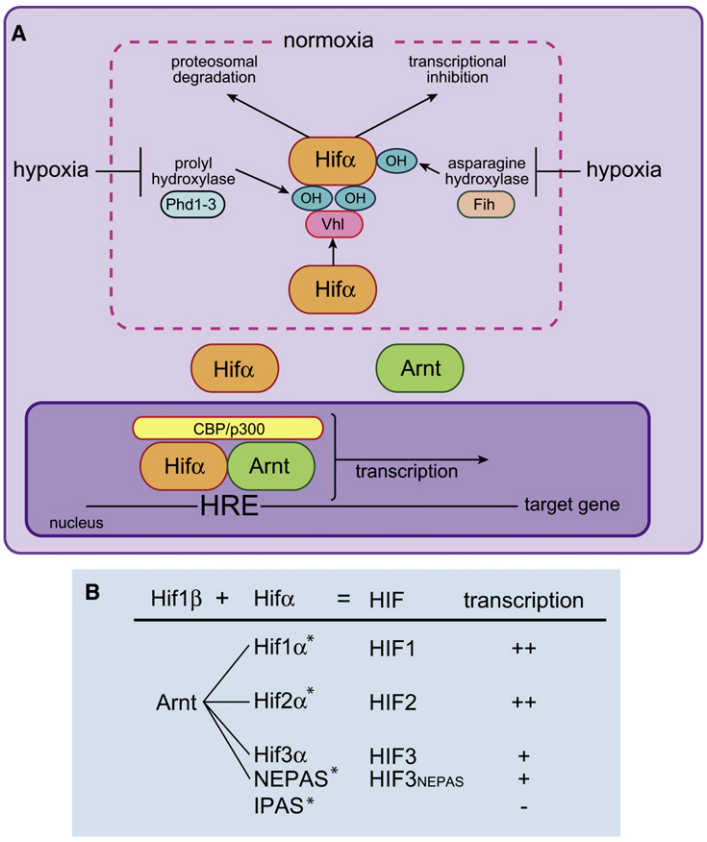

Figure 1. HIF Regulation under Aerobic and Hypoxic Conditions

(A) Oxygen is required for the hydroxylation of two proline residues of Hifα by prolyl hydroxylase domain proteins (Phd1–3). Proline hydroxylation is necessary for binding of the von Hippel-Lindau tumor suppressor protein (Vhl) and ubiquitin-mediated proteosomal degradation. Oxygen is also a cofactor for hydroxylation of an asparagine residue in the transcriptional activation domain of Hifα by the asparagine hydroxylase factor inhibiting HIF (Fih). Asparagine hydroxylation prevents the binding of the transcriptional coactivator CBP/p300. Hypoxia inhibits proline and asparagine hydroxylation, which allows Hifα to accumulate, dimerize with Arnt, form the HIF transcription complex, and activate transcription of target genes that carry functional hypoxia responsive elements (HREs).

(B) Arnt (Hif1β) dimerizes with Hif1α or Hif2α to form HIF1 and HIF2, respectively, which activate transcription (++). Hif3α encodes for three isoforms (Hif3α, NEPAS, and IPAS). Dimerization of Arnt and Hif3α or NEPAS forms HIF3 or HIF3NEPAS, which activate transcription to a lesser extent (+). IPAS binds Hif1α or Hif2α, preventing interaction with Arnt and inhibiting transcription (–). NEPAS, neonatal and embryonic PAS; IPAS, inhibitory PAS protein. (*) denotes protein stabilized by hypoxia.

nutrients. This requirement was met by altering the circuitry and increasing the capacity of the cardiovascular system, as excellently reviewed by Fisher and Burggren (2007). Despite the efficacy of the cardiovascular system in sensing and ameliorating systemic low oxygen, cells can still experience hypoxia, depending on their proximity to blood vessels, tissue architecture, and rate of oxygen consumption. When hypoxic conditions occur, HIF transcriptionally activates genes that, depending on the context, are involved in energy metabolism, autophagy, translation inhibition, erythropoiesis, and angiogenesis. These genes promote tolerance of hypoxia by decreasing the cellular requirement for oxygen and increasing the supply of oxygen (Anderson et al., 2009; Kaelin and Ratcliffe, 2008).

In mammals the HIF transcription system is not only involved in tolerance of, and rescue from, pathological hypoxia. Normal mammalian development occurs in a hypoxic environment, and here HIF activates genes that regulate cellular events, so hypoxia and HIF are therefore responsible for aspects of developmental morphogenesis. Oxygen concentrations range from 1% to 5% (pO_2 0.5–30 mmHg) in the uterine environment (Okazaki and Maltepe, 2006). Although the placenta and the embryonic cardiovascular system are conduits for oxygen delivery within the developing conceptus, they too are forming and so conditions of low cellular oxygen prevail. Cells low in oxygen can be identified using 2-nitromidazole drugs, such as pimonidazole and EF5, which bind protein and DNA at ≤2% oxygen (Mahy et al., 2003). There is significant colocalization of drug binding and Hif1α protein expression, demonstrating that these drugs are a useful marker of regions experiencing cellular hypoxia. In the mouse embryo, cells low in oxygen (≤2%) are widespread (Lee et al., 2001; Pringle et al., 2007) until the maternal and fetal blood interface around midgestation. After this time cells low in oxygen are still consistently detected in specific regions of the embryo, including the developing heart, gut, and skeleton (Figure 2). In part this is probably because the developing vasculature lacks the capacity to keep pace with the phenomenal growth and energy demands of the embryo during the second half of gestation. The HIF transcription system is used in embryos to enable cellular survival in a low-oxygen environment. However, during evolution HIF has been co-opted to direct many other cellular processes that ultimately enable the embryo to survive after birth.

A true appreciation of the role of HIF in the cellular response to low oxygen levels during embryonic development is still unfolding; its effects on stem cell function and on angiogenesis have been recently reviewed by Simon and Keith (2008) and Fraisl et al. (2009). Here I describe its impact on development of the placenta, heart, and bone in mouse. Through effects on cell proliferation, cell differentiation, and cell behavior, the response to low oxygen (or physiological hypoxia) ultimately contributes to embryonic morphogenesis. Throughout this review, the term "hypoxia" will be used to describe the naturally low (<2%) oxygen concentrations experienced during normal development. This type of hypoxia is distinct from "nonphysiological hypoxia," which can result from cardiovascular demise, placental insufficiency, or environmental insult.

Oxygen and HIF Activity Impact Placental Development

The placenta is a remarkable amalgamation of tissues derived from extraembryonic cells of the conceptus and the maternal decidua (uterine lining). The past decade has seen an astounding increase in our understanding of placental development, and in our appreciation of this organ's role in normal embryonic development. These advances are covered in excellent review articles (Simmons et al., 2007; Watson and Cross, 2005).

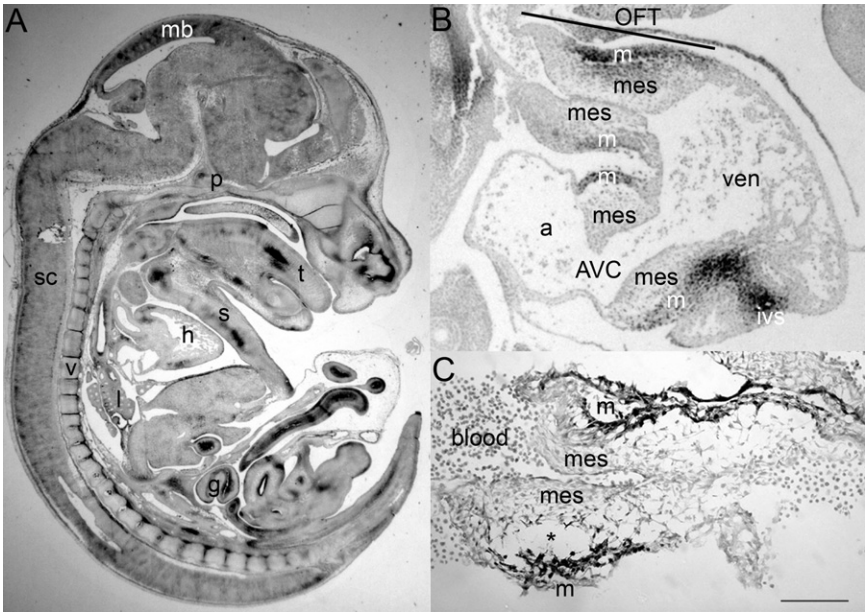

Figure 2. Cellular Hypoxia in the Embryo and Heart

Pimonidazole (2-nitromidazole compound) binds DNA and protein at <2% oxygen and identifies hypoxic cells (black) in parasagittal sections in the mouse embryo at E14.5 (A), E11.5 heart (B), and E11.5 atrioventricular endocardial cushion (C). Hypoxic cells are present at E14.5 in the heart (h), gut (g), lung (l), midbrain (mb), pituitary (p), spinal cord (sc), sternum (s), tongue (t), and vertebra (v); and at E11.5, also in the interventricular septum (ivs), cushion-associated myocardium (m) of the outflow tract (OFT), and atrioventricular canal (AVC). a, atrium; mes, mesenchymal cells; ven, ventricle. (*) denotes torn tissue between the myocardium and mesenchymal cells of the developing endocardial cushion. These data (unpublished) support and extend that published by Ream et al. (2008) and Xu et al. (2007). Scale bar: 108 mm (A), 130 mm (B), 13 mm (C).

Briefly, the placenta develops between embryonic day (E) 3.5 and 14.5 of gestation, and key features of its morphogenesis are summarized in Figure 3. In mouse at E3.5 the conceptus (blastocyst) consists of two cell lineages: the trophectoderm, which gives rise to trophoblasts that constitute the placenta, and the inner cell mass (ICM), which gives rise to the embryo, allantois (component of umbilical cord), and fetal vasculature of the placenta. At embryo implantation (E4.5), trophectoderm overlaying the ICM proliferates and produces the extraembryonic ectoderm, while mural trophectoderm not associated with the ICM ceases to divide and undergoes endoreduplication, forming primary parietal trophoblast giant cells (TGCs) that are polyploid. After embryo implantation a secondary wave of parietal TGCs forms, and these surround the entire conceptus and facilitate implantation and invasion into the decidua. As development proceeds the extraembryonic ectoderm expands to form the chorion and ectoplacental cone (EPC); these tissues provide a pool of diploid trophoblast progenitor cells that support further placental development.

At maturity, the murine placenta consists of three fetal layers (Figure 3): the labyrinthine layer is proximal to the embryo, and is where fetal blood vessels and trophoblast-lined maternal blood sinuses are brought into proximity to enable nutrient and gaseous exchange. Next is the spongiotrophoblast layer, which provides structural support to the expanding labyrinthine layer and is a source of trophoblast subtypes (Figure 4). The parietal TGC layer then lies distal to the spongiotrophoblasts and borders the decidua. The parietal TGCs facilitate embryo implantation and decidua invasion, and produce hormones to maintain the pregnancy. Fetal development requires that the placenta is integrated with the maternal decidua. Thus spiral arteries direct maternal blood from the decidua to the labyrinthine layer via central canals, and this organization requires decidual invasion by spiral artery TGCs. Glycogen trophoblasts also invade the decidua and are postulated to act as a source of stored energy (Coan et al., 2006). Because multiple cell types, derived from the conceptus as well as the mother, interact to produce a functional placenta, it is not surprising that at least 85 genes are required in mouse for normal placental formation and function (Watson and Cross, 2005).

One of the main functions of the placenta is to bring oxygenated maternal blood into proximity with deoxygenated fetal blood; paradoxically, however, the placenta develops in a low-oxygen environment and some placental cells remain hypoxic ($\leq$2% oxygen) later in development despite the flow of oxygenated maternal blood to the placenta (Okazaki and Maltepe, 2006; Withington et al., 2006). Hypoxic cells, and cells expressing Hif1α and Hif2α, are present in the decidua and placenta between E6.5–14.5 (Pringle et al., 2007; Schäffer et al., 2006; Withington et al., 2006).

HIF activity is required for normal development of the placenta, and loss of *Hif1*α, both *Hif1*α and *Hif2*α, or *Arnt* in mouse causes a catastrophic failure in placenta formation, resulting in embryo lethality by E10.5 (Abbott and Buckalew, 2000; Adelman et al., 2000; Cowden Dahl et al., 2005a; Kozak et al., 1997). Key features of the placental defects (Table 1 and discussed below) include: reduced or lessened interaction between the embryo and forming placenta (chorioallantoic interaction); reduced development of the labyrinthine layer; limited vascularization of the placenta; overarching disruption of trophoblast differentiation with the absence of syncytiotrophoblasts; a dramatic reduction in spongiotrophoblasts; and greater numbers of TGCs. These placental defects can be placed in context with our current understanding of the effects of hypoxia on trophoblast proliferation, differentiation, and gene expression.

HIF Regulates Branching Morphogenesis and Fetal Vascularization of the Placenta

Development of the labyrinthine layer of the placenta requires interaction between the allantois, which is the source of the fetal vasculature of the placenta, and the chorion, which is the source of diploid trophoblasts (Figures 3 and 4). This interaction involves reciprocal signaling between the allantois, which

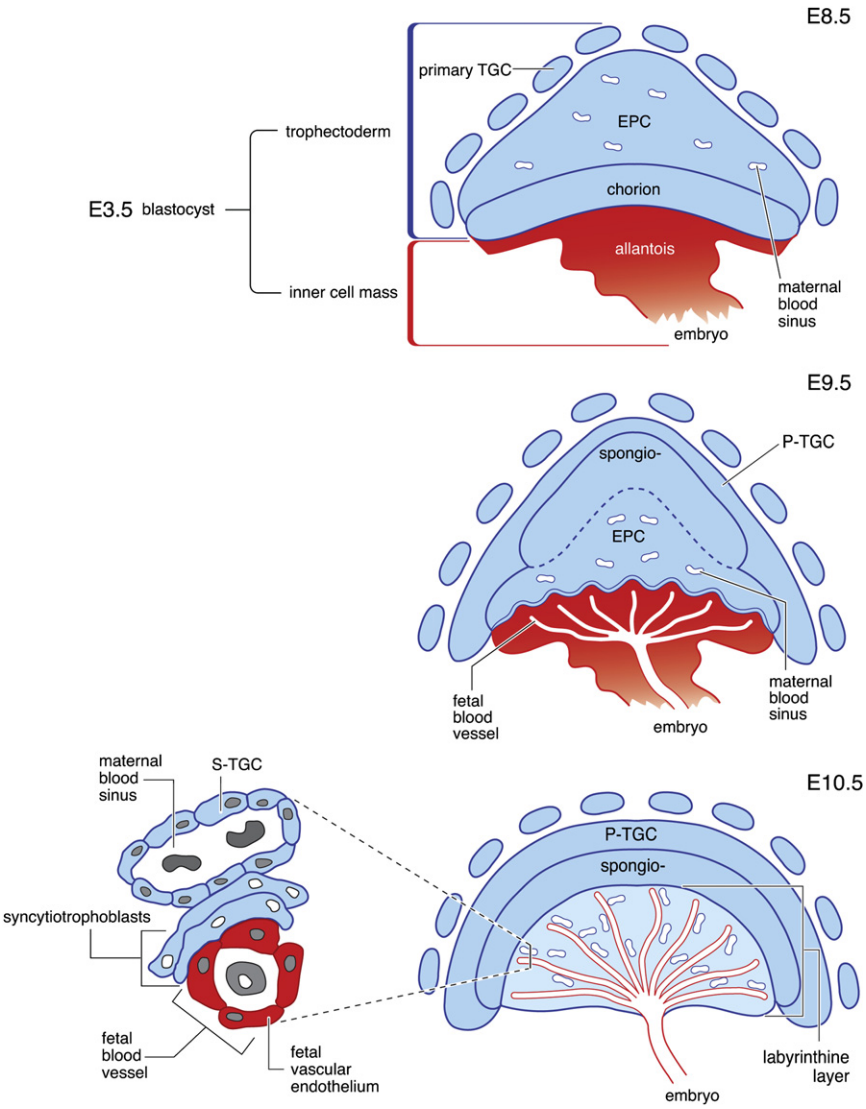

Figure 3. Placental Development in Mouse
Two distinct cell lineages of the blastocyst, the ICM and the trophectoderm, contribute to the placenta. The maternal decidua surrounds the conceptus (not depicted), and primary parietal trophoblast giant cells (P-TGC; oval-shaped) facilitate the invasion of the decidua as placental development proceeds. At E8.5 cells of the ectoplacental cone (EPC) are interspersed with sinuses lined with sinusoidal TGCs (S-TGC) and filled with maternal blood. The chorion and allantois interact; this induces folding of the chorion, which facilitates branching morphogenesis and vascularization of the developing labyrinthine layer. Trophoblasts of the chorion and EPC proliferate and differentiate into trophoblast subtypes (see Figure 4). At E9.5 spongiotrophoblasts (spongio-) and secondary P-TGC layers are evident as the labyrinthine layer expands. At E10.5 the density of fetal blood vessels and maternal blood sinuses increases in the labyrinthine layer. Fetal endothelial cells, syncytiotrophoblasts, and S-TGCs separate fetal and maternal bloods.

extends from the caudal aspect of the embryo, and the chorion, and it is essential for buckling of the chorion, for its penetration by fetal vascular endothelial cells, and for diploid trophoblast proliferation (Stecca et al., 2002). Further elaboration of the fetal vasculature requires branching morphogenesis of the trophoblast-derived epithelium and angiogenesis by fetal vascular endothelial cells.

HIF activity is required for effective chorioallantoic interaction and branching morphogenesis. Loss of HIF1α activity disrupts chorioallantoic interaction in 31% of null embryos, and accordingly fetal blood vessels are unable to invade the trophoblasts (Table 1). In these cases, no overt morphological deficiency in the allantois or chorion is evident, suggesting that HIF affects molecular components, such as integrins and their ligands (*Itga4*, *Cyr61*, and *Vcam1*), that are required for the interaction itself (Watson and Cross, 2005). In the majority of null embryos, chorioallantoic interaction does occur, but vascularization of the developing labyrinthine layer is reduced. This suggests that branching morphogenesis of the trophoblasts is impaired, that angiogenesis by fetal vascular endothelial cells is defective, or both.

The molecular mechanism by which HIF affects chorioallantoic fusion and fetal vascularization is unclear, but at least two avenues seem worthy of further investigation. First, chorioallantoic interaction and branching morphogenesis rely on integrins (*Itga4*, *Itga5*, *Itgb8*), and components of their signal transduction pathway (*Sos1*, *Raf1*, *Map3k3/Mekk3*, *Map2k1/Mek1*, *Mapk1/Erk2*, *Mapk7/Erk5*, and *Mapk14/p38alpha MAPK*) (Watson and Cross, 2005). Hypoxia induces the expression of some integrins and their ligands including *Itga5* (Cowden Dahl et al., 2005b; Kilburn et al., 2000), and Hif1α is stabilized by Mapk1 and Mapk3-induced phosphorylation (Berra et al., 2000). Second, vascularization of the placenta depends on angiogenesis, and some HIF target genes such as Kdr (Vegfr2), Angiopoietin1 (Ang1), and Tek (Tie2) are reduced in *Arnt* null placentas (Abbott and Buckalew, 2000). It will be interesting to discover through further investigation whether either or both of these pathways are involved in mediating the role that HIF plays in placental angiogenesis.

HIF Is Required for Trophoblast Proliferation and Differentiation

Trophoblasts of the chorion and EPC proliferate and differentiate into distinct trophoblast subtypes that populate the different layers of the placenta and play essential roles in its development and function. Cellular hypoxia is present as the placenta develops, and HIF activity is required for both trophoblast proliferation and the formation of specific cell subtypes. There are numerous differentiated trophoblast subtypes: diploid trophoblasts (spongiotrophoblasts and glycogen trophoblasts); diploid and multinucleated trophoblasts (syncytiotrophoblasts); and the polyploid TGCs (parietal, spiral artery, canal, and sinusoidal) (Figures 3 and 4) (Simmons et al., 2007). The maternal decidua is invaded by spiral artery TGCs and glycogen

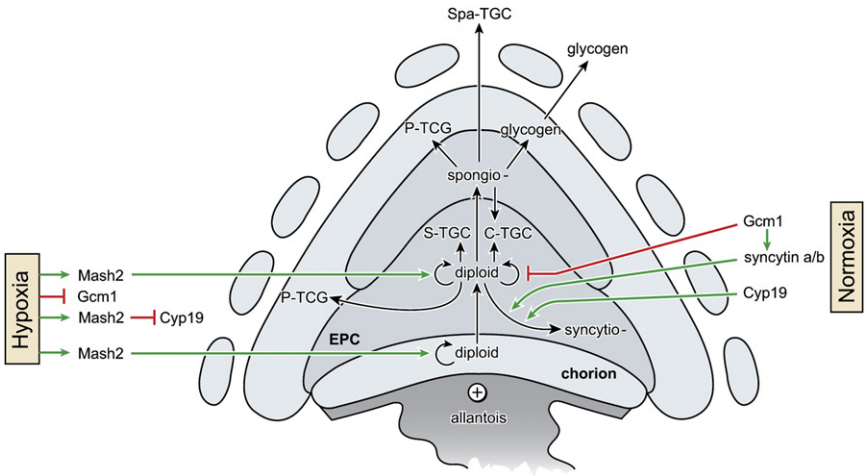

Figure 4. Trophoblast Lineages and the Effects of Hypoxia on Their Formation

Schematic diagram of the mouse placenta (decidua not shown) from E8.5–14.5 depicting lineage relationships of trophoblasts (black arrows). Diploid trophoblasts proliferate (semicircle with arrow) or differentiate (arrow) into various trophoblast subtypes. Hypoxia facilitates chorioallantoic attachment (+). Hypoxia induces Mash2 expression, supporting proliferation of diploid trophoblasts. Hypoxia inhibits Gcm1 and Cyp19 expression. Normoxia (absence of hypoxia) allows Gcm1, synctin-a/b, and Cyp19 expression; this supports the differentiation of syncytiotrophoblasts. Green arrows show stimulatory effects; red lines, inhibitory effects. The outer cells are primary parietal trophoblast giant cells (TGC; oval-shaped). Syncytio-, syncytiotrophoblast; S-TGC, sinusoidal TGC; C-TGC, canal TGC; spongio-, spongiotrophoblast; glycogen, glycogen trophoblast; P-TGC, parietal TGC; Spa-TGC, spiral artery TGC. (Adamson et al., 2002; Adelman et al., 2000; Cowden Dahl et al., 2005a; Simmons and Cross, 2005; Simmons et al., 2007).

trophoblasts, which are derived from spongiotrophoblasts. Spiral artery TGCs replace the maternal endothelial cells in the lumen of the spiral arteries and facilitate the funneling of maternal blood to the labyrinthine layer. Glycogen trophoblasts accumulate glycogen and localize near the spiral arteries, and their lysis prior to term may provide a substantial energy source for the final phase of fetal growth (Coan et al., 2006). Parietal TGCs line the wall of the implantation site separating the EPC from the decidua. They are formed in two waves—the primary parietal TGCs from mural trophectoderm, and the secondary parietal TGCs from the EPC and spongiotrophoblasts. These parietal TGCs are invasive and mediate embryo implantation; they also produce hormones that regulate the maternal endocrine and immune systems and promote blood flow to the implantation site. Spongiotrophoblasts, derived from the EPC, lie adjacent to the parietal TGCs. They provide structural support, secrete peptide hormones, and give rise to spiral artery TGCs, parietal TGCs, and glycogen trophoblasts. Canal TGCs line the central canals that bring maternal blood from the decidua to the labyrinthine layer, and are derived from the EPC and spongiotrophoblasts. A layer of fetal vascular endothelial cells (derived from the allantois), a layer of sinusoidal TGCs, and two layers of syncytiotrophoblasts separate the maternal and fetal blood spaces in the labyrinthine. Sinusoidal TGCs, which are derived from the EPC, line the maternal blood spaces; they secrete hormones and are likely to have an endocrine function. The syncytiotrophoblasts, also derived from the EPC, mediate trophic exchange between the mother and the fetus (Dupressoir et al., 2009).

Trophoblasts that border the decidua and labyrinthine layer (spongiotrophoblasts and parietal TGCs) are hypoxic from E9.5–14.5 (Pringle et al., 2007; Schäffer et al., 2006; Withington et al., 2006). During this time *Hif1α* and *Hif2α* transcripts are localized to these border cells. *Hif1α* is also highly expressed in the developing labyrinthine layer and *Hif2α* in the decidua. In addition, Hif1α protein is expressed in the decidua, spongiotrophoblasts, and parietal TGCs (Pringle et al., 2007; Schäffer et al., 2006; Withington et al., 2006).

Reduced HIF activity has a profound effect on the production of trophoblast subtypes; there are fewer spongiotrophoblasts and syncytiotrophoblasts, and an increase in TGCs (Table 1). These changes in trophoblast composition occur in the 69% of *Hif1α* null mutants that do undergo chorioallantoic interaction, indicating that HIF activity is also required at this later stage of placental development. A number of studies have indicated that the effect on trophoblast differentiation results from fewer diploid progenitor trophoblasts, and that proliferation of the progenitor diploid trophoblasts (resident in the chorion and EPC) intrinsically require HIF activity. In these studies, trophoblast stem (TS) cells have been a valuable tool for determining the roles that oxygen concentration and HIF activity have in trophoblast proliferation and differentiation. TS cells, although most readily derived from the blastocyst, are resident in the chorion. They can be propagated in culture, and differentiate into trophoblast subtypes (spongiotrophoblasts, TGCs, syncytiotrophoblasts) (Cowden Dahl et al., 2005a). Thus TS cells behave similarly to diploid progenitor trophoblasts. In the absence of *Arnt*, TS cells have a proliferation deficiency; conversely, low levels of oxygen promote proliferation of wild-type TS cells (Adelman et al., 2000). These results indicate that HIF activity regulates TS cell proliferation and by inference, that of diploid progenitor trophoblasts also.

In addition to stimulating the proliferation of progenitor trophoblasts, oxygen concentration and HIF activity also direct the differentiation of certain trophoblast subtypes. For example, there is evidence supporting a more direct role of HIF activity in spongiotrophoblast differentiation. In vitro, under low-oxygen conditions, differentiating TS cells have augmented expression of spongiotrophoblast markers such as *Mash2* and *Tpbpa* (Adelman et al., 2000; Cowden Dahl et al., 2005a; Maltepe et al., 2005). In addition, TS cells lacking *Hif1α*/*Hif2α* or *Arnt* are virtually incapable of differentiating into *Tpbpa*-expressing spongiotrophoblasts (Adelman et al., 2000; Cowden Dahl et al., 2005a; Maltepe et al., 2005). Therefore, trophoblast progenitors seem to require the HIF-mediated hypoxic response to drive spongiotrophoblast production in the developing placenta (Figure 4).

Table 1. Placental Defects in Mice Lacking Components of the HIF Transcription Complex

Gene	Allele (Type)	Mouse Model (Cells Affected)	Lethal	Key Aspects of Placenta Phenotype	Marker Gene Expression	HIF Target Gene Expression	Reference
Hif1α	Hif1α^{tm1Pec} (null)	homozygous (conceptus)	E10.5	no chorioallantoic attachment or vascularization (31%), reduced vascularization and spongiotrophoblasts (69%)	mPl1, Hand1, LIMK, Ada, Pl1, Mmp9, Id2, VE-cadherin, *Tpbp, Mash2*	Vegfa, Flt1, Flk1	Cowden Dahl et al., 2005
Hif1α Hif2α	Hif1α^{tm1Pec} Epas1^{tm1Pec} (null)	homozygous (conceptus)	E10.5	no chorioallantoic attachment or vascularization, reduced spongiotrophoblasts and syncytiotrophoblasts	**mPl1,** *Tpbp, Tfeb*	-	Cowden Dahl et al., 2005
Arnt	Arnt^{tm1Oha} (null)	homozygous (conceptus)	E9.5–10.5	E9.5 small chorioallantoic plate, reduced vascularisation, and cavities in labyrinth	*Pecam1*	-	Kozak et al., 1997
Arnt	Arnt^{tm1Mcs} (null)	homozygous (conceptus)	E9.5–10.5	chorionic plate present, reduced vascularization and spongiotrophoblasts, increased TGC	Mash2 (E8.5), **mPl1,** *4311, Tgfβ3 (E9.5)*	*Tgfβ3 (E9.5)*	Adelman et al., 2000
Arnt	Arnt^{tm1Mcs} (null)	chimera (embryo and vasculature of placenta)	E9.5–10.5	E9.7 placenta normal but yolk sac defect retained	Tgfβ1, Glut3, Mash2, p21, Tfeb, Pdgfβ, Pdgfrβ, Plf, Plfr, Pgf, CSF-1R,	Glut1, Vegf, Tie2, *Tgfβ3*	Adelman et al., 2000
Arnt	Arnt^{tm1Oha} (null)	homozygous (conceptus)	E10.5	reduced vascularization	-	Vegfa, *Vegfr2, Tie2, Ang1*	Abbott et al., 2000
Arnt	Arnt^{tm1Mcs} (null)	homozygous (conceptus)		no chorioallantoic attachment, reduced spongiotrophoblasts and syncytiotrophoblasts	**mPl1,** *Tpbp, Tfeb*	-	Cowden Dahl et al., 2005

Allele symbols are standard (http://www.informatics.jax.org). Conceptus includes embryonic and extraembryonic tissues. The publications use various gene names; Flt1 (Vegfr1), Kdr (Vegfr2, Flk1), Tek (Tie2), and 4311 (Tpbpa). Gene expression level: equal, Roman lightface text; up, boldface; down, italics. Initial analysis of conceptuses homozygous for the Arnt^{tm1Mcs} null allele did not describe placental defects (Maltepe et al., 1997). Hif2α (Epas) null conspectuses do not have placental defects (Tian et al., 1998; Cowden Dahl et al., 2005a; Peng et al., 2000).

Although loss of HIF activity leads to an increase in the number of TGCs in the placenta, two lines of in vitro evidence suggest that this defect is unlikely to reflect a direct role of HIF in TGC differentiation. First, TS cells grown in low-oxygen conditions do not preferentially differentiate into TGCs, given that they express reduced levels of *Hand1* (required for TGC differentiation) and *proliferin* (a TGC marker) (Cowden Dahl et al., 2005a). Second, TS cells null for *Hif1α*/*Hif2α* or *Arnt* do not overproduce TGCs; indeed, expression of TGC markers (*proliferin* and *placental lactogen1*) is greatly diminished (Cowden Dahl et al., 2005a; Maltepe et al., 2005). Therefore, the most parsimonious explanation for the increase in TGCs in placentas that lack *Hif1α*/*Hif2α* or *Arnt* is that diploid trophoblasts differentiate into parietal TGCs because they are unable to form spongiotrophoblasts.

Lack of HIF activity results in the loss of syncytiotrophoblasts in the placenta, but this effect is unlikely to be direct and instead is probably a result of the lack of progenitor trophoblasts caused by their diminished proliferation, as discussed above. Syncytiotrophoblasts are reduced in placentas lacking *Hif1α*/*Hif2α* or *Arnt* (Cowden Dahl et al., 2005a), suggesting that hypoxia and HIF might be required for syncytiotrophoblast production. However, this is unlikely to be the case because TS cells cultured in low oxygen do not form syncytiotrophoblasts, and their production from TS cells is in fact enhanced in the absence of *Hif1α*/*Hif2α* or *Arnt* (Cowden Dahl et al., 2005a).

The molecular framework that underpins progenitor trophoblast proliferation and syncytiotrophoblast differentiation is beginning to emerge (Figure 4). During trophoblast proliferation, low oxygen induces the expression of *Mash2* and *cyclin B*, which promote cell cycle progression in diploid progenitor trophoblasts; it also inhibits the expression of *Gcm1*, which promotes cell cycle exit (Hughes et al., 2004; Knerr et al., 2005). For syncytiotrophoblast formation, low oxygen and Mash2 repress the expression of Cyp19, which encodes the aromatase P450 that

promotes syncytiotrophoblast formation (Jiang and Mendelson, 2003). In addition, Gcm1 activates the expression of *syncytin-b*; because syncytin-a and syncytin-b promote cell fusion in culture, they might also promote the formation of syncytiotrophoblasts (Knerr et al., 2005; Simmons et al., 2008; Dupressoir et al., 2005). It appears that the overall effect of low oxygen and subsequent HIF activity is to lower syncytiotrophoblast numbers (Figure 4). In vivo, loss of HIF activity leads to a reduction in syncytiotrophoblasts because there is a loss of their progenitors (diploid trophoblasts). However, in vitro when there are sufficient progenitor trophoblasts, low oxygen and HIF activity prevent formation of syncytiotrophoblasts; conversely, when oxygen levels are high and HIF activity absent, syncytiotrophoblasts form (Cowden Dahl et al., 2005a).

In summary, placental development depends on an HIF-mediated response to the low oxygen environment in which the conceptus develops (Figure 4 and Table 1). Loss of HIF activity not only stalls placenta formation before it has the capacity to deliver oxygen or nutrients to the fetus, but has multiple other roles: (1) it induces expression of integrins and ligands in the allantois and chorion, facilitating chorioallantoic interaction and branching morphogenesis within the developing labyrinthine layer; (2) it induces expression of components of the MAP kinase pathway, facilitating branching morphogenesis; (3) it induces *Mash2* expression, trophoblast proliferation, and spongiotrophoblast differentiation; and (4) it inhibits *Gcm1* and *Cyp19* expression and thus prevents syncytiotrophoblast formation. This last point presents something of a paradox. Syncytiotrophoblast formation relies on expression of the transcription factor *Gcm1*, exit from the cell cycle, and cell fusion (Hughes et al., 2004; Jiang and Mendelson, 2005; Knerr et al., 2005). Hypoxia and HIF inhibit all these activities, so an increase in oxygen concentration would promote syncytiotrophoblast formation. How in an environment that is generally hypoxic do these syncytiotrophoblasts get exposed to higher oxygen levels, facilitating their differentiation and fusion? One explanation could be that oxygen is carried by maternal blood, which is "embedded" in an expanding domain of maternal blood sinuses resident in the developing labyrinthine layer (Figure 3). This microenvironment of higher oxygen could lead to cell cycle arrest and cell fusion to form syncytiotrophoblasts (Jiang et al., 2000a). High oxygen (or lack of hypoxia) derived from the maternal blood would also provide a trigger for syncytiotrophoblasts to form adjacent to maternal blood sinuses, and thus represents an exquisitely practical way of ensuring that these specialized trophoblasts form where they are required. Moreover, it further illustrates the ways in which oxygen can play a regulatory role in laying down the cellular topography required for the establishment of a functional placenta.

Placental insufficiency that develops as a result of reduced HIF activity reduces cellular oxygen levels throughout the embryo (Ryan et al., 1998). Consequently, studying the role that HIFs play during normal embryonic development is confounded by nonphysiological hypoxia generated as a result of an impaired placenta. Confusion resulting from the effects of placental insufficiency has been overcome with the use of tissue-specific deletion strategies, which have demonstrated that Hif1α is specifically required for development of the lung (Saini et al., 2008) as well as the heart and bone (see below).

The Effects of Hypoxia and HIF Activity on Heart Development

The mammalian heart consists of four specialized chambers that fulfill distinct roles. The atria collect blood and actively fill the ventricles, and the ventricles propel blood through the pulmonary and systemic vascular circuits. Valves and septa divide the four chambers to prevent blood regurgitation and the mixing of oxygenated and deoxygenated blood. The conduction system directs electrical impulses throughout the heart and coordinates chamber contraction. The coronary vasculature envelops and penetrates the myocardium (muscle tissue of the heart), and supplies oxygen and nutrients to this vital organ. Mouse heart development takes places from E7.75–15 (Figure 5), and is only covered briefly here. More detailed information can be found in recent reviews that highlight the key features of the process (Christoffels et al., 2004; Harvey, 2002; Hutson and Kirby, 2007).

A number of cell types with different origins contribute to the development of the heart. Cellular hypoxia and expression of HIF components (Hif1α, Hif2α, and Arnt) occur during the heart development process (Aitola and Pelto-Huikko, 2003; Jain et al., 1998; Krishnan et al., 2008; Lee et al., 2001; Ream et al., 2008; Xu et al., 2007), and HIF activity is absolutely required for normal development of the heart (Table 2, Figures 5 and 6A). More specifically, cardiac morphogenesis is abnormal in the absence of *Hif1α* or *Arnt,* but not *Hif2α* or *Hif3α.* In addition, studies outlined below place the HIF target gene *Vegfa* in a central position as a mediator of HIF function in heart development.

HIF Regulates Morphogenesis of the Developing Heart

As it forms, the mammalian heart undergoes a variety of morphogenetic changes (Figure 5). Cardiac progenitor cells (myocardial and endothelial) arise from mesoderm that coalesces at the anterior of the embryo. These cells adopt a crescent shape (E7.75), and then move ventrally and fuse into a linear heart tube (E8.25). This tube is composed of endothelial cells shrouded by a myocardial epithelium. The heart tube grows by division of myocardial cells and by addition of cells to both poles of the heart. Between E8.5 and E12 the heart undergoes looping morphogenesis and chamber formation. Initially the linear heart adopts a spiral form, and with further development the inflow portion that receives venous blood (primitive common atrium and sinus venosus) moves in an anterior and dorsal direction, bringing the inflow and outflow regions into proximity. Chamber formation occurs through local expansion of the myocardium. During the remodeling stage (E12.5–15), division of the heart chambers by septation is completed and valves ensure unidirectional blood flow. HIF activity is required for normal heart morphogenesis. Loss of *Hif1α* or *Arnt* leads to arrested morphogenesis at various stages from the cardiac crescent stage through to chamber formation (Table 2, Figure 5) (Adelman et al., 2000; Compernolle et al., 2003; Iyer et al., 1998; Ryan et al., 1998). Many of the mechanisms involved in these complex morphogenetic changes are not yet clear, and so the mechanistic basis of HIF contribution also remains to be established.

Loss of HIF Leads to Defective Development of the Myocardium

During heart development, the myocardium undergoes regional specialization (Figure 6A). Chamber formation requires the

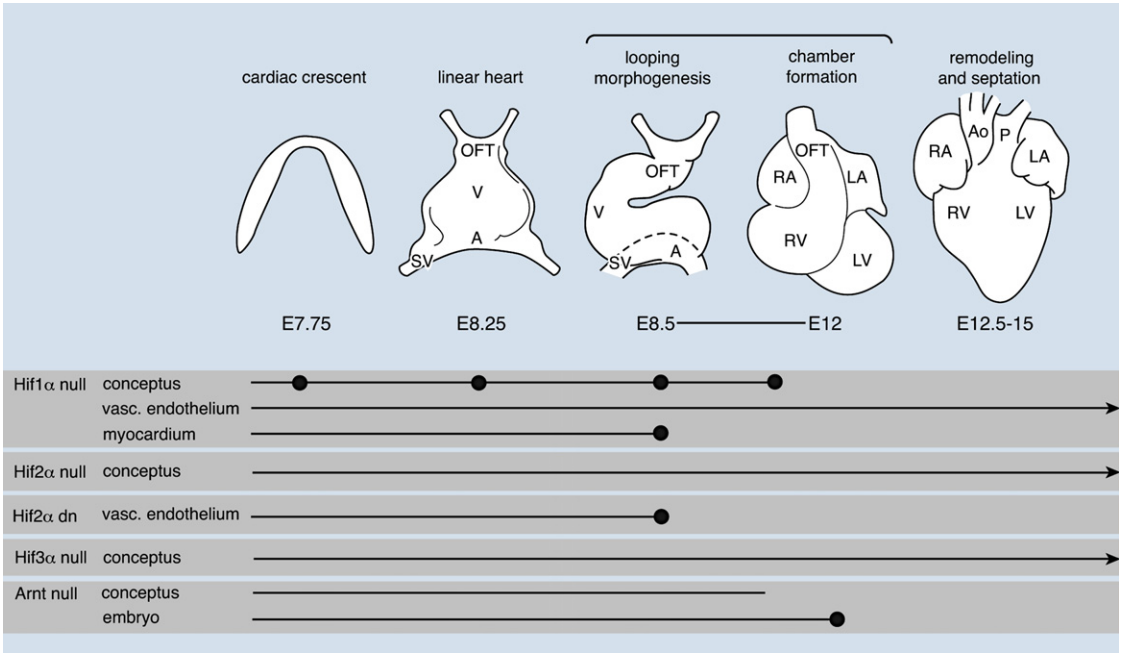

Figure 5. Heart Development and Loss of HIF Activity in Mouse

Stages of heart development (top) and the point at which cardiac morphogenesis arrests (circle) when HIF components are deleted or modified in mouse (bottom). HIF components (Hif1α, Hif2α, Hif3α, and Arnt) are deleted or modified (Hif2α dn) in the whole conceptus, the embryo, vascular (vasc) endothelium, or myocardium. OFT, outflow tract; V, ventricle; A, atrium; SV, sinus venosus; RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; Ao, aorta; P, pulmonary artery; dn, dominant-negative.

myocardium to balloon from the outer curvature of the heart tube in zones that presage the formation of the atria and ventricles. Subsequently, a sponge-like layer of myocytes (trabeculae) develops at the lumenal surface of the forming chambers. These are most prominent in the ventricles and are thought to increase the surface area to maximize oxygen uptake by the myocardium. At the same time, the outer layers of the ventricles thicken, as a result of cell proliferation, and form the ventricular compact layer. In addition the interventricular septum, which divides the ventricles, forms through polarized growth of myocardial cells at the junction of future left and right ventricles.

The myocardium is broadly hypoxic (≤2% oxygen) at E9.5, but by E13.5 the hypoxia is restricted to the myocardium of the outflow tract, interventricular septum, and atrioventricular cushions (Figure 2) (Krishnan et al., 2008; Lee et al., 2001; Ream et al., 2008; Xu et al., 2007). Hif1α protein localization mirrors the distribution of myocardial hypoxia during these stages (Krishnan et al., 2008; Lee et al., 2001; Ream et al., 2008; Xu et al., 2007). Immunohistochemical analysis with antibodies directed against Hif2α and Arnt has not been reported, but transcripts for *Hif1α*, *Hif2α*, and *Arnt* are present in the heart in mouse from E9 to day 1.5 (Aitola and Pelto-Huikko, 2003; Jain et al., 1998). In general, *Hif1α* is expressed in the myocardium of the ventricles and atrioventricular cushions, *Hif2α* in endothelial cells, and *Arnt* at low levels.

Development of the myocardium is severely disrupted in embryos that lack *Hif1α* (Table 2). Myocardial trabeculation is reduced or absent in embryos homozygous for either of two *Hif1α* null alleles (*Hif1αtm1Rsjo* and *Hif1αtm1Pec*), and this is consistent with the observed myocardial hypoplasia (Compernolle et al., 2003; Ryan et al., 1998). In striking contrast, mice homozygous for a third null allele, *Hif1αtm1Jhu*, display a remarkable myocardial hyperplasia (Iyer et al., 1998). In each of the *Hif1α* null alleles, exon 2, encoding the bHLH region of Hif1α required for DNA binding and protein dimerization, is deleted. However, neomycin is expressed using the phosphoglycerokinase (PGK) promoter in the *Hif1αtm1Jhu* allele, and so it is conceivable that the myocardial hyperplasia that is specific to this allele is dependent on the presence of PGK-Neo in the *Hif1α* locus rather than the loss of Hif1α per se. Myocardial hypoplasia also occurs in *Arnt* null embryos. Initially, heart defects were not reported in *Arnt* null embryos, which die at E10.5 (Kozak et al., 1997; Maltepe et al., 1997). However, subsequent analysis of tetraploid chimeras generated with *Arnt* null embryonic stem cells and wild-type (tetraploid) embryos revealed a role for *Arnt* in heart development (Adelman et al., 2000). Without the placental defect, embryo survival was improved and hypoplasia of ventricular myocardium and endocardial cushions was exposed. In addition to prolonged embryo survival, revealing heart defects, it is also possible that Arnt function was affected by modifiers, because *Arnt* null conceptuses and tetraploid chimeras had distinct mouse genetic backgrounds.

Loss of HIF Impacts the Vascular Endothelium of the Heart

Vascular endothelial cells are intrinsic to heart formation, and associate with both surfaces of the myocardium (Figure 6A). The lumenal endocardium develops in proximity to the myocardium from the cardiac crescent stage (E7.75), and the epicardium that envelops the heart from E10–13.5 is the source of

Table 2. Morphological Heart Defects in Embryos Lacking Components of the HIF Transcription Complex

Gene	Allele (Type)	Mouse Model (Cells Affected)	Lethal	Key Aspects of the Heart Phenotype	Marker Gene Expression	HIF Target Gene Expression	Reference
Hif1α	Hif1α^{tm1Rsjo} (null)	homozygous (all tissues)	E10	morphogenesis arrested at looping stage, small heart, reduced endothelial cells	*CD31*	-	Ryan et al., 1998
Hif1α	Hif1α^{tm1Jhu} (null)	homozygous (all tissues)	E10	morphogenesis arrested at linear heart tube stage, myocardial hyperplasia	Pecam1	-	Kotch et al., 1999, Iyer et al., 1998
Hif1α	Hif1α^{tm1Pec} (null)	homozygous (all tissues)	E9.5–10	morphogenesis arrested at various stages, reduced/ absent trabeculation, impaired neural crest cell migration	Hnf3, p75, NP-1, Nkx2-5, GATA-4, βMHC, MLC2a, SMA, desmin, Tgfβ1, Pdgfβ, *titin, Mef2c, eHAND, Id2, Et1, Sema3a, Ang1, Ang2, Tie2, VE-cadherin*	Anf, Vegfa, *ETa, Flk1, Flt1, Tie2*	Compernolle et al., 2003
Hif1α	Hif1α^{tm3Rsjo} (conditional null)	Hif1α^{tm3Rsjo}/ Hif1α^{tm1Jhu} and MLC2v-Cre (ventricular myocardium)	E11–12	morphogenesis arrested at looping stage, myocardial hyperplasia, increased myocyte proliferation	**cyclin D1, Cdk4,** *p27, p21, Nkx2-5, Tbx5, Mef2c*	-	Krishnan et al., 2008
Hif2α	Flk1-HIFdn (dominant negative)	hemizygous (endothelial cells)	E11.5	morphogenesis arrested at looping stage, myocardial hypoplasia, absent trabeculation	Pecam1, Tie1, VE-cadherin	*Tie2*	Licht et al., 2006
Arnt	Arnt^{tm1Mcs}	homozygous all tissues)	by E10.5	E9.5 "appeared normal"	-	*Vegfa* (embryo and yolksac)	Maltepe et al., 1997
Arnt	Arnt^{tm1Mcs}	chimera (embryo)	?	hypoplastic ventricular myocardium, reduced endocardial cushions, enlarged atrio-ventricular canal	-	-	Adelman et al., 2000

Allele symbols are standard (http://www.informatics.jax.org). Conceptus includes embryonic and extraembryonic tissues. The studies use various gene names; Flt1 (Vegfr1), Kdr (Flk1, Vegfr2), Tek (Tie2), CD31 (Pecam1), eHAND (Hand1), Anf (Nppa), SMA (smooth muscle actin), and ETa (Ednra). Gene expression level: equal, Roman lightface text; up, boldface; down, italics. Initial analysis of conceptuses homozygous for the Arnt^{tm1Mcs} null allele did not describe placental defects (Maltepe et al., 1997). Hif2α (Epas) null embryos are defective in catecholamine homeostasis and heart function; they do not have morphological heart defects (Compernolle et al., 2002; Peng et al., 2000; Tian et al., 1998). Hif3α null embryos survive but display enlarged right atrium and ventricle post birth (Yamashita et al., 2008).

the coronary vessels that lie on the outer myocardium and supply blood to the heart muscle. The epicardium is also the major source of the coronary microvasculature, which is embedded in the myocardium and is continuous with the coronary vessels. As stated, early in development the myocardium is generally hypoxic. This is followed by a dramatic reduction in the number of hypoxic cells between E13.5 and E15.5 in all regions except the atrioventricular cushions; this reduction correlates with the perfusion of the coronary vasculature following its connection to the aorta at E14.5 (Xu et al., 2007). Loss of *Hif1α* disrupts vascular endothelial development of the heart, and null embryos have a severely reduced endocardium (Ryan et al., 1998); however, the embryos die at E10 so it was not established whether *Hif1α* is required for formation of the epicardium or subsequent formation of the coronary vasculature. It is likely that *Hif1α* impacts formation of the coronary vasculature because HIF activity is required for normal vascular development in the embryo more generally, and angiogenesis in the embryo and yolk sac are affected in the absence of *Hif1α*, *Hif2α*, or *Arnt* (Compernolle et al., 2003; Duan et al., 2005; Iyer et al., 1998; Kozak et al., 1997; Maltepe et al., 1997; Peng et al., 2000; Ryan et al., 1998). For more information the reader is directed to a recent appraisal of the effects of oxygen and HIF on angiogenesis (Fraisl et al., 2009).

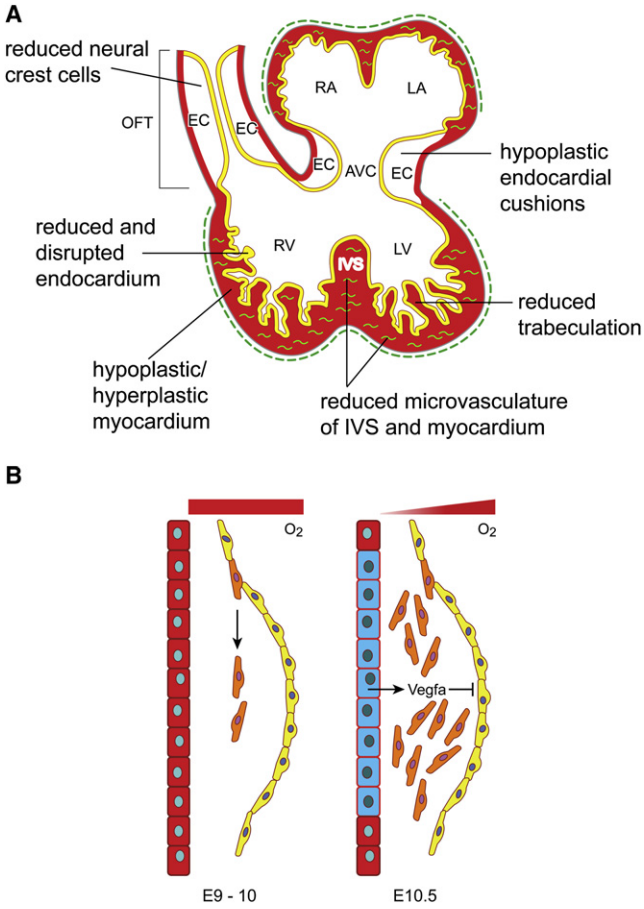

Figure 6. Features of Heart Development Impaired with Loss of HIF Activity

(A) Sectional representation of a normal mouse heart covering developmental stages E9–12 with a summary of defects that occur with the loss of HIF activity. Myocardium (red), epicardium (green dashed line), myocardial microvasculature (green ~), and endocardium lumenal layer (yellow) are shown. AVC, atrioventricular canal; EC, endocardial cushion; IVS, interventricular septum; LA, left atrium; LV, left ventricle; OFT, outflow tract; RA, right atrium; RV, right ventricle.

(B) Model of endocardial cushion formation. E9–10: myocardial cells support epithelial-to-mesenchymal transition. E10.5: mesenchymal cells populate the endocardial cushion and consume oxygen to an extent that renders the underlying myocardium hypoxic. Myocardial hypoxia results in vascular endothelial growth factor (Vegfa) production and inhibition of epithelial-to-mesenchymal transition. Myocardium (red), hypoxic myocardium (blue), endocardium (yellow), and mesenchymal cells (orange) are shown.

Is HIF Required in Both the Myocardium and the Associated Vascular Endothelium?

Development of the myocardium and vascular endothelial cells of the heart relies on their reciprocal interaction (Armstrong and Bischoff, 2004; Grego-Bessa et al., 2007; Tomanek, 2005). For this reason it is often difficult to establish if a gene is specifically required in one tissue or the other for heart development. Mice lacking HIF activity are defective in both myocardial and endocardial development, raising the question of whether HIF is required in the myocardium, the vascular endothelium, or both. Several lines of evidence indicate that normal heart morphogenesis requires *Hif1α* in the myocardium and not in the vascular endothelium of the heart. In addition, it is likely that expression of the HIF target gene *Vegfa* in the myocardium underpins the development of the vascular endothelium of the heart because Vegfa receptors (Flt1 [Vegfr1] and Kdr [Vegfr2]), expressed on vascular endothelial cells, affect survival, proliferation, migration, and differentiation. First, HIF activity is not required in vascular endothelial cells of the heart because deletion of *Hif1α* from these cells does not affect heart development (Tang et al., 2004). This indicates that the heart defects in *Hif1α* null embryos are likely to be derived from the loss of *Hif1α* in the myocardium. In support of this, deletion of *Hif1α* from the ventricular myocardium leads to an arrest of cardiac morphogenesis at the looping stage and to myocardial hyperplasia (Krishnan et al., 2008). However, this finding needs verification because the *Hif1α* null allele (*Hif1αtm1Jhu*) that was used in combination with the *Hif1α* conditional null allele uniquely causes myocardial hyperplasia; this is in contrast to the other *Hif1α* null alleles, which cause hypoplasia (Table 2), as previously discussed. In this study, the vascular endothelium of the heart was not examined; therefore, it is not clear what impact the myocardial-specific deletion of *Hif1α* might have on the vascular endothelial components of the heart during development. This is addressed in part by another study that also examines the role of *Hif1α* in the myocardium. Here, incomplete deletion of *Hif1α* from myocardial cells results in reduced microvascularity of the ventricular myocardium (Huang et al., 2004). This indicates that *Hif1α* in the myocardium affects development of vascular endothelial cells of the heart. It is likely that the HIF target Vegfa is responsible for this non-cell-autonomous effect, and this is supported by the finding that incomplete deletion of *Vegfa* from the developing myocardium results in myocardial and endothelial defects (Giordano et al., 2001). Although not as extensively studied in this context, it is also possible that another HIF target gene, *Ang2*, is expressed in the myocardium and affects vascular endothelial cell function because its receptor Tek (Tie2) functions in vascular endothelial cells (Simon et al., 2008; Suri et al., 1996). *Ang2* null embryos exhibit poorly developed myocardium and endocardium, like those null for *Hif1α*, and so the HIF1-Ang2 axis in the myocardium could also contribute to the heart defects in *Hif1α* null embryos.

Despite the evidence that HIF1 only acts in the myocardium during heart development, a function for HIF in vascular endothelial cells of the heart cannot be completely excluded. One study tested the role of HIF activity in endothelial cells by expressing a dominant-negative Hif2α (HIFdn) in vascular endothelial cells (Licht et al., 2006). HIFdn inhibits expression of HIF1 and HIF2 target genes in cultured cells, probably via binding to Hif1α, Hif2α, and Arnt. Embryos expressing HIFdn in endothelial cells develop heart defects that include a thin myocardium, a disorganized endocardium, and poorly established trabeculation. These results suggest that HIF transcriptional activity might be required in endothelial cells for normal heart development. Loss of either Hif1α from endothelial cells or Hif2α from the whole conceptus does not impair heart development (Table 2), but it is possible that HIF1 and HIF2 are performing redundant functions, and therefore only the loss of both would lead to a phenotype. Alternatively, HIFdn may interfere with Hif1α and Hif2α functions that are independent of Arnt, since Hif1α and Hif2α each also bind to other factors such as Notch1 and

β-catenin (see below). Deletion of Arnt from endothelial cells could test this possibility.

In summary, it is likely that *Hif1α* is required in the myocardium for normal myocardial and vascular endothelial development, and that Vegfa mediates the non-cell-autonomous function of HIF in vascular endothelial cells. Additional examples of myocardial-specific deletion of *Hif1α* will further clarify the situation.

HIF Is Required for Migration of Neural Crest Cells

Neural crest cells also contribute to heart development, and are affected by loss of Hif1α (Compernolle et al., 2003; Jiang et al., 2000b). Cardiac neural crest cells contribute to the outflow tract from E9.5, and if their numbers are reduced the outflow tract is misaligned by E12.5 and does not divide into the aorta and the pulmonary artery (Hutson and Kirby, 2007; Jiang et al., 2000b). Cardiac neural crest cells also provide all the parasympathetic innervation to the heart and thus are associated with maturation of the cardiac conduction system.

In *Hif1α* null embryos, neural crest cells form but do not migrate ventrally into the head mesenchyme and branchial arches (Compernolle et al., 2003). Neural crest cell migration is dependent on interaction between endothelin1, expressed by non-neural-crest cells in the branchial arches, and its receptor (Ednra), expressed by the neural crest cells (Hutson and Kirby, 2007). In the absence of *Hif1α*, expression of endothelin1 is upregulated, and this likely explains the observed defects in neural crest cell migration. Although *Hif1α* null embryos die before neural crest cells contribute to the heart (Table 2), their disrupted migration makes it highly likely that neural-crest-dependent heart development would be impaired by the loss of *Hif1α*. The role of *Hif1α* in cardiac neural crest cell migration could be addressed by specific deletion of *Hif1α* in neural crest cells (using Wnt1-Cre), and this would determine whether *Hif1α* acts in neural crest cells or plays a role in the mesenchyme to non-cell-autonomously direct neural crest function.

Hypoxia and HIF Are Features of Endocardial Cushion Formation

Myocardial hypoxia is a feature of endocardial cushion formation and there is evidence that loss of HIF activity affects cushion development. Endocardial cushions are swellings that form on the lumenal surface of the heart tube and give rise to the septa and valves (see Figure 6A) (Armstrong and Bischoff, 2004). They form in association with nonchamber myocardium and initially act to direct independent patterns of blood flow for the pulmonary and systemic circuits. In the atrioventricular canal, endocardial cushions are the precursors of the tricuspid and mitral valves, whereas in the outflow tract they form a scaffold for the aorticopulmonary septum that divides the outflow tract into the aorta and pulmonary artery and forms the aortic and pulmonary valves. Endocardial cushions develop from E9–10.5, and from E11 they are remodeled to form valves. Initially ECM (produced by the myocardium) is deposited between the myocardium and the endocardium, resulting in a swelling. Next, endocardial cells undergo epithelial-to-mesenchymal transition, producing mesenchymal cells that populate the ECM-rich swellings. Epithelial-to-mesenchymal transition then ceases, and signals from the endocardium promote remodeling and valve formation.

The hypoxia-HIF-Vegfa alliance is involved in development of the endocardial cushions in a few different ways. First, cushion myocardium is hypoxic from E11.5–14.5 (Xu et al., 2007 and Figures 2B and 2C) (earlier stages have not yet been examined). Second, epithelial-to-mesenchymal transition of the endocardium is inhibited if levels of the HIF target gene *Vegfa* are altered or if cushion explants are cultured in low oxygen (Armstrong and Bischoff, 2004; Dor et al., 2001; Dor et al., 2003). Other studies focused on Vegfa and nuclear factor of activated T cells cytoplasmic 1 (Nfatc1) indicate that Vegfa, produced by the myocardium, activates Kdr (Vegfr2) on endocardial cells, leading to nuclear localization of the transcription factor Nfatc1 and endocardial cell proliferation. Endocardial proliferation then facilitates epithelial-to-mesenchymal transition, which is necessary for mesenchymal cells to populate the developing cushion (Johnson et al., 2003). Although HIF plays a significant role in endocardial cushion formation, it is important to note that it is not the only regulator of *Vegfa* expression (Chang et al., 2004), and that factors other than Vegfa affect epithelial-to-mesenchymal transition in endocardial cushion formation. Interestingly, some of these factors are also linked to Hif1α: transforming growth factor β1, Notch1, β-catenin, and epidermal growth factor (Armstrong and Bischoff, 2004; Gustafsson et al., 2005; McMahon et al., 2006; Wang et al., 2009).

Why does the myocardium underlying the developing endocardial cushion become hypoxic, when adjacent myocardium does not? One possible explanation is illustrated in Figure 6B. Initially, when endocardial cushions are largely filled with ECM and few to no cells, the underlying myocardium is readily supplied with oxygen as blood passes between the developing cushions. As additional mesenchymal cells populate the endocardial cushions, they increasingly consume oxygen, and the cushion-associated myocardium is exposed to lower oxygen concentrations. Thus, cell density in the endocardial cushion inversely correlates with oxygen concentration. Moreover, oxygen depletion feeds back to prevent further epithelial-to-mesenchymal transition by triggering HIF activity to activate *Vegfa* expression. The effects of hypoxia on epithelial-to-mesenchymal transformation in the endocardial cushion could potentially serve as a paradigm for other locations in the embryo where epithelial-to-mesenchymal transitions occur.

In sum, hypoxia and HIF activity are features of normal heart development. Despite recent advances in our understanding, a number of key questions remain, including: to what extent are the heart defects secondary to impaired hemodynamics? What features of heart development specifically require HIF activity? What are the direct molecular effectors of HIF activity? Answering these questions will be crucial to understanding heart development, and will be best addressed by deleting HIF activity from myocardial progenitors (rather than just ventricular myocardium), or from epicardial cells or neural crest cells using appropriate Cre recombinase mouse lines such as Nkx2-5-Cre, Gata5-Cre, or Wnt1-Cre (Jiang et al., 2000b; Merki et al., 2005; Saga et al., 1999; Stanley et al., 2002).

Changing Oxygen Levels Impact Endochondrial Bone Formation

Endochondrial bone formation involves a progression from condensed mesenchyme to a bipotent osteochondro progenitor, to cartilage formation (chondrogenesis), and then to bone formation (osteogenesis) (Figure 7) (Karsenty, 2008). This

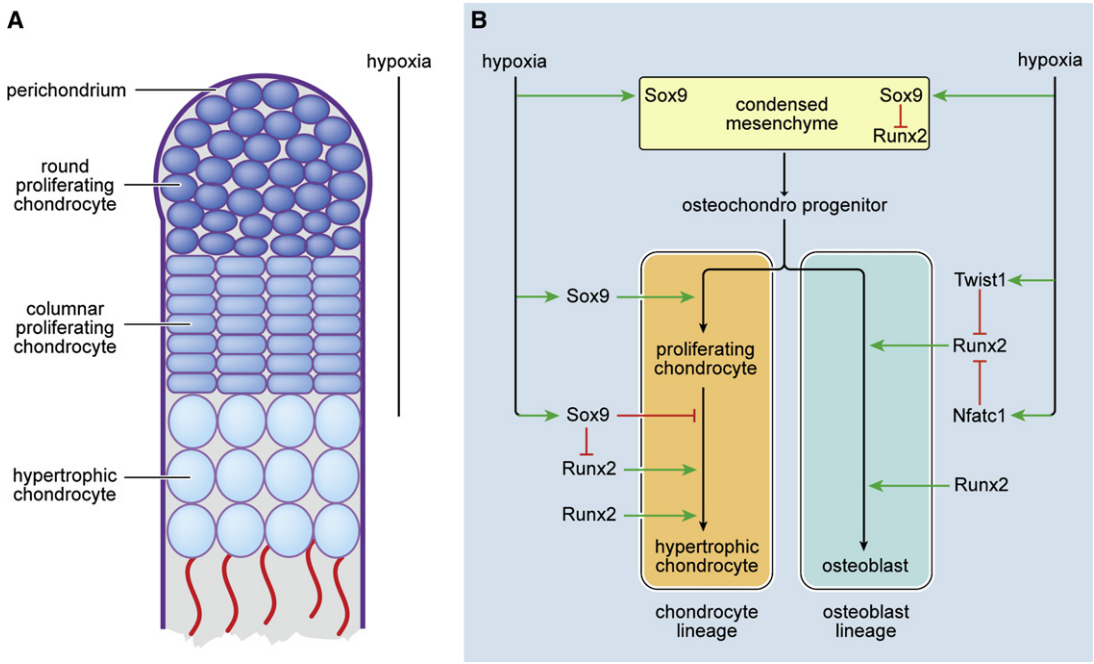

Figure 7. Hypoxia Directs Endochondrial Bone Formation

(A) Diagrammatic representation of chondrocyte proliferation and differentiation in the fetal growth plate of long bones (proximal at top). The vertical line depicts the extent of cellular hypoxia. Mesenchymal condensations differentiate into round and then columnar chondrocytes that proliferate and express collagen type II. As chondogenesis progresses, chondrocytes exit the cell cycle, undergo hypertropy, express collagen type X, direct mineralization of ECM, attract blood vessels, and then undergo apoptosis.

(B) Lineage relationships (black arrows) in endochondrial bone formation show condensing mesenchyme forming osteochondro progenitor cells, which differentiate under hypoxic conditions into chondrocytes or into osteoblasts once hypoxia is relieved. Hypoxia induces the expression of Sox9, Twist1, and Nfatc1, and inhibits Runx2 expression. Green arrows show stimulatory, and red lines inhibitory, interactions.

progression, which takes places in limb bones, ribs, and vertebrae, relies on bone forming on a template generated by the cartilage-producing chondrocytes. The chondrocytes that form from these progenitors undergo a program of proliferation, hypertrophy, ECM deposition, and subsequent apoptosis, which makes way for the bone-depositing osteoblasts. In mice the axial skeleton (vertebrae and ribs) forms from E10–18.5. The limbs also develop during this time with mesenchymal condensation occurring in the limb buds from E10.5 (Wright et al., 1995). There are a number of recent reviews that provide excellent appraisals of endochondrial bone formation (Dai and Rabie, 2007; Hall et al., 2006; Karsenty, 2008).

Many stages of endochondrial bone formation are sculpted by the availability of oxygen and the HIF-mediated transcriptional responses. The roles of hypoxia and HIF activity in endochondrial bone formation have been studied primarily in the context of the limb. Hypoxia ($\leq$2% oxygen) and a HIF-mediated response are apparent at the earliest stages (E10.5–12.5) of bone development in the mouse embryo (Amarilio et al., 2007; Provot et al., 2007). Moreover, tissue-specific deletion demonstrates that Hif1α is required during endochondrial bone formation for chondrogenesis (Amarilio et al., 2007; Provot et al., 2007).

Hypoxia and HIF Affect Mesenchyme Condensation and Chondrocyte Formation

The earliest stages of endochondrial bone formation require the HIF-mediated response to hypoxia. The limb anlage, which is highly vascularized, undergoes vascular remodeling, producing avascular areas that subsequently undergo mesenchymal condensation (Feinberg et al., 1986). It appears that hypoxia presages and therefore might also induce mesenchyme condensation. It also is possible that increasing cell density in an avascular environment would promote cellular hypoxia. As yet, specific studies to address these possibilities have not been performed. The condensing mesenchyme of the mouse limb bud and axial skeleton are hypoxic and express Hif1α protein (Amarilio et al., 2007; Provot et al., 2007). Deletion of *Hif1*α from E9.5 in limb bud mesenchyme using *Prx1-Cre* expression produces viable mice with dramatically shortened limbs (Amarilio et al., 2007; Provot et al., 2007). Because condensation of mesenchyme still occurs, the authors argued that mesenchymal condensation presages the hypoxia rather than being reliant on it. However, because the deletion of *Hif1*α only reached 75%, there could have been sufficient Hif1α to respond to the cellular hypoxia induced by the vascular remodeling prior to condensation. Complete deletion of *Hif1*α from this mesenchyme would be needed to address this question definitively.

Hypoxia and HIF Are Required for Chondrogenesis

Newly formed chondrocytes lie within the growth plates, which are located at the ends of long bones and are where growth occurs. Within the growth plate, distally located chondrocytes are round and proliferative; they then become organized into columns more proximally (Figure 7). These proliferating chondrocytes express angiogenic inhibitors (Moses et al., 1999)

and components of articular cartilage including aggrecan and type II collagen. Hypoxic cells and Hif1α are localized to the proximal growth plate where chondrocytes are undergoing proliferation (Schipani et al., 2001). As chondrogenesis progresses, chondrocytes exit the cell cycle, hypertrophy, and then undergo apoptosis. The hypertrophic chondrocytes are drivers of bone growth; they express collagen type X (which is subsequently replaced by bone and marrow), act as a regulatory cell type directing mineralization of the surrounding ECM, attract blood vessels through Vegfa expression, and attract chondroclasts (from the macrophage lineage) to digest ECM. In addition, before undergoing apoptosis hypertrophic chondrocytes instruct adjacent perichondrial cells to become osteoblasts. Osteoblasts differentiate on the plates of mineralized cartilage between columns of hypertrophic chondrocytes and lay down delicate spicules of woven bone (primary trabeculae), while osteoclasts remove mineralized ECM. Only the distal portion of the hypertrophic zone is hypoxic, and the transition in the growth plate from proliferating chondrocytes to hypertrophic chondrocytes coincides with a loss of hypoxia (Schipani, 2005).

Deletion of Hif1α early or late in chondrogenesis leads to massive cell death in the proximal limb bones (stylopod and zeugopod) and severe limb shortening (Schipani et al., 2001; Amarilio et al., 2007). However, the distal limb bones of the autopod are spared this cell death and instead exhibit delayed chondrogenesis (Schipani et al., 2001; Amarilio et al., 2007). This difference might reflect the variation in bone shape and thickness, which is likely to affect cellular oxygen concentrations (Provot et al., 2007). It also allowed the role of Hif1α in chondrocyte differentiation to be studied in the autopod, and revealed a delay in chondrogenesis (Provot et al., 2007). Normally, mesenchymal condensations of the developing limb differentiate into chondrocytes by E13.5, but in the absence of *Hif1α*, undifferentiated mesenchyme persists in the autopod. Differentiation to chondrocytes does still occur but it is clearly delayed, indicating that Hif1α is required for timely chondrocyte formation. Subsequent chondrocyte hypertrophy is also delayed, perhaps because there are fewer proliferative chondrocytes, or because *Hif1α* is specifically required for chondrocyte hypertrophy. This Hif1α-dependent delay in chondrogenesis is most apparent at joints (Amarilio et al., 2007; Provot et al., 2007). During joint formation the avascular perichondrium thickens, the underlying chondrocytes become hypoxic, and Hif1α protein is stabilized. The joints of the ankle and wrist, which are the most severely affected by the loss of *Hif1α*, are the regions in the developing limb with lowest levels of oxygen (Provot et al., 2007).

In sum, normal development of limb bones requires Hif1α. In its absence, the progression from condensed mesenchyme to proliferating chondrocytes and then to hypertrophic chondrocytes is delayed, leading to impaired chondrogenesis. Moreover, substantial cell death occurs in the growth plate, demonstrating that Hif1α is needed for chondrocyte survival. There are a number of potential mechanisms that could contribute to chondrocyte death in *Hif1α* mutants. Hif1α might act as a survival factor by activating the expression of *Vegfa,* which is required for chondrocyte survival (Dai and Rabie, 2007). Alternatively, Hif1α might promote the survival of chondrocytes through their dependence on ECM interaction. Hypoxia and Hif1α enhance the deposition of ECM (Pfander et al., 2003) by increasing the expression of procollagen hydroxylases (*P4ha1*, *P4ha2*, *Plod1*, and *Plod2*), which are necessary for the processing and secretion of collagen (Hofbauer et al., 2003; Takahashi et al., 2000). Thus, in the absence of Hif1α, a reduction in the ECM may lead to cell death. In a related manner reduced expression of procollagen hydroxylase leads to the accumulation of large amounts of unprocessed collagen, and the resultant unfolded protein response could trigger endoplasmic reticulum stress signaling (ERSS) and subsequently cell death (Wouters and Koritzinsky, 2008; Lai et al., 2007). Indeed, ERSS occurs and endochondrial bone formation is delayed in a mouse model where a mutant form of collagen type X is produced (Tsang et al., 2007). Another possibility is that impaired metabolic adaptation to hypoxia might promote chondrocyte apoptosis (Schipani, 2006). For example, Hif1α normally activates expression of PGK in the growth plate. PGK is a key enzyme of the anaerobic glycolysis pathway, so its absence might trigger apoptosis. Finally, autophagy represents an adaptive response to hypoxia. HIF inhibits mTOR and mTOR inhibits autophagy, so loss of Hif1α can lead to unrestrained autophagy, resulting in apoptosis (Srinivas et al., 2009). Determining the molecular trigger for apoptosis in this context will require in-depth analyses, and may well reveal that more than one of these programs is at play in directing cell death in the absence of Hif1α.

Hypoxia-Dependent Transcriptional Network in Bone Formation

Further insight into the role that oxygen and Hif1α play in endochondrial bone formation requires an appreciation of the broader transcriptional network involved. The transcriptional landscape of endochondrial bone formation is built around two transcription factors: Sox9, which is required for the formation of cartilage-forming chondrocytes (Akiyama et al., 2002; Bi et al., 1999), and Runx2, which is required for bone-forming osteoblasts and for chondrocyte maturation (Lefebvre and Smits, 2005) (Figure 7). Hypoxia activates *Sox9* expression and leads to the inhibition of *Runx2* expression. As a result, chondrogenesis and osteogenesis are spatially and temporally separated during bone formation by differing oxygen concentrations. Sox9, a DNA-binding protein of the high mobility group (HMG) family, is expressed in condensed mesenchyme and in proliferating chondrocytes (Lefebvre and Smits, 2005). Runx2 is a member of the Ig-loop family and is expressed in condensed mesenchyme and prehypertrophic chondrocytes, and at high levels in the osteoblast lineage (Karsenty, 2008; Lefebvre and Smits, 2005). Condensing mesenchyme becomes hypoxic, leading to Hif1α-dependent activation of *Sox9* transcription and the formation of osteochondro progenitor cells (Amarilio et al., 2007). *Sox9* expression, supported by hypoxia, drives these progenitors to differentiate into proliferating chondrocytes (Akiyama et al., 2002; Bi et al., 1999). At the same time Sox9 inhibits these proliferating chondrocytes from differentiating into hypertrophic chondrocytes (Bi et al., 1999). In addition, by inhibiting *Runx2* expression Sox9 also prevents the formation of hypertrophic chondrocytes (Zhou et al., 2006). More distally, release from hypoxia promotes a switch in expression from *Sox9* to *Runx2*, which causes proliferating chondrocytes to

exit the cell cycle and undergo hypertrophy (Lian et al., 2006). Concurrently, although hypoxia promotes early progression through the chondrocyte lineage with the formation of proliferating chondrocytes, it inhibits the development of the osteoblastic lineage. This inhibition occurs because osteochondro progenitors require *Runx2* expression for osteoblast formation, and hypoxia inhibits *Runx2* expression. Inhibition of *Runx2* expression by hypoxia is indirect, because Hif1α directly activates the expression of *Twist1* and *Nfatc1*, which in turn inhibit *Runx2* transcription (Seifert et al., 2008) (Yang et al., 2008) (Figure 7).

Endochondrial bone formation differs from heart development where the hypoxia-HIF-Vegfa nexus is central to morphogenesis. In endochondrial bone formation, hypoxia and HIF-dependent Sox9 and Runx2 expression directly drive the changing cellular landscape, and Hif1α-dependent Vegfa appears to act as a chondrocyte survival factor rather than a promotor of angiogenesis (Schipani, 2006). The hypoxic and Vegfa-expressing proximal growth plate remains avascular because proliferating chondrocytes express antiangiogenic factors, and the ECM captures the Vegfa, rendering it latent (Hall et al., 2006; Moses et al., 1999). Vascularization of the growth plate occurs more distally in response to relatively higher Vegfa levels, induced by Runx2 in hypertrophic chondrocytes, and presumably also by Vegfa that is released as the ECM is broken down (Dai and Rabie, 2007; Lian et al., 2006).

Perspectives

This review documents the occurrence of cellular hypoxia, describes the function of HIF during development, and synthesizes the molecular and cellular aspects of hypoxia and HIF in the mouse embryo and placenta. As a result it is possible to identify elements of hypoxia-HIF action that are both distinct and recurrent during development. In addition, this review demonstrates that the timing of hypoxia and release from it, as well as its spatial nature, are key to tissue morphogenesis. Finally, it raises the issue of whether the HIF-mediated response to physiological hypoxia differs from the nonphysiological response.

By considering the different ways in which hypoxia-HIF action contributes to development, it is possible to identify elements that are distinct and those that are recurrent. Hypoxia and HIF drive developmental morphogenesis by directing cell differentiation and cell behavior. Although cell differentiation and behavior are not always easily separable, it appears that HIF-dependent expression of transcription factors directs cell differentiation, while its promotion of Vegfa signaling affects cell behavior. For example, direct HIF-dependent expression of the transcription factors Mash2 and Sox9 promotes differentiation of spongiotrophoblasts in the placenta and chondrocytes in bone, respectively. Moreover, indirect effects of hypoxia and HIF on the transcription factors Gcm1 and Runx2 correspondingly inhibit syncytiotrophoblast formation in the placenta, and chondrocyte hypertrophy and osteoblast formation in bone. In comparison, the HIF-dependent expression of the secreted factor Vegfa primarily affects the behavior of vascular endothelial cells by impacting their survival, proliferation, and migration (Hall et al., 2006). In doing so it affects angiogenesis, which in terms of the systems discussed in this review, is a feature of fetal vascular development in the labyrinthine layer of the placenta and vascularization of the heart. In addition, Vegfa released by the myocardium affects endothelial cells of the endocardium, and thus regulates two processes: epithelial-to-mesenchymal transition during endocardial cushion formation, and myocardial trabeculation with Vegfa-activated endocardial cells signaling back to the Vegfa-producing myocardium (Ferrara et al., 1996; Lambrechts and Carmeliet, 2004). Furthermore, Vegfa signaling also appears to act on nonvascular endothelial cells, because it promotes the survival of proliferating chondrocytes during bone formation.

Development is driven by spatiotemporal differences in gene expression and cell behavior. HIF activity during development is controlled in space and time by the availability of oxygen. This is especially evident when hypoxia-induced HIF activity is switched between on and off states, as seen in the placenta, the endocardial cushion, and bone formation. In the placenta, oxygen from the maternal blood sinuses releases trophoblasts adjacent to these sinuses from hypoxia, allowing them to differentiate into syncytiotrophoblasts. During formation of the endocardial cushion, acquisition of myocardial hypoxia leads to inhibition of epithelial-to-mesenchymal transition of the endocardium, in response to high cushion cell density. Endochondrial bone formation requires that chondrogenesis proceed osteogenesis because bone forms in the ECM scaffold laid down by the chondrocytes. Hypoxia and then release from it drives this progression, because the initial hypoxia is required for chondrogenesis, and subsequent oxygenation, for osteogenesis.

Some 200 HIF target genes have been described in various cell types cultured under low-oxygen conditions (Manalo et al., 2005; Wenger et al., 2005). It seems unlikely that all these genes would be transcribed in vivo in cells exposed to either physiological or nonphysiological hypoxia. It is more plausible that the transcriptional response to hypoxia would be context-dependent; that is, it would depend on the cell type involved and the extent and duration of hypoxia. There is some initial evidence that physiological hypoxia in the conceptus induces the expression of distinct transcription factors in a cell-type-dependent manner; *Mash2* is expressed in diploid trophoblasts of the placenta, and *Sox9*, in proliferating chondrocytes during bone formation. Hypoxia and HIF-induced expression of *Mash2* and *Sox9* do not seem to be features of nonphysiological hypoxia. Despite these examples of context-dependent responses to physiological hypoxia, it is also clear that some genes are transcribed by HIF in response to both physiological and nonphysiological hypoxia in a variety of contexts in vivo. Vegfa is a case in point; it is induced during development, and also in response to ischemia in the mouse brain and heart (Bernaudin et al., 2002; Das and Maulik, 2006). In conclusion, there is evidence suggesting that HIF responds to hypoxia in a context-dependent manner, and understanding the details of this awaits the collection of a significant amount of additional data.

Nonphysiological Hypoxia Adversely Affects Embryonic Development

The hypoxia-dependent HIF transcription system has evolved the capacity to work with the physiological hypoxia present during normal embryonic development, and indeed, uses it to sculpt the embryo. However, it is important to note that this

system is limited in its ability to respond to nonphysiological hypoxia—that is, enhanced and/or spatially extended hypoxia—during development. For example, inducing nonphysiological hypoxia in mouse and rabbit embryos leads to a variety of developmental defects that occur in places including the heart and vertebral column (Clemmer and Telford, 1966; Loder et al., 2000; Ream et al., 2008). We are now at a point in our understanding of the effectors of the hypoxic response where we can begin to elucidate how this nonphysiological hypoxia induces morphological changes.

Nonphysiological hypoxia also results from placental insufficiency (Bozec et al., 2008; Navankasattusas et al., 2008; Sparrow et al., 2009), and it appears that the timing and extent of the deficiency influences the type of developmental process that is disrupted. For example, placental insufficiency at midgestation due to the loss of *retinoblastoma* (*Rb*) leads to midgestation embryonic lethality with widespread developmental defects and aberrant apoptosis and cell proliferation (Jacks et al., 1992; Lee et al., 1992). The defects in the embryo are due entirely to placental insufficiency, because *Rb* null embryos survive gestation when supported by a wild-type placenta (Wu et al., 2003). Placental insufficiency at a later stage (E14.5) resulting from loss of *Cited1* in the placenta specifically does not lead to embryo lethality, but rather to a more restricted developmental defect involving hypoxia-induced apoptosis and defective corticomedullary patterning in the kidneys at E17.5 (Sparrow et al., 2009). Eighty percent of the mice die at birth, and survivors have a deficiency in the ability to concentrate urine. In a third example, placental insufficiency at the end of gestation (E18.5) leads to hypoxia in the long bones of *Fosl2* (*Fra-2*) null mice (Bozec et al., 2008), resulting in bone defects through increased osteoclast size and number.

Crosstalk between Hypoxia and HIF with Signaling Pathways in Development

In this review I have only discussed activity of the HIF complex (Hif1α or Hif2α with Arnt) in relation to transcriptional activation of hypoxia-responsive genes, but components of the HIF transcription complex also interact with other signaling pathways that are active during development. For example, Hif1α binds to the activated intracellular domain of the Notch receptor (NICD) (Gustafsson et al., 2005). NICD complexes with the DNA-binding protein CSL to activate transcription of target genes. Interaction between Hif1α and NICD stabilizes NICD, and potentiates the transcriptional activation of target genes such as the transcription repressors *Hes1* and *Hey2*. This interaction, which has been well reviewed (Poellinger and Lendahl, 2008; Sainson and Harris, 2006), presents the exciting possibility that Notch signaling is positively modulated in hypoxic regions during development. As Notch signaling is a major factor in embryonic development, the effects of this crosstalk could potentially be extensive. A second point of interaction between these pathways involves binding of Fih to the Notch receptor (Coleman et al., 2007; Linke et al., 2008). Fih has a greater affinity for Notch than for Hif1α, so the presence of Notch may reduce asparagine hydroxylation of Hif1α and thus indirectly enhance Hif1α-mediated transcriptional activation of HRE-containing genes (Poellinger and Lendahl, 2008).

Hif1α also interacts with β-catenin and thereby connects hypoxia to Wnt signaling (Kaidi et al., 2007; Lim et al., 2008). Wnt-dependent transcription relies on dimerization of β-catenin with the transcription factor T cell factor 4 (Tcf4). Under hypoxic conditions Hif1α interacts with β-catenin. Additionally, hypoxia can cause destabilization of β-catenin because Hif1α binds human arrest defective 1 (hARD1), which acetylates and stabilizes β-catenin. In both cases, either HIF1α or hypoxia limits interaction between β-catenin and Tcf4, resulting in reduced transcription of the Wnt target gene *c-Myc* and cell growth arrest. Conversely, dimerization of Hif1α and β-catenin under hypoxic conditions potentiates transcription of Hif1α target genes including *Vegfa* (Kaidi et al., 2007).

These exciting examples of crosstalk reveal additional avenues through which hypoxia and Hif1α can impact cell behavior. However, the findings regarding Notch and Wnt rely on protein overexpression, and those concerning Wnt signaling were performed using cancer cell lines and not embryos. Their relevance to embryonic development therefore awaits validation.

Conclusion

The primary function of the HIF transcriptional system is to protect organisms against hypoxia. In mammalian embryos, which develop in a low-oxygen environment, HIF has been co-opted to play a role in embryo morphogenesis. Recently our understanding of the role that hypoxia and HIF activity play in embryo and placenta morphogenesis has increased significantly. However, we still need to define specific molecular interactions and outcomes, and link these with their cellular responses. To achieve this goal, tissue- and stage-specific gene deletions in combination with tissue culture studies will be essential. In particular we need to elucidate the primary roles of HIF activity in the developing cardiovascular system and placenta. These are key sites of HIF activity, and if either site is compromised, the whole of embryonic development is impaired. Next, a systems biology approach is needed to identify direct targets of the HIF1 and HIF2 transcription complexes. Understanding the roles of HIF targets in the context of development will be a real triumph. What is the goal of a comprehensive molecular and cellular understanding of HIF activity in embryonic development? At its simplest, it is to understand birth defects; more broadly, it is to provide an intellectual and molecular framework for stem cell manipulation and regenerative medicine.

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