The role of oxygen availability in embryonic development and stem cell function

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Abstract

Low levels of oxygen (O_2) occur naturally in developing embryos. Cells respond to their hypoxic microenvironment by stimulating several hypoxia-inducible factors (and other molecules that mediate O_2 homeostasis), which then coordinate the development of the blood, vasculature, placenta, nervous system, and other organs. Furthermore, embryonic stem and progenitor cells frequently occupy hypoxic 'niches' and low O_2 regulates their differentiation. Recent work has revealed an important link between factors involved in regulating stem/progenitor cell behaviour and hypoxia-inducible factors, which provides a molecular framework for hypoxic control of differentiation and cell fate. These findings have important implications for the development of therapies for tissue regeneration and disease.

Introduction

Joseph Priestley clearly demonstrated the importance of molecular oxygen (O_2) for animal life in 1774 when he placed a burning candle in a bell jar alongside a mouse. O_2 consumption by the candle had obvious deleterious effects on the unfortunate rodent, underscoring the potentially lethal outcome of exposure to low levels of O_2 (**hypoxia**). Even moderate hypoxia elicits immediate, transient responses, which range from rapid changes in the carbohydrate metabolism of tissues to more permanent changes in local blood vessel networks. Most organisms, including bacteria, yeasts, invertebrates and vertebrates, require O_2 for survival. O_2 is the primary electron acceptor in many intracellular biochemical reactions and is harnessed by mitochondria to generate ATP via aerobic metabolism.

What constitutes physiologically '**normoxic**' conditions for embryonic or adult cells varies widely, but largely falls in the 2–9% O_2 (14.4–64.8 mm Hg) range (ambient air is 21% O_2). However, there are some exceptions to this rule — for example, the thymus, kidney medulla, and bone marrow niches can exist at 1% O_2 (7.2 mm Hg) or lower due to their atypical blood vessel networks. Homeostasis of O_2 levels within an organism is maintained by multiple processes; many of these mechanisms have been characterized at the molecular level, including hypoxia-inducible transcription factors (HIFs), the environmental sensing mammalian target of rapamycin (mTOR), and the endoplasmic reticulum (ER) stress response [1–3].

Hypoxia is commonly associated with pathologies such as tissue **ischaemia** and inflammation, and occurs in solid tumours [4]. However, hypoxic microenvironments also occur in both the developing embryo and adult, and often create specific '**niches**' that regulate cellular differentiation [5,6]. Of note, stem cells reside in niches, or specific anatomic locations, which

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modulate their activities during development and tissue maintenance or repair. A connection between mammalian embryogenesis and O_2 levels was first appreciated in the 1970s, when Morriss and New demonstrated that successful development of the neural fold by *ex utero* mouse embryos was dependent on the creation of low O_2 culture conditions [7]. Since then, discrete molecular mechanisms through which O_2 levels modulate embryonic development have been elucidated by the cloning and subsequent characterization of HIFs, dimeric transcription factors (see Boxes 1 and 2) that regulate many hypoxic responses in cells and tissues [1,8]. Genetic analysis of HIF function in multiple species and the multiple developmental defects exhibited by HIF-deficient embryos have revealed the importance of O_2 as a key regulator of **ontogeny**. In this Reviews, we discuss the role of O_2 availability and HIFs in the regulation of development and stem cell behaviour. We also outline HIF-independent pathways that confer tolerance to hypoxia and also contribute to embryogenesis (e.g. mTOR).

The effects of O_2 on development

The development of oxygen delivery systems is directly dependent on subtle differences in tissue O_2 levels, and ensures that resident cells maintain proper metabolic activity. The genetic regulation of these responses has been conserved throughout the animal kingdom [9]. For example, the effects of O_2 on mammalian cardiovascular components share certain features with the *Drosophila melanogaster* respiratory organ, the trachea, as described below.

O_2 controls branching in tracheal development

The *D. melanogaster* tracheal system consists of a tubular epithelial network that delivers O_2 to internal tissues and develops by sequential sprouting of branches from epithelial sacs within larvae [10]. Sprouting of the main tracheal branches is simple, stereotyped, and controlled by predetermined developmental cues that involve Branchless (a homologue of the human fibroblast growth factor [FGF]), Breathless (a homologue of the FGF receptor), and Pointed (an **ETS** transcription factor), but the pattern of terminal branching is highly complex and variable [11]. Krasnow and colleagues have shown that **ramification** of fine terminal tracheal branches is in fact regulated by a local signal provided by O_2-starved cells [12]. This local signal is Branchless: O_2 deprivation stimulates larval cells to secrete Branchless, which then functions as a chemoattractant to guide new terminal branches to the Branchless-expressing cells (see Figure 1A). Importantly, this change in airway branch patterning is the result of a 'switch' from developmental to physiological control of Branchless expression. Specifically, environmental cues, like O_2 availability, "fine tune" Branchless expression to promote an optimal tracheal network that effectively delivers O_2 to the organism.

Mammalian cardiovascular morphogenesis is regulated by HIF

During mammalian vascular development (see Figure 1B), vascular endothelial growth factor (VEGF) is a dominant angiogenic growth factor produced by O_2-starved cells [13,14]. Like FGF, VEGF is used reiteratively during several steps of vertebrate vascular morphogenesis (as shown in Figure 1B), including **vasculogenesis** and **angiogenesis** [15,16]. VEGF, FGF2, and many other angiogenic factors (such as transforming growth factor-β, platelet-derived growth factor-β, angiopoietin-1, and angiopoietin-2) are direct transcriptional targets of HIF [17−19].

Before the circulatory system is established, mammalian development occurs in a relatively O_2 poor environment (3% O_2) [20,21]. It would seem logical that blood vessel patterning could be fine-tuned by local hypoxic microenvironments that are encountered during embryogenesis and organogenesis, in which existing vessels would sprout into regions containing O_2-starved cells. This hypothesis was tested by generating HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator [ARNT]) -deficient mice. These animals show lethality by embryonic day (E)10.5, due to vascular defects in the yolk sac, branchial arches, cranium,

somites, and placenta [22,23]. Reminiscent of the scenario in tracheal morphogenesis, the initial development of vascular beds is intact in $Arnt^{-/-}$ embryos, but vessel remodelling is subsequently compromised. Furthermore, ARNT -deficient embryos have decreased VEGF mRNA and protein levels, which implies that VEGF secretion is modulated by naturally low O_2 microenvironments in the early conceptus [22–24]. Consistent with these findings, Iyer *et al*. demonstrated that HIF-1α protein can be detected in E8–E18 mouse embryos [25]; HIF-1α - deficient embryos show similar phenotypes to $Arnt^{-/-}$ mice, with defects in blood vessel formation and neural fold closure [25,26].

Endothelial cells share a spatial and functional relationship with haematopoietic stem cells (HSCs). Analysis of $Arnt^{-/-}$ embryos revealed decreased numbers of yolk sac haematopoietic progenitors [27]. Early haematopoietic cell numbers are also substantially reduced in the aorta–gonad–mesonephros (AGM) domain of the embryo proper, and some of the vascular defects exhibited in the AGM domain probably occur as a consequence of decreased numbers of HSCs in $Arnt^{-/-}$ embryos [23]. Strikingly, the haematopoietic phenotype of the yolk sac and haematopoietic/vascular abnormalities associated with the AGM domain are all attributable to VEGF deficiency [23,27], which underscores the importance of VEGF regulation by hypoxic foetal microenvironments.

Mammalian placentation

Placental development is also clearly influenced by O_2 tension. Before E9.5, the murine embryo relies on glycolysis to supply ATP for metabolic demands. Establishment of the placental circulation by E10.5–E11.5 permits O_2 and nutrient delivery to the rapidly growing foetus. Mice in which *Arnt*, *Vhl* (the gene that encodes von Hippel–Lindau, a protein involved in oxygen sensing and vasculogenesis), *Phd2*, or a combination of *Hif-1α* and *Hif-2α* have been deleted exhibit aberrant placental architecture owing to reduced labyrinthine layers and markedly fewer foetal blood vessels. These observations clearly validate the hypothesis that O_2 levels regulate several steps of placentation [28–31].

Similar results were obtained in experiments designed to investigate the role of O_2 tension in controlling cell fate during human placental **cytotrophoblast** development [32,33]. The uterine surface experiences low O_2 levels (17.9 mm Hg, or 2.5% O_2) during early pregnancy. Cytotrophoblasts from the embryo invade into maternal spiral arterioles, an event essential for the generation of an utero–placental circulation. After establishing connections with the maternal vasculature, placental O_2 levels increase to a relatively rich 8.6% (60 mm Hg). Genbacev *et al*. demonstrated that human cytotrophoblasts proliferate *in vitro* under low O_2 conditions but at higher O_2 levels differentiate into a more invasive phenotype, mimicking the developmental transition they undergo as they invade the placental bed to establish the maternal–foetal circulation [32] (see Figure 2A). These results imply that O_2 can directly influence mammalian cell-fate decisions.

Mammalian cardiovascular–pulmonary development

Although targeted mutation of the genes that encode HIF-1α or ARNT results in early (E9.5–10.5) embryonic lethality, HIF-2α−deficient mice survive until mid-late gestation or, in some cases, birth. These animals succumb to one (or more) of several cardiovascular and pulmonary phenotypes that are not shared with *Hif-1α* mutants (see below). This indicates that the two proteins probably regulate overlapping, but not identical, target genes.

Surprisingly, the phenotypes observed in *Hif-2α*$^{-/-}$ embryos vary markedly depending on the mouse strain used. In one background strain (C57/129SvJ), McKnight and colleagues described embryonic lethality as a result of **bradycardia** owing to **catecholamine dysregulation**, whereas Carmeliet *et al*. demonstrated a perinatal lung maturation defect in Swiss/129Sv

animals owing to improper surfactant production by *Hif-2α*$^{−/−}$ type II pneumocytes [34,35]. *Hif-2α*$^{−/−}$ embryos generated in another background (129Sv/Sv-CP) developed severe vascular defects in both the yolk sac and embryo proper [36]. Finally, in yet another genetic background (129 x C57 F$_1$ hybrid), *Hif-2α*$^{−/−}$ mice showed pathologies including **retinopathy, hepatic steatosis, cardiac hypertrophy**, and **skeletal myopathy** [37]. Therefore, both HIF-1α–ARNT and HIF-2α–ARNT heterodimers regulate multiple, non-redundant developmental pathways. More recently, it was also shown that postnatal deletion of a conditional *Hif-2α* allele, but not *Hif-1α*, resulted in anaemia associated with decreased expression of erythropoietin [38,39].

The overall conclusion from these studies is that '**physiological hypoxia**' encountered *in utero* by developing embryos is essential for generating all components of an intact cardiovascular–pulmonary system. As observed in earlier studies of *D. melanogaster*, patterning and morphogenesis of the nutrient and O$_2$ delivery system of mammals is itself modified by O$_2$ availability. A 'switch' from purely developmental to physiological control of these processes is required to meet the metabolic needs of the rapidly growing conceptus. It is notable that mutations in the *D. melanogaster* gene *Tracheless*, which encodes a bHLH–PAS (for <u>b</u>asic-<u>H</u>elix-<u>L</u>oop-<u>H</u>elix-<u>P</u>er-<u>A</u>rnt-<u>S</u>im proteins, the original members of this transcription factor family) protein, cause defects in the tracheal O$_2$ delivery system, which implies that bHLH–PAS factors have been evolutionarily conserved to regulate O$_2$ delivery [40,41].

O$_2$ and bone morphogenesis

Another intriguing case of O$_2$ levels influencing development involves the growth plates of developing bones. Growth plates are constitutively avascular structures; therefore, low O$_2$ partial pressures experienced by the cartilaginous microenvironment were assumed to affect chondrocyte phenotypes as they evolved from a proliferative to a terminally differentiated state [42]. Growth plate chondrocytes progress through ordered phases of cell proliferation, differentiation and apoptosis [43]. Proliferating chondrocytes synthesize collagen type II and then differentiate into postmitotic hypertrophic cells that express collagen type X and VEGF. Targeted deletion of *Hif-1α* in murine growth plate chondrocytes results in cell death owing to defects in HIF-1α-regulated growth arrest [44]. Mice that lack HIF-1α in this chondrocyte population have markedly shorter limbs owing to increased apoptosis and a disorganized transition from hypertrophic chondrocytes to primary spongiosa. It has been postulated that physiological O$_2$ gradients in the cartilaginous growth plate have a role in modulating chondrocyte proliferation, differentiation, and growth arrest via HIF activity [REF 44]. HIF-1α is also critical for earlier steps in bone formation, such as the generation of cartilaginous primordial limb bud mesenchyme and chondrogenesis [45].

Adipogenesis is modified by O$_2$ levels

O$_2$ concentrations are also important regulators of **adipogenesis**. As fatty acid metabolism requires mitochondrial respiration, hypoxia limits fatty acid usage and the need for additional adipose tissue. Presumably, hypoxia inhibits adipocyte development from mesenchymal precursors by attenuating the expression of peroxisome proliferative activated receptor γ (PPARγ), a nuclear hormone that regulates many adipocyte-specific genes and promotes differentiation of mesenchymal cells to adipocytes [46]. Yun *et al*. have shown that fibroblasts from HIF-1α-deficient mouse embryos are refractory to the hypoxic inhibition of adipogenesis [47]. HIF regulation of DEC1/Stra13 (Drosophila hairy/Enhancer of split transcription factor family member; also known as Stra13), a repressor of the PPARγ promoter, provides an underlying molecular mechanism for O$_2$-mediated effects on adipogenesis. Low O$_2$ activates HIF-1α–ARNT heterodimers, which upregulate *Dec1* gene expression; DEC1, in turn, represses PPARγ transcription and inhibits the differentiation of preadipocytes into adipocytes. HIF-2α expression is induced during adipogenesis *in vivo* and *in vitro*, but plays a distinct role

from HIF-1α [48]. Therefore, O_2 availability directly controls the development of adipose tissue via HIF.

Context-dependency of O_2 on developmental programmes

It has become increasingly clear that O_2 influences specific cell fates in several developmental processes; however, the effect of O_2 levels on cell differentiation is context-dependent. For example, terminal differentiation of megakaryocytes into platelets is promoted by high O_2 concentrations [49]. By contrast, reducing O_2 levels of cultured rat peripheral and central nervous system stem cells from 21% to 3–5% (physiological normoxia for these cells) promotes their differentiation into neurons with specific neurotransmitter phenotypes [50],51. As O_2 can modulate cell fates in a concentration-dependent manner, it seems reasonable to consider O_2 as a developmental morphogen that influences cell fate in a manner that is similar to the more traditionally recognized gradients of secreted growth factors. The precise mechanisms by which HIFs, and other O_2-responsive transcriptional regulators (such as nuclear factor κB [NFκB] and activator protein-1 [AP1]), modulate differentiation in response to these gradients is a subject of ongoing research.

O_2 levels influence stem cell phenotypes

Stem cells, as well as multipotent progenitor cells and germ cells, reside in complex microenvironments or "niches" [52]. Several studies have revealed that O_2 levels might profoundly influence stem cell niches, and can promote the differentiation of certain types stem or progenitor cells, while inhibiting the differentiation of others. These differing results have been demonstrated in experiments in which stem cell populations have been cultured under hypoxic conditions *in vitro*. For example, murine placental trophoblast stem cells adopt a spongiotrophoblast cell fate as opposed to a trophoblast giant cell fate when cultured at 3% O_2 instead of 21% O_2 [28]. Rat bone marrow mesenchymal stem cells exhibit enhanced colony-forming capability and increased proliferation at 5% O_2 (as opposed to ambient air), similar to previous observations obtained by culturing embryonic haematopoietic progenitors [27],53. The rat mesenchymal stem cells cultured under low O_2 also produce more osteocytes when subsequently implanted *in vivo*. Human cytotrophoblasts and murine trophoblast stem cells are excellent examples of how numerous cells transit through a natural O_2 gradient as they migrate from one microenvironment to another, varying their spatial relationship with blood vessels [28,32] (see Figure 2B).

O_2 levels regulate hematopoietic and embryonic stem cells

HSCs in adult mammals reside in the bone marrow. The partial pressure of O_2 in human bone marrow is lower than in peripheral blood and the architecture of medullary sinuses and arterial blood flow patterns generate an O_2 gradient. It has been proposed that HSCs and their proliferating progenitors are naturally distributed along this gradient, with the HSCs occupying the most hypoxic niches [54,55]. Furthermore, Danet *et al*. demonstrated that bone marrow HSCs cultured at 1.5% O_2 promoted their ability to engraft and repopulate the haematopoietic organ of immunocompromised recipient mice [56]. Intriguingly, antibodies against markers that substantially enriched for HSCs during purification identify cells that are localized to the sinusoidal endothelium, as opposed to the more hypoxic endosteal lining [57]. The precise location of bone marrow HSCs remains controversial; however, it is possible that different stem and progenitor cell populations require different O_2 conditions, so that multiple niches characterized by different O_2 levels might exist in bone marrow. In this regard, it is interesting to note that spermatogonial stem cells [58] and brain tumour stem cells [59] also seem to be associated with vascular niches. In summary, it seems that some stem cells occupy hypoxic niches (see Figure 3A), whereas others occupy relatively well-oxygenated perivascular

microenvironments (see Figure 3B). Changes in O_2 tension probably influence stem cell quiescence, proliferation and differentiation.

Finally, embryonic stem (ES) cells also grow more efficiently under low O_2 conditions, as opposed to ambient air supplemented with 5% CO_2. It was previously noted that bovine blastocysts produced under reduced O_2 tensions exhibited significantly more **inner cell mass (ICM)** cells than those maintained at higher O_2 levels [60]. The ICM and their ES cell counterparts are **pluripotent**. Roberts *et al.* demonstrated that human ES cells proliferate at similar rates when cultured at 3–5% O_2 as they do under 21% O_2 [61]. However, the appearance of differentiated regions in these ES cultures, as assessed by morphology and loss of stem cell markers like stage-specific embryonic antigen (SSEA) and OCT-4 (see below), was substantially reduced under hypoxic conditions. The authors concluded that hypoxic conditions are required to maintain the full pluripotency of mammalian ES cells.

O_2, stem cells, and disease

O_2 effects on the function of stem and progenitor cells might also be important in pathophysiological settings, as suggested by recent investigations of neuroblastoma [62]. The sympathetic nervous system (SNS) develops from the neural crest and is composed of both neurons and neuroendocrine (chromaffin) cells. Neuroblastoma is a childhood cancer that originates from the developing SNS, and consists of tumour cells that exhibit several differentiation stages, with immature cells generating a more aggressive form of the disease. Some neuroblastomas contain both neuroblastic and neuroendocrine cell types, with spontaneous changes in cell differentiation status clearly affected by O_2 availability within these tumours. Pahlman and colleagues showed that hypoxia (1–5% O_2) induced the expression of markers associated with neural crest sympathetic progenitors, such as c-Kit and Notch, in cultured neuroblastoma cells, whereas it decreased the expression of SNS transcription factors HASH-1 and dHAND [62]. Similar changes in gene expression were also noted in O_2-starved regions of neuroblastoma xenografts grown in mice. Thus, hypoxia causes dedifferentiation of neuroblastoma cells both *in vitro* and *in vivo* and selects for cells with stem cell characteristics. Taken together, these findings implicate oxygenation levels as an important aspect of microenvironmental niches (along with stromal cell contacts, extracellular matrix proteins, growth factors, and temperature) that influence stem cell behaviour.

Hypoxic control of stem cell behaviour

Hypoxia clearly promotes the undifferentiated state in several stem cell and precursor cell populations, but the molecular mechanisms underlying these observations remained obscure until recently. A clear link has been demonstrated between hypoxia, HIFs and molecules that are crucial for the regulation of the differentiation of stem and/or progenitor cells, including Notch, β-catenin, OCT4, and c-MYC.

HIFs affect stem and progenitor cell differentiation

Optimal *in vitro* culture conditions for maintaining precursor cells in the desired state of differentiation probably reflect the physiological O_2 levels that these cells encounter in either the embryo or the adult. The fact that the developmental state of multiple stem or progenitor cell populations is influenced by oxygenation levels strongly implicates O_2-sensitive intracellular pathways, such as HIF-dependent pathways, in the regulation of cell fate. Genetic studies in mice have confirmed this idea *in vivo* [27,28,45].

Placentas from HIF-deficient mice (that lack both the HIF-1α and HIF-2α subunits or the ARNT subunit) exhibit aberrant cellular architecture owing to reduced labyrinthine and spongiotrophoblast layers and increased numbers of trophoblast giant cells [28,29]. These *in*

vivo findings are consistent with a role for hypoxia (via HIF) in promoting the *in vitro* differentiation of trophoblast stem cells into spongiotrophoblasts as opposed to giant cells [28]. As noted above for human cytotrophoblasts, murine trophoblast stem cells migrate through a natural O_2 gradient as they transit from the O_2-lacking region (in the chorionic plate) to the relatively O_2-rich region that surrounds maternal spiral arteries (Figure 2B). We have shown that these stem cells adopt the spongiotrophoblast fate at low O_2 concentrations (3%, close to their 'natural' oxygenation levels) and the giant cell fate at higher O_2 concentrations [28,29].

Similarly, the *in vivo* yolk sac haematopoietic progenitor phenotype of decreased cell numbers exhibited by $Arnt^{-/-}$ embryos can be recreated by three-dimensional **embryoid bodies** derived from $Arnt^{-/-}$ ES cells [27]. Of note, wild-type embryoid bodies grown at 3% O_2 generate significantly more erythroid and myeloid progenitors than those cultured at 21% O_2. Therefore, 'physiological hypoxia' encountered by embryos is important for the proliferation and/or survival of haematopoietic precursors during development.

Endothelial and haematopoietic cells emerge simultaneously in both the yolk sac blood islands and regions surrounding the dorsal aorta during organogenesis, which suggests they probably arise from a common mesodermal precursor known as the haemangioblast. The abundance of haemangioblasts within the early embryo also appears to be regulated by O_2 availability [63]. Haemangioblast proliferation within embryoid bodies is enhanced by hypoxia, which implies that the vascular and haematopoietic defects seen in HIF-deficient embryos are partly the result of depletion of a common progenitor pool.

O_2 availability regulates Notch activity

Notch signalling has been evolutionarily conserved to maintain stem or progenitor cell fates in multicellular organisms [64,65]; myogenic, haematopoietic, and neuronal precursor cell differentiation is inhibited by members of the Notch family [66–69]. Notch mediates cell-cell signalling between adjacent cells that express Notch receptors (Notch 1–4) and Notch ligands (Delta, Serrate, and Lag-2). When activated by ligand binding, Notch receptors undergo a series of proteolytic cleavages to liberate the Notch intracellular domain (ICD), which translocates to the nucleus and interacts with the DNA-binding protein CSL (C-promoter-binding factor/Suppressor-of-Hairless/Lag1) and coactivators such as CBP/p300 and Mastermind to activate targets such as Hes and Hey. Hes and Hey, in turn, negatively regulate the expression or activity of differentiation factors like Mash, MyoD, and Neurogenin [70,71].

Some hypoxic effects on progenitor cells correlate with the effects of Notch signalling in these cells. Gustaffson *et al*. have shown that hypoxia directly influences Notch activity [72]. Hypoxia (1% O_2), via the accumulation of HIF-1α, blocks the myogenic differentiation of C2C12 myoblast cells and the neuronal differentiation of P19 embryonic carcinoma cells. Reduced O_2 levels also inhibit the maturation of primary satellite cells obtained from muscle and neural stem cells derived from embryonic rat cortex. These effects are abrogated in the presence of γ-secretase inhibitors, which inhibit Notch signalling by blocking endomembranous Notch proteolysis. Hypoxia induces the expression of the Notch transcriptional targets Hes1 and Hey2. Hes1 levels are also elevated by hypoxia mimetics that stabilize HIF-1α. These results imply that HIF-1α directly mediates the hypoxic effects on Notch activity; indeed, HIF-1α has been shown to physically associate with Notch ICD, promoting its stability [72]. The authors propose a model in which HIF-1α interacts with Notch–CSL transcriptional complexes at Notch-responsive promoters in hypoxic cells to control the differentiation status of myogenic and neuronal precursors (see Figure 4A). HIF-1α also regulates the expression of the *APH-1A* gene, which encodes a component of the γ-secretase complex; this finding suggests a potential additional mechanism whereby hypoxia augments Notch signalling [73]. In this case, Notch ICD levels increase due to enhanced γ-secretase-mediated proteolysis. Both mechanisms result in elevated Notch ICD in hypoxic cells.

Hypoxia modulates Wnt activity

The Wnt signalling pathway is another important regulator of stem cell function in *D. melanogaster*, *Caenorhabditis elegans* and mammals. Hypoxia downregulates β-catenin (via its interaction with HIF-1α), which is stabilized in response to Wnt signalling and forms an active transcriptional complex with lymphoid enhancer factor/T-cell factor-4 (LEF/TCF4). Kaidi and colleagues demonstrated that HIF-1α competes with TCF4 for direct binding to β-catenin, resulting in hypoxia-mediated cell-cycle arrest and inhibition of transcriptional activity [74]. Intriguingly, β-catenin can also promote HIF-1α-mediated transcriptional activity, which might help cells to adapt to severe hypoxia [74]. It will be interesting to determine the degree to which these interactions affect specific stem-cell functions.

OCT4 regulation by O_2 levels

A third molecular pathway underpinning hypoxic control of stem-cell behaviour involves the POU-domain transcription factor OCT4 (also known as OCT3/4 or Pou5F1), which is directly activated by HIF-2α [75]. OCT4 is essential for maintaining the undifferentiated state of ES cells, ICM, the embryonic epiblast, and primordial germ cells (PGCs) [76,77]. *Oct4* expression is tightly controlled during embryogenesis and adult life: *Oct4* downregulation is required for differentiation of the trophectoderm lineage and subsequent gastrulation by the epiblast; however, *Oct4* expression is maintained in PGCs. In the adult, *Oct4* is exclusively expressed in germ cells, and had been detected in stem-cell populations such as bone marrow multipotent adult progenitors, haematopoietic stem cells, and stem cells that reside in epidermal basal layers [78,79]. More recent studies demonstrate that OCT4 is essential for germ-cell maintenance, but dispensable for somatic stem-cell self-renewal [80,81]. The importance of strictly maintaining *Oct4* expression levels has been demonstrated both *in vitro* and *in vivo*: even a two-fold change in OCT4 protein abundance can cause ES cells to lose pluripotency [82], and ectopic *Oct4* expression promotes epithelial dysplasia in transgenic mice [83].

We have shown that HIF-2α, but not HIF-1α, binds to the *Oct4* promoter and induces *Oct4* expression in hypoxic cells if the *Oct-4* locus is in an 'open' configuration and not embedded in heterochromatin [75,84]. There are several putative HREs in the promoter region of *Oct4* that are conserved between mice and humans [85]. Deletion of these HREs abrogates hypoxic induction of the *Oct4* promoter in transient transfection assays, indicating that they are functional [75]. Furthermore, chromatin immunoprecipitation (ChIP) assays demonstrated that endogenous HIF-2α occupies the *Oct-4* HREs in O_2-starved cells. By generating mice with an expanded region of HIF-2α expression, we found that early embryos exhibited elevated OCT4 levels and severe developmental patterning defects. HIF-2α-overexpressing ES cells also generated, in an OCT4-dependent manner, large subcutaneous teratomas characterized by altered cellular differentiation [75]. Of note, *Hif-2α$^{-/-}$* embryos display a striking reduction in the number of PGCs, which require *Oct4* expression for survival and/or maintenance [75]. Taken together, the data identify HIF-2α as an upstream regulator of *Oct4* expression (see Figure 4B), and indicate a potential novel pathway in which hypoxia directly influences stem-cell function.

Can O_2 levels influence adult cell 'reprogramming'?

The opposing effects of HIF-1α and HIF-2α on the activity of c-MYC have implications for stem-cell function. HIF-1α inhibits c-MYC activity [86,87], whereas HIF-2α has been shown to promote c-MYC-dependent proliferation [88]. The fact that HIF-2α enhances the levels of OCT4 and c-MYC activity is particularly intriguing, as these two factors can directly regulate murine ES cell identity. Several reports [89–92] demonstrate that enforced expression of OCT4, c-MYC and two other transcription factors, Krüppel-like factor 4 (KLF4), and SOX2 in differentiated murine fibroblasts alters DNA methylation, chromatin structure, and gene expression, giving rise to cells that are functionally indistinguishable from bona fide murine ES cells. These remarkable results provide strong support for the idea that 'stemness' can be conferred on more

differentiated cells. Moreover, they indicate a potential mechanism whereby hypoxia can regulate stem-cell function by modulating the expression or activity of OCT4, c-MYC, and possibly other proteins in a HIF-dependent manner.

Additional mechanisms regulating embryogenesis

While HIFs regulate a critical transcriptional response to low O_2, other pathways (as shown in Figure 5) also provide important hypoxic adaptations. In mammals, hypoxia results in both acute and more chronic responses. Rapid and reversible effects on cellular metabolism, cell mass, ion channel activity, and protein synthesis affect the balance between energy supply and demand in the face of reduced capacity for oxidative metabolism 93. Although HIFs promote cellular survival and vascular remodelling during chronic hypoxia, several HIF-independent pathways, engaged by acute hypoxia, are critical for ATP conservation by limiting energy-dependent processes such as cell division, ribosome biogenesis, mRNA translation, and ion flux. These have now been described, albeit to a limited degree, and include the "mammalian target of rapamycin" (mTOR) energy sensing [94], the "unfolded protein response" (UPR) 95, and soluble guanylate cyclase 96 pathways. O_2 sensation by the developing nervous system, while poorly understood, also ultimately impacts behavioural responses in adult animals [96]. In *C. elegans* this involves neural cGMP-gated channels and a soluble guanylate cyclase homologue, GCY-35 that directly binds O_2 via a haem domain. In this section, we discuss the regulation of mTOR and UPR pathways and their possible contribution to embryonic development.

mTOR regulates early development

mTOR is a highly conserved serine/threonine kinase that integrates multiple environmental cues to regulate metabolism, mRNA translation, cell survival, and actin organization in response to O_2, nutrient, hormone, and growth factor availability [94]. mTOR exists in two complexes; mTOR complex 1 (mTORC1), which also contains raptor, mLST8, and other associated proteins. mTORC1phosphorylates initiation factor 4E binding protein-1 (4E–BP1) and p70 ribosomal protein S6 kinase (p70[S6K]), resulting in decreased cellular protein synthesis, growth and proliferation, to conserve ATP[97]. By contrast, the second complex, mTORC2, contains mTOR, mLST8, and rictor. mTORC2 phosphorylates and activates the kinase AKT/ protein kinase B (PKB), which regulates cell proliferation, survival and metabolism [98]. By regulating the actin cytoskeleton, mTORC2 also controls cell shape and motility.

Germline disruption of *mTOR* in mice causes embryonic lethality during blastocyst implantation [99–101]. Explanted mTOR-null blastocysts appear normal, but the ICM and trophoblast giant cells fail to expand during culture. Explanted raptor-null blastocysts exhibit similar phenotypes: they stop growing by day 4 and by day 7, most cells detach and presumably die [101]. By contrast, explanted mLST8-null blastocysts exhibit no obvious phenotypes and grow normally. However, *mLST8*^{−/−} embryos die *in vivo* by day 10.5 of gestation due to cardiovascular defects. Although they exhibit a beating heart, the cardiac wall is slightly thinner and they are visibly smaller than wild type or heterozygous embryos [101]. Defective vascular development was observed in these embryos, wherein many blood vessels, particularly in the head, were dilated. The phenotype of *mLST8*^{−/−} mice is similar to the phenotype of embryos that lacking phosphoinositol 3-kinase (PI3K; p110α) and the endothelial kinase 2-receptor (TIE2) [102,103]. When TIE2 is stimulated by its ligand angiopoietin-1, it signals through PI3K to regulate vascular development. Results from analysing *mLST8*^{−/−} embryos suggest that mLST8 participates in TIE2-mediated endothelial cell signal transduction.

Rictor-deficient embryos look very similar to mLST8-deficient **concepti**[101], which suggests that mLST8 is necessary to maintain rictor/mTOR interactions but not raptor/mTOR interactions. Furthermore, both mLST8 and rictor (but not raptor) are required for

phosphorylation of AKT and protein kinase Cα (PKCα), but not p70^{S6K}. These results demonstrate that mTORC1 functions in early development and becomes essential by gestational day 5.5–6.5 shortly after implantation (i.e. the egg cylinder stage). By contrast, mTORC2 is necessary for later vascular development, possibly due to its role in TIE2-mediated endothelial cell signal transduction, or effects on endothelial cell cytoskeletal function. mTOR activity is clearly inhibited by O_2 deprivation [104]. Furthermore, HIF-α protein expression is dependent on mTOR in some cellular contexts [105,106]. However, it remains to be determined if mTOR modulation by low O_2 levels or a connection between mTOR and HIF in the developing conceptus promote normal development.

Hypoxic regulation of the endoplasmic reticulum (ER)

Nascent proteins enter the ER, which serves as a critical site for protein folding, disulfide bond formation, and glycosylation before peptides become secreted or translocated to the plasma membrane. ER "stress" occurs during variations in new protein accumulation or overabundance of unfolded proteins [107]. To alleviate ER stress, a series of cell defence mechanisms are activated, collectively known as the "ER stress response", the "integrated stress response", or UPR [107]. These mechanisms include the phosphorylation of eukaryotic initiation factor-2α (eIF2α) by the ER pancreatic eIF2-α kinase (PERK), which results in reduced protein translation to prevent further accumulation of unfolded polypeptides. Hypoxia activates PERK (by unknown mechanisms), resulting in increased eIF2α phosphorylation and decreased rates of translation initiation [108].

In contrast to mTORC1 inhibition in response to chronic hypoxia, eIF2α phosphorylation is transient and decreases after reaching a plateau at 2 hours [108]. Studies of PERK-deficient mice, and mice with a mutation in the eIF2α PERK phosphorylation site (S51A) reveal that eIF2α phosphorylation is connected to glucose metabolism [109,110]. PERK-deficient mice are viable but develop marked hyperglycemia at 4 weeks of age, while the eIF2α−S51A mutant mice appear normal at birth but die of severe hypoglycemia within 18 hours. Both mutant strains exhibit defects in pancreatic β-cell development. These defects are apparent in eIF2α−S51A embryos but only become apparent in PERK-deficient animals several weeks after birth. Such differences indicate that more than one kinase phosphorylates eIF2α in the β−cells of the pancreas. PERK is specifically required in the insulin-secreting pancreating β cells during the foetal and neonatal period to ensure β cell proliferation and differentiation [111]. In conclusion, pathways that involve the O_2 sensitive ER clearly regulate pancreatic morphogenesis. However, a link between O_2 availability, the ER, and development of the pancreas remains to be established.

Conclusions and future directions

As stated above, changes in O_2 availability occur naturally during embryonic development. O_2-starved cells respond to their microenvironment by stimulating several adaptive responses that include HIFs, mTOR, ER-associated kinases, and soluble guanylate cyclases (see Figure 5). Findings from *in vitro* and *in vivo* models (invertebrate and vertebrate) discussed herein convincingly demonstrate that molecular O_2 is not simply a fuel to maintain cellular bioenergetics and metabolism, but is also an essential signal that regulates cell fate. As stated, physiological 'normoxia' is usually much lower than ambient air. Although most cells are maintained in culture conditions at 21% O_2, this is unlikely to be optimal for maintaining their normal proliferative or developmental state. Derivation of novel stem and undifferentiated cell populations should therefore be enhanced by culture in the 3–5% O_2 range.

Genetic dissection of the HIFs in mammals is extensive and convincingly demonstrates that O_2 levels and gradients play a significant role in developmental processes, including but not limited to angiogenesis, haematopoiesis, placentation, cardiogenesis, bone formation, and

adipogenesis. While the role of mTOR and mTOR-associated proteins (raptor, rictor, and mLST8) are critical for embryonic development, their connection to changes in embryonic O_2 availability is not conclusive at this time. However, given the similarity of phenotypes between HIF-deficient and mTORC2-deficient embryos [22,26,101], it seems plausible that mTOR is sensing O_2 in the early embryo to also regulate cardiovascular differentiation. By contrast, O_2 sensation by soluble guanylate cyclases appears to regulate neuronal function as opposed to neuronal development in nematodes. It remains to be determined if O_2 sensation by cyclic GMP pathways regulate neuronal development in mammals.

Continued analysis of the role of hypoxia in embryonic development in general, and stem and/ or progenitor cell behaviour in particular should reveal additional interactions between O_2-sensitive regulators (like HIFs) and essential pathways that control differentiation. Furthermore, as HIFs are clearly active at the microenvironmental O_2 concentrations in which stem/progenitor cell populations commonly reside, it is important to determine if other developmental regulators, such as Fox-family members or ephrins, intersect with pathways that mediate O_2 homeostasis. It should be emphasized that many HIF-independent, O_2-regulated mechanisms (such as those involving mTOR and ER stress responsive kinases) that promote tolerance to hypoxia are also likely to control multipotency and differentiation and further investigation is required to address this.

In addition to regulating normal stem cell function, hypoxia might also modify the behaviour of so-called '**cancer stem cells**' and their progeny. If so, inhibiting HIF activity could reduce Notch or Oct-4 levels below a threshold required to maintain stem cell identity, and thereby promote tumour dormancy [112]. Hopefully, the recent discovery that hypoxia regulates factors that are crucial for stem/progenitor cell maintenance in normal development and tumour progression will guide the development of novel therapeutics for both tissue regeneration and cancer treatment.

Box 1 Hypoxia-inducible factors: subunit complexity

Hypoxia-inducible factors (HIFs) belong to a family of environmental sensors known as bHLH–PAS (basic Helix-Loop-Helix–per-Arnt-Sim) transcription factors [113], which regulate diverse biological processes. HIF-1 was cloned on the basis of its affinity for the hypoxia-response element (HRE, see Box 2) located within the enhancer region of the human *erythropoietin* gene [114,115]. The HIF-1 heterodimer consists of an α (HIF-1α) and a β subunit (HIF-1β; also known as the aryl hydrocarbon receptor nuclear translocator (ARNT)).

Both HIF-1α and HIF-1β/ARNT are bHLH–PAS contain two PAS domains of 100–120 amino acids, designated PAS-A and PAS-B (see figure), which are necessary for heterodimerization and DNA binding. PAS domains can mediate environmental sensing through direct ligand binding or by interacting with other cofactors such as heat shock protein 90 (HSP90) [113]. However, so far no ligands have been found to bind to the HIF PAS domains. HIF-1α contains two transactivation domains (TADs) bridged by an inhibitory domain.

Three genes — *HIF-1α, HIF-2α* and *HIF-3α* — encode mammalian HIF-α subunits. HIF-1α is ubiquitously expressed, whereas HIF-2α (also called EPAS (endothelial PAS protein), HLF (HIF-1α-like factor), and HRF (HIF-related factor)) and HIF-3α exhibit more restricted tissue distributions. HIF-2α is expressed primarily in the vasculature of the early developing embryo and subsequently in the lung, kidney interstitial cells, liver parenchyma, and neural crest cells [116–118]*HIF-3α* mRNA and protein are primarily detected in the thymus, kidney, cerebellar Purkinje cells, and corneal epithelium of the eye [119,120]. HIF-1β/ARNT is constitutively expressed and is largely insensitive to changes in O_2 levels,

whereas all three HIF-α subunits are acutely regulated by hypoxia (see Box 2). Two HIF-1β/ARNT homologues, ARNT2 and ARNT3 (also known as bMAL) have also been described; however, they largely participate in O_2-independent pathways such as development of the hypothalamus and regulation of circadian clocks, respectively [121,122]. ODD, oxygen-dependent degradation domain.

Box 2 Regulation of hypoxia-inducible factor by O_2 deprivation

The genes that encode hypoxia-inducible factor (HIF)-α are transcribed and translated at a high rate, but HIF-α proteins are rapidly degraded in the presence of sufficient O_2 levels. Under hypoxic conditions, the oxygen-dependent degradation domain (ODD), which comprises residues 403–602 of human HIF-1α (see Box 1), is hydroxylated on two conserved proline residues, P^{402} and P^{564} (see Box 1) [123–126] by a family of three HIF-specific prolyl hydroxylases, PHD1, PHD2, and PHD3 [127–129]. The residues that surround these two proline residues (30 amino acids each) are highly conserved between HIF-1α, HIF-2α, and HIF-3α. Hydroxylated HIF-α proteins are recognized by the von Hippel-Lindau (pVHL) tumour suppressor gene product (a component of a multisubunit ubiquitin-ligase complex), covalently tagged with polyubiquitin, and degraded by the 26S proteasome [130].

Under hypoxic conditions (3–5% O_2), HIF-α ODD hydroxylation and interaction with pVHL is inhibited. HIF-α subunits therefore accumulate in the cytoplasm of O_2-starved cells, and translocate to the nucleus, where they dimerize with HIF-1β/aryl hydrocarbon receptor nuclear translocator (ARNT) through their PER-ARNT-SIM (PAS) domains (see Box 1), and bind to HIF-response elements (which contain the core recognition sequence 5'-RCGTG-3') located within the promoters, introns, and 3' enhancers of a large number of O_2-regulated target genes. During this process the C-terminal transcriptional activation domain (TAD) also interacts with coactivators such as p300/CBP (CREB-binding protein; CREB is CRE-response element binding protein)[131] (see figure). This interaction is thought to be required for full HIF activity, but is also regulated by normoxic hydroxylation reactions; in this case at asparagine 803 (see Box 1)[132]. Asparagine hydroxylation is carried out by factor inhibiting HIF (FIH), and FIH activity is inhibited under hypoxic conditions in a manner that is reminiscent of the prolyl hydroxylases [133–135]. HIF targets include members of stress-response gene families that mediate acute and chronic hypoxic adaptations, such as glucose transporters, glycolytic enzymes, angiogenic factors, haematopoietic growth factors, and molecules that affect cell growth, survival, and motility [4,130,136,137]. This panel has been adapted from a review article written by Bruick (2003).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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GLOSSARY

Adipogenesis	differentiation of lipid producing and storage cells known as "adipocytes".
Angiogenesis	remodeling of blood vessels into the large and small vessels typical of mature networks containing arteries, capillaries and veins.
Arborize	to develop many branching parts or formations.
Bradycardia	a slowing of heart rate, usually measured as fewer than 60 beats per minute in humans.
Cancer stem cells	cancer-initiating cells capable of generating distinct cell types.
Cardiac hypertrophy	overgrowth of organ size via increased cell size rather than cell number.
Catecholamine dysregulation	mice lacking HIF-2α die by in utero due to decreased catecholamine (e.g. L-3, 4-dihydroxyphenylalanine [L-DOPA]) production by the organ of Zucker-Kandl (02) chromaffin cells. Catecholamines are required for normal cardiovascular function.
Conceptus	an embryo or fetus.
Cytotrophoblasts	outer cells of the developing embryo that adhere to the endometrium.
Embryoid body	a three dimensional structure consisting of differentiated derivatives of embryonic stem cells.
ETS	the founding member of a family of oncogenes and proto-oncogenes. ETS refers "E26 specific".
Hepatic steatosis	lipid accumulation in the liver.
Hypoxia	decreased O_2 levels relative to normal, which is 1%–9% for most mammalian cell types.
Inner cell mass	early cells in the embryo that generate all lineages of the mature organism but do not give rise to the placenta.
Ischaemia	a pathologic condition resulting from blood vessel occlusion involving oxygen, nutrient, and growth factor deprivation. This condition usually also leads to decreased tissue pH levels.
Niche	the natural, anatomic environment that supports stem cell behaviour.
Normoxia	although frequently defined in the literature as 21% O_2, physiologic normoxia is actually in the 2–9% O_2 range for most adult cells *in vivo*.
Ontogeny	development of the fetus during embryogenesis.

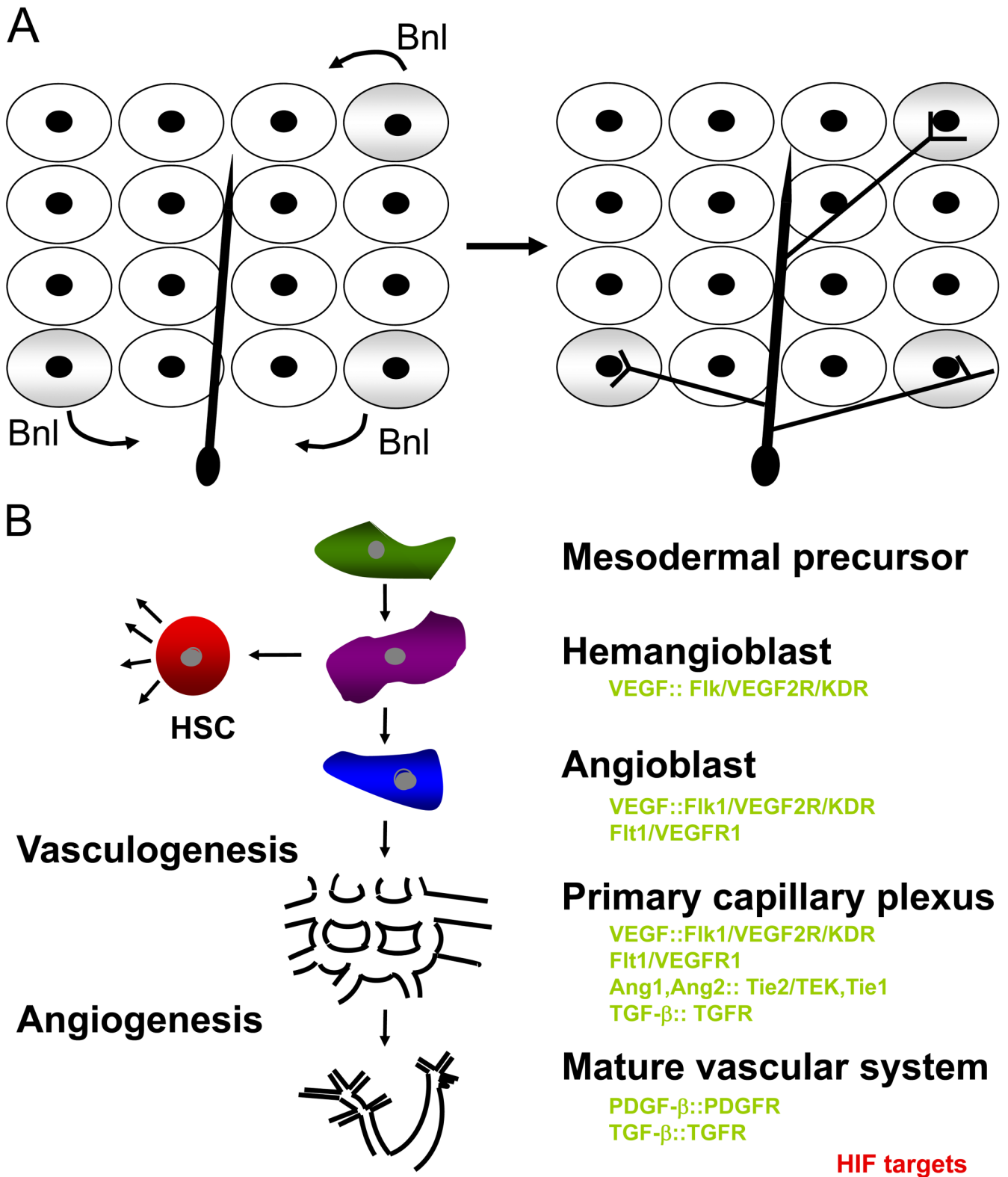| Physiological hypoxia	the natural low O_2 encountered by cells within the developing embryo, in particular prior to establishment of the utero-placental network.
Pluripotent	capable of differentiating into cell lineages of the developing organism.
Ramification	the process of dividing or spreading into branches.
Retinopathy	abnormal increase in retinal vascular networks.
Skeletal myopathy	any disease of muscle tissues, such as muscular dystrophy.
Somites	primordial tissue generating the vertebrae, dermis, and muscles.
Vasculogenesis	the formation of nascent blood vessels from newly generated endothelial cells.

A

Bnl

Bnl   Bnl

B

Mesodermal precursor

HSC

Hemangioblast

VEGF:: Flk/VEGF2R/KDR

Angioblast

VEGF::Flk1/VEGF2R/KDR
Flt1/VEGFR1

Vasculogenesis

Primary capillary plexus

VEGF::Flk1/VEGF2R/KDR
Flt1/VEGFR1
Ang1,Ang2:: Tie2/TEK,Tie1
TGF-β:: TGFR

Angiogenesis

Mature vascular system

PDGF-β::PDGFR
TGF-β::TGFR

HIF targets

Figure 1. Branch morphogenesis during *D. melanogaster* tracheal development and mammalian blood vessel formation are regulated by O$_2$ levels

(A) Models for O_2 sensing and patterning in *D. melanogaster*. Cells within a target tissue experiencing low O_2 (blue), due to their distance from an existing tracheal branch (red), begin expressing Branchless (Bnl, the orthologue of mammalian FGF). Branchless expression increases in these O_2-starved cells and the tracheal cells respond by sprouting terminal branches that grow toward each Bnl signalling centre. When the branch approaches the source, it starts to **arborize** (adapted from 12). *(B)* Model for vascular morphogenesis. Haemangioblasts are putative mesodermal progenitor cells giving rise to both haematopoietic stem cells (HSCs) and angioblasts, the forerunner of endothelial cells which line the vasculature. Vascular endothelial growth factor (VEGF) is required to generate haemangioblasts in the developing embryo. Vasculogenesis, the formation of a primary endothelial cell plexus, also depends on VEGF. Angiogenic remodelling into a mature vascular system (including arteries and veins), involves other important endothelial cell receptors and their ligands, such as Tie2, Tie1, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), and transforming growth factor-β (TGFβ)/TGF-receptor (TGFR) interactions. Of note, virtually all of these vasculogenic and angiogenic regulatory factors (VEGF, Tie2, angiopoietins, TGFβ, platelet-derived growth factor-β (PDGFβ), etc.) are regulated by both decreased O_2 levels and the HIFs.
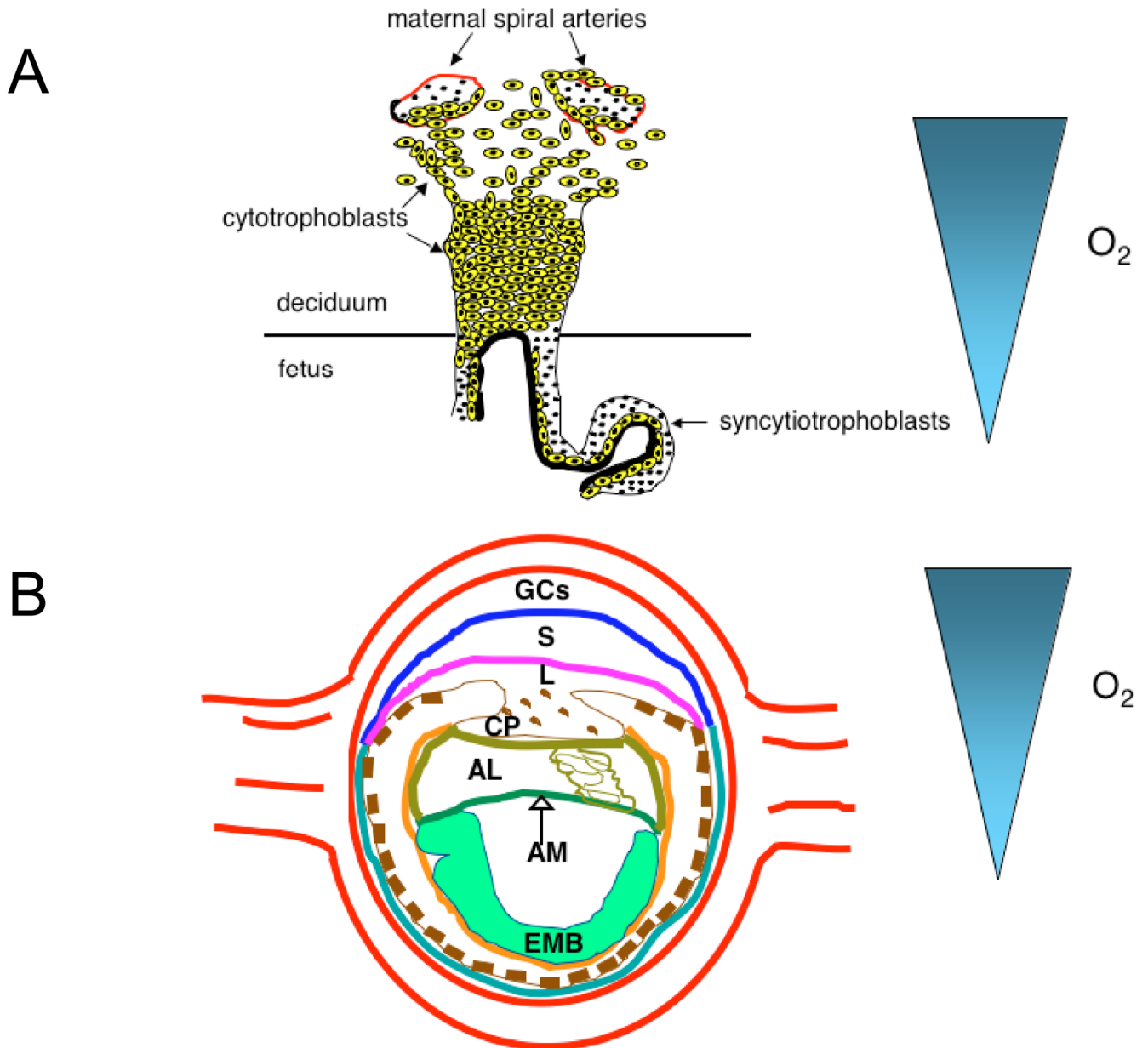
Figure 2. Oxygen gradients are generated in developing human and mouse placentae
(A) Diagrammatic representation of the differentiation pathway that cytotrophoblast stem cells undertake *in vivo*. These cells detach from the underlying uterine basement membrane and either fuse to form multinucleated syncytiotrophoblasts or columns of mononuclear cells that attach the conceptus to the uterine wall. A subset of these cells stops proliferating and differentiates into invasive cytotrophoblasts that breach and enlarge maternal blood vessels to generate an utero–placental circulation. The differentiation of proliferating cytotrophoblasts into invasive cytotrophoblasts is an O_2-dependent process, with O_2 levels increasing as cells migrate towards the maternal spiral arteries. *(B)* Placentation is regulated by changes in O_2 availability. An E8.0 mouse embryo is shown to illustrate the O_2 gradient generated during murine placentation. Similar to human placentae, the early murine placenta generates an O_2 gradient where cells that migrate dorsally experience increasing O_2 levels. Trophoblast stem

cells adopt specific cell fates in the placenta when they encounter discrete O_2 levels: low O_2 enforces a spongiotrophoblast cell fate, whereas higher O_2 levels enforce a giant cell fate.
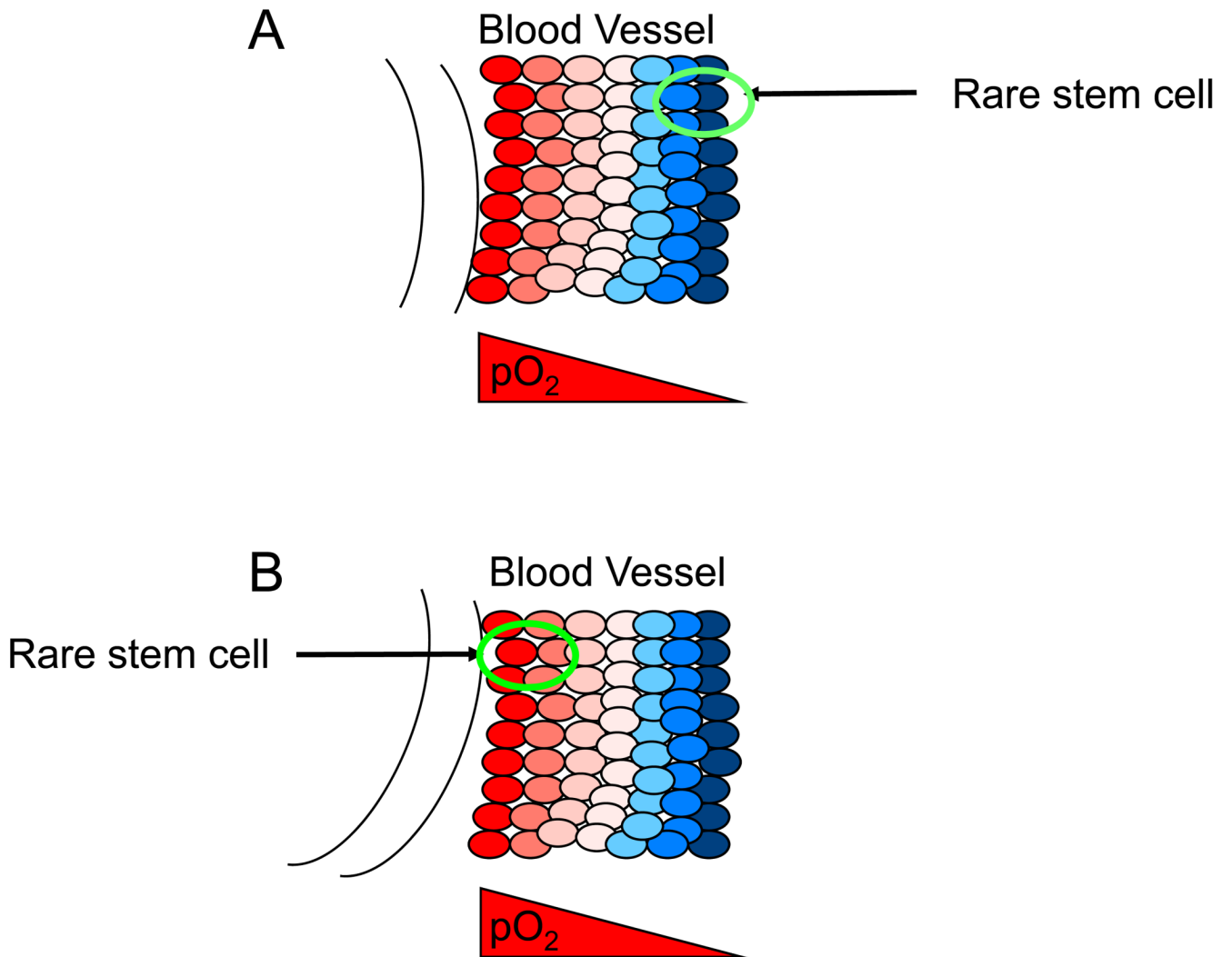
Figure 3. Distinct populations of stem cells occupy microenvironments that contain different O_2 levels

As described in the main text, some stem cells (such as those in the endosteal bone marrow compartment) occupy extremely low O_2 microenvironments (less than 0.5% O_2) as shown in *(A)*. Other stem cells (as those described as perivascular SLAM[+] (for Signalling Lymphocyte Activation Molecule) stem cells can occupy relatively well-oxygenated environments as they are in close proximity to blood vessel endothelial cells *(B)*. However, it should be noted that although stem cells can be perivascular, the vessels might be associated with venous structures and therefore be relatively hypoxic.
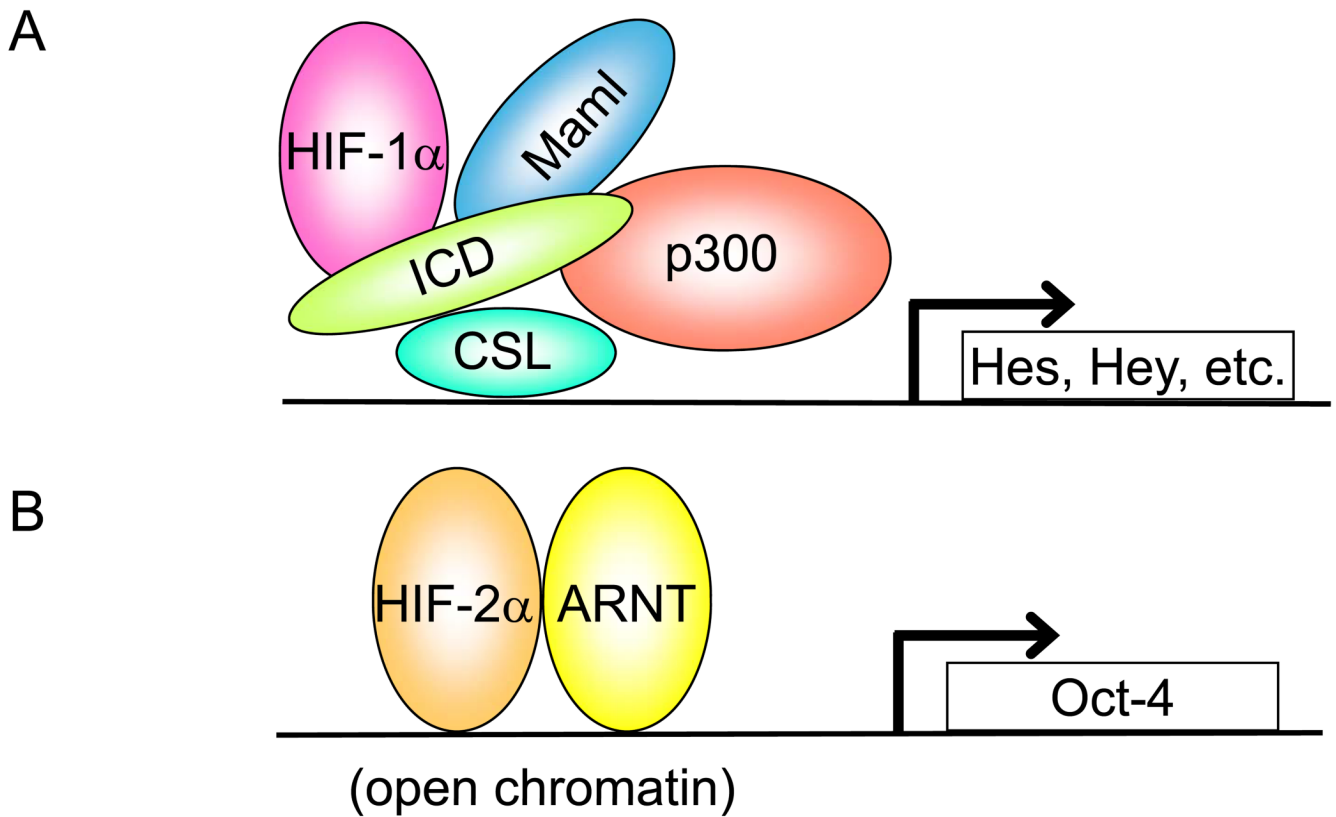
A

B

Figure 4. Models depicting O$_2$ availability and transcriptional activity
(A) Under hypoxic conditions, hypoxia-inducible factor-1α (HIF-1α) typically interacts with
HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator (ARNT)) to stimulate
target genes such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2
(FGF-2), and platelet-derived growth factor-β (PDGF- β). HIF-1α can also interact with the
intracellular domain (ICD) of Notch in the nucleus at Notch-responsive promoters. In the
nucleus, Notch interacts with the CSL (C-promoter-binding factor/Suppressor-of-Hairless/
Lag1) DNA-binding protein and coactivators such as CBP/p300 and Mastermind (Mam1) to
activate target genes such as *Hes* and *Hey*. It is currently not known if the initial HIF-1α–Notch
interaction occurs outside or within the nucleus. Furthermore, the actual relationship between
components of the Notch complex at promoters is unclear. HIF-1α could directly interact with
ICD, an unidentified 'bridging' protein, or with Mam1 [138]. *(B)* In cells where the *Oct-4* locus
is accessible as a result of open chromatin, its transcription is induced directly by HIF-2α–
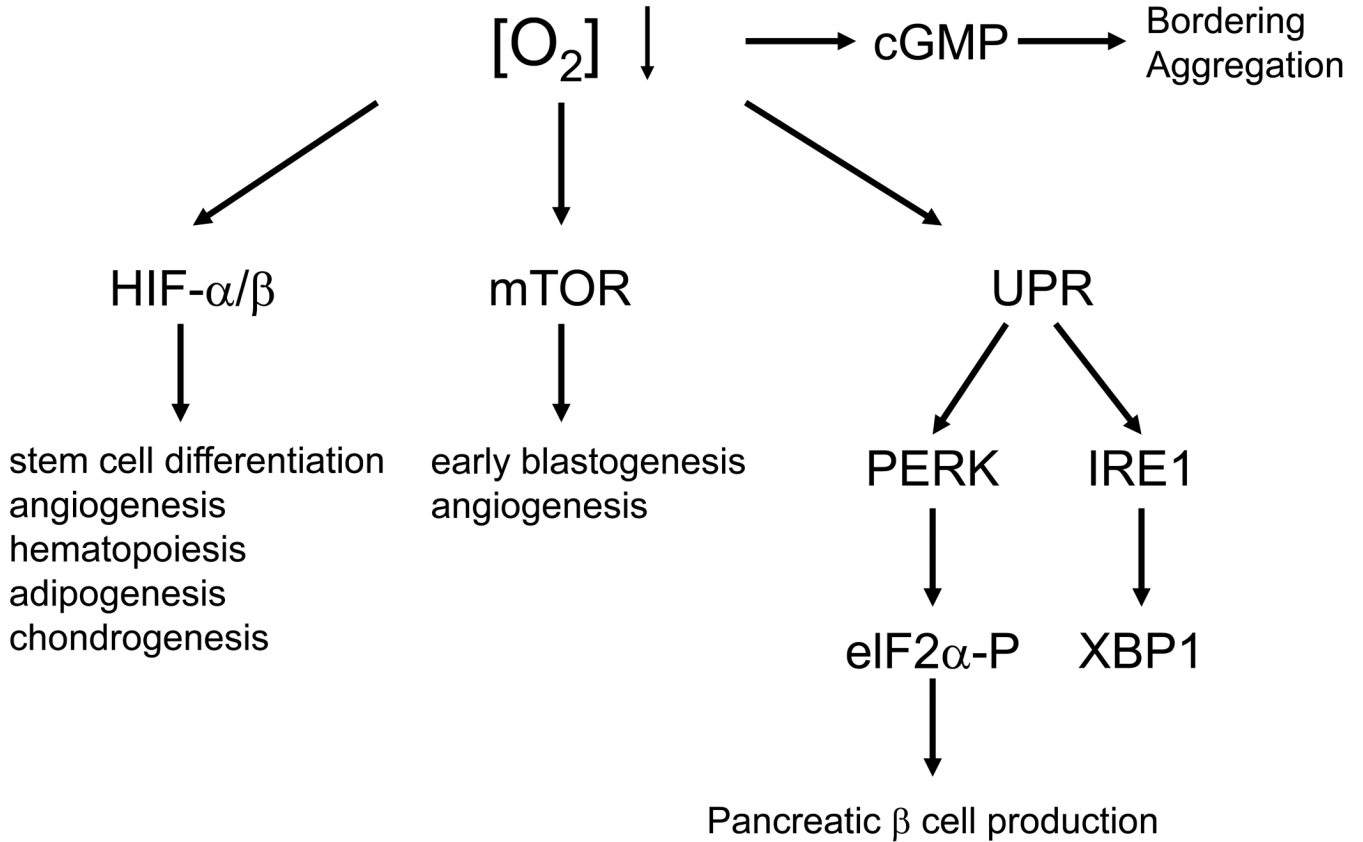ARNT dimers in response to hypoxia.

$$[O_2] \downarrow \longrightarrow cGMP \longrightarrow \text{Bordering Aggregation}$$

HIF-α/β mTOR   UPR

stem cell differentiation   early blastogenesis   PERK   IRE1
angiogenesis   angiogenesis
hematopoiesis
adipogenesis   eIF2α-P   XBP1
chondrogenesis

Pancreatic β cell production

Figure 5. Multiple pathways responding to changes in O$_2$ availability affect developmental processes as well as social behaviour

As described in the text, HIFs regulate many aspects of cardiovascular morphogenesis and stem and/or progenitor cell maintenance. Mutagenesis of mammalian target of rapamycin (mTOR) and its associated proteins, such as raptor, rictor, and mLST8, has revealed an important role for mTORC1 and mTORC2 during embryonic development. However, whether mTOR is responding to hypoxia in embryonic microenvironments to regulate development remains to be determined. The "unfolded protein response" (UPR)-regulated kinase PERK (and its substrate eIF2α) is necessary for pancreatic β cell production during development or shortly after birth. Inositol-requiring-1 (IRE1) is another ER-associated UPR effector that activates X box-binding protein 1 (XBP-1), promoting the transcription of ER chaperone genes such as BiP and c/EBP-homologous protein (CHOP) [139]. Finally, cyclic guanosine monophosphate (cGMP) regulation promotes neuronal activity and social feeding behaviour in *C. elegans*, allowing them to avoid O$_2$ levels outside the range of 5–12% O$_2$. This leads to specific appearances of nematode colonies, causing "bordering" or "aggregation".