

# Hypoxia-inducible factor-1-dependent mechanisms of vascularization and vascular remodelling

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The vascular system delivers oxygen and nutrients to every cell in the vertebrate organism. Hypoxia-inducible factor 1 (HIF-1) is a master regulator of hypoxic/ischaemic vascular responses, driving transcriptional activation of hundreds of genes involved in vascular reactivity, angiogenesis, arteriogenesis, and the mobilization and homing of bone marrow-derived angiogenic cells. This review will focus on the pivotal role of HIF-1 in vascular homeostasis, the involvement of HIF-1 in vascular diseases, and recent advances in targeting HIF-1 for therapy in preclinical models.

**Keywords** Angiogenesis • Cell therapy • Gene therapy • HIF-1 • Hypoxia • Vascularization

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## 1. Introduction: O<sub>2</sub> and vascular homeostasis

Development of the embryo and maintenance of adult homeostasis depend on the establishment of a functional vascular system that supplies O<sub>2</sub> and nutrients to approximately 10<sup>14</sup> cells in an adult human. Local O<sub>2</sub> delivery is regulated by the circulatory system through transient changes in tone of pre-existing blood vessels, establishment of new vessels (angiogenesis), and the remodelling of existing vessels to accept increased blood flow (arteriogenesis). Hypoxia is defined as a reduction in the ambient O<sub>2</sub> concentration. In ischaemic conditions, tissue perfusion is reduced such that O<sub>2</sub> availability is insufficient to meet tissue metabolic requirements. In this review, we will focus on recent studies delineating the role of hypoxia-inducible factor 1 (HIF-1) in regulating tissue perfusion under physiological and pathological conditions. In addition, we will discuss novel potential therapeutic approaches targeting HIF-1.

## 2. Hypoxia is transduced to the nucleus as HIF-1 transcriptional activity

HIF-1 is a ubiquitously expressed heterodimeric transcription factor that mediates adaptive responses to hypoxia/ischaemia in all nucleated cells of metazoan organisms. HIF-1 consists of O<sub>2</sub>-regulated HIF-1 $\alpha$  and constitutively expressed HIF-1 $\beta$  subunits.<sup>1</sup> HIF-2 $\alpha$  is a HIF-1 $\alpha$  paralogue that is

also involved in vascular responses to ischaemia.<sup>2,3</sup> In humans, HIF-1 $\alpha$  is targeted for proteasomal degradation under non-hypoxic conditions through hydroxylation of proline residue 402 and/or 564 by prolyl-4-hydroxylases, which utilize  $\alpha$ -ketoglutarate and O<sub>2</sub> as substrates.<sup>4</sup> Prolyl hydroxylation of HIF-1 $\alpha$  is required for binding to the von Hippel–Lindau protein and recruitment of an E3 ubiquitin-protein ligase, resulting in HIF-1 $\alpha$  ubiquitination and proteasomal degradation.<sup>5,6</sup> Hydroxylation of asparagine 803 of HIF-1 $\alpha$  by FIH-1 (factor inhibiting HIF-1)<sup>7</sup> prevents binding to the transcriptional co-activators CBP and p300.<sup>8</sup> Hypoxia-induced inhibition of prolyl and asparaginyl hydroxylase activity results in a rapid increase in HIF-1 $\alpha$  levels and transcriptional activity.<sup>4–7</sup> HIF-1 $\alpha$  translocates to the nucleus, dimerizes with HIF-1 $\beta$ , and binds to hypoxia response elements (HREs), which function as cis-acting elements that determine the target genes for activation by HIF-1. HREs contain the core HIF-1 binding site nucleotide sequence 5'-(A/G)CGTG-3'.<sup>9</sup> Several hundred direct HIF-1 target genes have been identified and many of these genes encode proteins that are involved in vascular homeostasis through effects on vascular tone, angiogenesis, and/or arteriogenesis.<sup>10,11</sup>

## 3. Regulation of vasculogenesis, angiogenesis, and arteriogenesis by HIF-1

Blood vessel formation and physiological remodelling occur according to three distinct mechanisms: (i) vasculogenesis, consisting of *de novo*

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organization of blood vessels from vascular progenitor cells (haemangioblasts), which give rise to endothelial cells, vascular smooth muscle cells, and pericytes during embryonic development; (ii) angiogenesis, the sprouting of new capillary branches from pre-existing vessels; and (iii) arteriogenesis, the remodelling of conduit vessels through an increase in luminal diameter, resulting in increased blood flow. Mouse embryos lacking HIF-1 $\alpha$  expression initiate vasculogenesis properly, but the initial vascular plexus subsequently degenerates, leading to embryonic lethality at midgestation.<sup>12,13</sup> Applying the definitions provided above, only angiogenesis and arteriogenesis occur in the adult. These processes depend on a network of angiogenic cytokines and cognate receptors that are expressed by multiple vascular cell types, as described below.

### 3.1 Angiogenesis: activation of local vascular cells

Hypoxia is the principal physiological stimulus that induces angiogenesis, which provides a stimulus–response pathway by which all cells are assured of adequate oxygenation. Expression of virtually all of the critical angiogenic growth factors is induced by hypoxia through the transcriptional activity of HIF-1, including vascular endothelial growth factor (VEGF), stromal derived factor 1 (SDF1), angiopoietin 2 (ANGPT2), placental growth factor (PGF), platelet-derived growth factor B (PDGFB), and stem cell factor (SCF).<sup>14–19</sup> The HIF-1-mediated transcriptional response to hypoxia is cell type specific<sup>16</sup> and involves an orchestrated expression of angiogenic growth factors by multiple cell types within the hypoxic tissue in a temporally and spatially regulated manner. These angiogenic factors bind to cognate receptors (VEGFR1/VEGFR2 for VEGF, CXCR4 for SDF1, TIE2 for ANGPT2, VEGFR1 for PGF, PDGFR $\alpha$ /PDGFR $\beta$  for PDGFB, and C-KIT for SCF), which are expressed on the surface of vascular endothelial cells and vascular pericytes/smooth muscle cells. Receptor–ligand interaction activates these cells and promotes the angiogenic budding of new capillaries from existing vessels. HIF-1 binds to and directly activates transcription of the *VEGF*, *SDF1*, *ANGPT2*, and *SCF* genes,<sup>14–17</sup> whereas for *PGF* and *PDGFB*, it is not known whether HIF-1 is a direct or indirect activator of gene expression. In addition to mediating the expression of secreted factors that bind to and activate endothelial cells, HIF-1 regulates the cell-autonomous expression in hypoxic endothelial cells of hundreds of genes, many of which encode cell surface receptors that allow endothelial cells to respond to hypoxia-induced angiogenic cytokines.<sup>18</sup> HIF-1 $\alpha$  mRNA and protein levels are highly induced in the ischaemic limb on day 3 after femoral artery ligation, coincident with the expression of mRNAs encoding multiple angiogenic growth factors.<sup>19</sup> All of these responses are markedly impaired in *Hif1a*<sup>+/-</sup> mice, which are heterozygous for a null (knockout) allele at the locus encoding HIF-1 $\alpha$ .<sup>19</sup>

### 3.2 Role of circulating angiogenic cells

In addition to activating cells in existing vessels, secreted angiogenic cytokines also serve as homing signals for the mobilization and recruitment of pro-angiogenic cells from distant sites, including bone marrow and the walls of vessels in other tissues. The circulating angiogenic cells (CACs) that are mobilized by hypoxia-induced angiogenic cytokines are a heterogeneous population of cells that include endothelial progenitor cells (EPCs), haematopoietic stem-progenitor cells, and mesenchymal stem cells (MSCs), but the vast majority are bone marrow-derived myeloid cells.<sup>20–24</sup> Most studies have characterized CACs by the

co-expression of (i) receptors for angiogenic cytokines, such as VEGFR2 or CXCR4, and (ii) progenitor cell markers, such as stem cell antigen 1 (Sca1), CD34, or CD117 (CKIT). Unlike bona fide EPCs and MSCs, which can differentiate into endothelial cells and pericytes/smooth muscle cells, respectively, and incorporate into blood vessels,<sup>25</sup> the bone marrow-derived myeloid cells are recruited to a perivascular location and appear to activate endogenous vascular cells through the paracrine secretion of additional angiogenic growth factors.<sup>21–25</sup> The recruitment of CACs appears to be critical for angiogenesis under conditions of massive tissue damage, as in the case of tissue infarction, or rapid cell proliferation, as in the case of tumour xenografts. In *Hif1a*<sup>+/-</sup> mice, ischaemia-induced mobilization of CD34<sup>+</sup>VEGFR2<sup>+</sup> and Sca1<sup>+</sup>CXCR4<sup>+</sup> CACs is impaired due to reduced VEGF and SDF1 expression in the ischaemic tissue.<sup>19</sup> The recruitment of CACs also plays an important role in vascular remodelling, as described below.

### 3.3 Arteriogenesis/collateral vessel remodelling

Although it is clear that hypoxia triggers a strong HIF-1-dependent angiogenic response in ischaemic tissue, the initiating stimuli for the arteriogenic response remain unclear.<sup>26</sup> There is some debate regarding the importance of hypoxia in arteriogenesis, because the tissues where vascular remodelling occurs usually are not hypoxic.<sup>26,27</sup> However, it should be emphasized that following experimental arterial ligation, angiogenesis occurs coincident with or prior to arteriogenesis.<sup>28</sup> Moreover, angiogenesis occurs in the same vascular bed, distal to the site of arteriogenesis.<sup>29</sup> Following femoral artery ligation, proangiogenic myeloid cells attracted to sites of ischaemia must pass through collateral blood vessels, which are remodelled to accommodate increased blood flow. The increased shear stress that is present prior to remodelling is believed to be an important inciting stimulus for vessel remodelling. Expression of monocyte chemoattractant protein 1 is highly induced in ischaemic calf muscle in mice subjected to femoral artery ligation,<sup>19</sup> yet it plays an important role in the recovery of perfusion by remodelling of collateral vessels present in the thigh.<sup>30,31</sup> Angiogenic factors such as VEGF and PGF are also arteriogenic.<sup>32,33</sup> As the restriction of blood flow through conduit vessels results in decreased perfusion in the downstream vascular bed, it is not surprising that signals from the ischaemic tissue play critical roles in the remodelling of collateral vessels. The mobilization and recruitment of bone marrow-derived angiogenic cells ((BMDACs) appears to play an important role in vascular remodelling following femoral artery ligation, and conditions that inhibit CAC mobilization, such as ageing and diabetes (see below), also inhibit vessel remodelling. Thus, current evidence suggests considerable overlap between the molecular mechanisms and physical stimuli that trigger angiogenesis and arteriogenesis. Furthermore, there is compelling evidence that HIF-1 contributes to both processes.<sup>14,16,18,19,29,34</sup> However, the extent to which acute events associated with femoral artery ligation in the mouse provide an appropriate experimental model for the chronic, progressive stenosis of vessels in patients with peripheral arterial disease is not known.

## 4. Ischaemia-induced coronary collateralization

Progressive atherosclerotic stenosis of one or more of the major arteries supplying the myocardium and their subsequent luminal

occlusion leads to ischaemia and myocardial infarction (MI). Approximately two-thirds of patients with coronary artery disease (CAD) develop collateral vessels that bypass the stenosis, and the presence of collaterals is associated with reduced infarct size, less severe functional deterioration, and reduced mortality following MI.<sup>35,36</sup> Monocytes from CAD patients with collaterals had higher levels of hypoxia-induced VEGF secretion compared with monocytes from CAD patients without collaterals, even after adjusting for covariates (age, sex, diabetes, smoking, and hypercholesterolaemia).<sup>37</sup> The frequency of a single-nucleotide polymorphism (SNP), which changes proline to serine at codon 582 of the human *HIF1A* gene (P582S) was five-fold higher in CAD patients without demonstrable collateral coronary circulation by angiography.<sup>38</sup> Another study showed that the frequencies of three *HIF1A* SNPs, including the P582S allele, were significantly increased in patients presenting with stable exertional angina compared with patients presenting with MI.<sup>39</sup> These *HIF1A* gene SNPs were associated with reduced HIF-1 activity, which may result in early-onset ischaemic symptoms, leading to clinical evaluation prior to collateral development or manifestation of advanced disease (i.e. MI). Most recently, increased HIF-1 $\alpha$  expression in leucocytes was associated with the presence of coronary collaterals in patients with CAD.<sup>40</sup>

## 5. Ageing and diabetes impair ischaemia-induced vascularization by inhibiting HIF-1

Patients over the age of 50 with atherosclerotic stenosis of peripheral arteries have a 1–2% risk of developing critical limb ischaemia (CLI), which is characterized by decreased perfusion that threatens tissue viability and is manifested by ischaemic pain at rest, ulceration, and/or gangrene, eventually requiring limb amputation.<sup>41</sup> Similar to human CLI, ageing in mice is associated with impaired recovery of limb perfusion following femoral artery ligation.<sup>19,42</sup> *Hif1a*<sup>+/-</sup> mice show impaired recovery of perfusion relative to wild-type littermates at all ages and suffer more severe ischaemic tissue damage with ageing, associated with impaired expression of HIF-1 $\alpha$  protein and of mRNAs encoding the angiogenic factors ANGPT1, ANGPT2, PGF, SCF, SDF1, and VEGF in the ischaemic limb following femoral artery ligation.<sup>19</sup> Impaired wound healing is an age-dependent manifestation of diabetes mellitus in humans and in the *Lepr<sup>db/db</sup>* mouse model of type 2 diabetes.<sup>43</sup> Exposure of mouse dermal fibroblasts to high glucose impairs the hypoxia-induced stabilization of HIF-1 $\alpha$  protein and reduced HIF-1 $\alpha$  levels are present in diabetic wounds when compared with non-diabetic chronic venous ulcers.<sup>44–47</sup> Excisional wounds of aged *Lepr<sup>db/db</sup>* mice expressed significantly lower levels of HIF-1 $\alpha$ , ANGPT2, PDGF-B, PGF, and VEGF mRNAs compared with young counterparts, resulting in further impairment of wound healing.<sup>48,49</sup> CACs are reduced in the blood of type 2 diabetics with vascular complications<sup>50</sup> and the mobilization of CACs and recovery of perfusion after femoral artery ligation is also severely impaired in *Lepr<sup>db/db</sup>* mice<sup>51</sup> and diabetic rats.<sup>52</sup> It is important to emphasize that unlike young healthy mice, which recover completely without any tissue damage, diabetic and aged mice suffer tissue damage ranging from soft tissue necrosis to the spontaneous amputation of a toe or the entire foot following femoral artery ligation<sup>19,51</sup> and thus represent animal models of CLI.

## 6. Therapeutic strategies to increase HIF-1 activity in ischaemic tissue

Given the critical role of HIF-1 in ischaemia-induced vascular remodelling and the evidence that ageing and diabetes impair HIF-1 activation in response to ischaemia, it is not surprising that there is great interest in devising therapeutic strategies to increase HIF-1 activity as a means of restoring the normal physiological responses to hypoxia.

### 6.1 Pharmacological strategies

HIF-1 $\alpha$  protein levels, HIF-1 DNA-binding activity, and HIF-1 transcriptional activity can be increased by exposure of cells to CoCl<sub>2</sub> or the iron chelator desferrioxamine.<sup>53,54</sup> These agents have been proposed to act through inhibition of prolyl hydroxylases: CoCl<sub>2</sub> may induce HIF-1 activity through replacement of Fe<sup>2+</sup> by Co<sup>2+</sup> at the catalytic site of these enzymes, whereas desferrioxamine is an iron chelator that induces HIF-1 activity by reducing Fe<sup>2+</sup> availability.<sup>4</sup> Dimethylxylglycine (DMOG) is a competitive antagonist of  $\alpha$ -ketoglutarate that inhibits prolyl hydroxylase activity and blocks the O<sub>2</sub>-dependent degradation of HIF-1 $\alpha$ .<sup>4</sup> Intraperitoneal administration of desferrioxamine to aged mice restored HIF-1 $\alpha$  expression in an ischaemic skin flap, ischaemia-induced mobilization of 'EPCs', and improved flap vascularization, leading to tissue survival that was comparable to young mice.<sup>55</sup> The reduced HIF-1 $\alpha$  and VEGF mRNA and protein expression in cutaneous wounds of diabetic *Lepr<sup>db/db</sup>* mice can be corrected by local administration of CoCl<sub>2</sub>.<sup>48</sup> Wound healing was also improved in *Lepr<sup>db/db</sup>* mice by local application of desferrioxamine or DMOG.<sup>44</sup>

### 6.2 Gene therapy

Electroporation-assisted transduction into the skin of *Lepr<sup>db/db</sup>* mice of a plasmid vector encoding a constitutively active form of HIF-1 $\alpha$  designated CA5 (containing a deletion of amino acids 392–520 and two missense mutations that render the protein resistant to O<sub>2</sub>-dependent degradation) significantly increased cutaneous HIF-1 $\alpha$ , ANGPT2, PDGF-B, PLGF, and VEGF mRNA levels; CACs in peripheral blood; and the vascularization and rate of healing of excisional skin wounds.<sup>50</sup> In another study, injection of a plasmid or adenovirus encoding various constitutively active forms of HIF-1 $\alpha$  also improved wound healing.<sup>44,48</sup> In *Lepr<sup>db/db</sup>* mice subjected to femoral artery ligation, intramuscular (IM) injection into the ischaemic calf and thigh muscle of AdCA5, an adenovirus encoding the same constitutively active HIF-1 $\alpha$  construct described above, significantly increased CACs in peripheral blood, limb perfusion, tissue viability, and motor function.<sup>51</sup> These changes were associated with increased vessel luminal area and vessel density in the AdCA5-transduced ischaemic limbs, demonstrating an arteriogenic effect,<sup>51</sup> as previously reported in a rabbit limb ischaemia model.<sup>29</sup> Moreover, AdCA5 treatment reduced the number of infiltrating CD3<sup>+</sup> (T-lymphocyte) and myeloperoxidase<sup>+</sup> (neutrophil) cells, whereas F4/80<sup>+</sup> (myeloid) cells were increased in the ischaemic limb of *Lepr<sup>db/db</sup>* mice treated with AdCA5 when compared with mice treated with AdLacZ.<sup>51</sup> F4/80<sup>+</sup> cells have been shown to possess pro-angiogenic properties.<sup>56</sup> Thus, AdCA5 treatment alters the composition of the inflammatory cell population that infiltrates the ischaemic muscle towards macrophage predominance,

contributing to arteriogenic and angiogenic effects that help conserve tissue viability. IM AdCA5 was sufficient to overcome the impaired recovery of perfusion in middle-aged mice<sup>19</sup> but did not improve recovery or prevent tissue damage in old mice.<sup>57</sup> In another study, injection of adeno-associated virus (AAV) encoding yet another form of constitutively active HIF-1 $\alpha$  resulted into non-ischaemic skeletal muscle induced marked capillary sprouting, whereas AAV-VEGF induced only endothelial proliferation without proper vessel formation.<sup>58</sup> IM administration of adenovirus encoding HIF-1 $\alpha$ /VP-16 fusion protein also stimulated collateral development in diabetic rats subjected to femoral artery ligation.<sup>59</sup> It should be emphasized, however, that this latter construct is likely to differ from HIF-1 $\alpha$  in its transcriptional properties and biological effects and did not show efficacy in clinical trials.<sup>60</sup>

### 6.3 Combined gene and cell therapy

There is considerable interest in utilizing bone marrow cell therapy for ischaemic disorders. Although the cell populations that have been used in many preclinical and clinical studies are described as EPCs,<sup>61</sup> we prefer the term BMDACs because there is no evidence that the majority of these cells are capable of differentiating into endothelial cells, whereas there is abundant evidence indicating that they promote angiogenesis and vascular remodelling. If the mobilization of CACs is impaired by diabetes<sup>49–52</sup> and ageing<sup>19</sup> due to impaired expression of angiogenic cytokines,<sup>42</sup> then the intravenous (IV) administration of autologous BMDACs might circumvent the impaired mobilization and thereby promote vascular remodelling. However, the absence of a major clinical response in many studies suggests that the autologous BMDACs from patients may be intrinsically impaired in their ability to support vascularization. Studies in mouse models have demonstrated impaired functional properties of BMDACs derived from aged donors.<sup>62</sup>

Although AdCA5 delivery to muscle improves production of angiogenic cytokines, it does not address the functional impairment of BMDACs that is also associated with ageing. To promote vascular remodelling, BMDACs must be recruited to, and retained in, the ischaemic tissue.<sup>63–65</sup> With these considerations in mind, we devised a strategy for promoting recovery following femoral artery ligation in old mice that involved three components.<sup>57</sup> First, AdCA5 was injected into the thigh and calf of the ischaemic limb, which served to mobilize CACs and recruit them to the ischaemic limb. Second, bone marrow cells from a donor mouse were cultured for 4 days in the presence of angiogenic growth factors plus DMOG to induce HIF-1 activity. Third, IV injection of these BMDACs into recipient ischaemic mice was performed 24 h after femoral artery ligation and AdCA5 injection. A significant improvement in recovery of perfusion and limb salvage was observed only in mice that received IM AdCA5 + IV DMOG-treated BMDACs and was not observed in mice that received only IM AdCA5 or only IV DMOG-treated BMDACs or IM AdCA5 + IV vehicle-treated BMDACs.<sup>57</sup> The synergistic effect of these treatments was due to increased recruitment of BMDACs to the ischaemic limb induced by IM AdCA5, whereas DMOG treatment promoted retention of recruited BMDACs by increasing the expression of cell surface CD11/CD18 ( $\beta_2$ ) integrins, which are known to interact with ICAM-1 and E-selectin on the surface of vascular endothelial cells.<sup>64</sup> These studies provide a preclinical foundation for the design of clinical trials involving patients with CLI.

## 7. HIF-1-mediated pulmonary vascular remodelling in response to chronic alveolar hypoxia

In contrast to systemic arterioles, which dilate in response to local tissue hypoxia in an effort to increase O<sub>2</sub> delivery, pulmonary arterioles constrict in response to alveolar hypoxia.<sup>66</sup> This represents an adaptive response to lobar pneumonia, in which a region of lung tissue becomes filled with inflammatory cells eliminating the possibility of gas exchange. Thus, pulmonary blood flow is regulated to ensure that tissue perfusion is matched to tissue ventilation. However, when gas exchanged is impaired throughout the lung, as in the case of end-stage chronic obstructive pulmonary disease, chronic hypoxia induces widespread increased pulmonary artery resistance, progressive haemodynamic dysfunction, and right ventricular failure.<sup>67</sup> The increased resistance of pulmonary arterioles is due to increased pulmonary arterial smooth muscle cell (PASM) tone as well as PASM hypertrophy and proliferation. HIF-1 modulates vascular reactivity by mediating decreased expression of voltage-dependent K<sup>+</sup> channels (K<sub>v</sub>1.5, K<sub>v</sub>2.1) and increased expression of transient receptor potential Ca<sup>2+</sup> channels (TRPC1, TRPC6) in PASM.<sup>68–71</sup> HIF-1 mediates increased expression of Na<sup>+</sup>–H<sup>+</sup> exchanger 1 in PASM, leading to intracellular alkalinization, which promotes hypertrophy.<sup>72,73</sup> HIF-1 also induces the expression of angiotensin converting enzyme and angiotensin receptor AT<sub>1</sub> in human pulmonary artery fibroblasts<sup>74</sup> as well as augmenting endothelin-1 (ET-1) mRNA expression. ET-1 is a potent vasoconstrictor peptide, known to enhance Ca<sup>2+</sup> influx in PASM,<sup>75,76</sup> in addition to profibrotic and mitogenic effects on PASM, contributing to hypoxic pulmonary vascular remodelling.<sup>77,78</sup> The central role of HIF-1 in these pathological responses to hypoxia is highlighted by the finding that hypoxia-induced depolarization, alkalinization, and hypertrophy of PASM is impaired in *Hif1a*<sup>+/-</sup> mice.<sup>79</sup> In contrast to peripheral arterial disease and wound healing, in which therapeutic strategies are designed to promote adaptive responses mediated by HIF-1, in the context of hypoxic pulmonary hypertension, pharmacological inhibition of HIF-1-mediated pathogenic responses may be therapeutic. In support of this hypothesis, a recent study showed that rapamycin inhibits hypoxia-induced pulmonary vascular remodelling and right ventricular hypertrophy in mice, an effect that may be due to blockade of HIF-1 $\alpha$  synthesis, which is positively regulated by the mammalian target of rapamycin.<sup>80–82</sup>

## 8. HIF-1-mediated tumour vascularization

Increased HIF-1 $\alpha$  expression is associated with a highly vascularized and aggressive tumour phenotype, whereas HIF-1 $\alpha$  loss-of-function leads to decreased tumour growth and vascularization.<sup>13,83–88</sup> Cancer cells express high levels of SDF1 and VEGF through HIF-1-dependent mechanisms leading to the mobilization and recruitment of CACs.<sup>86–88</sup> Screening of known drugs for novel HIF-1 inhibitors revealed that digoxin and other cardiac glycosides inhibit HIF-1 $\alpha$  synthesis,<sup>89</sup> doxorubicin and other anthracyclines block HIF-1 DNA binding,<sup>87</sup> and acriflavine blocks HIF-1 dimerization.<sup>88</sup> These drugs inhibited tumour growth, tumour-induced mobilization of VEGFR2<sup>+</sup>/CD117<sup>+</sup>, VEGFR2<sup>+</sup>/CD34<sup>+</sup>, and CXCR4<sup>+</sup>/Sca1<sup>+</sup> CACs, and tumour vascularization in mouse xenograft models.<sup>87–89</sup>



## 9. Pathogenic role of HIF-1 in retinal neovascularization

In retinopathy of prematurity, exposure to increased O<sub>2</sub> concentrations inhibits retinal vascularization; when supplemental O<sub>2</sub> administration is discontinued, the retina becomes ischaemic, thereby triggering excessive formation of new blood vessels (neovascularization) with increased vascular leakage, which ultimately results in retinal detachment and loss of vision.<sup>90</sup> Multiple HIF-1-regulated angiogenic factors have been implicated in the pathogenesis of retinal neovascularization including VEGF, PGF, ANGPT2, PDGFB, SDF1, and erythropoietin.<sup>91–96</sup> In a mouse model of O<sub>2</sub>-induced retinopathy, in which HIF-1 $\alpha$  is induced by retinal ischaemia,<sup>97</sup> intraocular or intraperitoneal injection of digoxin markedly reduced retinal levels of HIF-1 $\alpha$  protein and of mRNAs encoding VEGF, PGF, PDGFB, SCF, and SDF1; blocked the recruitment of pro-angiogenic F4/80<sup>+</sup> and CXCR4<sup>+</sup> myeloid cells to the ischaemic retina; and inhibited retinal neovascularization.<sup>98</sup> Although anti-VEGF therapy has revolutionized the treatment of ocular neovascularization, ranibizumab results in improved vision in less than half of all treated patients,<sup>99</sup> suggesting that angiogenic factors other than VEGF may be playing an important role in the non-responders. Digoxin therapy has the potential advantage of inhibiting the expression of multiple angiogenic factors. YC-1, a compound that inhibits HIF-1 $\alpha$  protein expression by unknown mechanisms, has also been reported to inhibit retinal neovascularization in the mouse.<sup>100</sup>

## 10. Conclusion

HIF-1 is a pivotal regulator of vascular responses to hypoxia and ischaemia. One important hallmark of ageing and diabetes is an impairment of ischaemia-induced HIF-1-mediated vascularization. Enhancement of HIF-1 $\alpha$  protein expression is advantageous over therapies that target single angiogenic factors, because HIF-1 $\alpha$  can induce multiple angiogenic targets in a coordinated manner. Moreover, preclinical experimental data suggest that transient local expression of a constitutively active form of HIF-1 $\alpha$  may be sufficient to induce a beneficial pro-angiogenic response in patients with CLI, diabetic ulcers, or non-healing wounds, thereby alleviating concerns of possible toxicity or adverse effects that might be associated with systemic HIF-1 activation. Conversely, inhibition of HIF-1 activity may block pathological vascular remodelling or angiogenesis associated with pulmonary hypertension, cancer, or retinopathy.

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