Review
Hypoxia

Hypoxia in the pathogenesis of systemic sclerosis

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Published: 21 April 2009
This article is online at http://arthritis-research.com/content/11/2/220

Arthritis Research & Therapy 2009, **11:**220 (doi:10.1186/ar2598)

Abstract

Autoimmunity, microangiopathy and tissue fibrosis are hallmarks of systemic sclerosis (SSc). Vascular alterations and reduced capillary density decrease blood flow and impair tissue oxygenation in SSc. Oxygen supply is further reduced by accumulation of extracellular matrix (ECM), which increases diffusion distances from blood vessels to cells. Therefore, severe hypoxia is a characteristic feature of SSc and might contribute directly to the progression of the disease. Hypoxia stimulates the production of ECM proteins by SSc fibroblasts in a transforming growth factor-β-dependent manner. The induction of ECM proteins by hypoxia is mediated via hypoxia-inducible factor-1α-dependent and -independent pathways. Hypoxia may also aggravate vascular disease in SSc by perturbing vascular endothelial growth factor (VEGF) receptor signalling. Hypoxia is a potent inducer of VEGF and may cause chronic VEGF over-expression in SSc. Uncontrolled over-expression of VEGF has been shown to have deleterious effects on angiogenesis because it leads to the formation of chaotic vessels with decreased blood flow. Altogether, hypoxia might play a central role in pathogenesis of SSc by augmenting vascular disease and tissue fibrosis.

Introduction

Oxygen homeostasis is a *sine qua non* for metazoan organisms. Reduction in physiological oxygen concentrations leads to metabolic demise because oxygen is the terminal electron acceptor during ATP formation in mitochondria and is a central substrate in many enzymatic reactions. Whereas lack of oxygen causes metabolic cell death, increased oxygen concentrations carry a risk for oxidative damage to proteins, lipids and nucleic acids, possibly initializing apoptosis or carcinogenesis. Thus, even slight changes in systemic and cellular oxygen concentrations induce a tightly regulated machinery of short-acting and long-acting response pathways to keep the supply of oxygen within the physiological range.

Molecular responses to hypoxia and endogenous hypoxia markers have been elucidated in detail during the past two decades. In this context, the molecular characterization of the transcription factor hypoxia-inducible factor (HIF)-1 and unravelling of its regulation were breakthroughs for our understanding of cellular adaptation to reduced oxygenation. HIF-1 protein accumulates under hypoxic conditions in many different cell types. It activates the transcription of genes that are of fundamental importance for oxygen homeostasis, including genes involved in energy metabolism, angiogenesis, vasomotor control, apoptosis, proliferation and matrix production [1].

Systemic sclerosis (SSc) is characterized by a triad of microangiopathy, activation of humoral and cellular immune responses and tissue fibrosis, affecting the skin as well as a variety of internal organs, including lung, heart and gastrointestinal tract [2]. Using nailfold capillaroscopy, alterations in the capillary network can be observed early in SSc. Vascular alterations include sac-like, giant and bushy capillaries, microhaemorrhages and a variable loss of capillaries that result in avascular areas [3]. The microangiopathy with progressive loss of capillaries leads to decreased blood flow followed by a lack of nutrients and tissue hypoxia. In advanced disease, fibrosis of the skin and of multiple internal organs, which results from excessive extracellular matrix production of activated fibroblasts, is the most obvious histopathological hallmark of SSc. Because the accumulation of extracellular matrix increases diffusion distances from blood vessels to cells, tissue malnutrition and hypoxia may be aggravated by fibrosis. In summary, severe tissue hypoxia is present in SSc and may even be involved in disease progression.

ARD = arrest defective; COL = collagen; CREB = cAMP response element binding protein; CTGF = connective tissue growth factor; HBS = HIF-1 DNA binding site; HIF = hypoxia-inducible factor; IGF = insulin-like growth factor; IGFBP = insulin-like growth factor binding protein; ODDD = oxygen-dependent degradation domain; PAS = Per/ARNT/Sim; P4H = prolyl-4-hydroxylase; PDGF = platelet-derived growth factor; PHD = prolyl hydroxylase domain; Po_2 = oxygen partial pressure; pVHL = Von Hippel-Lindau tumour suppressor protein; SSc = systemic sclerosis; TGF = transforming growth factor; TGF-βi = TGF-β-induced protein; VEGF = vascular endothelial growth factor.

The present review presents current knowledge of molecular signalling pathways in response to hypoxia and discusses the role that hypoxia plays in the pathogenesis of SSc.

Molecular structure of hypoxia-inducible factor-1

In 1995, Wang and coworkers cloned the transcription factor HIF-1, based on its ability to bind to the 3' enhancer region of the erythropoietin gene [4]. Structural analysis revealed two subunits: HIF-1α (120 kDa) and HIF-1β (91 to 94 kDa). Both HIF-1 subunits contain a basic helix-loop-helix domain, enabling them to recognize and bind to specific DNA sequences, called HIF-1 DNA binding sites (HBSs), within the regulatory regions of hypoxia-inducible genes. Both proteins are also charactarized by two Per/ARNT/Sim (PAS) regions located at the amino-termini. Using HIF-1α deletion mutants, Jiang and coworkers [5] demonstrated that the helix-loop-helix domain and the PAS-A region of HIF-1α are sufficient for heterodimerization with HIF-1β. The most intriguing structural element of HIF-1α is the oxygen-dependent degradation domain (ODDD), which links HIF-1α to the cellular oxygen sensor. Under normoxic conditions the hydroxylation of two proline residues within the ODDD results in ubiquitinylation and degradation of HIF-1α. In contrast, hydroxylation and degradation of HIF-1α are decreased in hypoxic milieus because oxygen is the critical substrate in hydroxylation reactions. Thus, lack of oxygen leads to HIF-1α accumulation [6].

Stabilization of hypoxia-inducible factor-1α protein

In contrast to the expression of HIF-1β, that of HIF-1α is tightly controlled by cellular oxygen levels. Cellular HIF-1α is not detectable under normoxic conditions because it is rapidly degraded after translation. After exposure to low oxygen concentrations, levels of HIF-1α increase exponentially. Maximal response is usually reached at oxygen concentrations of about 0.5% .

Hydroxylation of two proline residues within the ODDD (positions 402 and 564) triggers the oxygen-dependent regulation of HIF-1α. This hydroxylation is catalyzed by a family of 2-oxoglutarate dependent dioxygenases called prolyl hydroxylase domains (PHDs) [7]. During the hydroxylation process, PHDs split molecular oxygen and transfer one oxygen atom to one of the proline residues. The second oxygen atom reacts with 2-oxoglutarate, generating succinate and carbon dioxide. The co-substrate ascorbic acid keeps the ferrous ion of the catalytic site in its bivalent state. The ability of PHDs to modify HIF-1α depends on the concentration of its substrate oxygen. Under normoxic conditions, PHDs hydroxylate HIF-1α efficiently, leading to the rapid degradation of the HIF-1α subunit. In contrast, the rate of hydroxylation is reduced at low oxygen levels. Thus, PHDs function as intracellular oxygen sensors and provide the molecular basis for the regulation of HIF-1α protein concentrations by cellular partial pressure of oxygen [8].

The hydroxylation of HIF-1α is similar to the prolyl modification of collagens [9,10]. However, collagen prolyl hydroxylases are unable to hydroxylate the proline residues of HIF-1α [9]. Three human HIF-1α dioxygenases have been identified thus far [8,11,12]: PHD3 (HPH-1/EGLN3), PHD2 (HPH-2/EGLN1) and PHD1 (HPH-3/EGLN2). All three PHDs have the potential to hydroxylate HIF-1α. Nevertheless, PHD2 exhibits the greatest prolyl hydroxylase activity in normoxic cells [13]. It is the key limiting enzyme for HIF-1α turnover and its knockdown by small interfering RNA stabilizes HIF-1α levels, whereas single knockdown of PHD1 or PHD3 has no effect on the stability of hypoxic conditions. Appelhoff and coworkers [14] demonstrated that PHD3 activity exceeded the activity of PHD2 in MCF-7 breast cancer and BXPC-3 pancreatic cancer cell lines under hypoxic conditions. Inhibition of PHD3 in hypoxic cells led to higher HIF-1α levels than inhibition of PHD2.

Recently, an endoplasmatic prolyl-4-hydroxylase (P4H) with a transmembrane domain, which is more closely related to the collagen prolyl hydroxylases, has also been shown to hydroxylate HIF-1α *in vitro* [15].

An additional mechanism for the regulation of HIF-1α stability was demonstrated by Jeong and coworkers [16]. Arrest defective (ARD)1, an acetyltransferase, binds directly to the ODDD of HIF-1α in the cytoplasm and acetylates a single lysine residue at position 532. Acetylation of this specific lysine residue favours the interaction of HIF-1α and the E3 ubiquitin ligase complex, and stimulates the degradation of HIF-1α. As shown by vascular endothelial growth factor (VEGF) promoter-driven luciferase reporter gene assays, ARD1 not only destabilizes HIF-1α protein, but it also downregulates its transactivation activity in ARD1-transfected HT1080 human fibrosarcoma cells under hypoxic conditions. Mutation of lysine residue 532 to arginine or application of antisense ARD1 results in stabilization of HIF-1α even under normoxic conditions [16,17]. In contrast, levels of HIF-1α decreased when deacetylation was inhibited. Finally, mRNA and protein levels of ARD-1 are diminished under hypoxia, resulting in less acetylated HIF-1α [16].

Blocking hydroxylation of proline residues 402 and 564 as well as blocking acetylation of lysine 532 have been demonstrated to prevent degradation of HIF-1α under normoxic conditions, thus abolishing the oxygen-dependent regulation of HIF-1α signalling [6,9,16]. These findings suggest that both pathways - hydroxylation and acetylation of HIF-1α - are essential for the physiological regulation of cellular responses to hypoxia.

Upregulation of prolyl hydroxylase domain activity in chronic hypoxia

Interestingly, PHD2 and PHD3 are induced by hypoxia in a HIF-1α-dependent manner, thereby creating a negative feedback loop of HIF-1α signalling [14,18]. In this context, a functional hypoxia-regulated element has been identified in

the PHD3 gene, enabling direct regulation of PHD3 by HIF-1. Recently, Ginouvès and coworkers [19] reported increased PHD activity in response to chronic hypoxia. PHD2 and PHD3 protein levels reached a maximum after 24 hours of hypoxia, whereas PHD activity rose steadily for 7 days, indicating that further mechanisms besides induction of PHDs led to increased PHD activity. Consistent with these findings, PHD activity increased with prolonged hypoxia *in vivo*. Only low PHD activity but high HIF-1α levels were observed in mice exposed to 6 hours of hypoxia at 8% oxygen, whereas PHD activity increased markedly after 24 hours of hypoxia, resulting in a subsequent reduction in HIF-1α. After 24 hours of 8% oxygen, escalation of hypoxia to 6% oxygen concentration for another 2 hours caused a re-accumulation of HIF-1α [19]. Together these findings suggest that HIF-1α is induced in response to hypoxia, accumulates in acute hypoxia and is removed as the activity of PHDs increases in chronic hypoxia.

Ginouvès and coworkers [19] also suggested a mechanism that may lead to augmented PHD activity that is distinct from PHD gene induction. During hypoxia, HIF-1 induces pyruvate dehydrogenase kinase-1, which has been reported to decrease mitochondrial oxygen consumption by inhibiting mitochondrial respiration [20,21]. Inhibition of mitochondrial respiration may increase intracellular oxygen levels and accelerate oxygen-dependent HIF-1α hydroxylation by PHDs [19]. Therefore, augmented PHD activity in chronic hypoxia might create an effective negative feedback loop for HIF-1α signalling. Although this hypothesis must be confirmed with further experiments, separating acute from chronic hypoxia will certainly gain importance for future studies, especially when evaluating HIF-1α or PHDs as possible therapeutic targets for diseases in which hypoxia has been implicated, such as SSc.

Degradation of hypoxia-inducible factor-α

The rapid degradation of HIF-1α under normoxic conditions is mediated by the von Hippel-Lindau tumour suppressor protein (pVHL) [22]. The β-subunit of pVHL interacts directly with the ODDD of HIF-1α when proline residue(s) 402 and/or 564 are hydroxylated, but not without this modification. pVHL itself is part of the E3 ubiquitin ligase complex. Interaction of proline-hydroxylated HIF-1α with pVHL/E3 ubiquitin ligase complex activates the ubiquitination machinery, thereby promoting degradation of HIF-1α [1,9,23,24]. A similar mechanism of recognition is proposed for the acetylation of the lysine residue 532 [16]. Under hypoxic conditions, the ODDD is neither hydroxylated nor acetylated, pVHL cannot bind and HIF-1α is not ubiquitinated. Thus, degradation of HIF-1α in the proteasome is inhibited and HIF-1α protein accumulates.

Binding of HIF-1 to HIF binding sites, formation of the transcriptional complex and regulation of HIF-1 transactivation

After translocation into the nucleus HIF-1α dimerizes with ARNT/HIF-1β. The HIF-1 heterodimer then binds via its basic helix-loop-helix domain to the HBS within the hypoxia-responsive element of most hypoxia-regulated genes [25-27]. The HBS is essential but not sufficient for HIF-1 gene activation. Besides the HBS, a complete hypoxia-responsive element contains additional binding sites for transcription factors that are not sensitive to hypoxia. These co-stimulatory factors, including cAMP response element binding protein (CREB)-1 of the lactate dehydrogenase A gene [28] or activator protein-1 (AP-1) in the VEGF gene [29], are also required for efficient transcription of oxygen-sensitive genes. Multimerization of HBS can substitute for additional transcription factors in several HIF-regulated genes [30-33].

For efficient induction of HIF-1-regulated genes, HIF-1 must be activated. Simple blockade of HIF-1α degradation (for example with chemical proteasome inhibitors such as *N*-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norvalinal) results in accumulation of HIF-1α but is often not sufficient for trans-activation [34]. Two modifications of HIF-1α involved in the regulation of HIF-1α transactivation have been identified: hydroxylation of the carboxyl-terminal transactivation domain and protein phosphorylation by tyrosine kinase receptors.

At low oxygen concentrations the carboxyl-terminal trans-activation domain of HIF-1α recruits several co-activators, including p300 and CREB-binding protein, which are required for HIF-1 signalling [35,36]. Under normoxic conditions the enzyme FIH-1 (factor-inhibiting HIF-1) hydroxylates an asparagine residue at position 803, thereby preventing interaction of HIF-1α with p300 with CREB-binding protein [37]. Consequently, oxygen-sensitive asparagine hydroxylation, inhibiting HIF-1 transactivation, is part of the oxygen-sensing mechanism [37,38].

Other members of the hypoxia-inducible factor family

Two proteins closely related to HIF-1α have been identified and designated HIF-2α and HIF-3α [39,40]. HIF-2α and HIF-3α are both able to dimerize with HIF-1β and bind to HBSs [41,42]. HIF-2α is similar to HIF-1α with regard to its genomic organization, protein structure, dimerization with HIF-1β, DNA binding and transactivation [22,35,43,44]. Moreover, both proteins accumulate under hypoxic conditions [45-47]. However, experiments with knockout mice revealed that HIF-1α and HIF-2α could not compensate for loss of the each other [31,48,49]. This finding suggests that the different α subunits of HIF might not be redundant and possess different biological functions.

Hypoxia in systemic sclerosis

Hypoxia and its central mediator HIF-1 control a large variety of different genes. Upregulation of HIF-1 in response to hypoxia regulates erythropoiesis, angiogenesis and glucose metabolism, as well as cell proliferation and apoptosis [1,7]. Using DNA microarray studies on primary pulmonary arterial endothelial cells, Manalo and coworkers [50] observed that a

minimum of 2.6% of all human genes were regulated by hypoxia in a HIF-1-dependent manner. In theory, microangiopathy and tissue fibrosis should result in reduced tissue oxygenation and may provoke HIF-1-dependent response to hypoxia. The reduced capillary density and vascular malformations should lead to decreased blood flow with lack of nutrients and oxygen in involved organs in SSc patients [51]. Besides the microangiopathy, tissue fibrosis might further aggravate tissue malnutrition and hypoxia. The progressive accumulation of extracellular matrix proteins such as collagens, fibronectin and glycosaminoglycans [52] increases distances between cells and their supplying vessels and may impair diffusion. Hence, lack of functional capillaries as well as impaired diffusion implicate significant tissue malnutrition and chronic hypoxia in SSc patients (Figure 1).

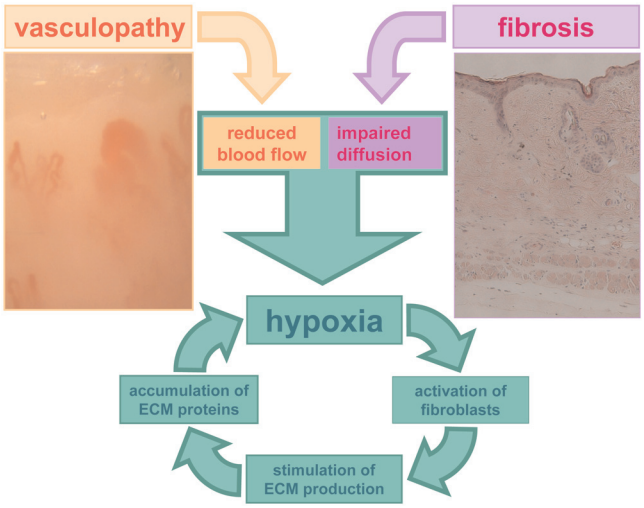
Indeed, two studies demonstrated severe hypoxia in lesional, fibrotic skin of SSc patients [53,54]. In both studies, low oxygen levels were only found in lesional skin of SSc patients, whereas the oxygen levels in nonfibrotic skin were not decreased compared with skin of healthy volunteers.

Using a noninvasive transcutaneous technique to measure oxygen levels, Silverstein and coworkers [53] showed that oxygen levels of fibrotic skin were inversely related to skin thickness. The lowest oxygen levels were measured in SSc patients with severely thickened skin. Indirect correlation of oxygen levels with dermal thickness supports the concepts of impaired diffusion due to accumulation of extracellular matrix in lesional skin of SSc patients. Patients suffering from primary Raynaud's disease did not exhibit hypoxic skin, and oxygen levels were similar to those in healthy individuals.

We quantified oxygen levels in the skin of SSc patients by applying an oxygen partial pressure (Po_2) histography method, involving introduction of a small polarographic needle electrode directly into the dermis [54]. To exclude systemic influences on local oxygen levels, we determined arterial oxygen saturation, haemoglobin content, blood pressure and heart rate, and patients rested for at least 10 minutes before the experiment. For each patient about 200 single measurements of Po_2 were taken at a predefined area on the dorsal forearm, and an individual Po_2 mean value was determined. Average Po_2 in the skin of healthy individuals was 33.6 ± 4.1 mmHg (4.4 ± 0.5% oxygen per volume), whereas involved skin of SSc patients exhibited significantly decreased oxygen levels, with a mean Po_2 value of 23.7 ± 2.1 mmHg (3.1 ± 0.3%). In contrast, the average Po_2 in nonfibrotic skin of SSc patients did not differ from that in healthy individuals (mean Po_2 37.9 ± 8.6 mmHg, corresponding to 5.0 ± 1.1%).

In summary, both studies demonstrated that hypoxia is a characteristic feature of involved, fibrotic skin of SSc patients. Although cutaneous blood flow, a potential confounding factor, was not determined in any of these studies, the inverse correlation of skin thickness with cutaneous Po_2 suggests that impaired oxygen diffusion due to extracellular matrix accumulation might contribute to tissue hypoxia in SSc.

Figure 1



Vicious circle of hypoxia and fibrosis in the pathogenesis of SSc. To the upper left is shown nailfold capillaroscopy from a patient with systemic sclerosis (SSc) with capillary rarification and vascular alterations, including sac-like, giant and bushy capillaries. Vasculopathy leads to reduced blood flow and causes tissue hypoxia in SSc. To the upper right is shown a haematoxylin and eosin stained skin section from an experimental mouse fibrosis model with increased skin thickness due to extracellular matrix (ECM) deposition. ECM deposition increases diffusion distances from blood vessels to cells and reduces tissue oxygenation. In the 'vicious circle', shown at the bottom of the figure, tissue hypoxia leads to activation of dermal fibroblasts and upregulation of ECM production. Further ECM deposition aggravates tissue malnutrition and hypoxia. Hypoxia once again stimulates ECM production in dermal fibroblasts.

Role played by hypoxia-inducible factor-1α in systemic sclerosis

Considering the presence of hypoxia, one would assume that HIF-1α is strongly upregulated in SSc [54,55]. This presumption is fortified by the fact that several cytokines and growth factors, upregulated in SSc, are able to stabilize HIF-1α under certain conditions. Examples include interleukin-1β, transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor 2 and insulin-like growth factors (IGFs) [56-58].

Despite severely reduced oxygen levels and despite the overexpression of these growth factors, protein levels of HIF-1α in the skin of SSc patients were even below the levels seen in healthy control skin [54]. Skin specimens from SSc patients did not exhibit increased expression of HIF-1α protein by immunohistochemistry. HIF-1α staining was moderate to high

in the epidermis of healthy individuals, whereas the expression of HIF-1α in SSc patients was restricted to single keratinocytes. HIF-1α protein was not detectable in the dermis of healthy individuals and SSc patients. Moreover, the HIF-1α expression pattern in involved skin in SSc patients did not correlate with upregulated VEGF, one of the main transcriptional targets of HIF-1α [54].

PHD-dependent HIF-1α negative feedback loops in chronic hypoxic conditions might be a plausible explanation for decreased HIF-1α levels in fibrotic skin of SSc patients. Considering the clinical course of SSc, lesional skin in SSc patients can be categorized as a chronically hypoxic tissue. In this context, low HIF-1α levels may be caused by negative HIF-1α feedback loops, even despite severe hypoxia. Increased PHD activity in response to chronic hypoxia [19] might lead to rapid HIF-1α degradation and decreased HIF-1α levels in fibrotic SSc skin. This theory is also supported by studies on the effects of prolonged hypoxia in murine organs. In mice exposed to 6% oxygen, HIF-1α protein reached maximum levels in the brain after 4 to 5 hours but declined afterward, attaining basal normoxic concentrations after 9 to 12 hours. Similar results were obtained for kidney and liver [59].

However, the low levels of HIF-1α in the skin of SSc patients *per se* do not argue against the persistent activation of oxygen-sensitive pathways in SSc. Marked and persistent upregulation of the oxygen-dependent gene VEGF is observed in lesional SSc skin even in late stages of SSc. Thus, the response to hypoxia appears to persist in chronic states, but might be driven by HIF-1α-independent pathways, for instance HIF-2α and HIF-3α. However, the role played by other members of the HIF family in the pathogenesis of SSc has not yet been investigated in detail.

Insufficient response to hypoxia: dysregulation of angiogenesis in systemic sclerosis

Angiogenesis and vasculogenesis are fundamental mechanisms in improving oxygenation of hypoxic tissue. HIF-1 promotes vascularization by inducing the expression of multiple angiogenic mediators such as VEGF, placental growth factor, angiopoietin 1 and 2, and PDGF-BB [60]. VEGF drives angiogenesis by activating endothelial cells in hypoxic tissue and vasculogenesis by mobilizing and recruiting endothelial progenitor cells [61-63]. In addition, VEGF exhibits synergistic angiogenic effects together with PDGF and fibroblast growth factor-2 [64].

Sufficient tissue vascularization depends on strict regulation of VEGF expression. Chronic and uncontrolled overexpression of VEGF induces the formation of chaotic vessels, characterized by glomeruloid and haemangioma-like morphology [65,66]. Dor and coworkers [67] demonstrated in pTET-VEGF$_{165}$/MHCα-tTa transgenic mice, in which VEGF expression can be conditionally switched off in an organ-dependent manner by feeding tetracycline, that time-dependent regulation of VEGF expression was essential for adequate vascularization. Although short-term over-expression of VEGF induced the formation of new mature and functional vessels in adult organs, prolonged exposure to VEGF without subsequently switching off its gene expression by tetracycline resulted in the formation of irregularly shaped, sac-like vessels leading to reduced blood flow. Irregularly shaped, sac-like vessels are reminiscent of the disturbed vessel morphology in SSc [3]. Hence, the microvascular defects in SSc might partly be caused by uncontrolled overexpression of VEGF.

VEGF levels are markedly upregulated in the skin of SSc patients compared with healthy volunteers [54]. As analyzed by *in situ* hybridization, the mean percentage of epidermal keratinocytes expressing VEGF was significantly increased in SSc patients compared with normal individuals. These findings were consistent with dermal expression levels of VEGF. In contrast, normal individuals did not exhibit VEGF expression in the dermis. VEGF was expressed in most SSc patients in a variety of different dermal cell types, including fibroblasts, endothelial cells and leucocytes [54]. VEGF was induced in dermal SSc fibroblasts in response to hypoxia, but expression levels did not differ significantly between fibroblasts from SSc patients and those from healthy volunteers [54]. However, as the oxygen levels are significantly lower in lesional skin of SSc patients than in control individuals, the induction of VEGF by hypoxia is only operative in SSc patients, but not in normal volunteers. Both receptors for VEGF, namely VEGF receptors 1 and 2, were also overexpressed in the skin of SSc patients. Therefore, enhanced activation of the VEGF/VEGF receptor axis may lead to typical changes in SSc vascularization, causing tissue malnutrition and hypoxia [54]. Because the expression of VEGF is stimulated by hypoxia, one might speculate that the hypoxia could augment vascular disease in SSc by contributing to persistent over-expression of VEGF. However, it remains to be demonstrated that chronic hypoxia alone is indeed sufficient to cause persistent upregulation of VEGF *in vivo*. Alternatively, the persistent over-expression of VEGF in SSc might also be driven by cytokines. Interleukin-1β, PDGF and TGF-β are all upregulated in SSc and can stimulate the expression of VEGF [54,68,69].

Induction of fibrosis by hypoxia

Microangiopathy with impaired angiogenesis and excessive accumulation of extracellular matrix may cause severe hypoxia in SSc [53,54]. However, what is the exact role played by hypoxia in the pathogenesis of SSc? Is it just the consequence of microangiopathy and fibrosis or does it contribute to the progression of SSc?

DNA microarray studies revealed the first causal links between hypoxia and fibrosis [50]. Manalo and coworkers [50] detected a striking number of genes encoding collagens

or collagen-modifying enzymes that were induced in pulmonary endothelial cells after 24 hours at 1% oxygen. These genes included collagen (COL)1A2, COL4A1, COL4A2, COL5A1, COL9A1 and COL18A1, as well as procollagen prolyl hydroxylases (P4HA1 and P4HA2), lysyl oxidase (LOX) and lysyl hydroxylases (procollagen lysyl hydroxylase and procollagen lysyl hydroxylase 2). Similar links between hypoxia and fibrosis have also been found in other models and organs, for example kidney [70,71], liver [72] and lung [73]. Together, these findings indicate that hypoxia could promote extracellular matrix production and that it may actively be involved in the pathogenesis of profibrotic disorders such as SSc.

We could demonstrate that hypoxia induced several extracellular matrix proteins, including fibronectin-1, thrombospondin-1, proα2(I) collagen (COL1A2), IGF-binding protein 3 (IGFBP-3) and TGF-β-induced protein (TGF-βi) in cultured dermal fibroblasts [74]. Type 1 collagens and fibronectins are the major matrix proteins within fibrotic lesions [52]. Thrombospondin-1 also accumulates in SSc and modulates angiogenesis. TGF-βi is an extracellular matrix protein that is known to be highly expressed in arteriosclerotic plaques [75] and in zones of thickened extracellular matrix in the bladder [76]. IGFBP-3 directly induces the synthesis of fibronectin in lung fibroblasts [77] and protects IGF-1 from degradation. IGF-1 itself stimulates collagen synthesis and downregulates the production of collagenases in fibroblasts [77].

Induction and production of these extracellular matrix proteins in response to hypoxia was time dependent and inversely correlated with oxygen levels [74]. Most of these proteins were significantly upregulated after 24 hours of oxygen deprivation, with a further significant increase after 48 hours. The expression of fibronectin-1, thrombospondin-1, COL1A2 and IGFBP-3 was significantly enhanced at 8% oxygen concentration and increased further with lower oxygen levels, reaching a maximum at 1% oxygen. Of note, severe and chronic hypoxia, as may be found in the skin of SSc patients [54], was associated with the most marked effects on the induction of extracellular matrix proteins.

These results were confirmed in a mouse model of systemic normobaric hypoxia [74]. Consistent with the results obtained *in vitro*, extracellular matrix proteins were upregulated in mice exposed to hypoxia after 24 hours compared with control mice breathing air with 21% oxygen. Prolonged exposure for 48 hours resulted in further upregulation of fibronectin 1, thrombospondin 1 and COL1A2, whereas TGF-βi and IGFBP3 mRNA levels decreased slightly. Because TGF-β is a major stimulus for the induction of extracellular matrix proteins in SSc [52,78], its role for hypoxia-dependent fibrogenesis was also studied in dermal SSc fibroblasts. Neutralizing antibodies against TGF-β completely abrogated the induction of COL1A2, fibronectin 1, thrombospondin 1 and TGF-βi in SSc fibroblasts that were cultured under hypoxic conditions for 48 hours [74]. These findings suggest that inhibition of TGF-β-dependent pathways may prevent the profibrotic effects of hypoxia.

Consistent with the results on TGF-β signalling, the expression of the fibrogenic cytokine connective tissue growth factor (CTGF) was also shown to be upregulated in SSc in response to hypoxia [79]. CTGF is a critical mediator of TGF-β-induced skin fibrosis in SSc [80]. Its serum levels are elevated in SSc patients and have been suggested to correlate with skin fibrosis [81]. Hong and coworkers [79] found increased levels of CTGF mRNA and protein in fibroblasts exposed to 1% of oxygen or treated with cobalt chloride, a chemical stabilizer of HIF-1α. The induction of CTGF in response to hypoxia depended on HIF-1α [79]. Because the authors concentrated on short-term hypoxia of up to 4 hours, it remains unclear whether CTGF is also induced by chronic hypoxia and by HIF-1α-independent mechanisms in SSc.

Thus, accumulating evidence suggests that hypoxia might be actively involved in pathogenesis of SSc by stimulating the release of extracellular matrix protein. This could result in a vicious circle of hypoxia and fibrosis. Hypoxia stimulates the production and accumulation of extracellular matrix. The resulting tissue fibrosis inhibits diffusion of oxygen, causing further tissue hypoxia, which stimulates further the production of extracellular matrix (Figure 1). Activation of TGF-β-dependent pathways appears to play a central role in the induction of extracellular matrix proteins by hypoxia, and inhibition of TGF-β signalling might prevent hypoxia-induced tissue fibrosis. However, further studies are needed to characterize further the role played by hypoxia in SSc and to identify the molecular mechanisms activated by hypoxia in SSc.

Conclusions

Capillary rarification and disturbed blood flow, as well as excessive extracellular matrix accumulation, cause chronic tissue hypoxia in SSc. However, levels of HIF-1α protein are decreased, probably due to PHD-dependent negative feedback loops. Interestingly, physiological mechanisms to overcome tissue hypoxia are impaired and dysregulated in SSc. Insufficient angiogenesis and vasculogenesis cannot abolish tissue malnutrition and hypoxia. Compensatory overexpression of VEGF might even result in a futile vascular response to hypoxia, characterized by the chaotic vessel formation. Hypoxia stimulates the production of several extracellular matrix proteins in SSc fibroblasts in a time- and concentration-dependent manner. The excessive deposition of matrix might impair further the diffusion of oxygen and cause a vicious circle of hypoxia and tissue fibrosis. Currently, there are no specific modulators of HIFs or PHDs available for clinical use. Thus, it is not yet possible to target hypoxia selectively in SSc patients. However, because inhibition of TGF-β prevented the induction of extracellular matrix by

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hypoxia, blocking of TGF-β signalling might be one approach to target at least in part the hypoxia-induced matrix production in SSc.

Competing interests

The authors declare that they have no competing interests.

References

1. Maxwell PH, Ratcliffe PJ: **Oxygen sensors and angiogenesis.** *Semin Cell Dev Biol* 2002, **13:**29-37.
2. Varga J, Abraham D: **Systemic sclerosis: a prototypic multisystem fibrotic disorder.** *J Clin Invest* 2007, **117:**557-567.
3. LeRoy EC: **Systemic sclerosis. A vascular perspective.** *Rheum Dis Clin North Am* 1996, **22:**675-694.
4. Wang GL, Jiang BH, Rue EA, Semenza GL: **Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension.** *Proc Natl Acad Sci USA* 1995, **92:**5510-5514.
5. Jiang BH, Rue E, Wang GL, Roe R, Semenza GL: **Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1.** *J Biol Chem* 1996, **271:**17771-17778.
6. Ivan M, Haberberger T, Gervasi DC, Michelson KS, Günzler V, Kondo K, Yang H, Sorokina I, Conaway RC, Conaway JW, Kaelin WG Jr: **Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor.** *Proc Natl Acad Sci USA* 2002, **99:**13459-13464.
7. Ke Q, Costa M: **Hypoxia-inducible factor-1 (HIF-1).** *Mol Pharmacol* 2006, **70:**1469-1480.
8. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ: ***C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation.** *Cell* 2001, **107:**43-54.
9. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim Av, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ: **Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation.** *Science* 2001, **292:**468-472.
10. Masson N, Willam C, Maxwell PH, Pugh CW, Ratcliffe PJ: **Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation.** *Embo J* 2001, **20:**5197-5206.
11. Bruick RK, McKnight SL: **A conserved family of prolyl-4-hydroxylases that modify HIF.** *Science* 2001, **294:**1337-1340.
12. Huang J, Zhao Q, Mooney SM, Lee FS: **Sequence determinants in hypoxia-inducible factor-1alpha for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3.** *J Biol Chem* 2002, **277:**39792-39800.
13. Berra E, Benizri E, Ginouves A, Volmat V, Roux D, Pouyssegur J: **HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia.** *Embo J* 2003, **22:**4082-4090.
14. Appelhoff RJ, Tian YM, Raval RR, Turley H, Harris AL, Pugh CW, Ratcliffe PJ, Gleadle JM: **Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor.** *J Biol Chem* 2004, **279:**38458-38465.
15. Koivunen P, Tiainen P, Hyvarinen J, Williams KE, Sormunen R, Klaus SJ, Kivirikko KI, Myllyharju J: **An endoplasmic reticulum transmembrane prolyl 4-hydroxylase is induced by hypoxia and acts on hypoxia-inducible factor alpha.** *J Biol Chem* 2007, **282:**30544-30552.
16. Jeong JW, Bae MK, Ahn MY, Kim SH, Sohn TK, Bae MH, Yoo MA, Song EJ, Lee KJ, Kim KW: **Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation.** *Cell* 2002, **111:**709-720.
17. Tanimoto K, Makino Y, Pereira T, Poellinger L: **Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein.** *Embo J* 2000, **19:**4298-4309.
18. del Peso L, Castellanos MC, Temes E, Martin-Puig S, Cuevas Y, Olmos G, Landazuri MO: **The von Hippel Lindau/hypoxia-inducible factor (HIF) pathway regulates the transcription of the HIF-proline hydroxylase genes in response to low oxygen.** *J Biol Chem* 2003, **278:**48690-48695.
19. Ginouvès A, Ilc K, Macias N, Pouyssegur J, Berra E: **PHDs overactivation during chronic hypoxia 'desensitizes' HIFalpha and protects cells from necrosis.** *Proc Natl Acad Sci USA* 2008, **105:**4745-4750.
20. Kim JW, Tchernyshyov I, Semenza GL, Dang CV: **HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia.** *Cell Metab* 2006, **3:**177-185.
21. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC: **HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption.** *Cell Metab* 2006, **3:**187-197.
22. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ: **The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis.** *Nature* 1999, **399:**271-275.
23. Salceda S, Caro J: **Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes.** *J Biol Chem* 1997, **272:**22642-22647.
24. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr: **HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing.** *Science* 2001, **292:**464-468.
25. Wenger RH: **Mammalian oxygen sensing, signalling and gene regulation.** *J Exp Biol* 2000, **203:**1253-1263.
26. Wenger RH: **Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression.** *FASEB J* 2002, **16:**1151-1162.
27. Camenisch G, Stroka DM, Gassmann M, Wenger RH: **Attenuation of HIF-1 DNA-binding activity limits hypoxia-inducible endothelin-1 expression.** *Pflugers Arch* 2001, **443:**240-249.
28. Ebert BL, Bunn HF: **Regulation of transcription by hypoxia requires a multiprotein complex that includes hypoxia-inducible factor 1, an adjacent transcription factor, and p300/CREB binding protein.** *Mol Cell Biol* 1998, **18:**4089-4096.
29. Damert A, Ikeda E, Risau W: **Activator-protein-1 binding potentiates the hypoxia-induciblefactor-1-mediated hypoxia-induced transcriptional activation of vascular-endothelial growth factor expression in C6 glioma cells.** *Biochem J* 1997, **327:**419-423.
30. Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, Semenza GL: **Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2.** *Cancer Res* 1999, **59:**3915-3918.
31. Kotch LE, Iyer NV, Laughner E, Semenza GL: **Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death.** *Dev Biol* 1999, **209:**254-267.
32. Rolfs A, Kvietikova I, Gassmann M, Wenger RH: **Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1.** *J Biol Chem* 1997, **272:**20055-20062.
33. Wood SM, Wiesener MS, Yeates KM, Okada N, Pugh CW, Maxwell PH, Ratcliffe PJ: **Selection and analysis of a mutant cell line defective in the hypoxia-inducible factor-1 alpha-subunit (HIF-1alpha). Characterization of hif-1alpha-dependent and -independent hypoxia-inducible gene expression.** *J Biol Chem* 1998, **273:**8360-8368.
34. Kallio PJ, Wilson WJ, O'Brien S, Makino Y, Poellinger L: **Regulation of the hypoxia-inducible transcription factor 1alpha by the**

ubiquitin-proteasome pathway. *J Biol Chem* 1999, **274:**6519-6525.
35. Ema M, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, Poellinger L, Fujii-Kuriyama Y: **Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300.** *EMBO J* 1999, **18:**1905-1914.
36. Kung AL, Wang S, Klco JM, Kaelin WG, Livingston DM: **Suppression of tumor growth through disruption of hypoxia-inducible transcription.** *Nat Med* 2000, **6:**1335-1340.
37. Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML: **Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch.** *Science* 2002, **295:**858-861.
38. Bruick RK, McKnight SL: **Transcription. Oxygen sensing gets a second wind.** *Science* 2002, **295:**807-808.
39. Ema M, Taya S, Yokotani N, Sogawa K, Matsuda Y, Fujii-Kuriyama Y: **A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development.** *Proc Natl Acad Sci USA* 1997, **94:**4273-4278.
40. Flamme I, Frohlich T, von Reutern M, Kappel A, Damert A, Risau W: **HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels.** *Mech Dev* 1997, **63:**51-60.
41. Gu YZ, Moran SM, Hogenesch JB, Wartman L, Bradfield CA: **Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha.** *Gene Expr* 1998, **7:**205-213.
42. Hogenesch JB, Chan WK, Jackiw VH, Brown RC, Gu YZ, Pray-Grant M, Perdew GH, Bradfield CA: **Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway.** *J Biol Chem* 1997, **272:**8581-8593.
43. O'Rourke JF, Tian YM, Ratcliffe PJ, Pugh CW: **Oxygen-regulated and transactivating domains in endothelial PAS protein 1: comparison with hypoxia-inducible factor-1alpha.** *J Biol Chem* 1999, **274:**2060-2071.
44. Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ, Maxwell PH: **Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha.** *Blood* 1998, **92:**2260-2268.
45. Kietzmann T, Cornesse Y, Brechtel K, Modaressi S, Jungermann K: **Perivenous expression of the mRNA of the three hypoxia-inducible factor alpha-subunits, HIF1alpha, HIF2alpha and HIF3alpha, in rat liver.** *Biochem J* 2001, **354:**531-537.
46. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL: **The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages.** *Am J Pathol* 2000, **157:**411-421.
47. Wiesener MS, Jürgensen JS, Rosenberger C, Scholze CK, Hörstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU: **Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs.** *FASEB J* 2003, **17:**271-273.
48. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL: **Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha.** *Genes Dev* 1998, **12:**149-162.
49. Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL: **The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development.** *Genes Dev* 1998, **12:**3320-3324.
50. Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG, Semenza GL: **Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1.** *Blood* 2005, **105:**659-669.
51. Distler JH, Gay S, Distler O: **Angiogenesis and vasculogenesis in systemic sclerosis.** *Rheumatology (Oxford)* 2006, **45(suppl 3):**iii26-iii27.
52. Varga J, Bashey RI: **Regulation of connective tissue synthesis in systemic sclerosis.** *Int Rev Immunol* 1995, **12:**187-199.
53. Silverstein JL, Steen VD, Medsger TA, Jr., Falanga V: **Cutaneous hypoxia in patients with systemic sclerosis (scleroderma).** *Arch Dermatol* 1988, **124:**1379-1382.
54. Distler O, Distler JH, Scheid A, Acker T, Hirth A, Rethage J, Michel BA, Gay RE, Müller-Ladner U, Matucci-Cerinic M, Plate KH, Gassmann M, Gay S: **Uncontrolled expression of vascular endothelial growth factor and its receptors leads to insufficient skin angiogenesis in patients with systemic sclerosis.** *Circ Res* 2004, **95:**109-116.
55. Jiang BH, Semenza GL, Bauer C, Marti HH: **Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension.** *Am J Physiol* 1996, **271:**C1172-C1180.
56. Haddad JJ, Land SC: **A non-hypoxic, ROS-sensitive pathway mediates TNF-alpha-dependent regulation of HIF-1alpha.** *FEBS Lett* 2001, **505:**269-274.
57. Hellwig-Burgel T, Rutkowski K, Metzen E, Fandrey J, Jelkmann W: **Interleukin-1beta and tumor necrosis factor-alpha stimulate DNA binding of hypoxia-inducible factor-1.** *Blood* 1999, **94:**1561-1567.
58. Richard DE, Berra E, Pouyssegur J: **Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells.** *J Biol Chem* 2000, **275:**26765-26771.
59. Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M, Candinas D: **HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia.** *Faseb J* 2001, **15:**2445-2453.
60. Kelly BD, Hackett SF, Hirota K, Oshima Y, Cai Z, Berg-Dixon S, Rowan A, Yan Z, Campochiaro PA, Semenza GL: **Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1.** *Circ Res* 2003, **93:**1074-1081.
61. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC: **Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1.** *Nat Med* 2004, **10:**858-864.
62. Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Landsman L, Abramovitch R, Keshet E: **VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells.** *Cell* 2006, **124:**175-189.
63. Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, Avecilla ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii S: **Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of $CXCR4^+$ hemangiocytes.** *Nat Med* 2006, **12:**557-567.
64. Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, Poellinger L: **Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression.** *Nature* 2001, **414:**550-554.
65. Drake CJ, Little CD: **Exogenous vascular endothelial growth factor induces malformed and hyperfused vessels during embryonic neovascularization.** *Proc Natl Acad Sci USA* 1995, **92:**7657-7661.
66. Sundberg C, Nagy JA, Brown LF, Feng D, Eckelhoefer IA, Manseau EJ, Dvorak AM, Dvorak HF: **Glomeruloid microvascular proliferation follows adenoviral vascular permeability factor/vascular endothelial growth factor-164 gene delivery.** *Am J Pathol* 2001, **158:**1145-1160.
67. Dor Y, Djonov V, Abramovitch R, Itin A, Fishman GI, Carmeliet P, Goelman G, Keshet E: **Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy.** *Embo J* 2002, **21:**1939-1947.
68. Kissin EY, Korn JH: **Fibrosis in scleroderma.** *Rheum Dis Clin North Am* 2003, **29:**351-369.
69. Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, Alitalo K: **Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells.** *J Biol Chem* 1994, **269:**6271-6274.
70. Orphanides C, Fine LG, Norman JT: **Hypoxia stimulates proximal tubular cell matrix production via a TGF-beta1-independent mechanism.** *Kidney Int* 1997, **52:**637-647.
71. Nangaku M: **Chronic hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure.** *J Am Soc Nephrol* 2006, **17:**17-25.

72. Corpechot C, Barbu V, Wendum D, Kinnman N, Rey C, Poupon R, Housset C, Rosmorduc O: **Hypoxia-induced VEGF and collagen I expressions are associated with angiogenesis and fibrogenesis in experimental cirrhosis.** *Hepatology* 2002, **35:** 1010-1021.
73. Karakiulakis G, Papakonstantinou E, Aletras AJ, Tamm M, Roth M: **Cell type-specific effect of hypoxia and platelet-derived growth factor-BB on extracellular matrix turnover and its consequences for lung remodeling.** *J Biol Chem* 2007, **282:**908-915.
74. Distler JH, Jüngel A, Pileckyte M, Zwerina J, Michel BA, Gay RE, Kowal-Bielecka O, Matucci-Cerinic M, Schett G, Marti HH, Gay S, Distler O: **Hypoxia-induced increase in the production of extracellular matrix proteins in systemic sclerosis.** *Arthritis Rheum* 2007, **56:**4203-4215.
75. O'Brien ER, Bennett KL, Garvin MR, Zderic TW, Hinohara T, Simpson JB, Kimura T, Nobuyoshi M, Mizgala H, Purchio A, Schwartz SM: **Beta ig-h3, a transforming growth factor-beta-inducible gene, is overexpressed in atherosclerotic and restenotic human vascular lesions.** *Arterioscler Thromb Vasc Biol* 1996, **16:**576-584.
76. Billings PC, Herrick DJ, Howard PS, Kucich U, Engelsberg BN, Rosenbloom J: **Expression of betaig-h3 by human bronchial smooth muscle cells: localization To the extracellular matrix and nucleus.** *Am J Respir Cell Mol Biol* 2000, **22:**352-359.
77. Pilewski JM, Liu L, Henry AC, Knauer AV, Feghali-Bostwick CA: **Insulin-like growth factor binding proteins 3 and 5 are overexpressed in idiopathic pulmonary fibrosis and contribute to extracellular matrix deposition.** *Am J Pathol* 2005, **166:**399-407.
78. Falanga V, Tiegs SL, Alstadt SP, Roberts AB, Sporn MB: **Transforming growth factor-beta: selective increase in glycosaminoglycan synthesis by cultures of fibroblasts from patients with progressive systemic sclerosis.** *J Invest Dermatol* 1987, **89:**100-104.
79. Hong KH, Yoo SA, Kang SS, Choi JJ, Kim WU, Cho CS: **Hypoxia induces expression of connective tissue growth factor in scleroderma skin fibroblasts.** *Clin Exp Immunol* 2006, **146:**362-370.
80. Duncan MR, Frazier KS, Abramson S, Williams S, Klapper H, Huang X, Grotendorst GR: **Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP.** *FASEB J* 1999, **13:**1774-1786.
81. Sato S, Nagaoka T, Hasegawa M, Tamatani T, Nakanishi T, Takigawa M, Takehara K: **Serum levels of connective tissue growth factor are elevated in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis.** *J Rheumatol* 2000, **27:**149-154.