Hypoxia-inducible factors (HIFs) and biological responses in hypoxia, inflammation and embryonic vascular development.

Maria Hägg

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ABSTRACT

Hägg, M. 2008. Hypoxia-inducible factors (HIFs) and biological responses in hypoxia, inflammation and embryonic vascular development. Göteborg. ISBN 978-91-628-7446-9.

Low oxygen tension (hypoxia) is a major inducer of neovascularisation and evidence emerging has indicated that oxygen tension and hypoxia-inducible factors (HIFs), a family of oxygen-regulated heterodimeric transcription factors, may play important roles in vascular and hematopoietic development. This thesis has focused on studying molecular and cellular mechanisms, by which cells and organisms sense changes in oxygen tension, and how this signal is transduced by HIFs into downstream signalling.
Cells, tissues, and organisms experience reduced oxygen (O_2) tension, or hypoxia under both physiologic and pathologic conditions. The total of all signals are integrated in the nucleus of the target cell, activating transcription of genes necessary for modulating cell function. HIF-1 is a key regulator of VEGF mediated angiogenesis, glycolysis and differentiation among a range of cellular responses to hypoxia. HIF-1 is also activated by non-hypoxic stimuli, like growth factors, hormones and cytokines i.e. IL-1 and TNF-α.
To investigate the possibility to induce HIF under non-hypoxic conditions, we made a construct, denoted the saturating domain (SD), based on a domain of HIF-1α. As hoped for, expression of the SD, saturated degradation of endogenous HIF-1α proteins, resulting in activation of HIF-1 mediated transcription in normoxia. Aggregates of differentiating embryonic stem (ES) cells, denoted embryoid bodies form blood vessels in a manner faithfully recapitulating vascular development and angiogenesis *in vivo*. By using ES cells lacking HIF-1α or HIF-2α, we show that HIF-1α is required for vascularisation. The related HIF-2α protein plays a role in regulating the number and activity of early endothelial and hematopoietic cells. Peritoneal dialysis (PD) induces fibrosis and angiogenesis in the peritoneal membrane, leading to ultrafiltration failure. The mesenteric-window angiogenesis model was used to study the role of the HIF-system in experimental PD. We showed that PD fluid exposure lead to hypoxia and activation of HIF-1α in various resident and inflammatory cell types. The increased HIF levels, either induced by hypoxia or inflammation, points out different cellular sources of VEGF.
The HIF-system is an interesting target for pharmacological intervention aiming at inhibition of VEGF-mediated angiogenesis in inflammatory disorders (PD) and at stimulation of blood vessel formation in ischemic diseases.

Keywords: Embryonic stem cell, embryoid body, HIF-1α, hypoxia, VEGF, angiogenesis, vasculogenesis, peritoneal dialysis

Maria Hägg, Department of Medical Biochemistry and Cell Biology, University of Gothenburg, SE-413 90 Göteborg, Sweden

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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. Activation of hypoxia-induced transcription in normoxia. (2005) **Hägg, M.** and Wennström, S. *Exp. Cell Res.* 306, 180-191

II. Hypoxia-inducible factor-2α (HIF-2α) modulates formation of vascular/hematopoietic progenitors in differentiating embryonic stem cells. ***Hägg, M.,** *Nilsson, I., Carmeliet, P., Claesson-Welsh, L. and Wennström, S.Manuscript
*Authors contributed equally to the manuscipt

III. Hypoxia-inducible factor-1α (HIF-1α) activation and hypoxia following catheter implantation and a single peritoneal dialysis dwell. **Hägg, M.,** Cavallini, N. and Braide, M. Manuscript.

CONTENTS

ABBREVIATIONS

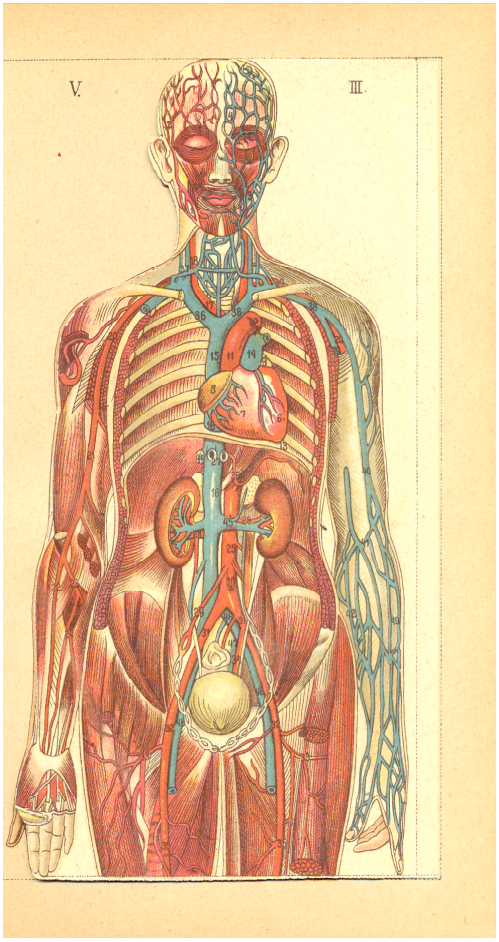
AGM	Aorta gonad mesonephros
bHLH	basic Helix-loop-helix
BL-CFC	Blast colony-forming cell
CAPD	Continuous ambulatory peritoneal dialysis
E	Embryonic day
EB	Embryoid body
EMT	Epithelial-mesenchymal transition
ES cells	Embryonic stem cells
HIF	Hypoxia-inducible factor
HRE	Hypoxia-responsive element
HSC	Hematopoietic stem cell
LIF	Leukemia inhibitory factor
NLS	Nuclear localisation signal
PAS	per-ARNT-Sim
O_2	Di-oxygen
ODD	Oxygen-dependent degradation
PD	Peritoneal dialysis
α-SMA	α-Smooth muscle actin
TAD	Transactivation domain
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	Von Hippel-Lindau

INTRODUCTION

The ability of cells to sense and respond to changes in oxygenation and the existence of a specific "oxygen-sensing" mechanism has long interested biologists, from a wide range of disciplines. Life on earth in multicellular organisms is based on the use of molecular oxygen (O_2) for effective generation of high-energy compounds. Cellular oxygen concentrations are tightly regulated to maintain ATP levels required for metabolic reactions in the human body. Hypoxia-inducible factor-1 (HIF-1) is a master regulator of oxygen homeostasis and its activity is essential for both embryonic development and postnatal physiology. An oxygen-sensitive signal controlling the activity of HIF-1 is provided by enzymatic hydroxylation reactions, which require molecular oxygen and modify specific prolyl and asparaginyl residues in the HIF-α subunit. Hydroxylation of two proline residues, serves as a recognition/binding site for the von Hippel-Lindau (pVHL) complex. Binding of pVHL targets HIF for ubiquitination and degradation by the proteasome [1]. One noteworthy aspect of the O_2-sensing system is its plasticity. Thus, the identification of the molecular components of the O_2-sensing system represents a milestone, rather than a finishing line, on the course to defining the physiology of oxygen homeostasis.

The earliest organ to become functional in mammalian embryogenesis is the vascular system and it is also the first organ system whose proper functioning is critical for the embryo´s homeostasis and continued growth. In the human embryo, the vascular system is formed during the third week of development and the heart starts to beat around embryonic day 23 (E23) [2]. The physiological and medical importance of the cardiovascular system has been apparent since William Harvey first described the circulation of blood in 1628 in *An Anatomical Study of the Motion of the Heart and of the Blood in Animals* [3].

A well functioning, and properly regulated cardiovascular system is essential to meet the metabolic and hemodynamic demands of various physiologic conditions. When dysregulated, the formation of blood vessels contributes to numerous malignancies, ischemic, inflammatory, infectious and immune disorders. The identification and characterisation of the genes that govern vascular functions within various organs may be beneficial for the treatment of these diseases. The vascular biology field seems to have evolved into a distinct discipline still having its own original set of scientific questions waiting for its answers. To increase progress into medical treatment we must start by expanding our knowledge of the basic regulatory mechanisms underlying vascular biology.



The cardiovascular system is composed of a heart and the vessels that carry our blood. Arteries carry oxygen-rich blood away from the heart. Veins carry oxygen-poor blood back to the heart and then further to the lungs. A system of capillaries, transports blood between small arteries and small veins. In the capillary bed O_2 and nutrients pass from the blood into fluids surrounding cells, and at the same time carbon dioxide and waste products are removed. As in the adult, proper growth and development of the embryo depends on the circulation of blood to maintain tissue homeostasis. While fine capillaries contains a single layer of endothelial cells, larger vessels are surrounded/supported by perivascular cells, such as pericytes and smooth muscle cells. The endothelial cells regulate transport processes involving fluid, molecules and cells such as leukocytes and are important for blood vessel formation.

Figure 1. Schematic illustration of the cardiovascular system. Picture adopted from [4].

The vascular system is formed by two distinct processes, vasculogenesis and angiogenesis (Fig.2).

Vasculogenesis

Vasculogenesis is the *de novo* formation of endothelial cells from primitive mesenchymal precursors and their organisation into vascular channels [5, 6]. As soon as the early mesoderm has formed via the process of gastrulation, blood islands form in the mouse yolk sac around E7-7.5 [7]. The internal core of blood islands develop further into hematopoietic precursors while the external ring, which is composed of endothelial precursors, give rise to the yolk sac vasculature, respectively [8].

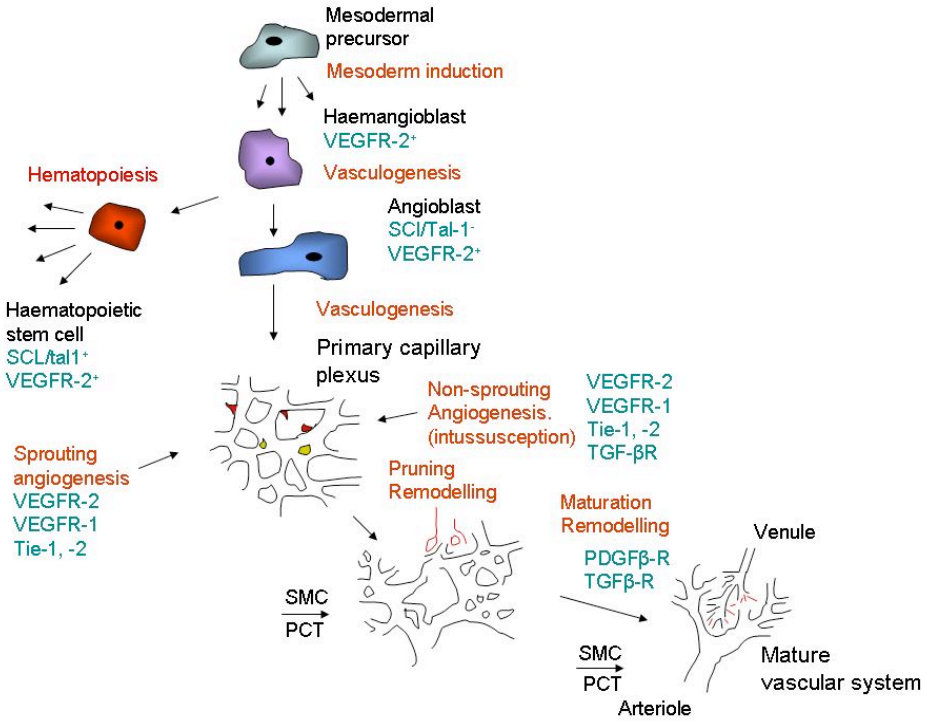


Figure 2.Vascular development. Mesodermal precursor cells, proliferate, sprout, and interconnect to form a loose meshwork forming the primary vascular plexus. The vessel network expands, via angiogenesis, which involves the remodelling of the initial vascular plexus into a more mature appearing vascular network followed by recruitment of supporting cells. Genes of importance for specific processes are shown in green. Red tips in the primary plexus represent sprouts, yellow circles represent splitting tissue pillars. SMC, smooth muscle cells, PCT, pericytes.

Eventual fusion of blood islands results in the formation of a primitive vascular network consisting of immature capillaries. In mice, vasculogenesis is completed by E8.5. In the embryo proper, certain major vessels like the aortic arches, the dorsal aorta and the cardinal veins are formed by direct assembly of endothelial cells without prior formation of primary plexa.

The close developmental association of the hematopoietic and endothelial lineages within the blood islands has led to the hypothesis that they arise from a common precursor, the hemangioblast [8, 9].The early progenitors of these lineages also share expression of a number of genes including vascular endothelial growth factor receptor-2 (VEGFR-2, Flk-1 in mouse and KDR in human [10], VEGFR-1 (Flt1 in mouse) [11], Stem cell leukemia/T-cell acute lymphocytic leukemia protein SCL (TAL-1 or TCL-5) [12], Runx1 (AML1 or Cbfa2)[13] GATA-2 [14], CD34 [15] and CD31/platelet endothelial cell adhesion molecule [16].

Although it has been difficult to isolate hemangioblast from embryos, cells with hemangioblast properties have recently been isolated from mid streak mouse embryos [17]. Furthermore, a morphologically distinct cell, the blast colony-forming cell (BL-CFC), has been described in embryonic stem (ES) cell-derived embryoid bodies (EBs) [8, 18]. BL-CFCs are likely to represent hemangioblasts, as they produce both endothelial and blood cells *in vitro.* This capacity, combined with the fact that BL-CFCs express genes common to both endothelial and hematopoietic lineages, suggests that these cells represent the *in vitro* equivalent of the hemangioblast [19]

Angiogenesis

Angiogenesis refers to the formation of new blood vessel from pre-existing vessels [9] [20]. Angiogenesis is a fundamental process in embryonic development and in certain physiological conditions, such as in wound healing and in the female menstruation cycle [21]. Angiogenesis also plays an important role in several pathological conditions, like in solid tumour growth, diabetic retinopathy, and rheumatoid arthritis [22]. There are at least two different types of angiogenesis; true sprouting of capillaries form pre-existing vessels, and non-sprouting angiogenesis or intussusception (Fig. 2). The first step in sprouting angiogenesis is proteolytic degradation of the extracellular matrix, which is followed by chemotactic migration and proliferation of endothelial cells, formation of a vessel lumen and functional maturation of the endothelium. New vessels also form by intussusception, a process characterised by splitting of existing vessels by insertion of tissue pillars [23]. Stabilisation of the vascular network involves the reversal of these processes, with re-establishment of the basement membrane, cessation of cell proliferation, functional complex formation, and recruitment of pericytes to support the newly established vessel wall. The emerging vascular plexus is rapidly remodelled to resemble a mature system with larger and smaller vessels.
The process responsible is termed pruning, as the resulting pattern resembles a tree. Angiogenesis is induced and modulated by various environmental cues, such as low oxygen tension (hypoxia), hypoglycaemia or pressure generated by proliferating cells.

Hematopoiesis

The hematopoietic system is composed of a vast variety of blood cells with different morphology and functions (Fig. 3). These functions include oxygenating the body, blood clotting in response to injury and immune defence. Mature blood cells have a relatively short life span, and consequently the progeny of stem cells must continuously replace the population. This process depends upon the presence of suitable microenvironmental conditions and growth factors [24]. Formation of blood cells occurs in successive waves during embryonic development. In mice, hematopoiesis is first detected in the blood islands of the yolk sac around E7.5. Primitive erythrocytes are the main precursors produced at that stage, but precursors of the definitive lineages can also be detected in the yolk sac, although they do not mature there and are most likely exported [25]. Around day 10 of gestation, hematopoiesis shifts to an embryonic site, the aorta gonad mesonephros (AGM) region, where definitive hematopoietic precursors are generated [26]. The first long-term hematopoietic stem cells (HSC) are also generated in the AGM region.

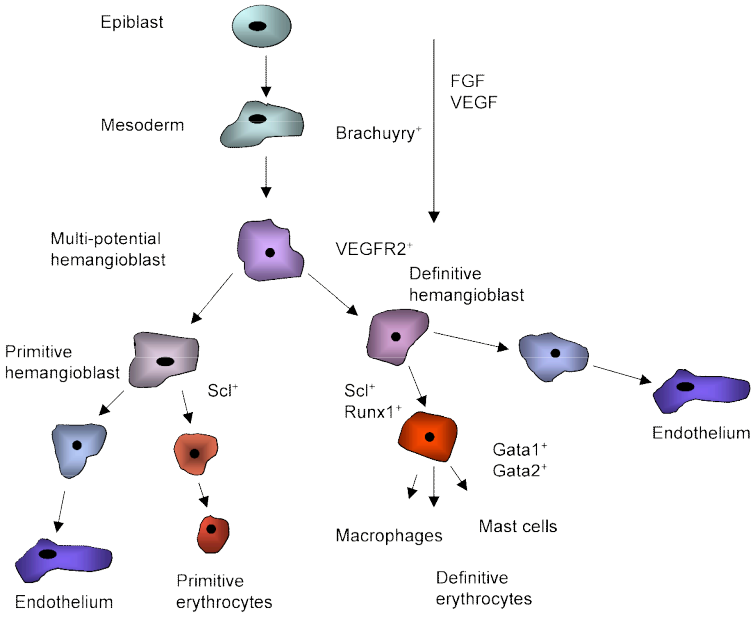


Figure 3. Schematic picture of blood lineage formation and some of the critical genes involved in the process.

Definitive hematopoietic precursors generated both in the yolk sac and in the AGM region are thought to migrate to the liver where they develop further and mature into all blood lineages. From day 12 until birth, the liver becomes the main site of hematopoietic development. After birth and during adult life hematopoiesis occurs in the bone marrow.
The HSC can give rise to all blood cell types, and is called pluripotential stem cell. These cells proliferate and form one cell lineage that will become lymphocytes and another lineage that will form myeloid cells. Myeloid progenitors are committed to differentiate into cells of the macrophage, granulocyte, eosinophil, basophil, mast cell, erythrocyte and megakaryocyte lineages, while the lymphoid progenitors give rise to T- and B-cells. The decision of stem cells to self-renew or differentiate, are intrinsic properties of the progenitors and are stochastic in nature. In contrast, subsequent clonal expansion, maturation and survival can be greatly influenced by signals generated by e.g. infections or injury [24].

Vascular endothelial growth factor (VEGF): a key regulator of vasculogenesis and angiogenesis

Numerous molecules are implicated in induction or inhibition of blood vessel formation, and a fine balance of positive and negative signals is required to ensure proper regulation of this process. Members of the vascular endothelial growth factor (VEGF) and angiopoietin of growth factors are relatively specific for endothelial cells. In contrast, mitogens such as fibroblast growth factors (FGF)-1 and -2 have a broader spectrum of target cells. Finally, other factors, such as transforming growth factor β (TGF-β), platelet-derived growth factor-B (PDGF-B) and tumour necrosis factor α (TNF-α), indirectly affect blood vessel formation via regulation of extracellular matrix production, recruitment of perivascular cells and induction of VEGF.

VEGF-A, originally described as a vascular permeability factor (VPF) [27], belongs to a gene family that includes placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and the parapoxyvirus VEGF-E [28] (Fig. 4).

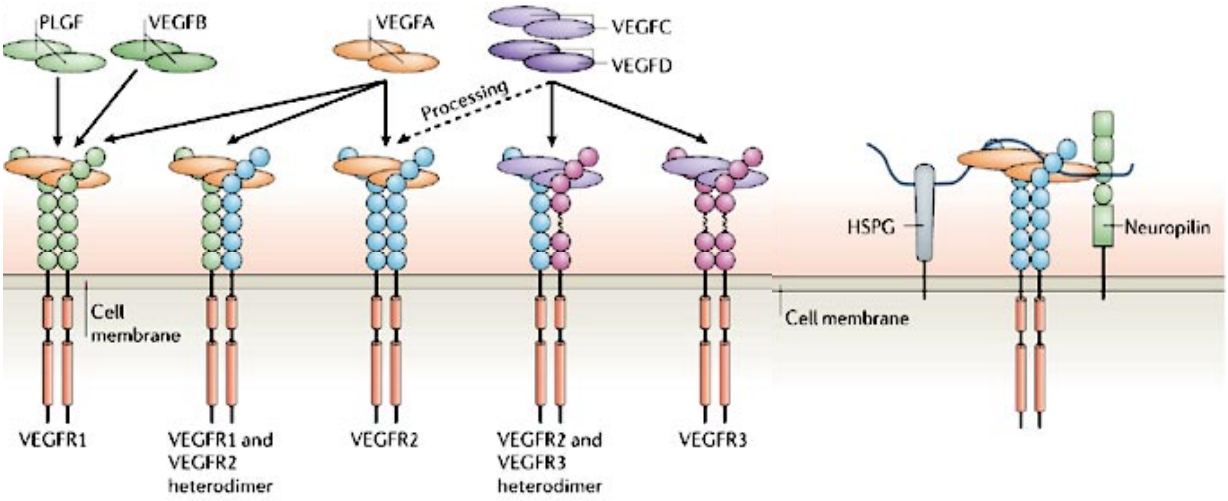


Figure 4. The VEGF family: ligands, and receptors. The VEGFs bind the VEGFRs leading to receptor dimerisation and signalling events resulting in cellular responses such as vasculogenesis, angiogenesis, cell survival and permeability. Arrows point at binding partners. Figure adopted from [29].

The VEGF ligands are glycoproteins usually secreted as homodimers. The gene for VEGF-A is organized into eight exons, which undergo alternative splicing events, giving rise to five different protein isoforms containing 121, 145, 165, 189 and 206 amino acids (aa) (murine forms are one amino acid shorter). The most prevalent form $VEGF_{165}$, lacks the residues encoded by exon 6, whereas $VEGF_{121}$ lacks the residues encoded by exons 6 and 7, does not bind to heparan sulfate and is freely diffusible [30]. VEGF-A is strongly induced by hypoxia and most cell types produce this factor under conditions of low oxygen tension [31].

VEGF receptors

The biological effects of VEGF are mediated by three plasma membrane bound receptor tyrosine kinases, VEGFR-1 (also denoted Flt-1), VEGFR-2/KDR/Flk-1 and VEGFR-3/Flt-4. There is also a soluble form of VEGFR-1 (sVEGFR-1), which lacks the transmembrane and intracellular part of the receptor.
VEGFR-1 and VEGFR-2 are characterised by the presence of seven Ig-like domains in the extracellular part of the receptor, while VEGFR-3 has one loop replaced by two peptides bound by a disulfide bridge [32]. All the receptors have a single transmembrane domain and an intracellular tyrosine kinase domain, the activity of which is critical for transmission of the signal produced by VEGF binding [33]. In addition to VEGFRs other molecules such as heparan sulphated proteoglycans, neuropilins, cadherins and integrin $\alpha v\beta 3$-, may also serve as co-receptors for certain, but not all VEGF proteins [34]. All VEGF-A isoforms bind to both VEGFR-1 and VEGFR-2 (although with 10-fold higher affinity to VEGFR-1). VEGF-B and PlGF bind only to VEGFR-1. VEGF-C and VEGF-D are expressed as propeptides and are selective ligands to VEGFR-3; processed mature ligands bind to VEGFR-3 with higher affinity and can in humans also bind to VEGFR-2 [30]. VEGF-E is a selective ligand for VEGFR-2.

VEGFR-1 is expressed in vascular endothelial cells, haematopoietic stem cells, monocytes/macrophages and smooth muscle cells; SMCs. VEGFR-2 is expressed in both vascular endothelial cells and lymphatic endhothelial cells. Its expression has also been demonstrated in several other cell types such as megakaryocytes, hematopoietic stem cells and neuronal cells [35].The expression of VEGFR-2 is eventually down-regulated in mature hematopoietic cells, but it resides in the endothelial lineage. VEGFR-3 is expressed at high levels in lymphatic endothelial cells, but also in certain normal and tumour vasculature [32].

Biology of VEGF

Mice heterozygous for inactivated VEGF-A alleles die at E8-E9 from defects in blood island formation, endothelial cell development and vascular formation [28]. Even inactivation of one of the VEGF-A alleles leads to embryonal death around E11-12 [36]. Based on these findings, VEGF-A is now accepted as a key regulator of vasculogenesis and angiogenesis, particularly during embryogenesis [37]. VEGFR-1-/- mouse embryos die at day 8.6-9 due to obstruction of vessels by an overgrowth of endothelial cells, caused by an increased proliferation of endothelial precursors [11]. The key role of VEGFR-2 in developmental angiogenesis and hematopoiesis is evidenced by a lack of both endothelial and hematopoietic cells, and failure to form blood islands and organized blood vessels in VEGF-2-null mice, resulting in death between days E8.5-9.5 [38]. No blood islands or vessels can be detected in the yolk sac of these animals, potentially due to an inability of precursor cells to properly migrate and populate the yolk sac. The process of vasculogenesis therefore requires the activity of VEGFR-2. Inactivation of *Vegfr-3* gene leads to a defective blood vessel development and is embryonic lethal at E9.5. Larger vessels are disorganized, leading to fluid accumulation and cardiovascular failure [39].
Of the three VEGF receptors, VEGFR-2 appears to be the major signalling receptor regulating many of the most important endothelial cell functions/responses including proliferation, differentiation, migration, survival and vascular permeability. These effects are likely to be mediated by activation of classical signalling pathways including the phospholipase C-γ, the Raf-MEK-Erk and the PI3-kinase pathway [30].

Embryonal stem cells and embryoid bodies

Embryonal stem (ES) cells are pluripotent cells derived from the inner cell mass of the early blastocyst [40] (Fig. 5). The inner cell mass is surrounded by a monolayer of epithelial cells, the trophoectoderm. The trophoectoderm produces leukaemia inhibitory factor (LIF) and inner cell mass cells have LIF receptors [41]. In the laboratory ES cells are maintained in a proliferating undifferentiated state, by co-culture with feeder cells or in the presence of LIF. The rationale, *in vivo* may be to inhibit differentiation in case of delayed implantation of the embryo [42]. LIF is also essential for implantation of the embryo into the uterus [43] . Upon withdrawal of LIF, ES cells have the capacity to differentiate into a number of cell types including endothelial and hematopoietic cells as well as neuronal cells, cardiomyocytes and adipocytes [44] [45]. Differentiation of ES cells into three-dimensional structures, called embryoid bodies (EBs), recapitulates development of post implantation embryonic tissues. For instance, early stages of vasculogenesis and initial vascular maturation can be studied using EBs [46], making it useful model system for examination of molecular and cellular processes involved in the formation of the embryonic circulatory system.

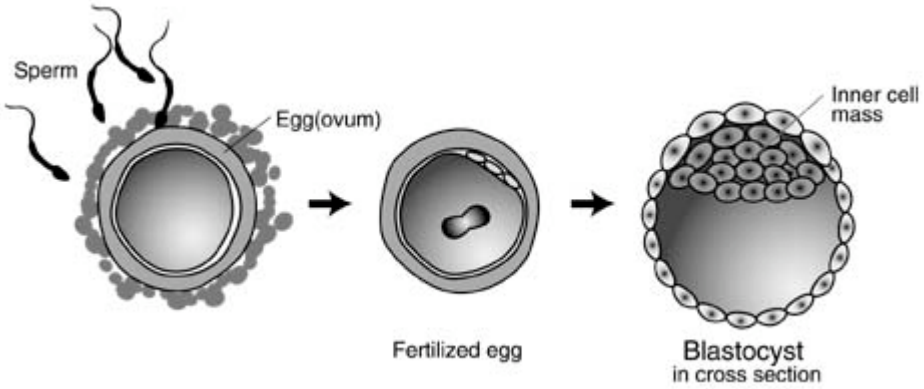


Figure 5. The blastocyst and the inner cell mass. Picture adopted from the National Health museum.

Hypoxia and Hypoxia-inducible factors (HIFs)

Hypoxia is defined as the subphysiological oxygen level at which biological (cellular) responses are elicited. During early embryogenesis, before E9.5 the embryo develops in a low O_2 environment and relies primarily on diffusion of oxygen and on glycolytic pathways to satisfy its metabolic demands. Around that time, the requirement for O_2 increases dramatically so that a functioning circulatory system, which permits efficient O_2, nutrient and waste exchange between mother and fetus, has to be established for further development. Most cells undergo growth arrest or apoptosis in response to severe hypoxia. However, certain cells, such as vascular cells and hematopoietic precursors, proliferate in response to low oxygen levels [47]. The observation that hypoxia can inhibit differentiation but instead keep various stem and precursor cells proliferating has been described for e.g. neuronal cells [48]. Therefore, physiological hypoxia, as encountered by the mammalian embryo, may be an essential signal for the proliferation and/or survival of blood cells and vascular precursors during development.

Cells and tissues, faced with a hypoxic challenge under which oxidative phosphorylation by the TCA cycle cannot proceed, must respond by switching to anaerobic metabolism. Increased glucose metabolism in the cell will here compensate for the reduced ATP, generating- capacity of glycolysis.

Cells in affected tissues will also promote neovascularisation, via the liberation of pro-angiogenic factors, and an increased number of erythrocytes will enhance the O_2 carrying capacity of the blood that supplies them.

The transcription factor hypoxia-inducible factor-1 (HIF-1) is the master regulator of responses to reduced oxygen tension, and several dozen HIF-1-regulated target genes have been identified that play essential roles in these processes [49](Fig. 6).

HIF-1 induces various glucose transporters and enzymes responsible for glycolysis under hypoxic conditions. It also actively represses mitochondrial function and oxygen consumption directly by inducing pyruvate dehydrogenase kinase (PDK). PDK phosphorylates and inactivates the pyruvate dehydrogenase enzyme complex that converts pyruvate to acetyl coenzyme A, thereby inhibiting pyruvate metabolism by the TCA cycle [50, 51]. Induction of LDH (lactate dehydrogenase) is also increased in hypoxia by HIF leading to the removal of excess pyruvate as lactate and also regenerates NAD+ for increased glycolysis. In combination, the HIF-dependent expression of PDK and LDH in hypoxia leads to inhibition of the TCA cycle.

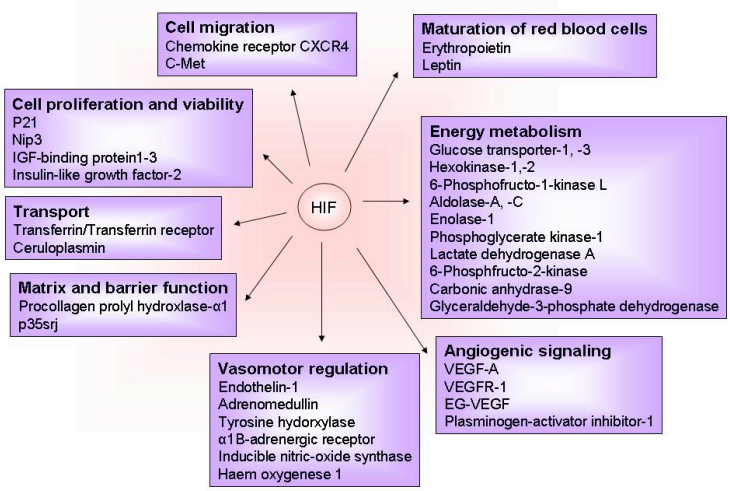


Figure 6. HIF-1-induced cellular responses and target genes.

Hypoxia-inducible factors structure and function

Hypoxia-inducible factors (HIFs) are heterodimeric transcription factors composed of one α and one β subunit [52]. Both subunits are members of the basic helix-loop-helix (bHLH)-PAS family of transcription factors [53], and HIFs induce gene expression by binding to a 50 base pair hypoxia response element (HRE), containing a core 5´ACGTG-3´sequence [52]. HIF-1α was first purified and characterised during investigation of the erythropoietin gene [53] Whereas HIF-1β (also known as the aryl hydrocarbon nuclear translocator, ARNT) is constitutively expressed, HIF-1α is induced in hypoxic cells with an exponential increase in expression, as cells are exposed to O_2 concentrations of less than 6 % [54]. By this virtue, HIF-1α functions as the critical regulator of HIF-1-induced gene expression under hypoxic conditions.

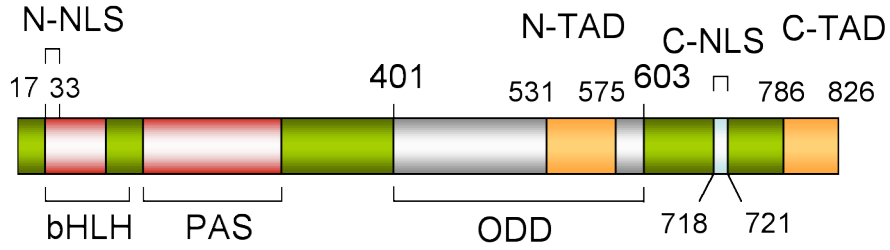


Figure 7. The structure of HIF-1α. The domains of HIF-1α are: basic helix-loop-helix (bHLH), Per-ARNT-Sim (PAS), oxygen-dependent degradation (ODD), and transactivation domains (N-TAD and C-TAD). Also, the position of nuclear localisation signals (NLS) is shown.

Human HIF-1α is an 826-amino acid (120-kDa) protein (Fig. 7). The N-terminal part contains the bHLH and the PAS domains, which are required for dimerisation with HIF-1β and for DNA binding [53]. Two transactivation domains (TAD), N-terminal and C-terminal TADs (also termed NAD and CAD), are localised in the C-terminal half of HIF-α (aa 531-575 and 786-826, respectively). Moreover, the N-TAD overlaps with a larger domain, denoted the O_2-

dependent degradation (ODD) domain (aa 401-603), which is responsible for degradation of HIF-1α under normoxic conditions [55]. The main function of C-TAD is to recruit and bind co-activators including CBP, p300, SRC1, and TIF-2. Additionally, HIF-1α contains two nuclear localisation signals, N-NLS and C-NLS.
Three HIF-α isoforms have been identified, whereas HIF-1α is expressed ubiquitously, the other two isoforms HIF-2α (also known as EPAS1, HLF, HRF or MOP2) and HIF-3α appear to have tissue/cell specific expression pattern [56] [57] [53]. For instance HIF-2α is predominantly expressed in endothelial cells and in catecholamine producing cells in the organs of Zuckerkandl [57]. The expression of both HIF-1α and HIF-2α in endothelial cells suggests an important role for the two HIF-α subunits in endothelial cell biology and vascularisation.

The O_2-sensing pathway-and regulation of HIF activity

In normoxia, the HIF-1α subunit undergoes proteolytic degradation that is dependent on the central ODD domain, which, in turn, is composed of two specific prolyl residues (Pro402 and Pro564 in human HIF-1α) in NODD and CODD mediates interaction of HIF-1α with the von Hippel-Lindau tumour suppressor protein (pVHL) [58]. pVHL, is the recognition component of an E3 ubiquitin ligase complex, composed of pVHL, elongin B and C, Cul2 and Rbx, that targets HIF-1α for ubiquitination and subsequent degradation by the proteasome [1].
Four human enzymes, prolyl hydroxylase (PHD) 1-4 have been identified as being responsible for prolyl hydroxylation of HIF-1α [59]. These enzymes are members of the 2-oxoglutarate-dependent hydroxylase superfamily, whose activity requires O_2, iron, 2-oxoglutarate and ascorbate as cofactors, implying that these enzymes directly function as O_2 sensors [59]. In addition to sensing oxygen, these enzymes are also in some way acting as metabolic sensors, able to sense the accumulation of bioenergetic intermediates and reactive oxygen species. Indeed, 2-oxoglutarate, a co-substrate of the reaction, is an intermediate metabolite of the TCA cycle. Furthermore, both succinate (a product of the hydroxylase reaction) and fumarate are products of the TCA cycle enzymes, and both can compete with 2-oxoglutarate to inhibit PHD enzyme activity [60, 61]. Lactate stabilised HIF-1α, and increased VEGF levels in aerobically cultured human endothelial cells [62]. Lactate and pyruvate bind to and inhibit the HIF prolyl hydroxylases that would otherwise hydroxylate HIF-1α for degradation [63] [64]. Regarding the effects of high glucose levels on HIF-1 activation, available data are inconsistent [65] [66].
To obtain full transcriptional activity, HIF-1 must bind to the DNA target sequence and recruit transcriptional co-factors, a phase which also is subjected to hypoxia-dependent regulation. HIF-1α and HIF-2α contain a specific asparaginyl residue in their C-TAD (Asn803 in HIF-1α) [67] that under normoxic conditions is hydroxylated by factor inhibiting HIF (FIH) [68]. This modification silences the transactivation domain by blocking the interaction between HIF-α and transcriptional co-activators CBP/p300.

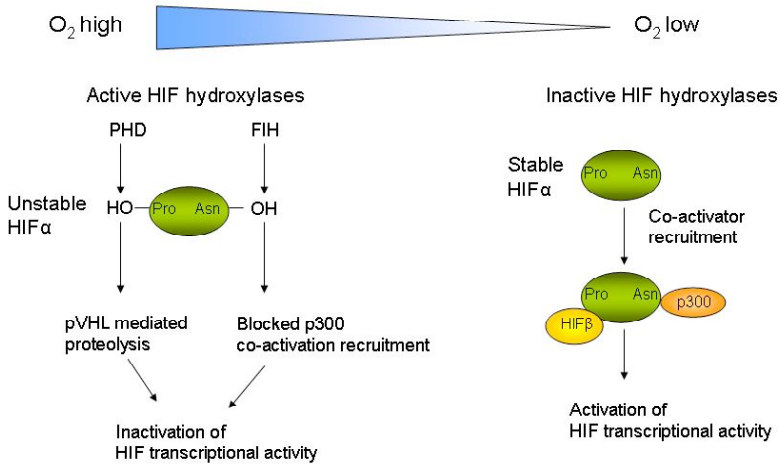


Figure 8. Dual regulation of HIF-α subunit stability and activity by prolyl and asparaginyl hydroxylation.

Other levels of HIF-1 regulation

Besides prolyl and asparaginyl hydroxylation of HIF-1α, the activity of HIF-1 may also be regulated by additional mechanisms including other types of posttranslational modifications, nuclear translocation, dimerisation and interaction with other protein. For instance the ARD1 acetylase has been shown to acetylate lysine 532 in the ODD domain of HIF-1α, thereby promoting interaction with pVHL, resulting in enhanced degradation of HIF-1α [69].

Nuclear localisation of HIF-1α

Despite the term ”nuclear translocator”, HIF-1β is not required for import of HIF-1α into the nucleus as HIF-1 autonomously translocates due to the presence of nuclear localisation signals [70]. It has been suggested that translocation is induced by hypoxia [70]. However, when HIF-1α is overexpressed the translocation occurs even under normoxic conditions [71]. Therefore, this process seems to be hypoxia-independent and the nuclear fraction of HIF-1α may simply reflect the overall level of this protein in the cell [71].

Non-hypoxic activation of HIF-1

Many growth factors, hormones and cytokines i.e. IL-1 and TNF-α are known to stabilise HIF-1α under normoxic conditions, and phosphorylation mediated by certain signalling pathways has been shown to trigger activation of HIF-1 [72]. In contrast to the hypoxic activation by reduced posttranslational hydroxylation, the induction of HIF-1α by non-hypoxic, proinflammatory stimuli seems to be much more diverse. It includes modification at the transcriptional, the translational, and the posttranslational levels.

Interplay between the oxygen-sensing and inflammatory pathways

Blood vessels play a central role in the inflammatory response, and physiological alterations of vascular function are a major characteristic of acute inflammation. Inflammation is a localised response to a perceived injury with the purpose of clearing the body of pathogens

and heals damaged tissue. The process of inflammation involves a vast amount of molecular mediators and many cellular mediators. In higher organisms the process makes use of the vascular system to recruit and deliver leukocytes and fluid to affected extravascular tissues (Fig. 9). Inflammation is hard to separate from tissue repair, beginning during the early phases of inflammation.
Acute inflammation has a relatively short duration including oedema and leukocyte infiltration. Chronic inflammation is associated with many inflammatory diseases and has a longer duration. Characteristics are the presence of active macrophages and lymphocytes, proliferation of blood vessels, and fibrosis.
The onset of inflammation is due to a release of inflammatory mediators that activate the response. The mediators can be released from granules (e.g. histamine from mast cells), synthesised *de novo* (e.g. IL-1 from macrophages) or activated through proteolytic cleavage (e.g. C3a from the complement system).
These mediators diffuse and are involved in the following cascade, directly or indirectly: Arterioles are dilated which leads to an increased blood flow into the vascular bed of the inflamed tissue. The microvasculature becomes more permeable which increases the deposition of serum proteins in the surrounding tissue. The mediators also activate nearby endothelium. P-selectin is exported from Weibel-Palade bodies to the plasma membrane on the lumenal-side. Selectins bind certain carbohydrates that are expressed on leukocytes. This process allows rolling of leukocytes on endothelium.

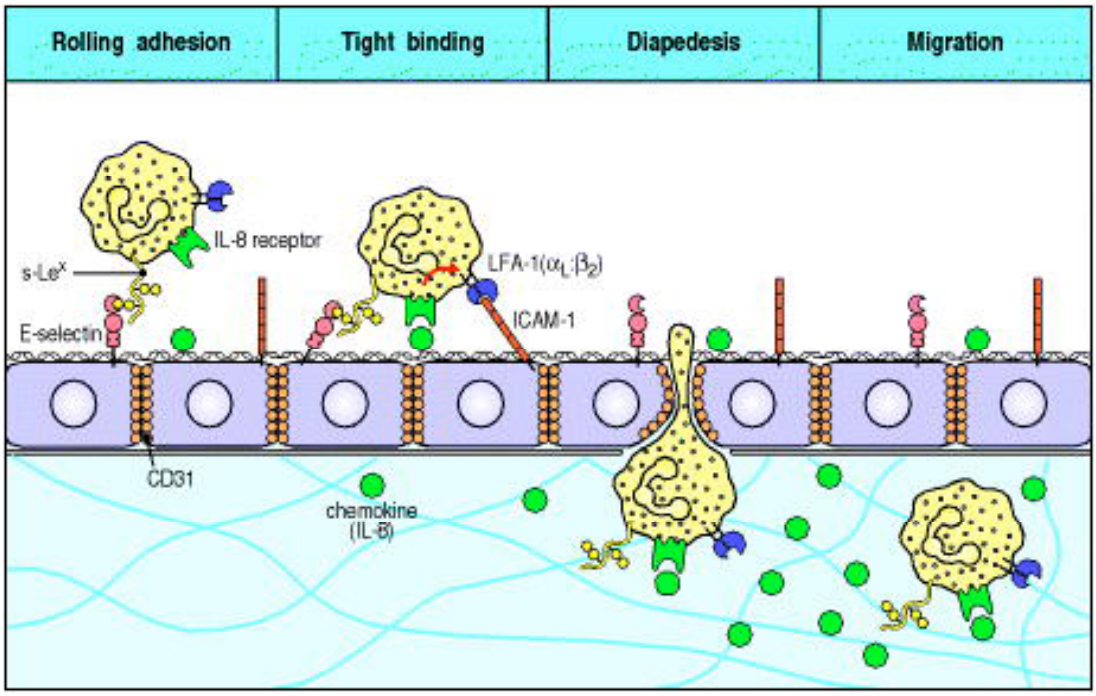


Figure 9. Order of events in inflammation. Picture from Immunology, 1999 [73].

Activated endothelium expresses other adhesion molecules which further anchors the leukocytes to the endothelium. Signals from the tissue activate the leukocyte, which also increases its adhesion molecules leading to a tight binding to the active endothelium. Activated leukocytes express CD31, which is the principal mediator of the transmigration through the endothelium-diapedesis. The leukocyte then migrates through a chemotactic gradient towards sites of action.
Monocytes differentiate to macrophages when they have reached their theatre of action in the extravascular tissue. Upon further activation they secrete a wide variety of biologically active

products. Normally, after the irritant is eliminated the macrophage leaves via the lymphatics or goes through apoptosis. In chronic inflammation this is not the case.
Low O_2 and glucose levels and high concentrations of inflammatory mediators, reactive oxygen species, (ROS) and nitrogen species often characterise the microenvironment of inflammatory sites [74]. This extreme environment arises from a combination of significant changes in metabolic activity, and increased diffusion distance, and disruption of blood flow through phagocytic plugging and damage of capillaries.
Neutrophils and mononuclear cells produce most of their ATP through glycolysis, rather than oxidative metabolism [75]. For that reason, from an energy supply viewpoint, these cells are well placed for function at sites of tissue injury and low oxygen availability. What drives glycolytic metabolism in these cells? Cramer *et al*, provide evidence that the transcription factor HIF-1 is the master switch for induction of genes relevant to myeloid cell glycolysis and generation of ATP for energetic metabolism. Deletions of HIF-1α selectively in myeloid cells does not affect total white cell and neutrophil counts, and the mice have a normal phenotype and viability. However, in the absence of HIF-1α, there is a dramatic reduction in cellular ATP pools, also seen under normoxic conditions. The dramatic reduction in ATP pools in the HIF-1α deficient myeloid cells is accompanied by an impairment in myeloid cell aggregation, motility, invasiveness, and bacterial killing examined in response to inflammation, both *in vitro* and *in vivo*, in functional assays of acute and chronic inflammation [76]. Inflammation symptoms and joint swelling could be avoided in these mice when subjected to an arthritis-induced treatment. The same study showed that inactivation of the negative regulator protein pVHL in the myeloid lineage caused a hyperinflammatory response, in the same model. Loss of the down-stream target gene VEGF eliminated tissue oedema, during inflammation but not the other measures of myeloid cell inflammation. This study thus illustrates the importance of the HIF-1α pathway for inflammatory cell recruitment.

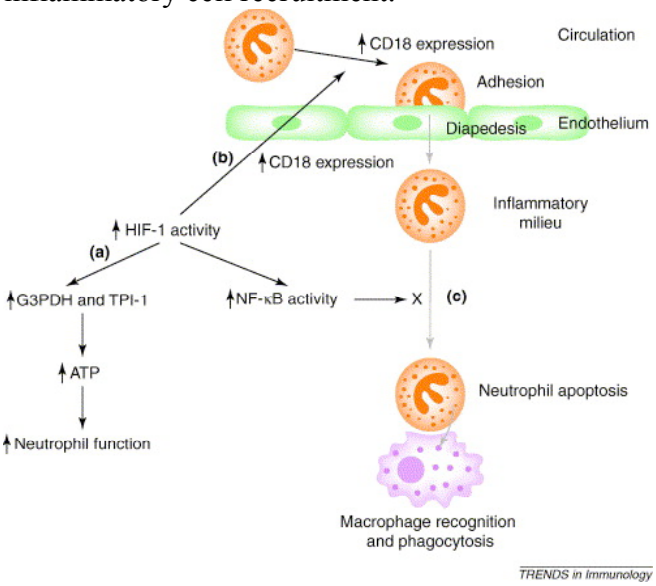


Figure 10. HIF-1-dependent mechanisms involved in myeloid cell-mediated inflammation. Three discrete mechanisms have been identified a) HIF-dependent regulation of key glycolytic enzymes, intracellular energy homeostasis and enhanced neutrophil function b) HIF-mediated upregulation of CD18 expression, neutrophil recruitment at the very earliest stages of inflammation c) HIF-dependent inhibition of constitutive neutrophil apoptosis. Abbreviations G3PDH, Glyceraldehyde-3-phosphate dehydrogenase, TPI, triosephosphate isomerase-1. Picture adopted from Walmsley, 2005 [74].

HIF-1 promotes neutrophil recruitment to hypoxic areas of inflammation by increasing diapedesis of leukocytes through the endothelial barrier. This process is dependent on rolling and adhesion of neutrophils to β_2-integrins expressed on the endothelial cell, and which contain a common subunit, encoded for by the CD18 (leukocyte-adhesion deficiency) gene that contains a HRE within its promoter [77]. HIF-1 also mediates the recruitment of monocytes and lymphocytes. The chemoattractant molecule (SDF-1), and its receptor, chemokine receptor CXCR-4 has been reported to be HIF-1 inducible.
A further regulator of neutrophil function is the ability of these cells to undergo constitutive apoptosis [78]. This event triggers the phagocytosis and clearance of apoptotic neutrophils by local macrophages and is vital for the limitation of tissue damage *in vivo* [78]. The mechanism(s) by which HIF-1α regulates neutrophil survival remains to be fully elucidated though work has pointed to a potential role of HIF-1α dependent regulation of IKKα and NF-κB, known regulator of apoptotic thresholds in neutrophils, and also PGK, a key enzyme involved in glyscolysis [74]. Thus HIF-1α is required for the maintenance of intracellular energy homeostasis in myeloid cells. It is also critical for infiltration at the very earliest stages of inflammatory cell recruitment, making it to a potential target for modulation of inflammation.

Cytokines-the" biologic response modifiers".

HIF is a redox-sensitive transcription factor, and so are cytokines. Cytokines are a group of small proteins secreted by white blood cells and a variety of other cells in the response to a number of inducing stimuli. The cytokines bind to specific receptors leading to a variety of signalling events such as development of cellular and humoral immune response, regulation of hematopoiesis, control of cellular proliferation and differentiation, and induction of wound healing. Mainly lymphocytes and macrophages, but also other granulocytes, endothelial cells and fibroblasts secrete the cytokines. Cytokines released from these cells activate an entire network of interacting cells. Human endothelial cells are capable of expressing a broad spectrum of pro- and anti-inflammatory cytokines including IL-1, (interleukin-1), IL-5, IL-6, IL-8, MCP-1 (monocyte chemotactic protein-1) and CSFs (colony-stimulating factors). Inflammation directly and hypoxia via HIF-1α promote the secretion of chemokines that attract neutrophils and monocytes [50].
It is clear that reactive oxygen species (ROS) mediates inflammative damage and controls apoptosis and cell proliferation. ROS including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH·), etc., is generated mainly by NADH oxidase and mitochondrial systems. They have a close relation with oxygen content in cells and extensive biologic activities. It is uncertain whether the level of ROS changes and participates in hypoxia as a part of signal transduction under hypoxia. Results from investigations in this field were different, even completely opposite [79, 80].
Recently, cytokines emerged as major regulators of HIF, under normoxic conditions. This evolving association between HIF and immunity is based on the established linkage that bonds oxidative stress and inflammation, the cytokines. The mechanism of cytokine-dependent regulation of HIF-1α is currently unknown. Accumulating evidence indicate that cytokines can increase ROS generation followed by HIF stabilisation. Data from multiple groups have suggested a role for ROS generated at the mitochondrial respiratory chain in regulating HIF-1α stabilisation. [81, 82]. The cytokines mediate their ROS-sensitive mechanisms through participating in some signal transduction ways (kinases and/or phosphates activities) governing HIF-1α translocation/activation. ROS may also interfere with hydroxylase activity. Furthermore, the macrophage-secreted cytokines interleukin 1 (IL-

1) or tumour necrosis factor-α (TNF-α) favour the expression and DNA binding of HIF-1α via the Mitogen-activated protein kinase (MAPK) and PI3 pathways [83].

Peritoneal dialysis

In medicine, peritoneal dialysis (PD) has been an established form of renal replacement therapy since 30 years. It is a method for removing waste such as urea and potassium from the blood, as well as excess fluid, when the kidneys are incapable of this. There are more than 130 000 patients on PD worldwide, representing approximately 15 % of the global population with end-stage renal failure.
PD is based of the principle that the peritoneal membrane that surrounds the intestine, can act as a dialysing membrane and if a dialysis fluid is instilled via a catheter then dialysis between the circulation and the PD fluid can occur by diffusion, and ultrafiltration (osmosis) (Fig.11). In conventional PD fluids, glucose is employed as an osmotic agent to generate a concentration gradient that moves excess fluid from the circulation into the peritoneal cavity. During continuous ambulatory peritoneal dialysis (CAPD), peritoneal dialysis fluid is instilled into the peritoneal cavity via a permanent catheter and exchanged regularly.

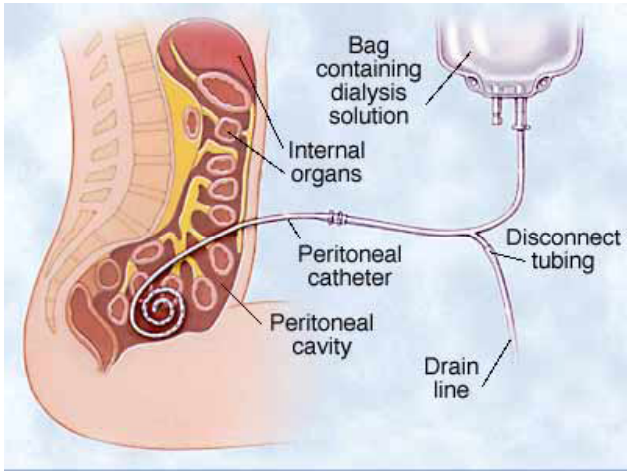


Figure 11. Peritoneal dialysis. Picture adopted from: MayoFoundation for Medical Education and Research.

One of the advantages with PD is that the patient can largely maintain the treatment. Although peritoneal dialysis (PD) has a number of advantages, the development of peritoneal dysfunction and peritoneal sclerosis limits its usefulness [84]. PD induces a mild inflammatory reaction characterised by fibrosis, neoangiogenesis, and alterations of microvascular ultrastructure.

Peritoneal cavity and structure of the peritoneal membrane

The natural functions of the peritoneum are to maintain homeostasis by allowing exchange of molecules and production of peritoneal fluid, to facilitate motion, to minimise friction and to conduct vessels and nerves to the viscera. The peritoneum is a serous membrane lining the abdominopelvic walls and the viscera. It is a strong colourless membrane with a smooth surface, and forms a double-layered sac that is closed in the male and is continuous with the mucous membrane of the uterine tubes in the female. Under physiological conditions, the predominant cell type (80 %) in the abdominal fluid is the macrophage. The remaining cell population consists of peritoneal lymphocytes, eosinophils, dendritic cells, mast cells and mesothelial cells.

Functionally, the peritoneal membrane consists of three main anatomical layers, that represent three sequential barriers to transport; the peritoneal and mesenteric capillary walls, the interstitial tissue and the highly permeable mesothelium [85].
The abdominal cavity is lined by the peritoneal membrane and in continuity, via the lymphatic system, with the pleural fluid in the thoracic cavity and the vascular system.
The mesothelium is a monolayer of specialised pavement-like mesothelial cells that line the body's serous cavities and internal organs, resting on a basement membrane [86]. The mesentery also contains supporting connective tissue elements such as collagen, elastin, and elastic fibers of varying caliber. Under normal conditions, mesothelial cells play a crucial role in peritoneal homeostasis, including anti-adhesive and fibrinolytic mechanisms, regulation of local blood circulation and vessel permeability, regulation of matrix formation and breakdown, and local defence mechanisms [87] [88]. The luminal surface of the mesothelial cell has a well-developed microvillous border with occasional cilia. Mesothelial cells are loosely attached to the basement membrane and can be readily detached by the slightest trauma [89]. There is a line of evidence, suggesting a role for mesothelial cells in the development and progression of peritoneal angiogenesis and fibrosis during PD. The interstitial, or submesothelial, space is occupied by connective tissue, the most common cells being fibroblasts, macrophages, mast cells, macrophages and occasional eosinophils and lymphocytes.

Angiogenesis and the inflammatory response of the peritoneum

Formation of new blood vessels has been found in PD patients [90], ([84]. The mechanisms behind the induced tissue alterations are not fully identified, however an angiogenesis inhibitor TNP-470, suppressed the progression of peritoneal fibrosis in a mouse model [91]. During peritoneal dialysis, the peritoneal membrane is exposed, through the PD fluid, to both proinflammatory stimulation and to factors that can alter metabolism. Lactate buffer, glucose, glucose degradation products, hyperosmolarity and low pH have been identified as important factors to promote VEGF mediated peritoneal angiogenesis in PD.
VEGF-A has been suggested to be the main growth factor mediating [92] the angiogenesis associated with ultrafiltration failure in PD. Several interdependent processes may promote angiogenesis in peritoneal dialysis. These include shear stress on the endothelial wall as a result of increased blood flow. Furthermore, many soluble products of a range of inflammatory cells, including macrophages, lymphocytes, mast cell, and fibroblasts, promote angiogenesis. Among these products are the proinflammatory cytokines TNF-α, IL-1 and IL-8.

Epithelial-mesenchymal transitions EMT, during embryogenesis and in peritoneal dialysis

It has been shown that peritoneal dialysis leads to epithelial-mesenchymal transition (EMT) of mesothelial cells. EMT is characterised by the loss of cell adhesion and loss of apical-basolateral polarity typical of epithelial cells, acquisition of a myofibroblast-like phenotype with the expression of α-smooth muscle actin (α-SMA) and increased cell mobility [93]. The myofibroblast is a key cellular regulator of extracellular matrix and is in between a fibroblast cell and a smooth muscle cell in differentiation. They are actively migrating and proliferating spindle shaped, α-smooth muscle actin (α-SMA)-positive cells. Myofibroblastic conversion of mesothelial cells is closely associated with EMT in peritoneal fibrosis [94] with the induction of the transcription factor snail and a dramatic down regulation of E-cadherin and cytokeratins [95]. These cells can invade the submesothelial stroma were they contribute to peritoneal fibrosis and angiogenesis associated with peritoneal membrane failure[96]. It is unclear whether myofibroblasts originate from differentiation from resident fibroblasts, or

from nearby epithelial cells through an EMT, or from circulating CD34+ cells (fibrocytes) recruited to injured tissues.
EMTs are programs essential in many stages of development including gastrulation, in which the embryonic epithelium gives rise to the mesoderm and the development of neural crest cells from a small portion of dorsal neural tube [97] (Fig.13).

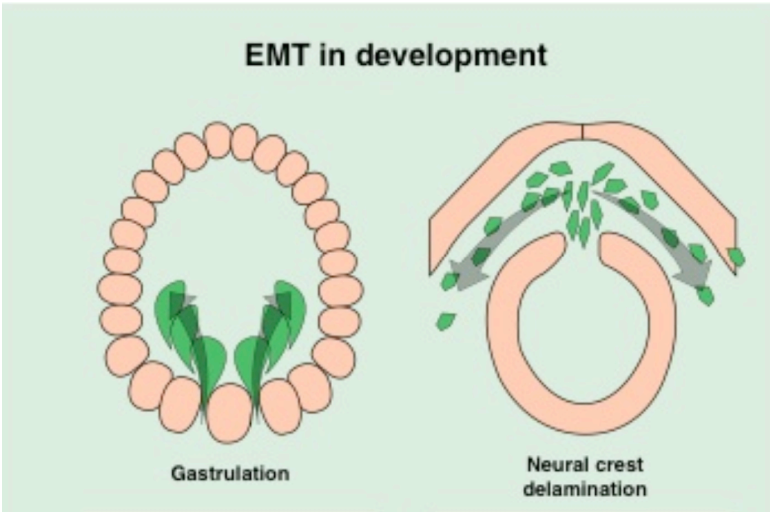


Figure 13. Illustration representing formation of the mesoderm during gastrulation, and the formation and migration of the neural crest cells later during embryonic development. Picture adopted from [98].

PRESENT INVESTIGATION: AIMS

The general aims of the present thesis were to study molecular and cellular mechanisms by which cells sense changes in oxygen tension and how the signal is transduced by Hypoxia-inducible factors (HIFs) into the nucleus and alters gene transcription and downstream protein synthesis.

More specifically, we wanted to investigate:

The possibility of activating HIF-1α under non-hypoxic conditions.
The function of the construct we made based on a region surrounding Pro564 in HIF-1α, and its effect on HIF-1 and HIF-1-regulated responses and compare it to inhibitors of HIF-1 modification and degradation.

The effect of low oxygen tension (hypoxia) on early vascular/hematopoietic development. And particularly, the role of the transcription factors HIF-1α or HIF-2α in this process.

Whether HIF-1α is activated by experimental peritoneal dialysis (PD) and if so characterise the relation to hypoxia and to identify the cells involved.

METHODS

Transfection of the SD and pVHL constructs to cultured cells

We have created a construct, denoted the saturating domain (SD), capable of inducing the HIF-1α protein in normoxia. The cDNA encoding the saturating domain (SD), based on amino acids 505 to 730 in HIF-1α, was generated by PCR from the HIF-1α cDNA using

primers
5´GCGGATCCACCATGTACACTGATATCGAGATGAATAGATTGGGAAAGGGA
GGAAGCACTAGACAAAGTTCACC-3´and
5´GCGGATCCTCATTGAAAAAGTGAACCATC-3´ incorporating Bam HI sites and an amino-terminal VSV-G tag. The PCR product was digested with Bam HI and ligated into the Bam HI-cleaved expression vector pSG5. This domain contains a proline residue (Pro564) which, when hydroxylated, targets HIF-1 for ubiquitination and proteasomal degradation. To test its specificity mutation of a proline to a glycine residue in the SD, corresponding to Pro564 in HIF-1α, was performed using site-directed mutagenesis (QuickChange, Stratagene).
The pVHL cDNA was amplified by PCR from a 293T cDNA library using primers
5´GCGGATCCACCATGGAGGCCGGGCGGCCG-3´and 5´-
GCGGATCCTCAAGCGTAATCTGGCACATCGTATGGGTATCCTCCATCTCCCATCC
GTTGATGTGC-3´ incorporating Bam HI sites and a carboxy-terminal HA tag. The PCR product was digested with Bam HI and ligated into Bam HI-cleaved pCDNA3. Both constructs were confirmed by DNA sequencing.
The expression vector pCDNA3 containing HA-tagged ubiquitin, FLAG-tagged Smad4 or dominant-negative E2 ubiquitin-conjugating enzymes Ubc(CS)s were kind gifts from Anita Morén and Aris Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). The reporter construct, pPUR/PGK-luciferase containing six copies of the phosphoglycerate kinase-1 hypoxia response element (HRE), and full-length human HIF-1α in pCDNA3 were kind gifts from Patrick Maxwell (Imperial College, Renal Section, Hammersmith Campus, London, United Kingdom).
African green monkey COS-7 cells and human 293T cell lines were used in the studies of the SD. 293T cells and COS-7 cells are extensively studied, viable, easy to grow, and can readily be transfected with expression vectors for different gene-constructs. Cells transfected with the wt/mutant SD was compared with cells transfected with empty vector, cells treated with dimethyloxalylglycine (DMOG, 1mM), or MG-132 (10μM) or cells subjected to normoxia or hypoxia. For hypoxia treatment, cells were placed in a humified chamber and flushed with a 95 % nitrogen/5 % carbon dioxide mixture until 0.5 % O_2 was reached, as measured by a Pac III instrument (Dräger) placed inside the chamber. The chamber was sealed and incubated at 37° C.

The embryoid body model.

Investigations aimed at discovering early stages of vasculogenesis and initial vascular maturation have been greatly facilitated by the use of embryonic stem ES cells. ES cell lines with inactivated *Hif-1α* or *Hif-2α* genes were generated as described previously [99]. ES cell clones with randomly integrated HIF-1α or HIF-2α-targeting vectors exhibiting functional alleles of the corresponding HIF-α subunit were used as controls. The different cell lines were cultured on mitomycin C-arrested mouse embryonal fibroblasts in ES medium containing LIF. ES cells were then resuspended in medium without LIF and allowed to aggregate and differentiate into embryoid bodies in hanging drops. After 4 days, embryoid bodies were transferred to chamber glass slides and cultured in normoxic (20.9 %) or hypoxic (1 %) conditions or treated with VEGF-A for different time periods. Morphological effects, and changes in gene and protein expression were analysed by immunohistochemical and immunofluorescent staining techniques, and by real-time PCR and FACS analysis.

The mesenteric-window angiogenesis assay

As a model system for studies of experimental angiogenesis with relevance for peritoneal dialysis the mesenteric-window angiogenesis assay was chosen (Fig. 14). The transparent thin

membranous ”window”-like part of the small-gut mesentery is a good tissue for studies of angiogenesis. It is natively largely avascular; the assay has the advantage that angiogenesis when it occurs, is obvious.

Figure 14. The mesenteric-window angiogenesis assay. The intact tissue are spread on objective slides and analysed by immunohistochemistry, or western blot. Picture adopted from Norrby *et al,* 2006.

The mesenteric test tissue is a 5-10 μm-thick membrane, sandwiching a tissue space that contains fibroblasts, mast cells, macrophages as well as occasional eosinophils and lymphocytes [100]. The tissue is pure peritoneum and also suited for i.e. *in situ* hybridisation and PCR. The same technique is now adopted for whole mounts from other regions, e.g. the parietal peritoneum.
By using cell-specific antibodies we were able to identify the common cell types of the tissue, and the co-staining provided additional data on each cell type. Originally, the model was used without fixation to demonstrate the formation of vascular networks under long-term exposure to PD fluids. In the present study we developed fixation techniques that conserve morphology on the cellular level, allowing functional studies of single cells. 99 % alcohol was instilled in the intra-abdominal cavity for 15 minutes to fixate peritoneal tissue *in situ*. Mesenteric windows were collected and stored frozen until use. The intact tissue mesenteric-window preparations were thawed and fixed in 2 % formaldehyde in phosphate buffer for 25 min at room temperature. Some antigens required 10 min fixation in acetone at room temperature. Fluid from the intraperitoneal fluid space was collected in to a tube containing heparin, to avoid aggregation of cells. Samples were kept on ice, and cells were obtained by centrifugation.
In our study control animals with or without PD catheter were compared to rats exposed to a single four-hour dwell of lactate buffered 3.9 % glucose fluid through an implanted catheter or through intraperitoneal injection. Control animals were subjected to a flush with this fluid at time point 0.

Nuclear extracts and western blot

Nuclear extracts were prepared from cultured cells (paper I), from intraperitoneal cell suspensions or from collagenase treated tissue specimen (paper III) as described. Tissue from mesenteric windows was incubated in collagenase (C0773; Sigma) for 30 min at 37°C prior to cell lysis. Cells from the intraperitoneal fluid space were obtained by centrifugation of fluid samples at 1000 rpm for 6 min. Cultured cells were washed with PBS and treated directly with lysis buffer. 1*10^6 cells from intraperitoneal fluid or material from 2-3 mesenteric windows were lysed in 0.5 ml cell lysis (CL) buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 100 mMEGTA, 100 μM Na_3VO_4, 10 μg/ml aprotinin, 0.1 mM PMSF, 1mM DTT and 1 %

NP-40 alternative). Lysates were transferred to Eppendorf tubes, incubated on ice and nuclei pelleted by centrifugation. Nuclear proteins were extracted by vortexing nuclei for 15 min at 1400 rpm in 100 µl nuclear extraction (NE) buffer (20 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 µg/ml aprotinin and 400 mM NaCl) and cleared by centrifugation. Coomassie staining determined the total protein content prior to dilution with NE buffer without NaCl and immunoprecipitation with HIF-1α antibodies (Abcam) followed by immunoblotting. One tenth of non-diluted nuclear extracts and immunoprecipitates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and separated proteins were electrophoretically transferred onto Hybond-C extra membranes. The membranes were blocked in PBS/3 % bovine serum albumin and incubated with the indicated primary antibodies, respectively. Equal loading of nuclear extracts were confirmed using antibodies, respectively. Membranes were finally incubated with appropriate secondary antibodies, conjugated to horseradish peroxidase and immune reactivity was visualised using the enhanced chemiluminescence (ECL) detection system.

Immunohistochemistry

EBs were fixed in zinc buffer, then subjected to chromogen or fluorescent staining. Endogenous peroxidase activity was quenched with 3 % H_2O_2 in methanol and EBs were incubated with antibodies against the endothelial marker CD31 (Becton Dickinson). To investigate early stages of hematopoietic development expression of CD41 (Becton Dickinson) and CD45 (Becton Dickinson), markers for early hematopoietic and panhematopoietic cells, respectively were analysed by fluorescent staining. EBs was also incubated with antibodies against endothelial marker vascular endothelial-cadherin VE-cadherin (R&D Systems) in order to stain blood vessels.
Some cells in paper II were transfected with GFP alone or in combination with wild-type or mutant SD. Cells were fixed in paraformaldehyde (3 % in PBS), permeabilised with ice-cold acetone and stained with HIF-1α antibodies followed by appropriated secondary antibodies.
Mesothelial cells, fibroblasts, ED-1 and ED-2 macrophages, eosinophils neutrophils, and mast cells were identified by their position in mesenteric-window tissue, shape of nucleus and expression of cell-specific antigens. (Table 1.)
Mouse anti-human mast cell tryptase, recognises human mast cell tryptase, both α and β isoforms. It is an excellent marker for mast cells. Mouse anti rat CD68, recognises a single chain glycoprotein that is expressed on the lysosomal membrane of myeloid cells. Weak cell surface expression also occurs. The antigen (also known as ED-1) is expresses by the majority of inflammatory macrophage and weakly by peripheral blood granulocytes and is a good marker for inflammatory macrophages. To detect resident macrophages we used an anti-rat CD163 antibody that recognises the rat CD163 cell surface glycoprotein, also known as ED2. CD163 is expressed by 50 % of peritoneal macrophages, a subset of splenic macrophages, and by resident macrophages in most other tissues. The Rat prolyl 4-Hydroxylase antibody is used as a marker for staining fibroblasts. Collagen prolyl 4-Hydroxylases play an essential role in the synthesis of all collagens. The antibody specifically reacts with the β-subunit of rPH. In order to detect neutrophils we used Rabbit to Myeloperoxidase. Myeloperoxidase is a protein abundantly expressed in neutrophils and secreted during their activation neutrophil. It possesses potent proinflammatory properties and may directly contribute to tissue injury.
Mesothelial cells, keratin positive epithelial cells, were identified by a rabbit Anti-Cow Keratin (Wide spectrum screening). Low molecular weight cytokeratins (40-54 kD) are distributed in nonsquamous epithelium. High molecular weight cytokeratins (48-67) are found in the squamous epithelium. OBT1612, stains both resting and activated eosinophils and may

be considered a ”pan eosinophil marker”. The antibody recognises the Eosinophil Major Basic Protein (EMBP), a 117 aa protein of Bone marrow proteoglycan.

Cell type	Antibody	Clone (no.)	Raised in	Source	Dilution
Inflammatory macrophage	CD68	ED1(MCA341R)	mouse	AbD Serotec	1:100
Resident macrophage	CD163	ED2(MCA342R)	mouse	AbD Serotec	1:100
Mesothelial cell	Keratin (wide spectr. scr.)	(N1512)	rabbit	Dako	Prediluted
Fibroblast	Prolyl 4-Hydroxylase	6-9H6(AF5110-1)	mouse	AcrisAb.	4ug/ml
Mast cell	Mast Cell Tryptase	AA1(MCA1438)	mouse	AbD Serotec	1:200
Neutrophil	Myeloperoxidase	(ab15484)	rabbit	Abcam	Prediluted
Eosinophil	MBP	BMK-13(OBT1612)	mouse	AbD Serotec	1:20

Table 1. Antibodies directed against cell-specific antigens were used in the study to identify specific cells.

Statistical analysis.

Parametric statistics were used to evaluate the results. Immunoblot and reporter gene data are representative results from experiments repeated three times. Reporter gene data are given as mean induction levels of duplicates ±SE compared to levels induced by hypoxia (set to 100 %). Real-time PCR data show representative results and are given as the mean induction level of triplicates ±SD compared to control (set to 1.0). The expression of each gene were analysed using at least two different RNA preparations.
Immunostainings show representative results from experiments repeated at least two times. Quantification of vascular area was performed on five EBs per condition and is given as mean values ±SD.
In the study of experimental PD in the mesenteric window model, differences between treatments were evaluated by Students t-test. The chosen level of significance was $p=0.05$.

RESULTS AND COMMENTS

Paper I: Activation of hypoxia-induced transcription in normoxia.

As a complementary molecular tool to investigate the possibility of activating HIF under non-hypoxic conditions, we made a construct, denoted the saturating domain (SD), based on a region surrounding Pro564 in HIF-1α. We hypothesised that overexpression of the SD would saturate and thereby block degradation of HIF-1α under normal (normoxic) culture conditions. To test the specificity, we also made a mutant SD, in which Pro564 was replaced by a glycine (P564G). The SD constructs were transiently transfected into COS-7 or 293T cells and the effect on HIF-1α protein levels and activity was analysed by western blotting and immunofluorescent staining techniques, and by reporter gene assays and quantitative real-time PCR.
As hoped for, expression of the SD saturated degradation of HIF-1α leading to accumulation of endogenous HIF-1α proteins under normoxic conditions. The SD was only detected in the

cytosolic fraction of transfected cells. This is consistent with the fact that the SD only contains one of the two nuclear localisation signals which both are required for nuclear import of HIF-1α [101]. The mutant SD failed to induce HIF-1α, whereas cells treated with the prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG) or the proteasome inhibitor MG132, accumulated HIF-1α proteins to levels seen in hypoxic cells. Furthermore, by using confocal microscopy we verified that HIF-1α proteins stabilised by the SD, or by DMOG or MG132, were localised to the nuclear compartment in intact normoxic cells.
The HIF-1α proteins, induced by the SD, were transcriptionally active as they efficiently induced expression from a hypoxia response element (HRE)-regulated luciferase reporter construct. Furthermore, expression of the SD induced activation of two endogenous HIF-1-regulated target genes in transfected cells, VEGF-A and carbonic anhydrase 9 (CA9) [102] [31]. In contrast, the mutant SD had no effect on either reporter gene expression, or on VEGF-A or CA9 mRNA levels.
To investigate the mechanism by which the SD exerted its function, we studied whether or not the SD interfered with ubiquitination of HIF-1α. We could not detect ubiquitination of the SD, neither by endogenous nor by co-expressed ubiquitin. The SD is therefore likely to saturate degradation at a level prior to ubiquitination. To further investigate possible mechanisms, we examined if the SD could be recognised by and bind to the von Hippel-Lindau protein (pVHL). The SD was co-expressed together with pVHL and immunoprecipitation of the SD followed by immunoblotting for pVHL confirmed interaction between the two proteins in intact cells. This interaction was dependent on Pro564 and prolyl hydroxylase activity since it could not be seen with the mutant SD, and since it was blocked by DMOG. The SD is expected to bind pVHL in a 1:1 ratio and competition for pVHL binding capacity could be the mechanism underlying the saturating effect of the SD, as well as for other domains or peptides containing either of the two proline residues [103]. Although pVHL remains the candidate target for the effect of the SD, other mechanisms of action, such as saturation of prolyl hydroxylase activities, cannot be ruled out.
In summary, we show that blocking HIF-1α degradation, either by saturation, or by inhibition of prolyl hydroxylases or proteasomal degradation, leads to nuclear localisation of active HIF-1 proteins.

Paper II: Hypoxia-inducible factor-2α (HIF-2α) modulates formation of vascular/hematopoietic progenitors in differentiating embryonic stem cells.

To investigate what role low oxygen tension and oxygen sensing components play in the formation of the circulatory system during early development, we employed mouse embryonic stem cells lacking HIF-1α or HIF-2α. Upon withdrawal of leukaemia inhibitory factor, ES cells can be made to differentiate into three-dimensional structures called embryoid bodies (EBs). As EBs support formation of several differential cell types, including endothelial and hematopoietic cells, in a manner resembling that *in vivo*, EBs have become a useful model for studying early stages of vascular and hematopoietic development [46].
In agreement with recent data [104], we found, by staining for the endothelial marker CD31, hypoxia to potently promote formation of a vascular plexus in control EBs between day 6 and 8. Also the formation of erythroblasts inside lumenised vessels and contracting cardiomyocytes reflecting the similarities with embryo development could be seen.

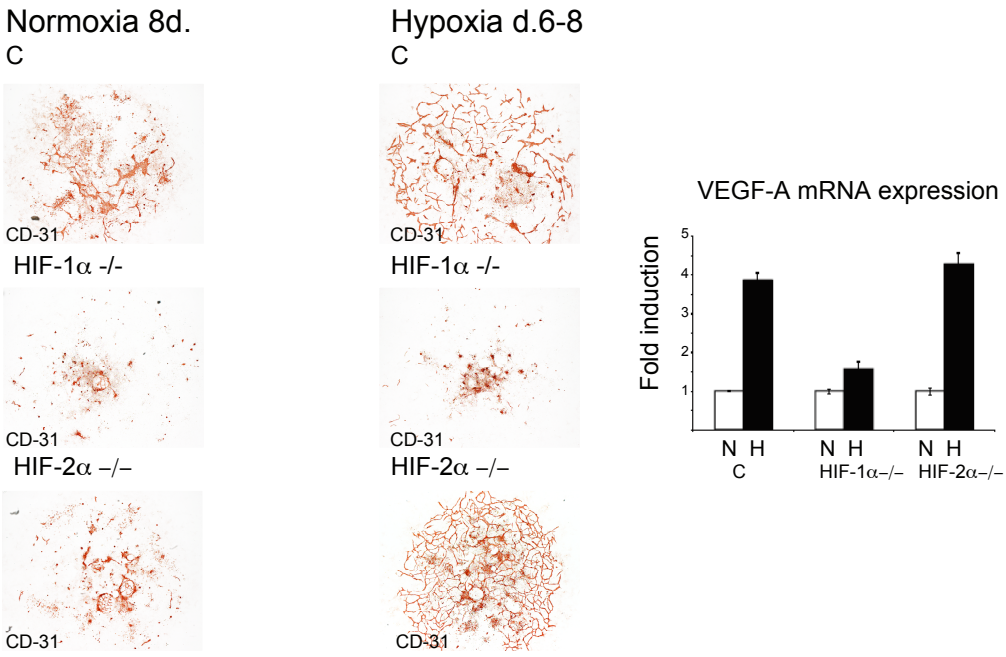


Figure 15. Lack of HIF-α subunits affect vascular plexus formation in response to hypoxia.. Controls EBs, C were cultured under normoxic conditions (20.9 %) for 8 days, or subjected to hypoxia (1 %) or VEGF-A treatment between day 6 and 8 days. Endothelial cells were stained by immunohistochemical staining for CD31. HIF-1α-/- and HIF-2α-/- EBs were cultured and analysed as described for control EBs. Expression of VEGF-A in control (C) or HIF-α-negative 8 day-old EBs cultured in normoxia (N) or hypoxia (H) was quantified by real-time PCR. Changes are expressed as fold induction relative to normoxic levels.

In contrast, ES cells lacking HIF-1α failed to form CD31-positive vascular structures in hypoxia, (Fig. 15) lending further support for an important role for this transcription factor in vascular development. Interestingly, ES cells lacking HIF-2α displayed elevated levels of CD31-positive cells and enhanced development of vascular structures under similar conditions (Fig. 15). Expression of VEGF-A, which was severely compromised in hypoxic HIF-1α-/- EBs, appeared to be normal in HIF-2α-/- EBs. Therefore, the phenotype of HIF-2α-negative EBs seemed not to result from hyperactivation of hypoxia-induced genes. The poor induction of VEGF-A in HIF-1α-negative EBs falls in line with several reports performed on HIF-1α-/- ES cells [105], and is likely to explain, at least to some extent, the lack of vascularisation in these EBs.

Our finding that hypoxia induced hypervascularisation in HIF-2α-/- EBs led us to investigate early stages of hematopoietic development in this genetic background. For this purpose, we analysed expression of CD41 and CD45, markers for early hematopoietic precursors and panhematopoietic cells, respectively. The numbers of CD41 transcripts were markedly elevated in normoxic HIF-2α-negative EBs at day 8, compared to control.

Furthermore, expression of CD45, which is extremely low at day 8 in normoxic control EBs, was clearly increased in HIF-2α-/-EBs. Expression of CD41 and CD45 was modulated by oxygen tension as both hypoxic control and HIF-2α-/- ES cells contained lower levels of he two transcripts than normoxic EBs. By employing immunofluorescent staining and FACS analysis, we confirmed that HIF-2α-negative EBs contained an increased number of hematopoietic cells and that hypoxia-induced vascularisation coincided with a reduced number of hematopoietic precursors.

As an increased number of early vascular/hematopoietic progenitors could explain the effects seen in HIF-2-negative background, we analysed expression of VEGFR-2 and SCL, during early stages of EB differentiation. VEGFR-2 and SCL, which are expressed on

hemangioblasts, are crucial components for many aspects of endothelial cell biology, including vasculogenesis and angiogenesis, and for primitive and definitive hematopoiesis, respectively [106] [10]. Analysis of VEGFR-2 and SCL mRNA levels revealed no major difference in expression between HIF-2α-/- and control EBs differentiated for 4, 6 or 8 days. If anything, expression of these two genes seemed to be slightly decreased in the former at earlier time-points (day 4 and 6). At day 8, HIF-2α-/- EBs expressed elevated levels of SCL mRNAs, presumably reflecting the increased number of hematopoietic cells observed at this stage. Thus, despite the effects on the endothelial and hematopoietic cell pools, HIF-2α-/- ES cells appeared not to produce an increased number of common progenitors for the two lineages.

Several studies have suggested a link between HIF-2α and VEGFR-2 expression [107] [108], and a peculiarity we noted was the deregulated expression of the *vegfr-2* gene in hypoxic HIF-2α-/- EBs. The effect on VEGFR-2 expression could, in conjunction with increased VEGF-A expression, perhaps explain the reduced number of hematopoietic progenitors and the concomitant enhanced hypervascularisation observed under this condition.

In summary, we found that while HIF-1α was required for formation of a vascular system under hypoxic conditions, the related HIF-2α protein appears to play a role in regulating the number and activity of early endothelial and hematopoietic cells.

Paper III: Hypoxia-inducible factor-1α (HIF-1α) activation and hypoxia following catheter implantation and a single peritoneal dialysis dwell.

Inflammations in combination with angiogenesis are adverse reactions to PD. An experimental model of PD was used to study the role of the HIF system in this situation. Rats were exposed for a single 4-hour dwell of lactate buffered, 3.9 % glucose PD fluid by infusion or through intraperitoneal injection ”PD” and ”Catheter+PD” groups. Control animals with or without catheter were observed during the same period of time (“Control” and “Catheter” groups). Before the treatment pimonidazole was injected intravenously, preparing for detection of hypoxia from the formation of intracellular pimonidazole-protein adducts. Cather implantation and PD fluid exposure, each or in combination, both significantly increased the density of cells with expression of HIF-1α in the mesenteric tissue and cavity, compared to the control group. However the difference between the Catheter and the Catheter +PD group was insignificant.

Rises of nuclear HIF-1α concentration in the tissue showed the same trend, quantified by western blot from nuclear extract. Changes are expressed as fold induction compared to untreated rats (set to 1.0). Catheter implantation and PD increased the levels of activated HIF-1α.

Microscopic analysis of the mesenteric window from the three groups where HIF-1α expressing cells were present, enabled the visualisation of cell-specific antigens to determine the contribution of each cell type. The HIF-1α positive cell types in the Catheter and the Catheter+PD groups showed similar distribution over the cell types, antibodies listed in table 1. HIF-expressing cells were largely neutrophils (34-40 %) and cytokeratin-expressing cells outside the mesothelial layer (26-27 %). Fibroblasts (16-18 %), mast cells (7-9 %), and ED-1 macrophages (0-2 %) made up smaller fractions. ED-2 macrophages and eosinophils were evaluated but did not make up significant fractions. The subcellular localisation of HIF-1α was generally nuclear, but some of the mast cells and neutrophils showed cytoplasmic or intragranular localisation, suggesting that nuclear fraction of HIF-1α may reflect the overall level of HIF-1α in the cells [71].

Immunohistochemistry of HIF-1α positive cell types from Catheter+PD animals demonstrate the expression of mesothelial markers, cytokeratin, in fibroblast-like cells in the stroma layer. Possibly these cells were mesenchymal cells of mesothelial origin, converted by EMT. It is unclear whether new fibroblasts originate from differentiation of resident fibroblasts, or from nearby epithelial cells through an EMT, or from circulating CD34+ cells (fibrocytes) recruited to injured tissues [109]. Eosinophils were only occasionally found in the tissue, but were invariably positive for HIF-1α in the Catheter and Catheter+PD groups. Mast cells and macrophages are known from the literature to express HIF-1α [110] [111] [112] [113], and this was seen in mast cells and some of the ED-1 macrophages but not among ED-2 cells in our study.
Regions with a high density of hypoxic cells (pO_2 <10 kPa) were observed after PD fluid exposure and the density of hypoxic cells was significantly higher in the PD and the Catheter+PD group than in the Control and the Catheter groups respectively (Fig. 16).

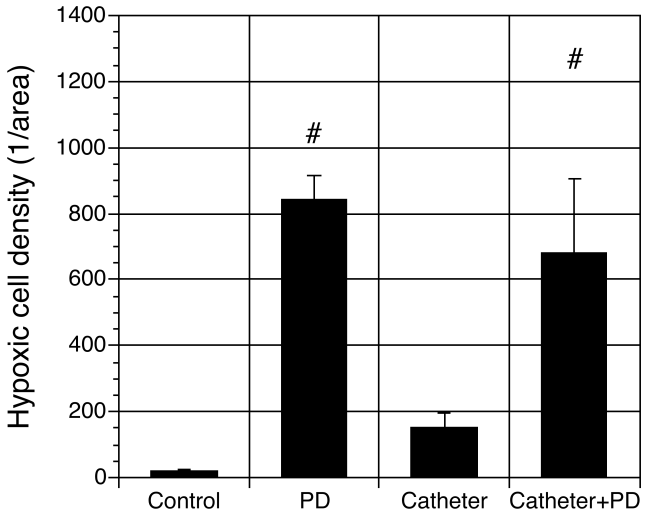


Figure 16.Occurrence of hypoxia.

Pimonidazole immunoreactive regions were detected in confined small areas in the tissue. We also found that HIF-1α was spatiotemporally co-localised with hypoxic regions in PD and to a less degree in animals with catheter (Fig.17).

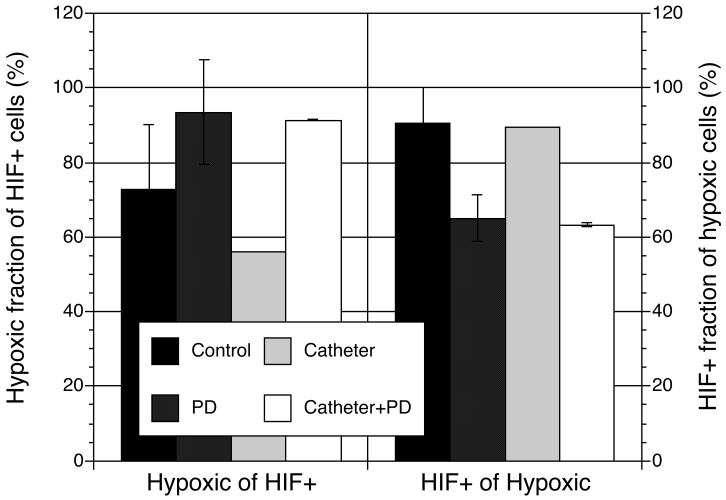


Figure 17. Co-localisation of HIF-1α and intracellular hypoxia.

Thus hypoxia was induced by peritoneal dialysis and may act as a signal for blood vessel formation in patients undergoing PD.
We show that cells expressing nuclear HIF-1α, are recruited to the intraperitoneal fluid following Catheter implantation and, significantly more, by PD. In the case of PD neutrophils, all expressing CD18 on the cell membrane accounted for the entire increase of cell number. The average total intraperitoneal cell numbers in the different groups were Control: $21.4\pm2.0*10^6$; PD: $31.9\pm5.4*10^6$; Catheter: $19.1\pm3.1*10^6$; Catheter+PD: $41.5\pm8.9*10^6$ cells.
Next, we focused on cellular sources of VEGF induced by hypoxia or inflammation. High VEGF-A immunoreactivity was observed following Catheter implantation and PD, both intra- and extracellularly in cells of deeper cell layers. VEGF-A protein expressing cells increased and co-localised with HIF-1α. The strong evidence for increased secretion of VEGF and the parallel co-localisation of HIF-1α and VEGF suggested that HIF activation mediated this response. In PD injected animals a fraction of the VEGF-A expressing cells stained for nuclear HIF-1α. VEGF-A was seen extracellularly, surrounding a small number of cells co-localised to the superficial mesothelial cell layer. Thus, VEGF-A expression could be connected to the inflammatory reaction to the PD catheter.

GENERAL DISCUSSION AND CONCLUDING REMARKS

Discussion of the present results

We showed in paper I that expression of the generated construct, SD, or treatment with prolyl hydroxylase or proteasome inhibitors, under normoxic conditions leads to stabilisation of HIF-1α and is *per se* sufficient for the localisation of endogenous HIF-1α to the nuclear compartment. This construct represents a new tool to stabilise HIF-1α and to correctly relocate it to the nucleus. The construct was intended to increase HIF-1α levels by saturating the degradation mechanisms. The specificity of the construct was studied by replacing the Pro564 with a glycine (P564G). Expression of wild-type SD promoted nuclear accumulation of HIF-1α in GFP-positive cells, whereas no expression could be detected in cells expressing the mutant SD.
The effect of the SD is clearly dependent on Pro564, indicating that hydroxylation of this residue is involved. The SD contains both binding sites for pVHL [114], and competition for pVHL binding capacity could be the underlying mechanism for the saturating effect of the SD.
Analysis of where overexpressed HIF-1α proteins are localised has yielded conflicting results; HIF-1α-GFP fusion proteins were reported to translocate to the nucleus only in hypoxia [70], whereas wild-type HIF-1α was fully localised to the nucleus in normoxia [71]. Interestingly, ubiquitinated forms of HIF-1α and complexes have been found solely in nuclear compartments of reoxygenated cells [115]. This has led to the suggestion that degradation of HIF-1α requires nuclear-cytoplasmic trafficking of pVHL and ubiquitination of HIF-1α in the nucleus [115]. Therefore, if ubiquitination of HIF-1α is a nuclear process then the SD might interfere with proper relocalisation of pVHL/E3 ubiquitin ligase complexes, i.e. by retaining them in the cytoplasm, thereby preventing degradation of nuclear HIF-1α. The possible requirement for nuclear localisation and ubiquitination of HIF-1α could perhaps explain why the cytosolic SD that contains the amino- but not the carboxy-terminal basic motif of the bipartite NLS, although recognised by pVHL, is not ubiquitinated.
The effect of the SD on transcription of endogenous and exogenous genes indicates that induced HIF-1α proteins are correctly localised to the nuclear compartment.

Immunofluorescent staining and confocal microscopy, in cells co-transfected with the SD and GFP, verified this.

The question about nuclear localisation is also important in the study of HIF-1α positive cells observed after peritoneal dialysis. In some of the cell-types, for example in mast cells, the increased HIF-1α levels were localised to the cytoplasm and not to the nucleus. HIF-activation is known to occur in mast cells [110] [111]. HIF-1 is not stored in any cells, but is constantly synthesised and removed by proteolysis. Under resting conditions neutrophils and mast cells do not have detectable levels of HIF. Under activating conditions cell-type-specific differences in stabilising HIF-1 protein levels could perhaps be explained by differential subcellular distribution and compartmentalisations of degradation of HIF-1[116]. It has been shown that HIF-1β is indispensable for HIF-1 DNA-binding and transactivation function [117]. Also during isolation of the nuclei both HIF-1α and ARNT tend to leak from the nuclei in the absence of either subunit, suggesting that heterodimerisation is required for stable association within the nuclear compartment [117]. However, heterodimerisation with HIF-1β is not necessary for induction and nuclear translocation of HIF-1α. Nuclear translocation is intrinsic to HIF-1α, due to the presence of a nuclear localisation signals. It has been observed, however, that HIF-1α accumulates in peroxysomes in hepatocytes on reoxygenation [118]. The functional importance of this is not known, but peroxisomal sequestration might provide a mechanism for keeping nuclear levels of HIF-1α in the liver in check when not needed.

Embryonic stem cells lacking HIF-1α or HIF-2α will give different and specific differentiation patterns of progenitor cells during early vascular development. Since HIF-1α-/- and HIF-2α-/- mice die at midgestation, the embryoid body model was used in paper II in order to study how low oxygen tension and oxygen-sensing components effect differentiation of vascular and hematopoietic progenitors.

HIFs play an important role in a remarkable variety of biological processes. HIF-1α-/- mice display neural tube defects, cephalic mesenchymal cell death, and defects in vascularisation, particularly in the yolk sac and cephalic regions [119] when they die at midgestation. The existing vessels also appear to be dilated and lacking branches, indicating that while proper vasculogenesis occurred, angiogenesis was not initiated. HIF-1β-/- mice also exhibited embryonic lethality due to defective vascularisation of the yolk sac and branchial arches, indicating a role of HIF-1β in development, particularly in angiogenesis [120-122]. In addition, a decreased number of hematopoietic progenitors were also observed in these mice. Although the two HIF-α subunits have many similarities, a number of observations indicate that they are not functionally redundant. For instance, the *Hif-1α* and *Hif-2α* genes are differentially expressed during development and the two factors may also affect transactivation of target genes in different manners [123] [124]. Our results confirm the non-redundancy since ES cells lacking HIF-1α failed to form CD31-positive vascular structures in response to hypoxia whereas ES cells lacking HIF-2α displayed deregulated expression of vascular endothelial receptor-2 (VEGFR-2), elevated levels of CD31-positive cells and enhanced development of vascular structures under the same conditions. There were clear differences between HIF-1α-/- and HIF-2α-/- EBs, for example the expression of *Vegf-a*, gene appeared to be normal in hypoxic HIF-2α-/- EBs whereas compromised in HIF-1α-/-. Moreover, HIF-2α-/- stem cells supported an enhanced formation of hematopoietic cells as increased numbers of CD41- and CD45-positive cells were observed in this genetic background.

The effect on vascularisation in HIF-1α-/- ES cells is in line with this phenotype. The compromised VEGF-A expression in HIF-1α-negative EBs is in agreement with results from experiments performed on HIF-1α-/- ES cells [125] [105] [119]. Surprisingly, HIF-1α-/- embryos demonstrated increased VEGF-A expression compared to wild-type embryos,

possibly due to induction by glucose deprivation independent of HIF-1α [125]. Still, the poor induction of VEGF-A in HIF-1α-negative EBs in our study can, at least in part, explain the inability to develop vascular networks under hypoxic conditions.

Mice with a targeted inactivation of *Hif-2α* gene is embryonic lethal and results in quite different and variable phenotypes [126] [127]. Though one study showed a defect in vascular remodelling [128], other studies have shown a defect in catecholamine production [57], or a defect in lung maturation involving surfactant deficiency [129]. The unexpected and variable phenotypes presumably reflect the complexity of these responses, as well as differences in strain background. Studies on knockout ES cells have demonstrated the potential of the embryoid body model system, complementing the *in vivo* gene knockout analyses [130]. We showed that HIF-2α-negative EBs responded to hypoxia with induction of hypervascularisation. HIF-2α-negative EBs had the following important characteristics. They contained an increased number of hematopoietic cells at day 8, compared to control, as increased analysis of CD41- and CD45-positive cells were observed in this genetic background. Interestingly, hypoxia-induced vascularisation coincided with a reduced number of hematopoietic precursors. Despite these effects on cell pools of the hematopoietic and endothelial lineage, HIF-2α-/- ES cells do not appear to produce an increased number of a hemangioblast. A peculiarity we noted in the hypoxic HIF-2α-/- EBs was the deregulated expression of the *Vegfr-2* gene.

The vascular phenotype in HIF-2α-/- mice is characterised by a failure of small vessels to merge properly in the yolk sac, resulting in the formation of extensive endothelial sheets. Large vessels in the yolk sac failed to seal completely, leaving openings where blood contents leaked out [128]. These effects that could be interpreted as aberrant activity of endothelial cells bear resemblance with the hyperactive vascular cells we observed in HIF-2α-negative EBs. We show that while hypoxia-induced vascular development requires HIF-1α, ablation of HIF-2α protein appears to play a role in regulating the number and activity of early hematopoietic and endothelial cells.

Hypoxia has been found to enhance formation of BL-CFCs in differentiating ES cells [47]. BL-CFC, Blast colony-forming cells have the capacity to give rise to both endothelial and primitive/definitive hematopoietic cells *in vitro*.

Moreover, HIF-1β, the major dimerisation partner for HIF-1α and HIF-2α, is required for development of endothelial and hematopoietic progenitors in EBs and in the mouse yolk sac [121]. Based on these findings, we argued that the HIF-2α-/- phenotype could stem from enhanced formation of hemangioblast-like cells. However, analysis of cells expressing the two genes *vegfr-2* and *scl*, did not indicate an increase of a hemangioblast-like cell under normoxic conditions. The expression of VEGFR-2 and SCL were in fact slightly reduced at earlier time points (day 4 and 6).

We found an increased amount of CD41- and CD45-positive cells in HIF-2α-/- EBs at day 8 compared to control, this effect may represent a shift in favour of hematopoietic versus endothelial cells at this stage. The hypervascular activity seen in hypoxic HIF-2α-negative EBs could be explained by conditions favouring expansion of endothelial cells. If HIF-2α-negative EBs do not form more endothelial/hematopoietic progenitors, what then is the underlying cause for the observed phenotype in HF-2α EBs? It is known form previous studies that, mice deficient in VEGFR-2 fail to develop blood vessels and yolk sac blood islands, and is embryonic lethal due to endothelial and hematopoietic defects [38]. It has been suggested that VEGFR-2 is required for survival and migration of endothelial and hematopoietic precursors, rather than for the initial commitment of the hemangioblast to either of the two lineages[10]. Thus, it is evident that the expression and activity of VEGFR-2 needs to be tightly regulated for proper endothelial and hematopoietic development. Our

observation that HIF-2α-negative EBs show altered expression of VEGFR-2 in hypoxia could perhaps explain the enhanced hypervascularisation under such conditions.
An alternative explanation is that HIF-1α in this situation rather competes with HIF-2α for a limited amount of the binding partner HIF-1β. In the absence of HIF-2α, more HIF-1α/HIF-1β heterodimers would form leading to increased expression of genes regulated by HIF-1. We failed to detect abnormal expression of hypoxia-regulated genes, such as the *vegf-a* gene, in HIF-2α negative EBs. Still, we cannot rule out that the HIF-2α-/- phenotype could originate from subtle differences in expression of HIF-1-regulated genes.
The EB model defines how loss of HIF-1α or HIF-2α affects the biology of early endothelial and hematopoietic cells and supports distinct and important functions for the two genes during establishment of the circulatory system. Careful examination of hematopoietic and endothelial precursor cells in HIF-2α null embryos could help to determine the exact role of HIF-2α in development of these two lineages.
Using immunohistochemistry and western blot we show in paper III that the levels of active HIF-1α increased after catheter implantation and a single PD dwell, each or in combination dramatically in the peritoneal tissue and cavity. It is known from the literature that VEGF is one important factor to promote peritoneal angiogenesis in PD [92], but as our study is the first attempt to describe HIF-1α activation during PD, there is no comparable data available. The precise role of VEGF in the induction of angiogenesis during PD is still unclear. Some of the HIF activation may be due to hypoxia (PD) and some may be due to inflammatory reaction to the catheter since the presence of a PD catheter increases the inflammatory response also to sterile solutions [131].
We also provided evidence, for the first time, that hypoxia exists in PD exposed rats. Hypoxia, is a finding in the microenvironment of inflamed and injured tissue [132]. Contributory factors to hypoxia in peritoneal dialysis include the high metabolic demands of inflamed tissue. Other factors include movement and accumulation of fluid containing high concentrations of lactate and glucose. This environment stimulates VEGF-A production and formation of new blood vessels in an attempt to restore oxygen homeostasis. VEGF-A production can be independently upregulated by proinflammatory cytokines and hypoxia, but *in vivo,* these factors are interdependent. It is not known whether the accelerated development of new vasculature in the peritoneal tissue, compensates for an initial hypo perfusion and hypoxia.
The mesenteric window preparation, earlier used to study angiogenesis, was further developed in order to allow studies of the activation of HIF-1 that controls VEGF secretion in a number of different cell types. We have shown that it is possible to study macrophage subtypes, mesothelial cells, mast cells, fibroblasts, eosinophils, HIF-1α, hypoxia and VEGF-A by confocal microscopy and immunohistochemistry in whole mount preparation of thin peritoneal tissue segments. Several cell types demonstrated HIF-1 expression, the most frequent being neutrophils. Activated neutrophils, migrating in the extra vascular space [133], secrete VEGF and VEGF secretion by neutrophils is mediated by HIF-1[76]. The recruitment of HIF-1 positive neutrophils to the peritoneal tissue was therefore expected, and finally this leads to the intraperitoneal accumulation of these cells.
It is well known, that the recruitment of myeloid cells to sites of inflammation is coordinated by the β_2-integrin family of adhesion receptors. As a transcriptional regulator of β_2-integrin expression, HIF may function to control the myeloid leukocytes to inflammatory lesions [77]. Indeed we show that after PD, recruited neutrophils in the intraperitoneal fluid space all expressed CD18 on the cell membrane and nuclear HIF-1α.
It is known from the literature that mesothelial and endothelial cells produce substantial amounts of growth factors such as VEGF in response to PD fluid [134, 135]. Regarding other

cell types, data is scarce. In our study, mesothelial cells were expressing VEGF only when exposed to PD fluid by injection. Mesothelial cells that have undergone an EMT has been found to invade the submesothelial stroma were they contribute to peritoneal fibrosis and angiogenesis associated with peritoneal membrane failure in patients undergoing PD [96] [94] A recent study demonstrated that transdifferentiated mesothelial cells are an important source of VEGF in PD patients and that the underlying mechanism of VEGF upregulation in mesothelial cells is the mesenchymal conversion of these cells [136]. We observed the expression of mesothelial markers, cytokeratin, in HIF-1 positive fibroblast-like cells in the stroma layer. A possibility is that these cells were mesenchymal cells of mesothelial origin, converted by EMT. A recent study showed that HIF-1α could induce colon cancer cells EMT [137] and evidence for similar correlation in prostate cancer cells were shown in a different study [138].
Eosinophils were expressing nuclear HIF-1α but constituted a small fraction of the HIF-1α positive cell population in the Catheter and Catheter+PD groups. No eosinophils were observed in the absence of PD catheter. There is also increasing evidence for the association of mast cells with angiogenesis, and HIF-activation is known to occur in mast cells [110, 111].

Methodological considerations:

We investigated whether or not the SD was ubiquitinated and destroyed by the proteasome. We could not detect ubiquitination of the SD, neither by endogenous nor by co-expressed ubiquitin. SD was immunoprecipitated from transfected cells and ubiquitination analysed by immunoblotting using ubiquitin antibodies. Using this approach, we could not observe any modification of the SD by endogenous ubiquitin (data not shown). To increase the sensitivity, cells were co-transfected with the SD and ubiquitin, tagged with the HA epitope. In order to prevent destruction of ubiquitinated SD, transfected cells were pre-treated with MG-132 for up to 12 h prior to lysis. Ubiquitination of immunoprecipitated SD was determined by immunoblotting using anti-HA antibodies. Despite using this modified protocol, we were still unable to detect any measurable ubiquitination of the SD. As a positive control we transfected cells with wild-type Smad4, an essential component for transcription induced by transforming growth factor-β (TGF-β), and a mutant Smad4 (L43S), known to be heavily ubiquitinated. In contrast to the SD Smad4 was clearly modified following co-transfection with ubiquitin. These findings, together with the observation that the SD exhibits a rather slow turnover, strongly suggest that most of the expressed SD escapes modification and destruction by the ubiquitin-proteasome pathway and exerts its effects prior to that.
When asking the question what the underlying cause for the observed phenotype in HIF-2-/-α EBs in paper II, it is worth mentioning that the different effects of HIF-1α-/- or HIF-2α-/- ES cells under normoxic or hypoxic conditions were not caused by alterations of cell viability, as judged by FACS analysis, nor by clonal variation, as similar responses/morphologies could be detected in three independent clones for each genetic manipulation (data not shown).
In a review of the current methods of hypoxia detection in the clinic, which include haemoglobin i.e. oxygen microelectrode, oxygen-dependent DNA strand break, and hypoxia marker techniques, it was concluded that the immunohistochemical hypoxia marker technique was ideally suited to correlative studies [139]. Until recently it was difficult to measure oxygen gradients at the cell level. However the 2-nitroimidazole hypoxia markers, pimonidazole, is a robust and effective marker of tissue hypoxia in both animal and human studies [140]. Because the marker detects hypoxia at a cellular level, it is not only the spatial patterns of hypoxia that can be investigated relative to adjacent histological landmarks, but also the role of oxygen gradients for different cell function can be studied using cellular and molecular markers.

A single intraperitoneal injection of any solution will reach all the micro vessel cells, as well as all non-vascular cells, in the test tissue. This is because the mesothelial layer of cells is highly permeable to compounds within a wide range of molecular weights. When no catheter is implanted, the test tissue is untouched and unaffected by wound-healing-induced inflammation and angiogenesis [141]. Repeated injections possibly induce some degree of inflammation. The different experimental models used, in the PD society leaves no consensus on a preferential model [142]. Earlier studies have shown that compared with PD in humans, rats react strongly to PD catheters [143-145]. According to the only published comparative study, dialysate cell counts, submesothelial fibrosis and peritoneal transport properties were identical for injections and catheters after one month of daily exposure to hypertonic PD solution [128]. In the study of acute effects of single PD fluid exposure injections may offer a more sensitive technique than catheters.

Importance of the present results

The cardiovascular system plays a key role in O_2 homeostasis. The work in this thesis demonstrates the biological role of hypoxia and HIFs in the early events of progenitor cell generation, endothelial or hematopoietic commitment and development, as well as in sites of hypoxia and inflammation in the adult organism. The thesis also demonstrates a new technique to intervene with the activation of the HIF system on the subcellular level. HIF-1α is an attractive target for the treatment of ischemic diseases and inflammation.

It is becoming increasingly evident that changes in oxygen tension and the activity of HIFs are important components for development of endothelial and hematopoietic lineages. In this context, many important questions remain to be answered. We have shown that HIF-1α and HIF-2α have unique functions during formation and differentiation of the vascular system under hypoxic conditions. It remains to show if HIF-1α or HIF-2α stimulates production of growth factors that influence mesoderm differentiation into early stem cells or mediate hemangioblast proliferation or differentiation during development of the cells of circulatory system. Understanding the differences between the α subunits and their relative contribution to the early events of progenitor cell generation, lineage commitment, and development of the vascular system under hypoxia is critical to assess O_2 delivery during embryo development.

In the adult organism the possibility of either stimulating or inhibiting the activity of HIFs, holds great promises for development of new therapies against common, yet severe pathological conditions including cancer and ischemic diseases. To this date, therapeutic angiogenesis, the induction of neovascularisation has focused on the delivery of, in particular, the *Vegf-a* genes, either as naked plasmid DNA or as an adenovirus [146] [147] [148] [149] to ischemic tissues. The disadvantage with this methodology is the formation of leaky, immature and irregular blood vessels. The HIF system appears as an attractive target for the treatment of ischemic diseases as activation of a ”master gene switch” would result in a broad and coordinated downstream reaction. Some of the approaches currently pursued to increase HIF activity include the development of prolyl hydroxylases inhibitors with greater specificity, and hence less side effects[150].

Several strategies have successfully been used for experimental activation of HIF-1. Deletion of the central oxygen-dependent degradation domain results in a stable and constitutively active HIF-1α molecule. Transgenic expression of this construct in skin, using a keratin K14 promoter, resulted in marked activation of HIF-1 transcriptional targets and growth of functional blood vessels [151]. An alternative approach has used DNA encoding a HIF-1α-VP-16 hybrid, lacking the ODD domain of HIF-1α, as gene therapy. In a rabbit hind-limb ischemia model, administration of this construct resulted in increased local blood supply [152].

Other investigators have focused on the possibility of inhibiting degradation of endogenous HIF-1α. PR-39, a proline- and arginine-rich macrophage-derived polypeptide, has been shown to interact with the proteasome and to stabilise HIF-1α [153]. Transgenic mice expressing PR39 did exhibit increased myocardial vascularisation. Therapeutic approaches aimed at increasing HIF activity will, most likely have to operate in near/non-hypoxic conditions, we have therefore made a construct denoted the saturating domain (SD), capable of inducing the HIF-1α protein in normoxia. A major problem with the concept of a saturating domain lies in delivering the construct (gene or protein) to the affected tissue. Although gene therapy using viral vectors could be one way to ensure delivery, the use of small stable peptides based on regions around the hydroxylated prolines is likely to be a more straightforward approach. Overexpression of peptides corresponding to either of the two prolyl hydroxylation sites, Pro402 or Pro564, in human HIF-1α has been shown to block degradation of endogenous HIF-1α, promote HIF-1 transcriptional activity and to generate an angiogenic response [103].The mechanism(s) underlying saturation of HIF degradation must also be elucidated; does it include saturation of pVHL binding, prolyl hydroxylase activity or some yet unknown mechanism?

On the other hand, blocking HIF activity could be one way to combat problems associated with tumour hypoxia or inflammation. Small molecular inhibitors of HIFs are being developed and these could in the future perhaps be used to correct/block unwanted responses induced by hypoxia and/or HIFs.

The findings in paper III indicate that HIF is involved in the persistence of inflammation and progression of neovascularisation during PD. The question is if the angiogenesis associated with PD is appropriate. And if so should it be inhibited? Conditions found in inflamed and injured tissues are characterised by high concentrations of inflammatory mediators and metabolites and low levels of oxygen and glucose. As HIF controls VEGF secretion in a number of different cell types, it seems probable that HIF enables interplay between the oxygen sensing and inflammatory pathways, also involved in peritoneal dialysis induced angiogenesis. In recent studies, accumulating evidences link inflammation to angiogenesis in a variety of chronic inflammatory diseases and the benefits of anti-angiogenic agents have been demonstrated [154]. Evidence has accumulated that inhibition of HIF-1 activity could also act to prevent inflammation, by virtue of its essential role in the activation and infiltration of macrophages and neutrophils into affected tissues.

In chronic inflammation, anti-angiogenic therapy results in anti-inflammatory responses, and its effect is probably mediated through several mechanisms. Decreasing the vasculature and endothelial cells in vessels may reduce cell infiltration and delete a potent source of proinflammatory cytokines and chemokines in inflammatory sites. Direct inhibition of HIF could target not only angiogenesis but also cell migration and tissue homeostasis. HIF inhibitors are being evaluated as potential therapeutic agents for the treatment of diseases such as cancer, but until downstream targets of HIF-1α have been fully characterised, the potential to use HIF-1α antagonists remains limited.

CONCLUDING REMARKS

Since its discovery, the HIF signalling cascade has been studied in detail, and its importance appears clearly evident in both physiological and pathological processes, such as embryogenesis, tissue repair, ischemia and inflammation.

The studies in this thesis has lead to the following:

Blocking HIF-1α degradation, either by saturation, or inhibition of prolyl hydroxylases or proteasomal degradation, leads to nuclear localisation of active HIF-1α proteins.

The phenotypic features of the knockout mice for *hif-1α* and *hif-2 α* show the central importance of the HIF signalling pathway in the embryonic development. The studies of the EB model has shown that HIF-1α is required for hypoxia-induced vascularisation during early stages of development.
HIF-2α modulates the activity and number of early endothelial and hematopoietic cells.
HIF-2α-/- stem cells displayed deregulated expression of vascular endothelial growth factor receptor-2, (VEGFR-2), and hypervascularisation under conditions of low oxygen tension.
HIF-2α-/- stem cells supported an enhanced formation of hematopoietic cells under basal (normoxic) conditions.
The effect on endothelial and hematopoietic cell pools was not caused by an enhanced formation of common progenitor cells.

PD fluid exposure leads to hypoxia and induction of HIF-1α in resident and inflammatory cells.

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