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PATHOGENIC MECHANISMS BEHIND DYSREGULATED ANGIOGENESIS WITH FOCUS ON HIF AND IGF-I SIGNALING

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To my wonderful family and friends

ABSTRACT

Angiogenesis is a complexly regulated process activated to assure cells with normal supplies of nutrients and oxygen. Playing such an essential role in homeostasis of the tissues it is critical to understand its physiology and pathology to be able to design therapies for several diseases where angiogenesis is dysregulated (either excessive or diminished).
We aim to better characterize the angiogenesis during chronic complications of diabetes and tumors, focusing on the roles of two pathogenic factors common for both diseases: hypoxia inducible factor (HIF) and insulin-like growth factor (IGF).

Chronic complications of diabetes significantly increase the mortality and morbidity in patients with diabetes and lack for the moment efficient therapies. Hypoxia along with hyperglycemia has been relatively newly identified as a pathogenic factor for complications in diabetes. We have therefore investigated in our studies the cross-talk between hyperglycemia and hypoxia and we have demonstrated that cells fail to properly adapt to hypoxia due to repression of HIF's stability and function in the presence of high glucose. Moreover we have shown that hyperglycemia leads to HIF destabilisation through a VHL-mediated mechanism and complexly affects the HIF transactivation. In agreement with the *in vitro* data, we have detected repressed HIF in ulcers of diabetic mice. Local stabilization of HIF, either pharmacologically or by adenovirus mediated transfer, improves wound healing rate in diabetic mice, which indicates the pathogenic relevance of the hyperglycemia-induced HIF repression for diabetes complications. We further studied the consequences of the HIF repression in diabetes and identified that it is also responsible for increased mitochondrial radical oxygen species (ROS), which are essential for the development of chronic complications of diabetes. In consequence the stabilization of HIF is followed by normalization of ROS production, both *in vitro* and *in vivo*, even under the persistence of the high glucose concentrations.

In a third study we investigated the role of IGF-I for diabetic wound healing. IGF-I, a growth factor and regulator of angiogenesis, is secreted into the blood stream by the liver but also produced locally in the tissues. The relative contributions of local vs systemic IGF for wound healing is still unclear. This is even more relevant for diabetic wounds where reduced IGF-I levels were detected. We demonstrated here that liver-derived IGF-I does not affect wound healing in mice with or without diabetes. This indicates that local therapy with IGF-I is sufficient for improving wound healing in diabetes, avoiding the potential side effects of a systemic therapy.

Dysregulated angiogenesis is also essential for tumor development. Kaposi's sarcoma (KS) is a highly vascularized tumor and its biology is dependent on angiogenic stimuli. We demonstrated here that the vascularized phenotype characteristic for KS is highly dependent on the interplay between IGF-I and HIF. We showed that IGF-I induced accumulation of both HIF-1α and HIF-2α paralogues. IGF increased also HIF activity as demonstrated by the HRE reporter gene assay and by induction of VEGF(classic target gene of HIF). We have further described that IGF induces HIF accumulation by increasing the translation of the HIF-α subunits. The biological relevance of the HIF signaling in KS biology was highlighted by its expression through all the characteristic progressive stages of the disease. Moreover, we demonstrated that blocking the IGF-IR signaling decreases HIF accumulation and blunts the VEGF expression, offering a promising therapeutic option in the management of KS.

In conclusion, we identified new mechanisms of dysregulated angiogenesis in diabetes and tumors and proposed new therapeutic strategies based on our findings.

LIST OF PUBLICATIONS

I. **Botusan IR***, Sunkari VG*, Savu O, Catrina AI, Grünler J, Lindberg S, Pereira T, Ylä-Herttuala S, Poellinger L, Brismar K, Catrina SB. Stabilization of HIF-1alpha is critical to improve wound healing in diabetic mice. *Proc Natl Acad Sci U S A. Dec 9;105(49):19426-31.*

* These authors contributed equally.

II. **Botusan IR***, del Sole M*, Zheng X, Grünler J, Sunkari VG, Solaini G, Brismar K, Catrina SB. Hypoxia Inducible Factor (HIF) repression is responsible for Radical Oxygen Species (ROS) overproduction during exposure to combined hyperglycemia and hypoxia. *Manuscript.*

* These authors contributed equally.

III. **Botusan IR**, Calissendorff FS, Grünler J, Sunkari VG, Ansurudeen I, Svensson J, Hansson JO, Ohlsson C, Brismar K, Catrina SB. Deficiency of liver-derived insulin-like growth factor-I (IGF-I) does not interfere with the skin wound healing rate. *Manuscript.*

IV. Catrina SB, **Botusan IR**, Rantanen A, Catrina AI, Pyakurel P, Savu O, Axelson M, Biberfeld P, Poellinger L, Brismar K. Hypoxia-Inducible Factor-1alpha and Hypoxia-Inducible Factor-2alpha are expressed in Kaposi Sarcoma and modulated by Insulin-like Growth Factor-I. *Clin Cancer Res. 2006 Aug 1;12(15):4506-14.*

LIST OF PULICATIONS NOT INCLUDED IN THESIS

I. Gu HF, Zheng X, Abu Seman N, Gu T, **Botusan IR**, Sunkari VG, Lokman EF, Brismar K, Catrina SB. Impact of the hypoxia-inducible factor-1 α (HIF1A) Pro582Ser polymorphism on diabetes nephropathy. *Diabetes Care. 2013 Feb;36(2):415-21.*

II. Zheng X, Zheng X, Wang X, Ma Z, Gupta Sunkari V, **Botusan I**, Takeda T, Björklund A, Inoue M, Catrina SB, Brismar K, Poellinger L, Pereira TS. Acute hypoxia induces apoptosis of pancreatic β-cell by activation of the unfolded protein response and upregulation of CHOP. Cell Death Dis. 2012 Jun 14;3:e322.

III. Savu O, Sunkari VG, **Botusan IR**, Grünler J, Nikoshkov A, Catrina SB. Stability of mitochondrial DNA against reactive oxygen species (ROS) generated in diabetes. *Diabetes Metab Res Rev. 2011 Jul;27(5):470-9.*

IV. Catrina SB, **Botusan IR**, Sunkari VG. Hyperglycemia and hypoxia inducible factor, a multifaceted story. *Cell Cycle. 2010 May;9(9):1856.*

V. Catrina SB, Rotarus R, **Botusan IR**, Coculescu M, Brismar K. Desmopressin increases IGF-binding protein-1 in humans. *Eur J Endocrinol. 2008 Apr;158(4):479-82.*

CONTENTS

LIST OF ABBREVIATIONS

ALS	Acid labil subunit
ARNT	aryl hydrocarbon receptor nuclear translocator
bHLH	Basic helix-loop-helix
CBP	CREB-binding protein
CHX	Cycloheximide
CTAD	C-terminal transactivation domain
DFX	Deferoxamine
DMOG	Dimethyloxalylglycine
EPC	Endothelial precursor cells
FIH	Factor inhibiting HIF-1
GH	Growth hormone
HDF	Human dermal fibroblasts
HDMEC	Human dermal microvascular endothelial cells
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus
HRE	Hypoxia responsive element
IGF	Insulin-like growth factor
IGFBP	Insulin like growth factor binding protein
IGF-IR	IGF-I receptor
KS	Kaposi's sarcoma
MGO	Methylglyoxal
miRNA	MicroRNA
mTOR	Mammalian target of rapamycin
NTAD	N-terminal transactivation domain
ODDD	Oxygen dependent degradation domain
PAD	Peripheral arterial disease
PARP	poly(ADP-ribose) polymerase enzyme
PAS	Per-ARNT-sim protein
PCBP	poly (rC) binding protein
PDGF	Platelet derived growth factor
PGC	Peroxisome proliferator-activated receptor
PHD	Prolylhydroxylase
PKC	Protein kinase C
RACK	receptor for activated C-kinase
RAGE	Receptors for AGE
ROS	Reactive oxygen species
SDF	Stromal derived factor
STZ	Streptozotocin
SUMO	Small ubiquitin like modifiers
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau protein

“We shall not cease from exploration and the end of all our exploring will be to arrive where we started and know the place for the first time”

T. S. Elliot

1 RATIONALE FOR THE PROJECT

Angiogenesis, the formation of new blood vessels, is essential for the survival of the new tissue formed during regenerative or proliferative processes. Moreover dysregulation of angiogenesis plays an important role in pathology. It is therefore essential to have a good understanding of its physiology and pathology for designing more effective therapies.

Diabetes and tumors are two diseases with high prevalence, resulting in significant morbidity and mortality. Even though big steps have been taken in the last years in the management of these diseases, unsolved issues still remain, including lack efficient strategies for development and treatment of chronic complications of diabetes and control of metastatic potential of tumors.

Both diseases are characterized by dysregulated angiogenesis.

The angiogenic phenotype in these diseases covers a large range. At one extreme, there are the tumors where the hypoxia signal generates a cascade of events resulting in increased angiogenesis. At the other, hypoxia signal could be inefficiently transduced due to hyperglycemia resulting in impaired angiogenesis. IGF and HIF are two important regulators of angiogenesis and are also factors relevant for pathogenic mechanisms of both diseases.

We aim in this thesis to characterize new pathogenic mechanisms responsible for the dysregulation of angiogenesis based on IGF and HIF signaling and to suggest potential therapeutic targets that could enter clinical research for the benefit of the patients.

2 BACKGROUND

2.1 ANGIOGENESIS

The normal function of cells in organisms is dependent on blood flow which provides the nutrients and oxygen and also removes the products resulting from metabolic processes. The distance of a cell from the blood vessel is limited by the diffusing capacity of the oxygen to 150-200 μm[1]. Therefore, blood vessels are a prerequisite for any developing process either regenerative or neoplastic[2,3].

Initial blood vessels develop as early as day 7 of embryonic life from multipotent cells which originate in the mesodermal layer in a process called vasculogenesis[4]. The subsequent ramification and specialization of the vascular network as well as the neovascularization during adulthood happens via new blood tube formation from the preexistent vessels in a process called **angiogenesis**.

In adult life the blood vessel endothelium is mostly quiescent with few exceptions such as physiologic angiogenesis during menstrual cycle or wound healing. However, endothelial cells preserve the capacity to divide, migrate and form new vessels in response to hypoxia or other stress conditions[5].

When an angiogenic signal is released, a complex reaction develops which involves: degradation of the basement membrane of the vessels by proteases which results in detachment of pericytes, loss of cell junction between pre-existent endothelial cells, sprouting, migration and proliferation of individual cells and finally remodeling of the extracellular matrix to form new tubule structures[6]. An efficient neovascularisation needs a fine-tuned interplay between pro- and anti-angiogenic mediators.

2.2 REGULATORS OF ANGIOGENESIS

2.2.1 Hypoxia inducible factor (HIF)

Hypoxia is an important signal for angiogenesis and plays important role in the pathology of a wide spread diseases like cardio-vascular disease and ischemia, cancer, inflammation, anemia and chronic obstructive pulmonary diseases[7].

Hypoxia is defined as the condition when the delivery of the oxygen does not meet the demands of the tissues.

The partial pressure of oxygen in the air is 20% (140 mmHg), while the level of oxygen across the tissues varies between 1 and 14 % (10 to 110 mmHg)[8,9]. Hypoxia is therefore defined when the oxygen concentration is below these levels.

Tumors present a hypoxic environment where oxygen levels are less than 10-15 mmHg, equivalent to 2% oxygen[10].

The transport of oxygen to the peripheral tissues is done by erythrocytes in the blood stream. Adaptation to hypoxia involves different mechanisms including increasing erythropoietin levels which is followed by increased capacity of oxygen delivery to tissues by increasing the number and improving the function of red blood cells[11]. Research on the molecular mechanisms of hypoxia-induced upregulation of erythropoietin resulted in the characterisation of a part of the erythropoietin's promoter where a protein bound and transduced activation of its transcription[12]. Afterwards the protein mediating this hypoxic response was identified and named hypoxia inducible factor- HIF[13].

Now it is accepted that the molecular reaction to hypoxia is mainly mediated by HIF. HIF activates many genes that adapt cells to the compromised levels of oxygen, and the function of many of these genes is to increase angiogenesis (figure 1)[7].

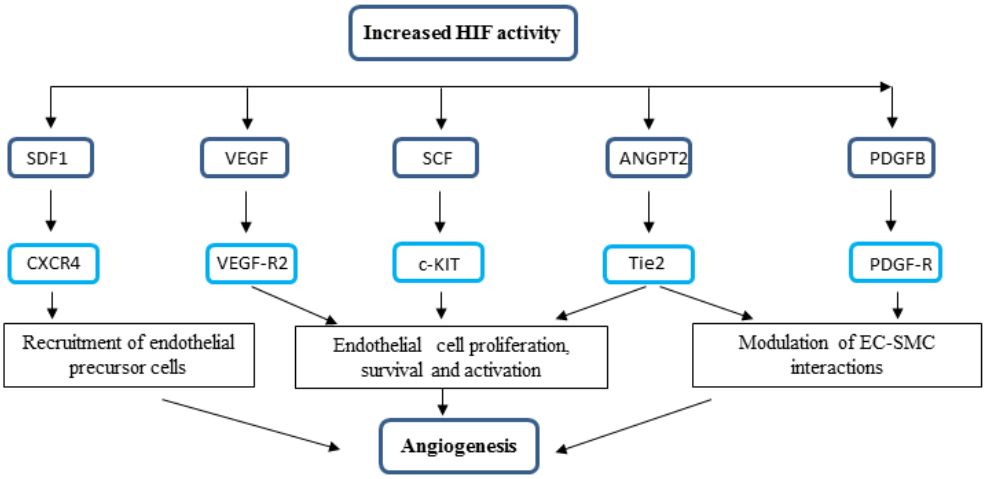


Figure 1: **HIF roles in upregulating angiogenesis.**
HIF activates the transcription of genes encoding secreted factors (row 1) and their receptors (row 2) that are important for angiogenesis. Adapted from G. Semenza, NEJM, 2011
Abreviations: SDF1 stromal derived factor 1, VEGF vascular endothelial growth factor, SCF stem cell factor, ANGPT2 angiopoietin2, PDGFB platelet-derived growth factor B, EC endothelial cells, SMC smooth muscle cells

2.2.1.1 HIF subunits

Initially, 60µg of highly purified HIF was obtained from 120 liters of HeLa cell culture and this allowed characterization of HIF as a heterodimeric transcription factor composed of two subunits: HIF-1α and HIF-1β [14]. HIF-1β, also called aryl receptor nuclear translocator (ARNT) was first cloned as the binding partner to the Ah (dioxin) receptor[15].

The two subunits of HIF belong to the family of proteins essential for development and homeostasis[16]. They contain a basic-helix-loop-helix and a PAS (bHLH-PAS) domain[17]. The two subunits of HIF bind to DNA only after hetero-dimerization[14]. The bHLH domain mediates both dimerization and DNA binding, whereas the PAS domain increases dimerization efficiency and confers DNA binding specificity[18-21].

Human HIF-1α subunit is an 826 aminoacids protein (Figure 2) with both bHLH and PAS domains located at the N-terminal ending, within the aminoacids sequence from 1 to 390[19]. The sequence between aminoacids 391 to 826 includes the oxygen dependent degradation domain (ODDD) which is responsible for HIF-1α degradation in the presence of oxygen[22] and two transactivation domains NTAD (N-terminal

transactivation domain) and CTAD (C-terminal transactivation domain) which are essential for HIF activity[23,24].

The HIF-1β subunit, contains a transactivation domain but with no importance for HIF function[25]. Moreover, HIF-1β lacks ODDD resulting in expression of HIF-1β even in the presence of oxygen.

Three HIF-α subunits (HIF-1α, HIF-2α /EPAS1, HIF-3α/IPAS) and two HIF-1β/ARNT isoforms (774 and 789 aminoacids)[14] have been described to date (Figure 2). Further, there are another two ARNT paralogues ARNT2 and ARNT3 (also known as bMAL/MOP3) which could function as alternative binding partners for HIF-2 alpha and HIF-3 alpha[26].

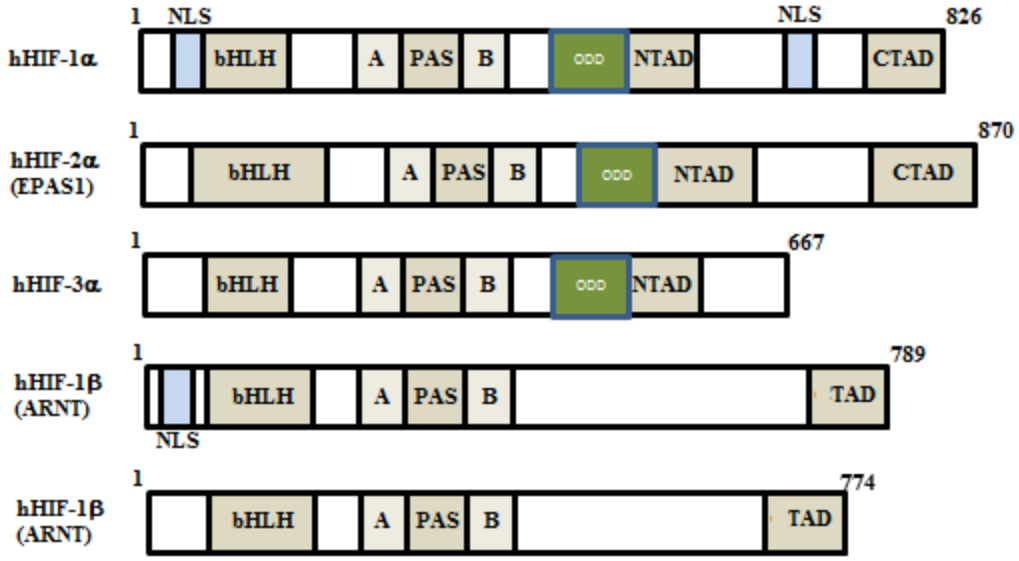


Figure 2: **Schematic representation of the HIF subunits.**
Abreviations: HIF-hypoxia inducible factor, bHLH- basic helix-loop-helix; PAS- Per-ARNT-sim, ODD- oxygen dependent, NTAD- N-terminal transactivation domain CTAD- C-terminal transactivation domain

The structure of HIF-2α resembles that its paralogue HIF-1α, with a 70% similarity of the N terminal part containing the bHLH and PAS domain[27,28] and high sequence homology within the C terminal transactivation domain[29]. Despite such a big similarity in structure and degradation pathways the two alpha paralogues are not redundant and have specific functions as well[30,31].

The third member of the alpha subunits (HIF-3α), shares high similarity with the other two alpha subunits, but has no C-terminal transactivation domain[32] and actually functions as a dominant negative regulator of HIF-1α[33] and HIF-2α[34]. HIF-3α has been identified as a HIF-1 target gene[35] being induced at mRNA levels in hypoxia[36].

The expression pattern of HIF-1α and ARNT is ubiquitous, while the other members have a restricted pattern of expression[26]. HIF-2α is expressed in endothelial cells, heart, liver, kidney, brain, and duodenum[27,28,37] while HIF-3 α is expressed in heart, brain, eye, skeletal muscles, lung, kidney and adult thymus[32,38].

Furthermore, cell-type specific pattern of expression have been noticed e.g. in kidney, where HIF-1α is expressed by tubular cells whereas HIF-2α is expressed mainly by endothelial cells and fibroblasts[39].

Availability of ARNT is crucial for HIF alpha actions, but is usually not an issue since it is present in large excess[26] .

After hetero-dimerisation, HIF binds to a core DNA sequence A/(G)CGTG within the hypoxia responsive elements (HRE) in the promoter region of the target genes, thereby exerting its activity[40].

2.2.1.2 HIF regulation

HIF function is mainly modulated by the oxygen-dependent regulation of available protein levels[40] with no HIF mRNA variation in response to hypoxia[41,42].

The canonical degradation pathway: PHD directed and VHL dependent

In normoxia, HIF protein level is kept low by the degradation of the HIF-1α subunit. The molecular basis of its degradation is the O_2-dependent hydroxylation of the proline residues[43-45] in the oxygen dependent degradation domain (ODDD) of HIF 1α. The proline residues are conserved between species and they locate in the aminoacid position 402 and 564 (HIF-1α), and 405 and 531 (HIF-2α and HIF-3α).

The reaction takes place under the control of a family of iron (II) – and 2-oxoglutarate dependent dioxygenase which hydroxylate the prolyl residues (PHD prolyl hydroxylases domain-containing protein) in the presence of oxygen[46,47]. There are three PHD paralogs important for the hydroxylation of HIF-α subunits, PHD1, PHD2 and

PHD3[46]. PHD2 is also called EGLN after the name of its gene first described in an abnormal egg laying phenotype in caenorhabdites elegans[47]. From experiments where the three PHD were knocked down individually by siRNA techniques, it turned out that the essential paralogue for HIF degradation in normoxia is PHD2[48]. Moreover, PHD2 not only controls the HIF-α degradation in normoxia but also degradation after re-oxygenation events[49]. However, the picture is more complex, since prolonged PHD2 inhibition induces PHD-1 which in turn degrades HIF-α protein in normoxia[48]. PHD3 might be an important regulator of HIF-2α subunit[50,51]. All three PHDs are expressed ubiquitously but the abundance level for their mRNA is cell and tissue specific[52].

The PHD- enzymes activity is conditioned by the presence of iron, 2-oxoglutarate and ascorbic acid. Chemical substances that compete or interfere with these co-factors such as iron chelators (desferoxamine- DFX), transition metals (cobalt in cobalt-chloryde) or oxoglutarate analogs (dymethyloxalylglycine-DMOG) induce potent PHD inhibition and consequently stabilize HIF, being called "hypoxia mimetics"[53,54].

In addition, an iron transporter called PCBP1 (poly (rC) binding protein 1) is responsible for the proper delivery of the iron to the PHD. Absence of PCBP1 reduces in consequence the HIF degradation by decreasing PHD efficiency[55].

PHD activity can be also decreased in normoxia by a low alpha-ketoglutarate to fumarate ratio[56]. Both fumarate and alpha-ketoglutarate are metabolites in the Krebs cycle which underscores the involvement of metabolic pathways in HIF modulation along with oxygen availability.

Interestingly, PHD2 is a HIF-1 regulated gene product and this creates a negative feedback loop by which HIF regulates its own stability[48].

Few other factors that interfere with the HIF prolyl-hydroxylation have been described. For example OS-9, a protein amplified in "osteosarcoma-9" has been shown to interact both with HIF-1α and with the PHD -2 and -3 and form a ternary complex which accelerates HIF hydroxylation[57]. However the role of this interaction in HIF degradation is controversial since there is no significant energy transfer between OS-9, HIF and PHD[58].

Oncogenes like RasV12 and v-Src induce HIF via inhibition of prolyl hydroxylation on residue Pro564 or via Akt-induced stabilization[59].

Factors such as ING4 from the growth inhibitors family are recruited in hypoxia by PHD resulting in a repressed HIF transcriptional activity[60].

Hydroxylated HIF-1α is polyubiquitinilated and targeted for proteosomal degradation. Ubiquitination involves the concerted action of ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin-protein ligase E3. E3 binds to the protein substrate and to E2, allowing the transfer of ubiquitin from E2 to the substrate. The von Hippel-Lindau suppressor protein (pVHL) acts as an E3 ubiquitin ligase and targets HIF-1α for 26S proteasomal degradation[61-64]. pVHL was first described and characterised in connection with the von Hippel Lindau syndrome that is an autosomal dominant inherited human tumor syndrome characterised by renal clear cell carcinomas (RCC), hemangioblastoma of the central nervous system, retinal hemangiomas and pheochromocytoma[65,66]. All these tumors express a high angiogenic phenotype and overexpress HIF which linked HIF with VHL.

VHL has 2 domains: α and β, and it binds through its β domain to the hydroxylated form of HIF-1α subunit[67] and, through its alpha subunit serves as binding partner to the elongin C/elongin B and Cul2 Rbx1 proteins forming the VBC-CR complex[68,69]. It is through the VBC-CR complex that HIF-1α is ubiquitinated and thus labelled for proteosomal degradation[63,70].

This complex is stabilized by SSAT2 (Spermidine/Spermine-N1-Acetyltransferase 2) which binds to HIF-1α, VHL and elongin C and further promotes HIF ubiquitination[71].

VHL-dependent HIF degradation could be accelerated by additional mechanisms. For example, one lysine residue in the 532 position can be acetylated by an acetyltransferase enzyme called ARD1 (Arrest Defective Protein-1) which results in an increased affinity for pVHL and subsequent increased HIF-1α degradation[72].

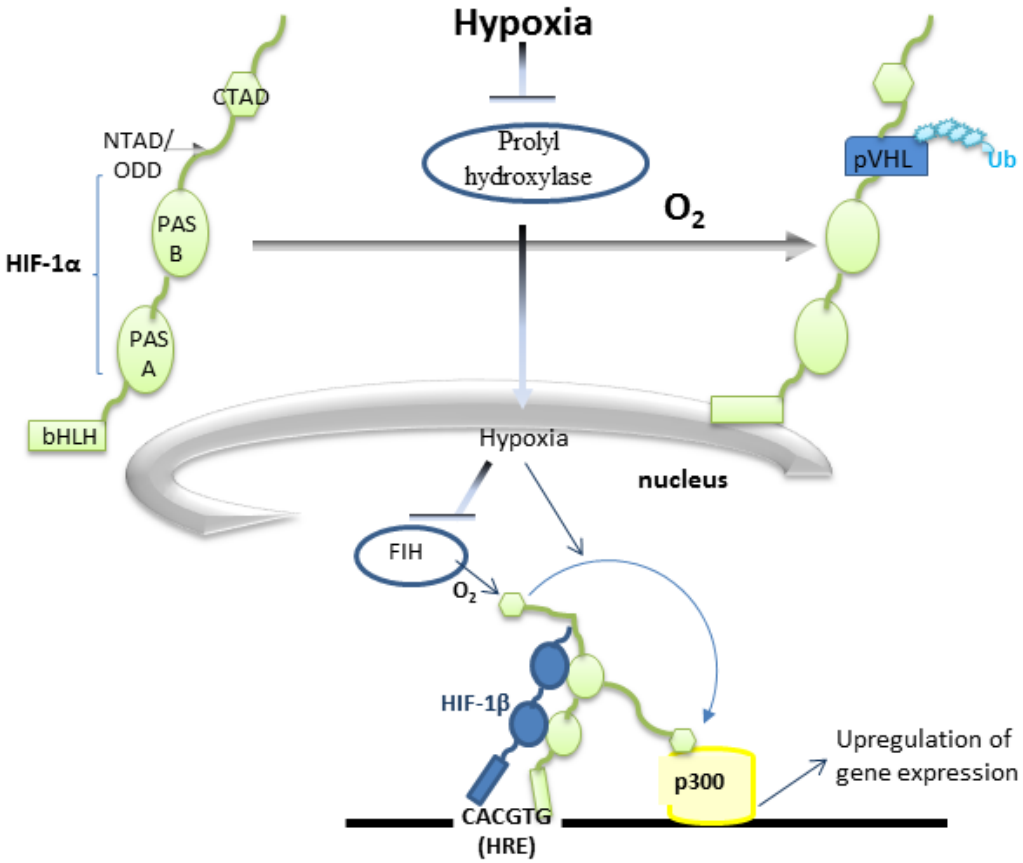


Figure 3:**Oxygen dependent regulation of HIF-1 α**
(adapted from Bruick, R and McKnight, S.L, Science 2002[73])
Abreviations:HIF- hypoxia inducible factor, FIH -factor inhibiting HIF, HRE- hypoxia responsive element,HIF domains: bHLH, PAS-A, PAS-B, ODD, NTAD, CTAD

pVHL dependent but PHD independent degradation pathway

Even though, the canonical model of HIF-1α degradation involves prolyl-hydroxylases activity, HIF degradation in normoxia can be registered independent of them. The mechanism still involves VHL dependent ubiquitination but takes place on a HIF variant which is resistant to prolyl hydroxylation as a consequence of the mutation of both prolyl residues to alanine[74].

Candidate proteins involved in this degradation pathway are the small ubiquitin like modifiers (SUMO), a family of ubiquitin-like proteins reported to affect many biological functions and required for cell viability[75,76], with three isoforms SUMO1, 2 and 3.

SUMOylation system is similar to the ubiquitin pathway containing an activating enzyme E1, a conjugating enzyme E2 (Ubc9) and a ligating enzyme E3. Ubc9 directly binds to the substrate protein and in its bound form recruits the E3 ligase and the pVHL and directs the protein to proteosomal degradation.

SUMOylation is a dynamic, reversible process and it happens almost simultaneously with de-SUMOylation of the same proteins. De-SUMOylation is mediated by SENP (SUMO-specific proteases), and there are 6 SENPs that have been described to date[77]. For most proteins SUMOylation results in an enhanced activity. However, in case of HIF- 1α the results are ambiguous. Both activation[78,79] and repression of HIF following SUMOylation have been reported[80,81]. The function of SENPs is still unclear since increased SUMOylation via down-regulation of SENP-1 is reported to be involved in pVHL-dependent destabilization of HIF[81],while SENP-3 can increase HIF transactivation via de-SUMOylation of p300, leading to angiogenesis[82].

pVHL independent HIF degradation

HIF degradation occurs even in hypoxic conditions or in cells defective of VHL (VHL-/- cells)[83] suggesting alternative degradation pathways independent of the canonical VHL system.

It has been shown that p53 binds directly to HIF-1α and mediates ubiquitination via Mdm-2 (mouse double minut 2 homologue) which function as an E3 ligase[63,84]. This interaction between HIF and p53 is blocked in the presence of Jab-1, a co-activator involved in cell- proliferation, cycle control and inflammatory response pathways[85] or by Kruppel-like factor 5 as recently shown[86].

Rack-1(receptor for activated C-kinase-1), a scaffolding protein, has been validated as interacting protein for HIF by proteomics approach[87]. RACK-1 induces HIF degradation even when both proline residues in the ODDD are mutated to alanines suggesting a PHD - independent pathway[87]. RACK1 binds to Elongin-C and recruits Elongin-B and other components of E3 ubiquitin ligase to HIF-1α directing HIF-1α to a pVHL independent proteosomal degradation.

Hsp90 has been also shown to associate to the PAS domain of HIF-1α and prevents the pVHL independent proteososmal degradation[88,89]. However, RACK-1 and Hsp90

compete for the same binding site on HIF, therefore maintaining a balance in the level of HIF degradation[87].

NEDD8 mediates an alternative mechanism for HIF stabilisation which acts at the degradation level and is reactive oxygen species (ROS)-dependent and pVHL-independent. NEDD8 is required for HIF stabilization in hypoxia and approximately 30% of HIF stabilization in hypoxia is NEDD8 dependent[90].

Interestingly, another alternative degradation pathway for HIF-1α has been recently described, which is independent of proteosomal degradation, but instead takes place in the lysosomes through chaperone-mediated autophagy[91].

Modulation of HIF activity by posttranslational modification of transactivation domain

HIF activity in hypoxia is not only modulated at the protein level, but also regulated by posttranslational modification of its two transactivation domains, NTAD and CTAD [92]. The function of HIF is therefore not increased by blocking the proteosomal degradation since this does not modulate the transactivation domains[93,94]. The NTAD overlaps with ODDD[62] thus its transcriptional activity is largely coupled to protein stability. However the CTAD transcriptional activity is mainly regulated by the recruitment of transcriptional coactivator complexes through factor-inhibiting HIF-1 (FIH-1) [95]. In the presence of oxygen an asparagine residue in the CTAD region is hydroxylated through a reaction catalysed by FIH-1, which is another iron and oxoglutarate-dependent oxygenase[96] which interferes with the recruitment of co-activators. In hypoxia, FIH is not active and co-activators such as CBP/p300 interact with both HIF-1 alpha transactivation domains to activate gene transcription[94,95,97]. Moreover, the reaction is enhanced by accessory coactivators, SRC-1, TIF2 and Ref-1[98].

It is interesting to note that the *Km* of FIH-1 is approximately 100μM [99] while for PHDs it is 200μM [100], which suggests a range of oxygen levels, where there is not enough oxygen to promote HIF degradation but low enough oxygen to limit the transactivation. The multiple levels of regulation therefore allow graded responses to subtle changes in O2 concentration.

The availability of the co-activators limits HIF transactivation. For example, CITED 2 and CITED4 compete with HIF for the binding of CBP/p300 and interfere with HIF activity[101,102]. The binding of CITED 2 to CBP/p300 increases in clinical situations like chronic kidney disease which impairs the adaptation of the kidney to hypoxia with pathogenic consequences [103].

The HIF transcriptional activity can also be increased by binding to Jab1[85].

RTEF-1 (related transcriptional enhancing factor-1) enhances HIF-1α transcription. By inducing HIF-1α transcription in endothelial cells, RTEF-1 accelerates endothelial tube formation and enhanced cell aggregation in matrigel models. In addition, accelerated ischemia recovery is observed in endothelial cell-specific RTEF-1 transgenic mice[104].

Sirtuins (Sirt) are regulators of metabolism which function as NAD^+-dependent proteins deacetylases and/or ADP-ribosyl-transferases. Some Sirtuins regulate HIF function.

Sirt-1 deacetylates HIF-1α and in this way modulates the HIF-1α accumulation and activity in hypoxia[105]. Sirt-1 gene expression increases in a HIF-dependent manner during hypoxia and augments HIF-2α transcriptional activity[106], [107].

Sirt-3, which mainly acts on mitochondrial metabolism, is a negative regulator of HIF1α[108],[109]. This effect has been attributed to the function of sirtuin3 to reduce mitochondrial ROS production, which inhibits PHD hydroxylase activity.

Sirt7 (another member of the same sirtuin family) impairs the function of both HIF- α subunits[110].

Several hormones and growth factors increase HIF activity, e.g. insulin[111], IGF-I[112,113], IGF-II[114], EGF[115] , angiotensin II (Ang II), thrombin, and platelet-derived growth factor[116]. They stabilize HIF-1α independent of its oxygen regulation. The main pathways activated by the growth factors are dependent on signaling via mitogen-activated protein kinase (MAPK)[117] or phosphoinositol 3-kinase (PI3K)[118]. The same pathways are used by receptor tyrosine kinases (RTKs) and Ras, and some tumour suppressors, such as phosphatase and tensin homologue (PTEN) during oxygen independent regulation of HIF[119].

MicroRNA (miRNA) are small, non-coding, single stranded RNA molecules containing only 22-23 bp, which couple to the 3' UTR region of target RNA and inhibits their translation. miRNA are generated from larger, several kilobase pair structures (pri-miRNA) which are transcribed under the control of RNA polymerase II. The pri-miRNA are capped and polyadenylated and contain a hairpin structure, the stem-loop. The stem-loop structure is cleaved in the nucleus by an RNAase, Drosha and the products are released into the cytoplasm as pre-miRNA[120]. The pre-miRNA are cleaved in the cytoplasm by an enzyme called Dicer with the release of mature miRNA [121].

miRNA are essential for development[122] and their impaired expression has been correlated with cardiovascular and inflammatory diseases and cancers.

Recently microRNAs have been suggested to mediate some of the HIF-1 functions[123]. miRNA-199a and miRNA-155 modulate HIF reaction to hypoxia[124], [125]. Interestingly, under prolonged hypoxia HIF-1 induces miRNA- 155 resulting in a negative feedback mechanism. MiR17-92, directly represses HIF-1 in normoxia but not in hypoxia[125,126]. MiR 424 is induced by hypoxia, and downregulates CUL2 which is a scaffolding protein critical to the assembly of the ubiquitin ligase system, and thus regulates the degradation of HIF alpha isoforms and promotes angiogenesis[127].

2.2.1.3 HIF function

The main function of HIF is to act as a sensor for oxygen levels, being able to bind to or dissociate from its binding sites on the target gene DNA in less than 1 minute[40].

HIF binds to the HRE in the promoter region of over 100 target genes and adapts the cells to hypoxia by regulating processes like red blood cell production (erythropoietin), angiogenesis (vascular endothelial growth factor-VEGF, angiopoietin 1 and 2), cellular survival and proliferation or cell metabolism[128,129] (Figure 4).

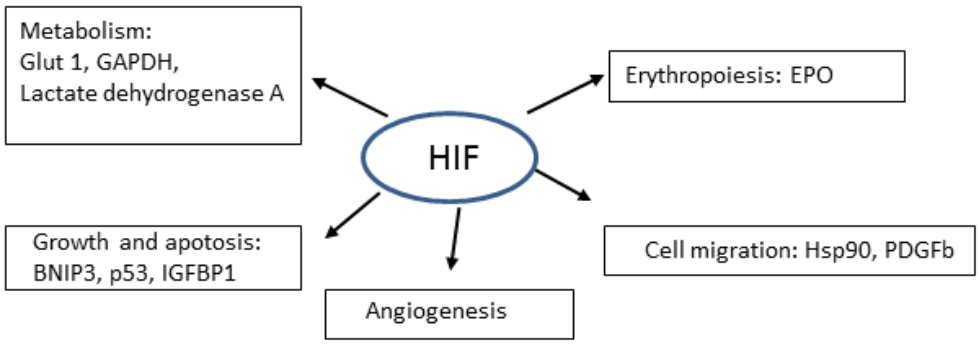


Figure 4**: HIF functions.**
Abreviations:HIF- hypoxia inducible factor, Glut-1- Glucose transporter-1, GAPDH- Glyceraldehyde-3-PDH, IGFBP- insulin growth factor binding protein, EPO- erythropoietin, Hsp90- heat shock protein 90

Many of the target genes are common for the two paralogs, HIF-1α and HIF-2α. However, some genes are regulated just by one of the isoforms such as BNIP3 for HIF-1α and VEGF, Oct4 by HIF-2α[130]. The target gene specificity seems to be related to NTAD whereas CTAD is mainly controlling the expression of common targets[130].

Moreover, the coactivator CBP/p300 has been related to the selectivity for target genes since p300 modulates the transcription activity of HIF-1α, while CBP is involved mainly in translating the signals from HIF-2α[107].

HIF is essential for angiogenesis and regulates directly or indirectly more than 2% of all human genes in endothelial cells[131]. This role is also underscored by the phenotype of the knockout mice which lack different components of the system.

Mice that are completely deficient of either HIF-1α or HIF-1β die during embryonic life principally due to vasculature defects. Mice deficient in HIF-1α die in E11 due to severe cardiovascular and neural malformations and complete lack of cephalic vascularisation[132,133]. Similarly ARNT knockout mice die in day E10.5 due to defective angiogenesis and failure of the embryonic component of the placenta to vascularize[134,135].

HIF-2α knockout mice show a variation of phenotypes, while some models die during embryonic development with vascular disorganization or catecholamine deficiency[136],

other models die shortly after birth due to respiratory distress syndrome related to inefficient production of VEGF[137].

HIF functions related to tumorigenesis are detailed in chapter 3.

2.2.2 IGF-I

IGF-I (Insulin like growth factor-I), the major component of the insulin-like peptides family (somatomedins) plays a central role in development through its growth promoting effects[138]. IGF-I is expressed by virtually all tissues.

Other components of the IGF system include IGF-II and insulin, their receptors IGF-IR, IGF-IIR and insulin receptors. Furthermore to the system belong the insulin like growth factor binding proteins (IGFBPs) which represents a family of 6 proteins that bind with great affinity both IGF-I and IGF-II thus regulating their availability for the receptors[139].

The IGFs signals are transduced after coupling of the agonist to the receptors, which belong to the tyrosine kinase class of membrane receptors[140]. Because IGFs have 50% homology with insulin, they could also bind to the insulin receptors.
Insulin- receptor and IGF-R have a complex structure composed of two extracellular alpha chains which bind to the ligand and two trans-membranary beta chains which have tyrosin-kinase activity**.** One alpha and one beta chain form a half-receptor which will dimerise to another half to form a complete receptor. The two dimers are bound by disulfide bonds[141].
Moreover, there is a 60% similarity between IGF-IR and insulin receptor which gives the possibility for hetero-dimerisation (one IGF-IR-$\alpha\beta$ complex and one IR-$\alpha\beta$ subunit complex) with formation of hybrid receptors important mainly in tumorigenesis[142]. However, the affinity of the IGF receptor is 1000 fold greater for IGF-I than for insulin and the insulin receptor has a 100 fold greater affinity for insulin than for IGF-I.

After binding with the ligand, the tyrosin-kinase is activated and auto-phosphorylates the receptor, which will recruit substrates like insulin receptor substrates 1 to 4 (IRS 1-4) and Shc (colagen domain protein) and this will initiate the cascade of reactions for IGF signal transduction[143]. IRS will further activate additional substrates like the p85

subunit of PI-3kinase which in turn activates Akt also known as protein kinase B (PKB) (figure 5)[144].

Akt signaling on the mTOR (mammalian target of rapamycin) pathway is conserved in all eukariotes and it transduces the signal for protein synthesis[145]. Akt can also activate Bad-Ccl2 pathway resulting in the inhibition of apoptosis.

Additionally, the coupling between IRS-1 and Shc activates the Ras-Raf-1/MEK pathway which controls the celullar proliferation.

An IGF-IIR (Manoso-6 phosphate receptor) has been described which binds IGF-II which internalizes and targets IGF-II to degradation without signal transduction. However, IGF-II could also bind to IGF-IR but with lower affinity than IGF-I.

The availability of the agonists to the IGFR is also conditioned by IGFBPs which controls their bioavailability[146]. IGFBPs can be modified in function by specific proteases[147]. Moreover, they are susceptible to post-translational modifications such as phosphorylation which influence their binding capacity for IGF-I[148].

IGFBP-3 is the principal binding protein in serum and forms binary complexes with IGF-I or ternary complexes which also involves the acid labil subunit (ALS). Both IGFBP-3 and ALS are under the control of Growth Hormone (GH). IGFBP-5 forms similar complexes with IGF-I[146]. The ternary complex (150kDa) is too large to cross the vascular wall and therefore the half time of IGF-I in serum is prolonged from minutes to several hours.

The binary complexes (40-50kDa) could however cross the vascular wall and deliver the IGF to the targeted tissues.

IGFBP-1 and 2 form only binary complexes with IGF-I, that contribute marginally to the half time of serum IGF-I. However, IGFBP-3 and 5 are saturated under normal circumstances but not IGFBP-1 and 2, which makes that change in their level influence markedly the free IGF-I levels and in consequence the biological response[149] . The levels of IGFBPs are also modified by specific proteases[150]. Every IGFBP is specifically regulated by different factors i.e. IGFBP-1 is centrally regulated by insulin but can be also influenced by hypoxia, cytokines, stress[151-154] and DDAVP[155].

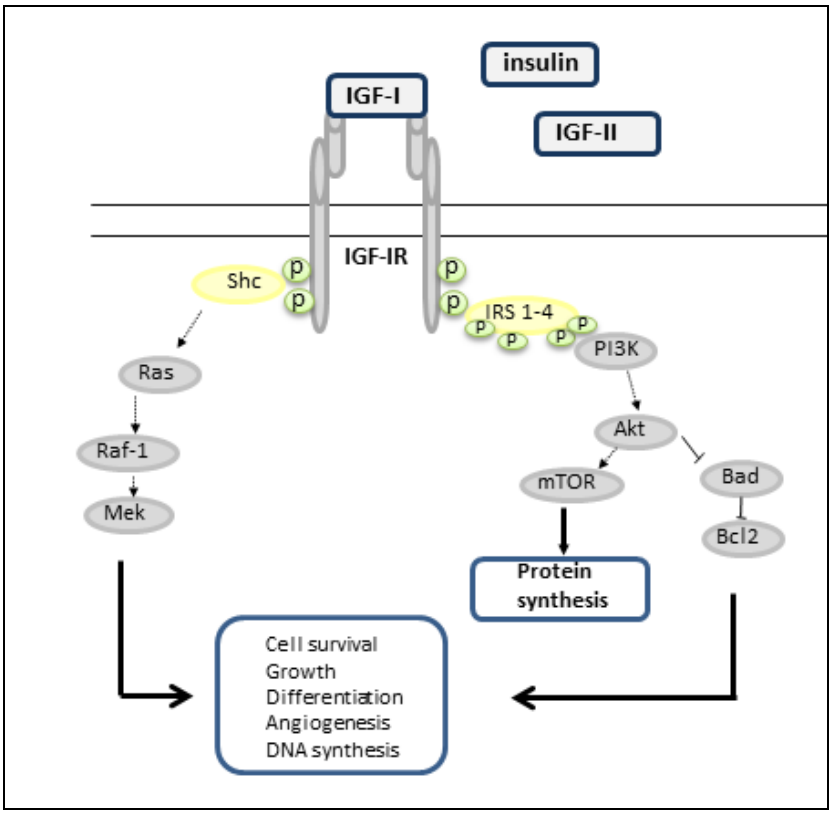


Figure 5: **IGF- receptor and its intracellular pathways**

Canonically, it was considered that circulating IGF-I is produced in the liver under the control of growth hormone (GH) and plays the main role in controlling the body growth[156].

However this theory was challenged by the finding that mice lacking the liver secretion of IGF-I have unaffected postnatal body growth[157-159]. Moreover, a local secretion of IGF-I has been demonstrated in multiple tissues[160].

Beyond the key role as body growth regulator, IGF-I plays critical roles to maintain the normal function of several organs e.g. kidneys, cardiovascular system, brain[161].

2.2.2.1 IGF and angiogenesis

IGF-I is an essential factor for vasculogenesis and angiogenesis in embryonic life, as it is involved in the development of mesodermal layer, maintaining of stem cells precursors and differentiation of the endothelial cells from the embryonic mesodermal layer cells[162]. Moreover, deficiency of IGF-I leads to impaired retinal development even in the presence of VEGF[163].

Due to its role in the vascular system, IGF is also involved in the proper development of the different organs. For example IGF-I is a potent angiogenic signal for fetal lung endothelial cells and by this affect the morphology of the lung as well[164].

The IGF system is also essential for the homing of the endothelial precursor cells during neovascularization process[165]. The neovascularization after ischemic injury of the retina in diabetes, is also under the influence of IGF-I together with other factors (FGF-2, VEGF etc) secreted by the retina cells.

IGF-I and IGF-IR are expressed by endothelial cells[166-168] and protects them from atherosclerosis through their anti-apoptotic[169] and anti-inflamatory properties. IGF-I stimulates the migration and angiogenesis of endothelial cells[170]. Moreover, IGF-I signaling via the PI3/akt pathway[171,172], phosphorylates NOS which results in NO synthesis and vasodilation [173] .

In cardiomyocytes IGF-I has protective effects and stimulates neovascularisation[174].

IGF-I secretion from brain microvascular endothelial cells enhances in response to ischemic injury and increases the survival of neurons making IGF-I a potential therapeutic target for ischemic stroke[175].

Recently the IGF system has been coupled with angiogenesis via activation of αvβ3-integrin, which are expressed especially by the activated endothelium during angiogenesis[176,177] with protective effect against apoptosis. IGF-IR and αvβ3- integrin forms complexes with SDC-1 (syndecan-1) that is a family of cell-surface proteoglycans and accelerate endothelial cells migration[178].

Apart from its direct effects on endothelium, IGF-I maintains and potentiates the cross-talk with other pro-angiogenic factors such as VEGF and FGF-2[179].

2.2.3 Other regulators of angiogenesis

IGF and HIF interact in normal vasculogenesis and angiogenesis modulating the secretion of angiogenic factors[162]. VEGF has been related with angiogenesis induced by hypoxia since its discovery[180]. IGF-I increases endothelial differentiation by increasing HIF function which results in enhanced secretion of VEGF[162].

VEGF (VEGF-A) was discovered as a vascular permeability factor [181,182] and is part of a family of growth factors which are key effectors and regulators of physiological and pathological angiogenesis acting through tyrosine kinase receptors[6]. The other members are VEGF-B, -C,-D and placental growth factor (PLGF). These factors bind to the three receptors VEGFR-1 (previous Flt-1), VEGFR-2 (former Flk-1/KDR) and VEGFR-3 (previous Flt-4)[183]. VEGFR-3 binds only VEGF-C and D and all these three members are related with lymphatic angiogenesis[184]. In addition, VEGF interacts with a family of coreceptors called neuropillins which are necessary for correct VEGF signaling, especially during vascular morphogenesis[185].

VEGF binds to both VEGFR-1 and VEGFR-2, but it is the VEGFR-2 receptor which mediates the main functions of VEGF related to its angiogenic and vascular permeability activity.

VEGF's essential role in angiogenesis is highlighted by knockout mice models: the VEGF knock-out mouse dies at embryonic day 11 with abnormalities related to defective angiogenesis[186,187]. Similarly, the mouse with VEGFR-2 deficiency dies in the embryonic day 8-9 due to deficient vasculogenesis[188]. Moreover, VEGF is required for normal growth and survival[189] and maintains vascular homeostasis[190] and promotes proliferation[191], migration and has an anti-apoptotic role for the endothelial cells[192-194].

VEGF has been extensively investigated in relation with diabetes. There is no clear information about the serum VEGF levels in patients with diabetes since both increased and unmodified levels have been reported[195-198]. These differences could reside either in methodological differences in inhomogeneity of selection of the patient group, e. g: duration of disease, type and duration of diabetes complications, treatment etc.

VEGF has been also investigated in relationship with diabetic nephropathysince it is important for the function of the kidney[199].

Augmented expression of VEGF and its receptors has been demonstrated in both type 1 and type 2 models of diabetes in animals[200-202] and therapeutically VEGF inhibition has proven clinical benefits[202].

VEGF plays a central role in the vascular lesions observed in diabetic retinopathy, ranging from the occlusion and leakage of retinal vessels, which lead to macular edema, to the highly permeable vessels in the proliferative phase of retinopathy[203]. However, recently a new concept emerged regarding diabetic retinopathy, which involves not only the retinal vascularization, but postulates a tight communication between endothelial cells, neurons, glial cells and pericytes within the so called "neurovascular unit", all the unit participating in VEGF secretion dysfunction. A more complex therapy that targets several of these factors could result in better control of the diabetic retinopathy[203].

Expression of VEGF and its receptors is seen in almost all tumors and is associated with poor prognosis. Moreover, some tumor cells secrete VEGF, which acts as a growth factor for the tumor[204].

Because VEGF has many roles in normal and pathological angiogenesis, therapies targeting VEGF are now developed for the treatment of diseases with dysregulated angiogenesis.

VEGF inhibitors are already used in the clinic for controlling tumor angiogenesis[5] and the excessive vascular leakage in diabetic retinopathy[203] while therapies that provide VEGF are explored for the treatment of ischemic events[205]. However the clinical results are not as impressive as expected partially because VEGF is just one member of a large complex of factors that regulate angiogenesis, and partially because drug resistance develops in many cases[206]. It is therefore highly important to make a better characterization of the angiogenic events to be able to design more efficient therapies.

Although VEGF is the most prominent angiogenic promoter it is not sufficient for the neovascularization process. Other factors are required, stimulators as well as inhibitors of angiogenesis.

The VEGF signaling activates the endothelial cells (EC) and contributes to the degradation of the basement membrane that will create the environment for EC to migrate. The selection of the endothelial cells towards tip cell or stalk cell is determined by the interplay between VEGF and Notch[207]. The tip cells will become the migrating endothelial cells during neovascularization which is mainly under the control of Dll4 whereas stalk cells will become the proliferating EC during the same process under the

control of Jagged1. Overall, DLL4 and Notch signaling restricts branching but generates perfused vessels[207] .

Fibroblasts growth factors (FGF) have been the first described as pro-angiogenic factors [208]. They exert their function after the degradation of the matrix in synergy with VEGF[209].

Angiopoetins represent another family of angiogenetic factors. Together with VEGF they have a high specificity for the endothelial cells. They act via tie-2 receptors and interfere with the later phases of angiogenesis, mainly during vessels ramification and remodeling or to promote the capillary stability[210].

Platelet derived growth factors (PDGF) and their tyrosine kinase receptors are important for migration and proliferation of the endothelial cells [211] during normal angiogenesis but also for the recruitment and regulation of tumor fibroblast during pathologic angiogenesis[212]. PDGFB and PDGFR-β are essential for vascular maturation, and inactivation of either PDGFB or PDGFR-β leads to pericyte deficiency, vascular dysfunction, micro-aneurysm formation, and bleeding[211].

Recently, the metabolic sensor peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) has been shown to stimulate angiogenesis in ischemic tissues and after exercise[213,214]. PGC-1α a potent regulator of metabolic processes[215] also controls angiogenesis adapting in this way the oxygen supply to the demand of the cells[216]. PGC-1α regulates angiogenesis and VEGF, independent of HIF[213]. This underscores again the multiple control levels for regulation of angiogenesis.

Other stimulators of angiogenesis include P1GF, TGF-β, SDF-CXCL12 system and sphyngosine 1 phosphate receptor[5,6].

On the other site are the inhibitors of angiogenesis, e.g. endostatin, trombospondin and caplostatin[217] that also have been proposed as therapeutic targets for normalizing angiogenesis[217].

2.3 HIF AND IGF-I SIGNALING IN A DISEASE MODEL WITH REDUCED ANGIOGENESIS RATE

2.3.1 Angiogenesis and diabetes complications

Diabetes has reached epidemic proportion with a worldwide incidence of over 300 million affected people and the number is predicted to raise dramatically[218,219]. The complications associated with prolonged exposure to hyperglycemia contribute to the increased mortality[220] and morbidity[221,222] in patients with diabetes compared with patients without diabetes.

Endothelial dysfunction and aberrant angiogenesis have been associated with all chronic complications of diabetes including both macrovascular complications, i.e. cardiovascular disease, stroke, peripheral arterial disease and microvascular complications as in diabetic nephropathy, diabetic neuropathy, retinopathy and diabetic foot ulcers.

Moreover, it has been shown that angiogenesis is impaired even after successful glucose control due to the fact that tissues preserve a memory of hyperglycemia[223].

Interestingly, the dysregulation of angiogenesis in diabetes complications is in both directions, ranging from deficient angiogenesis in wound healing and myocardial perfusion to overshooting angiogenesis as in retinopathy or atherosclerotic plaque.

Therefore characterizing the mechanisms for vascular dysfunction in diabetes is a promising field which could lead to the development of alternative therapies in the management of diabetes and its complications. In fact, therapies targeting angiogenesis, mainly directed against VEGF are already current medical practice especially for diabetic retinopathy[203]. However, the results are not optimal all the time as in the case of diabetic nephropathy[202,224-226] which suggests that the angiogenesis in diabetes is a complex field which is only partially understood and warrants further exploration.

2.3.2 Diabetic foot ulcers

Diabetic foot ulcer is a debilitating complication of diabetes associated with decreased angiogenesis. Almost 25 % of the patients with diabetes are at risk of developing foot ulcers[227]. Despite progress in the control of hyperglycemia, delayed wound healing in

diabetes remains a common complication and every 30 seconds one diabetic foot is lost in the world by amputation[228].

The therapeutic options besides the metabolic control include off-loading, treatment of infections and improvement of blood flow. The available therapies, however, are insufficient and almost 10% of patients eventually undergo amputation[229,230]. About 85% of cases of major lower limb amputations in patients with diabetes are due to preceding ulcers[231].

The development of diabetic foot ulcers is multifactorial and includes peripheral vascular disease, microangiopathy[232,233], neuropathy and even a reduced blood flow due to hyperglycemia[234].

Due to neuropathy, the patients lose the sensation of pain, which would normally trigger the avoidance of injuring factor and raise awareness of the existent lesion[235,236]. Moreover, due to pain insensitivity the patient applies repetitive stresses on a preexistent lesion which leads to poor healing and ulcer chronicity [237].

Motor neuropathy affects the small muscles of the foot and results in disturbances of the normal movement and weight distribution during walking creating areas of high pressure where calluses develop. These areas will be more prone to ulcer development. In addition, there is also autonomic neuropathy affecting the sympathetic tone with altered function of the sweat glands and consequent dryness, fissuring and ulcerations of the skin[238].

In the end, the existence of peripheral neuropathy results in almost 3 times increased risk to develop foot ulcer in patients with diabetes[239,240].

Peripheral arterial disease (PAD) is present in many cases of diabetic foot ulcers [241] and is a major risk factor for amputation [235,242]. Furthermore, the coexistence of sensory and autonomic neuropathy delays PAD diagnostic in patients with diabetes. The severity of the PAD predicts wound healing potential[241]. The severity of the PAD could be appreciated by markers like transcutaneous oxygen pressure (TcPO2). It is generally accepted that a wound will heal if the TcPO2 is higher than 50mmHg whereas values under 30mmHg will severely impair healing[243].

A recent meta-analysis of the algorithms for stratification of the risk to develop diabetic foot ulcer has identified diabetic neuropathy, peripheral vascular disease, foot deformity, previous ulcer and previous lower extremity amputation as the most common predictors[244].

Wound healing is a complex and a well-coordinated succession of events which include clot formation, inflammation, re-epithelialization, angiogenesis, granulation tissue formation, and tissue remodeling[245,246]. These events need the coordinated actions between different cell types like fibroblasts, keratinocytes, endothelial cells and macrophages under the stimulation of growth factors and cytokines[224,225].

Diabetes has a repressive effect on most of these processes [247,248] including growth factors secretion[249], cell migration [250], macrophages functions [251], the capacity of metalloproteinases to remodel the extracellular matrix[252] and angiogenesis [253,254]. There is also a reduced production of SDF-1 and CXCR4 which will impair the recruitment and function of EPC and will also contribute to the deficient angiogenesis[255-257].

Furthermore, the cellular functions are impaired by high glucose and reduced proliferation and adhesion of endothelial cells or vascular smooth muscle cells have been shown [258].

2.3.3 Mechanisms of chronic complications of diabetes. Radical oxygen species (ROS) in diabetes.

Hyperglycemia causes organ failure by affecting the functions of cells that are unable to maintain constant level of intracellular glucose. The endothelial cells important for angiogenesis are such an example, along with mesangial cells or cells in the peripheral nerves.

Several mechanisms have been proposed to explain how the increased intracellular glucose influx results in cellular damage. The first mechanism is through increased polyol pathway. It implies that the excess intracellular glucose is degraded to sorbitol by aldose reductase. Sorbitol is further oxidized to fructose in a reaction dependent on NADPH. However, NADPH is also necessary for maintaining the reduced form of the antioxidant glutathione which further means that in hyperglycemia the glutathione level

is not maintained and the cells are vulnerable to oxidative stress[259]. Indeed, overexpression of aldose reductase results in glutathione deficit[260].

The second mechanism involves the production of AGE (advanced gylcosylated end products) which is responsible for modifications and impaired activity of intracellular or extracellular proteins. The extracellular proteins as for example albumin, bind after glycosylation to the receptors for AGE (RAGE) and determine the secretion of cytokine and growth factors, thereby initiating inflammation cascade and vascular pathology. In addition, there is an enhanced RAGE expression in response to high glucose[261].

Another pathway is the protein kinase C α, β, δ (PKC) pathway, which is activated by diacylglycerol produced from excessive intracellular glucose[262]. It modulates the gene expression for proteins involved in vascular contraction (eNOS and endotelin-1), angiogenesis and vascular permeability (VEGF), vascular occlusion (PAI-1 and TGF-β) or inflammatory processes via activation of NF-kB[263,264]. Moreover, activated PKC determines PDGF receptor-b dephosphorylation which results in pericytes apoptosis[265].

The last mechanism involves an increased flux through the hexosamine pathway with posttranslational modifications of proteins and deleterious effect on diabetic blood vessels[266,267]. In this pathway glucose-6 phosphate is metabolized to fructose-6 phosphate and further diverted to UDP (uridine diphosphate) N-acetyl glucosamine via glucosamine-6 phosphate. N-acetyl glucosamine in turn posttranslationaly modifies proteins such as SP1, TGF-α and TGF -β1[267].

All these mechanisms have been unified into a single theory which states that radical oxygen species (ROS) overproduction in mitochondria in hyperglycemia is responsible for the activation of all the above mentioned mechanisms[268]. The increased ROS production in mitochondria causes DNA strand breaks that activate poly(ADP-ribose) polymerase enzyme (PARP) which further decreases glyceraldehyde-3 phosphate dehydrogenase (GAPDH)[269].

This event is followed by activation of all the other pathogenic pathways suggested to underlie chronic complications in diabetes e.g. PKC activation, AGE products formation, increased hexosamine activity and increased flux through the polyol pathway[266,268] .

2.3.3.1 ***HIF and ROS***

Hypoxia is an additional pathogenic factor in diabetic complications beside hyperglycemia [270]. In hypoxia, in presence of normal glucose concentration HIF is activated and controls expression of different genes involved in the maintenance of ROS production within the normal levels.

Activated HIF controls mitochondrial ROS generation at a multi-level process: it represses mitochondrial biogenesis and respiration[271], it decreases mitochondrial mass by autophagy[272], it shunts pyruvate away from the mitochondria by activating PDK1 gene[273,274], it increases the efficiency of cytochrome c oxidase which decreases ROS formation from complex IV[275]. HIF increases also the lactate dehydrogenase activity which increases the flow of glucose through anaerobic glycolysis decreasing in this way its access to the aerobic glycolysis and to the secondarily ROS production[276]. The effect of hyperglycemia on these control points is not known, but is part of this thesis investigation.

2.3.4 IGF-I in diabetes

IGF-I plays important roles in diabetes which is underlined by the fact that mice who present only 25 % of the normal serum IGF-I levels develop impaired glucose tolerance associated with increased insulin resistance and are prone to develop diabetes easier [277,278]. Lower levels of IGF-I are also present in type 1 diabetes patients[279,280], mainly due to the inadequate liver IGF-I secretion due to the lack of insulin[281,282]. In type 2 diabetes, the IGF-I levels are more related to the levels of IGFBP[283]. In the beginning of disease the IGFBP-1 levels are reduced due to an increase of insulin secretion in response to insulin resistance which results in higher level of circulating IGF-I[151,284]. However as the disease progresses, the liver becomes resistant to insulin induced suppression of IGFBP-1 and consequently the circulating IGF-I levels decrease[285,286].

IGF-I correlates also with insulin resistance[287]. Systemic IGF-I administration has been tried as complementary therapy to insulin and was associated with enhanced insulin sensibility, decreased insulin requirements and better glucose control[288-290]. However, side effects e.g. edema, worsening of retinopathy, headache, arthralgias, jaw pain, significantly has limited its use in diabetes.

IGF-I signaling is associated with many chronic complications of diabetes, such as diabetic retinopathy[291-293], diabetic nephropathy[294,295] and diabetic wound healing[296]. In the diabetic kidney, IGF-I and GH are related to the increased kidney volume, increased glomerular filtration rate and microalbuminuria and also with the presence of tubular injury [297].

Patients with higher serum IGF-I levels have more severe forms of diabetic retinopathy[298,299]. This observation was not confirmed later, probably due to methodological differences[279]. However, there is a general consensus that local IGF-I levels are higher in diabetic patients undergoing vitrectomy than controls[300]. Moreover inhibition of IGF-I could result in some positive effects in the management of retinopathy[301].

IGF-I is also important for diabetic wound healing. Lower levels of IGF-I are reported at the wound level [246,296,302] and furthermore, the deletion of IGF-IR is accompanied by reduced angiogenesis and granulation tissue formation.[303]

2.4 HIF AND IGF-I SIGNALING IN A DISEASE MODEL WITH INCREASED ANGIOGENESIS RATE

2.4.1 Kaposi's Sarcoma

Kaposi sarcoma (KS) is a vascular tumor that has first been described in 1872 by Moritz Kaposi[304]. Nowadays they are most commonly associated with AIDS. Based on population demographics and risks, Kaposi sarcoma is divided in four classes[305]:

- *Chronic KS* (classic or European) presents with multiple red to purple skin plaques or nodules, frequently localized in the distal lower extremities. The lesions could increase in number and spread, but some cases of spontaneous disappearance have been also reported. They grow on the skin and subcutaneous tissue and are usually asymptomatic. The chronic form of KS could be associated to another malignancy but not with human deficiency virus (HIV).
- *Lymphadenopatic KS* (endemic or African) is most prevalent in the South African children from Bantu. It presents as sparse skin lesions with lymphadenopathy. This form is very aggressive and could also affect viscera.

- *AIDS- associated* (epidemic) KS it is the most common tumor in AIDS patients. Due to antiretroviral therapy its prevalence decreased from almost 30 % to circa 1% in patients with AIDS. Together with the lymphadenopatic form it is the most common tumor in Africa, and it could be found in almost 50% of men in some African countries.
- *Transplant associated KS* occurs in association with immunosuppressant therapy after organ transplantation. It is usually aggressive with nodal, mucosal and visceral involvement. The skin lesions could be absent.

There are three different stages in the evolution of the disease: from the initial *patch* stage which presents as red to purple flat lesions -macules, going through the stage of *plaque,* when the lesions raise, become larger and more violaceous to the final stage, the *nodular* stage characterized by bigger, prominent lesions. The nodular stage often associates nodal and visceral involvement especially in the limphadenopatic and AIDS associated forms.

The spindle cell- the characteristic cell type for KS is present from the early patch stage and becomes predominant towards the later stages. The vascular tumors grow due to spindle cell proliferation.

The most frequent pathogenic agent identified in relationship with KS development is a type of herpes virus called KS-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8)[306]. The finding of KSHV DNA in all KS lesions, the distribution of KSHV infection similar to that of KS together with the fact that KSHV is identified in the spindle cells offer strong arguments that KSHV is an important pathogen for the development of KS[307].

There are few mechanisms which explain the role of KSHV in KS tumorigenesis. The most clearly defined mechanism is the existence of latent viral genes which promote viral replication and interfere with the normal cellular anti-apoptotic check points resulting in increased survival of KS cells. The proteins encoded by these genes are latency associated nuclear antigen (LANA), viral cyclin C (v-cyclin), v-Flice inhibitory protein (v-FLIP), kaposins A, B and C[307] and they induce cell growth, block apoptosis, downregulate the host immune responsiveness and control angiogenesis. Furthermore, the latent viral genes encode also a 12 pre-miRNA miRNAs which produce for example, miRNA 155 which has been related to the development of KS[308,309]. In the

genome of KSHV are also few open reading frames (ORF) which are similar to cyclins. Cyclins activate cyclin dependent kinases (CDKs) which will further create deregulations of cellular proliferation a mechanism used by KSHV in influencing KS tumorigenesis[310].

Initially it was considered that the spindle cells are of endothelial origin and are the only important cell type in the progression of the disease. However, lately the participation of myofibroblast-like cells was proposed as an important event. Accordingly, both endothelial and myofibroblast like cells come from common pluripotent mesenchimal cells which are modified by the KSHV, immunodeficiency, viral G protein coupled receptors or viral interleukin-6 and LANA[311].

The tumorigenesis process also includes inflammation and angiogenesis, apart from the proliferation discussed above. The KSHV infection alone is not able to maintain the KS growth without a proper local environment with pro-inflammatory[312] and pro-angiogenic factors.

The angiogenesis in KS is particular since it initiates before the tumor mass is established, which is opposite to the "classical" neovascularization in tumors, where the process is initiated, (so called "angiogenetic switch"[313]) by a critical tumoral mass that reaches a certain level of hypoxia.

Angiogenesis in KS is regulated by VEGF[314] and its receptors. Furthermore the viral proteins involved in the pathogenesis of KS modulate the secretion of other strong angiogenic factors like angiogenin[315] and angiopoietin 2[316].

2.4.2 HIF and tumorigenesis

The tumor growth is strictly dependent on the existence of blood supply and often the tumors develop around a blood vessel. Above a critical volume, the distance from the blood vessels overcomes the diffusing capacity for oxygen and generates areas within tumors that are hypoxic. Moreover, hypoxia is entertained by irregular, aberrant vessels which often characterize the tumors. The hypoxic environment in the tumor correlates with the progression and aggressiveness of the tumors[317] and even with the response to radiotherapy[318].

As HIF adapts the cells to hypoxia, its role in tumor biology have been extensively investigated in relation with the tumoral progression, metastatic capacity and the response to therapies[319]. It has been shown that HIF promotes tumor cell proliferation by activating growth factors like PDGF, IGFII and EGF[319]. It controls the angiogenesis as previously described. Moreover, in hypoxia HIF adapts the cells to the metabolic demands by controlling the switch from aerobic to anaerobic glycolysis[320]. On top, HIF promotes gene instability and regulates cell apoptosis[7].

HIF-1α and its isoform HIF-2α are up-activated in most of the tumors studied and their expression correlates with the clinical evolution and with the sensitivity of the tumors to irradiation[321]. Although both alpha subunits harbor the same stabilization pathway, their potential in tumorigenesis is different[322]. HIF-2α could be expressed even at near normal oxygen tension[323] and seems to have a special pathogenic role in tumors. Several factors probably contribute to the oncogenicity of HIF2α relative to HIF1α. HIF2α is less sensitive than HIF1α to inhibition by FIH1 and both the NTAD and CTAD of HIF2α are active under normoxia[324].

Both HIF-1α and HIF-2α could also function as tumor suppressors, but in different tumor types e.g.HIF-1 α in renal cell carcinoma and HIF-2 α in lung adenocarcinoma[30]. This is an interesting observation which suggests that for a specific tumor it matters which of the HIF α isoforms is expressed and to which level and this decides their progressing potential.

2.4.3 IGF and tumorigenesis

The IGF system plays a critical role in cancer biology[325] underscored by the epidemiological studies where high serum IGF-I levels increase[326] the risk of a person developing a cancer [327]while reductions of circulating IGF-I levels decrease the risk for cancer development[328].

Moreover cancer cells secrete IGF-I and IGF-II[325] that can function auto/paracrine for tumor progression since most of the tumors have increased expression of IGF-IR[329],[330].

IGF-IR regulates the cell cycle thus controlling the tumor growth[331]. Independent of the malignancy potential of a cell a tumor cannot metastasize in the absence of IGF-IR[332]. Its expression is associated with an enhanced metastatic capacity of the tumors[333] and

also with the resistance to chemo- and radiotherapy[334,335]. Recently it has been shown that IGF-IR could also translocate to the nucleus and function as transcription factor[336], mainly through binding to the promoter of cyclin D1 contributing to tumor progression[337].

During neoplastic transformation, IGF-I couples not only to IGF-IR but also to insulin receptors and to hybrid variants of receptors formed between IGF-I and insulin receptors. There are two types of insulin receptors: IRA and IRB. A special role in tumorigenesis is played by the insulin receptor type A mainly expressed in cancers[338] [339]. Both IGF-I and IGF-II have increased affinity for the hybrid receptors[142].

Due to its pluripotent roles in tumorigenesis, the IGF system is investigated as a potential target for anticancer therapies. The most successful of preclinical studies are the therapies that specifically block the IGF-IR. One problem arising with these therapies is development of resistance to therapy. However, some compounds such as the specific tyrosine kinase inhibitor – picropodopylin (PPP) develop less resistance[340]. Preliminary results from the phase III clinical studies debate the benefit of IGF-IR blocking therapies as single therapy due to their side effects[141]. However the final results are not yet published.

3 AIMS

3.1 GENERAL AIM

The general aim of the thesis is to investigate the role of HIF and IGF-I as pathogenic mechanisms of two diseases with dysregulation of angiogenesis namely diabetes and Kaposi Sarcoma (KS) to enable suggestion of new therapeutic targets based on these findings.

3.2 SPECIFIC AIMS

- To investigate the interaction between hyperglycemia and hypoxia as pathogenic mechanisms for the development of chronic complications of diabetes
- To investigate the consequences of hyperglycemia dependent HIF repression for the development of chronic complications of diabetes
- To investigate the mechanisms responsible for HIF repression in diabetes
- To establish the therapeutic effect of HIF stabilization in hyperglycemia in general and in diabetic wound healing in particular
- To establish the contribution of systemic IGF-I for the wound healing process, in normoglycemia and hyperglycemia
- To investigate the relationship between IGF and HIF in Kaposi's Sarcoma
- To study the mechanisms of HIF accumulation in Kaposi's Sarcoma
- To investigate the efficiency of IGF-IR blockade as potential therapy in Kaposi's Sarcoma

4 RESULTS

Diabetes complications and tumors are two diseases with impaired angiogenesis that nowadays have limited therapeutic options. Our focus was to investigate the mechanisms of impaired angiogenesis in these clinical situations to be able to propose novel rational therapeutic strategies.

Paper I and Paper II

We investigate in our studies the cross-talk between hyperglycemia and hypoxia as main pathogenic factors in the development of chronic complications in diabetes. We base our research on previous results from our group showing that hyperglycemia impairs HIF-1α stability and function and we hypothesize that this is a main cause for the defective wound healing seen in diabetes. For this study we use the db/db mice as model of diabetic ulcers because of similarities with the wound healing process in humans[341].

Moreover, given the central role played by ROS in the development of chronic complications of diabetes, we proposed to study the consequence of the impaired response to hypoxia induced by hyperglycemia on ROS production.

4.1 HYPERGLYCEMIA DESTABILIZES HIF AND IMPAIRS ITS FUNCTION

Hypoxia was recently identified as an additional pathogenic factor in diabetic complications beside hyperglycemia[270,342]. It is a consequence of several mechanisms e.g. deficient blood supply due to micro- and macro-vascular disease, poor local diffusion of oxygen due to local oedema, but also an increase of oxygen consumption induced by hyperglycemia[343].

In response to hypoxia HIF is up-regulated and activates the transcription of several genes involved in metabolism, angiogenesis, proliferation and apoptosis which will adapt the cells to hypoxia.

We demonstrate here that, in the presence of high glucose, the cells fail to properly adapt to hypoxia due to a repression on HIF stability and function with important consequences on the tissue's ability to heal after wounding (Paper I).

Knowing that fibroblasts are important effectors in wound healing, we first investigated the effects of combined hyperglycemia and hypoxia on mouse fibroblasts derived from skins of db/db mice. Hyperglycemia destabilized HIF-1α in hypoxia which was followed by repression of HIF target genes that are important for the wound healing such as VEGF involved in angiogenesis, SDF-1 and SCF involved in recruitment and homing of endothelial progenitor cells (EPC) and Hsp90 important for cells migration (Paper I). The same HIF repression induced by high glucose has been observed by others and us in other types of primary cells[344-349]. Moreover, fibroblasts isolated from the skin of diabetic patients are unable to increase the VEGFA production in response to hypoxia exactly like fibroblasts from diabetic mice[348]. However, it seems that the HIF repression induced by hyperglycemia is specific for primary non-tumor cells since it is not constantly seen in tumor cells[350].

The negative regulatory effect of hyperglycemia on HIF-1α stability and function is further confirmed *in vivo,* in diabetic wounds of db/db mice. We demonstrated that despite a more hypoxic environment generated in diabetic wounds (evaluated by staining with the "hypoxia dye" pimonidazole hydrochloride) HIF-1α expression is reduced compared to the wounds in normoglycemic mice. This is highly relevant for human diseases since HIF-1α levels are repressed in biopsies from patients with diabetic ulcer as compared to venous ulcers that share the same hypoxic environment but are exposed to normal blood glucose levels [344]. mRNA levels of HIF-1α target genes involved in wound healing (VEGF, Tie-2, Hsp90, SDF-1α) are also downregulated in the wounds of db/db mice (Paper I).

4.2 HIF STABILISATION IS CRITICAL FOR IMPROVING WOUND HEALING IN DIABETIC MICE

The hypoxic environment of skin wounds in diabetes is a consequence of multifactorial processes. In the first place there it is general hypoxia of the skin, especially at the epidermis level due to the distance from the blood supply[351]. Secondly, it associates with acute hypoxia following any injury with an increased demand of oxygen from the

cells recruited to regenerate the tissues and finally, the oxygen supply is limited due to the micro and macro angiopathy present in diabetes [237,352,353].

On the other hand, HIF is essential for wound healing during all phases of the wound healing process[354] through the control of angiogenic growth factors [7], recruitment of EPC [355] and cell motility[356]. However, we have shown that hyperglycemia impairs the tissues' reaction to hypoxia in general and that diabetic wounds express less HIF-1α. Therefore we hypothesised that the defect in the wound healing seen in diabetes is a consequence of HIF-1α repression and we aimed to stabilise HIF-1α as potential therapy.

For this purpose we used two prolylhydroxylase (PHD) inhibitors, dimethyloxalylglycine (DMOG) and desferoxamine (DFX)[53]. HIF-Hydroxylases contain Fe(II) in their catalytic centers and use -ketoglutarate as a co-substrate[47] and they can therefore be inhibited by iron chelators, such as DFX and by competitive antagonists of α-ketoglutarate, such as DMOG. Both substances efficiently counteract the repressive effect of hyperglycemia on HIF whether used *in vitro* or *in vivo*, as topical treatment of the wounds. Moreover, the HIF stabilisation was followed by a significant improvement in wound healing in diabetic mice despite persistent chronic hyperglycemia. Accordingly, processes important for wound healing (angiogenesis, granulation, epidermal regeneration, homing of the EPC) were improved by PHD inhibitor treatment (Paper I).

The central role of HIF in diabetic wound healing was brought by gain-of-function studies with adenovirus-mediated expression of constitutively stable forms of HIF-1α which had the same positive effect on wound healing in diabetic animals as PHD inhibitors.

4.3 MECHANISMS FOR HYPERGLYCEMIA INDUCED HIF REPRESSION IN HYPOXIA

We next considered the mechanisms by which hyperglycemia interferes with HIF regulation. Previous work in our group indicated that HIF repression induced by hyperglycemia takes place posttranslationally, at the degradation level as it is canceled by proteasome inhibitor, MG132[344]. Since VHL has a central role in HIF degradations we first analyzed the effects of hyperglycemia on HIF after VHL inactivation.

Using two different approaches we demonstrated that the repressive effect of high glucose on HIF is dependent on a VHL-mediated degradation mechanism. First we showed that HIF-1α modulation by hyperglycemia was abolished in a renal carcinoma cell line that that lacked functional VHL and second that classic HIF target genes like VEGF were no longer suppressed by high glucose after siRNA silencing of VHL (Paper I).

Furthermore, since HIF activation in hypoxia is modulated by complex mechanisms involving the two transactivation domains: NTAD and CTAD[92], we pursued our investigation and studied the effect of hyperglycemia on each of the two transactivation domains. The negative regulatory effect of glucose affected both NTAD and the CTAD. Consistent with our results, HIF transactivation repression by hyperglycemia has been confirmed by other groups[348]. The relevance of this multiple level regulation of HIF transactivation remains to be evaluated. For the moment we could not find any additional benefit of a constitutive activated form of CTAD over the double prolyl mutated HIF construct for promoting wound healing in diabetes using different adenovirus mediated constructs. It is however unclear if using VP16 in the adenovirus construct would not hide the contribution of CTAD, being known that VP16 has a highly active transactivation activity[357].

In conclusion, hyperglycemia- induced HIF repression in hypoxia is at multiple levels, including the stabilization of the protein but also the transactivation activity.

However, VHL expression is not induced by hyperglycemia which suggests that hyperglycemia exerts its effects by enhancing the sensitivity of HIF-1α to VHL dependent degradation.

Canonically, VHL binds to HIF-1α in normoxia, after hydroxylation of the proline residues mediated by PHDs. We therefore decided to investigate if the PHDs are responsible for the increased VHL-mediated degradation of HIF-1α. Indeed, both PHD inhibitors used, DFX and DMOG counteracted the repressive effect of hyperglycemia on HIF stability and function. The effect was however only partial but to a level sufficient to improve the reaction of the tissues to hypoxia.

In addition, our preliminary data using a NTAD construct with both critical prolines mutated demonstrated that hyperglycemia activates an additional mechanism beside

PHD modulation since high glucose still represses the NTAD construct resistance to PHDs activity (Figure 6). This effect is in agreement with the observation that HIF is only partially rescued by PHD inhibitors. Similar HIF-1α degradation has been described even in hypoxia by a mechanism dependent on VHL but independent on PHD activity[74].

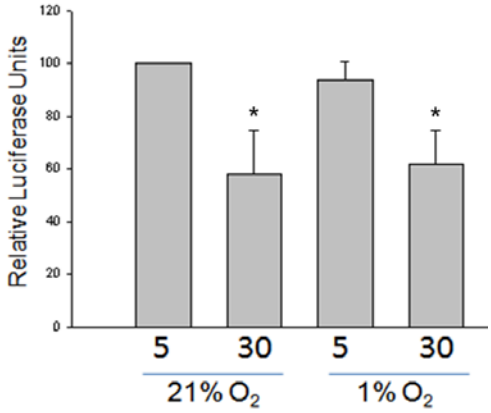


Figure 6: **Hyperglycemia represses the function of HIF construct resistant to PHDs activity**
Relative luciferase activity in the extract of 3T3 cells exposed to different oxygen and glucose concentrations (5 mM and 30mM) after co-transfection of NTAD-PPA (PPA-both prolyl residues mutated to alanine) and Gal4-responsive reporter gene plasmid

One possible candidate to mediate the effect of hyperglycemia is SUMO (small ubiquitin like modifiers) which functions as ubiquitin and use the same VHL system for targeting SUMOylated proteins to degradation.

Since SUMOylation is an extremely dynamic process with a high turnover rate of SUMOylation and deSUMOylation, we used SUMO constructs which are resistant to the action of SUMO-specific proteases SENP, the proteins that are responsible for deSUMOylation. Indeed as seen in figure 7 we could detect an enhanced SUMOylation of the HIF protein in hyperglycemia (figure 7).

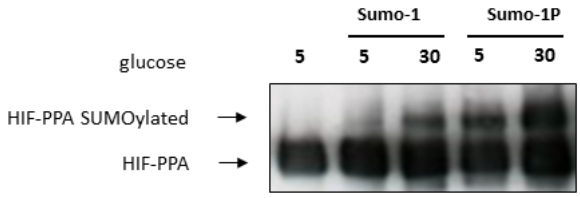


Figure 7: **Enhanced SUMOylation of the HIF protein in hyperglycemia**

SUMOylation of proteins has been reported in relation to hypoxia[358,359] or hyperglycemia[360,361]. , The significance of HIF sumoylation in hyperglycemia is not clear and further investigation is needed since SUMOylation has such discordant reported effects on HIF function[78-81].

Other mechanisms, independent of VHL ubiquitination may be of significance for HIF repression in hyperglycemia.

Methylglyoxal (MGO) accumulates intracellularly in diabetes as a result of glycolysis[362] and leads to HIF-1α ubiquitination through a mechanism independent of VHL and PHDs hydroxylation. Instead, MGO uses a chaperone binding ligase- CHIP (Carboxy terminus of Hsp70-Interacting Protein) such as the E3 ligase which targets HIF-1α for proteosomal degradation. The interaction between HIF-1α and CHIP is mediated by Hsp70[363].

Moreover, MGO affects HIF-1 functional activity not only through HIF-1α destabilization but also through modifying the coactivator p300, which in turns leads to decreased HIF-1a/p300 interaction during HIF transactivation[348,363].

However, it has been demonstrated that Hsp70, by recruiting the ubiquitin ligase CHIP, promotes the ubiquitination and proteasomal degradation of only HIF-1α but not HIF-2α[364] whereas we were able to detect degradation of both paralogs in hypoxia following high glucose exposure (data not shown).

This may also explain why overexpression of glyoxalase I, which normalizes the intracellular levels of MGO, only partially rescues the hyperglycemia-induced HIF destabilization[363] whereas deferoxamine administration which abrogates methylglyoxal

conjugation and also stabilizes HIF against PHD mediated degradation has more pronounced effects[348].

Another interesting molecule in the context of HIF repression in hyperglycemia is p53 which is activated in response to high glucose[365]. p53 contributes to both HIF degradation by recruiting ubiquitin ligases such as CUL-1 or mdm-2,[84] and also represses the HIF transcriptional activity by competing with HIF for the co-activator p300[366]. It is however unlikely to be involved in hyperglycemia induced HIF repression since HIF destabilization in hyperglycemia is observed in fibroblasts lacking p53 gene[344].

4.4 THE IMPACT OF HIF REPRESSION ON DIABETES COMPLICATIONS

We have demonstrated that repression of HIF induced by hyperglycemia is an important pathogenic mechanism for defective wound healing in diabetes. The same pathogenic effect played by HIF repression by hyperglycemia is also seen in other tissues affected in the development of chronic complications of diabetes.

HIF-1α is destabilized by high glucose as early as after 6hrs exposure to hypoxia (Paper I) which highlights the potential relevance for the early cell reaction to hypoxia during acute ischemic events (acute myocardial infarction, stroke).

Moreover, during cardiovascular ischemic conditions, hyperglycemia precludes adaptation to hypoxia, which results in increased myocardial infarction size[367,368] as a consequence of a reduced angiogenic capacity[369], [370] which leads to poor collateral vessel formation. This is confirmed in human left ventricular biopsies of diabetic patients with acute coronary events which express lower HIF and its pro-angiogenic target gene VEGF[371]. The repression of VEGF has been correlated with in an increased risk of cardiovascular morbidity and mortality in patients with diabetes[372]. However, stabilization or overexpression of HIF-1α in hyperglycemia has been proven an efficient therapeutic tool in myocardial capillary network improvement following myocardial injury[373] or in increasing limb perfusion and function in diabetic mice after ischemic events[374]. The observed beneficial effects were mainly due to an enhanced angiogenic potential through normalizing VEGF, enhancing the recruitment of EPCs, etc.

In diabetic nephropathy, hypoxia can be detected by MRI in the outer medulla of diabetic kidneys very early in the development of the disease pointing towards its primary pathogenic role[375], [376]. Due to the hypoxic environment, HIF and its targets gene expression are enhanced[377]. The reaction to hypoxia, is however impaired due to hyperglycemia which is shown by incomplete overlap between pimonidazol staining (a marker of hypoxia)[378] and HIF-1α and HIF-2α expression in kidneys of STZ induced diabetic rats[379] and db/db mice[378]. In addition, VEGF induction is reduced[345]. HIF overexpression in kidneys has therefore also been proposed as therapeutic approach in diabetic nephropathy and has been demonstrated to protect against progression to end stage renal disease[380-382].

Furthermore, the role of HIF signaling in the development and progression of diabetic nephropathy is indicated by the finding that a polymorphism of HIF-1α (P582S) which confers relative resistance to the repressive effect of hyperglycemia is associated with protection for nephropathy in patients with type2 diabetes[378,383].

Biopsies from patients with diabetic foot ulcers, as demonstrated before, express less HIF-1α compared to biopsies from patients with venous ulcers that share the same hypoxic environment but are not exposed to hyperglycemia[344]. In addition, HIF stabilisation by pharmacological substances and overexpression by a genetic approach cancel the deleterious effect of high glucose (paper I,[347,349]).

In conclusion, hyperglycemia-induced repression of HIF is an important pathogenic mechanism in the development of diabetes complications and overexpression or stabilisation of HIF is an efficient therapeutically approach against development and progression of diabetes complications.

Hypoxia and hyperglycemia also affect the success of pancreatic islets transplantation. Diabetes induces a more pronounced hypoxic environment for the transplanted islets[384]. This results in a poor revascularization of the transplanted islets[385-387] and in a reduced transplantation success rate[388]. A better revascularization pattern is associated with a higher rate of beta cell proliferation and superior beta cell function[389].

4.5 ROS AND DIABETES COMPLICATIONS

Hyperglycemia dependent damage of tissues has been explained by several mechanisms which include PKC activation, increased advanced glycation end (AGE) products formation and increased expression of AGE receptors, increased hexosamine activity and increased glucose flux through the polyol pathway[266,268].

All the mechanisms have been demonstrated to be the consequence of an upstream common event, **increased mitochondrial ROS production,** due to enhanced glucose flux during glycolysis through oxidative phosphorylation[390,391]. The increased ROS production causes DNA strand breaks that activate poly(ADP-ribose) polymerase enzyme (PARP) which further decreases glyceraldehyde-3 phosphate dehydrogenase (GAPDH)[390] with consequences on all 5 other pathogenic mechanisms. The more ROS is produced, the quicker the diabetes complications progress[392].

Mitochondrial ROS production is tightly regulated by HIF. It increases within minutes in hypoxia but chronic hypoxia leads to lower levels of ROS as a consequence of HIF stabilization and activation[7]. Because in hyperglycemia, HIF expression and function are repressed as demonstrated above, we hypothesised that one mechanism which contributes to the overproduction of mitochondrial ROS in diabetes is a consequence of hyperglycemia- induced HIF repression.

We tested our hypothesis in *in vitro*, on two primary cell culture models relevant for diabetes complications, Human Dermal Fibroblasts (HDF) and Human Dermal Microvascular Endothelial Cells (HDMEC) and were able to show that indeed, concomitant exposure to high glucose and hypoxia leads to an increased mitochondrial ROS production which is not present when cells are exposed to the same level of hypoxia in normal glucose levels.

The exposure to 30 mmol mannitol (used as osmotic control) did not affect mitochondrial ROS production. This is expected with the background that mannitol is metabolically inactive, but still a relevant observation because we observed a HIF protein destabilisation after cell exposure to mannitol in hypoxia[344].

Endothelial cells are one of the main targets for the deleterious effects of hyperglycemia and their dysfunction is the cause of micro- and macroangiopathy which

are associated to all diabetes complications. One pathogenic mechanism by which ROS overproduction contributes to complications is induction of apoptosis[393,394] which is observed in endothelial cells in diabetes[395] .

Therefore, our next step was to investigate the consequences of ROS overproduction on apoptosis in HDMEC and we demonstrated that the apoptosis rate increases after exposure of the cells to both high glucose and hypoxia.

4.6 HIF STABILISATION IN HYPERGLYCEMIA RE-ESTABLISHES THE NORMAL ROS LEVELS

We have previously shown that HIF overexpression or stabilisation in hyperglycemia has essential benefits for the prevention and treatment of diabetes complications. This effect is observed in almost all the tissues affected by chronic complications of diabetes: skin, heart, kidneys and arteries. Since an excess of ROS production is accepted to be the pathogenic mechanism for all these complications, we decided to further evaluate the consequences of HIF stabilisation on mitochondrial ROS production.

We investigate d whether restoration of HIF stabilization by a pharmacologic approach or by siRNA silencing of VHL would lead to normalization of mitochondrial ROS production in cells exposed to combined hypoxia and hyperglycemia.

In the first approach we used DMOG as a PHD inhibitor, which efficiently rescues HIF function in the presence of hyperglycaemia as previously shown (Paper I). DMOG normalized the ROS levels in cells exposed to combined hyperglycemia and hypoxia to the levels observed in normoxia and normal glucose, suggesting that HIF repression in diabetes is “the missing link” that explains the ROS overproduction in diabetes. Consequently the apoptosis rate of the endothelial cells was decreased by exposure to DMOG treatment.

We demonstrated in paper I that HIF-1α destabilisation in hyperglycemia and hypoxia is mediated by pVHL. We therefore investigated the effect of HIF induction through VHL siRNA silencing on mitochondrial ROS production. siRNA silencing of VHL was followed by normalisation of mitochondrial ROS production, consistent with HIF stabilization through DMOG treatment.

We further investigated whether HIF repression induced by hyperglycemia contributes to excess ROS production *in vivo.* Since the nephropathy is one of the major complications in diabetes[396,397] we analysed the ROS production in the kidneys in streptozocin (STZ) induced diabetic mice (for at least 5 weeks).The ROS production was evaluated by -4-hydroxynonenal (4-HNE), which is a stable compound of lipid peroxidation and accepted marker of oxidative stress.

As seen in figure 9, 4-HNE levels in kidneys from STZ induced diabetic mice are significantly higher than those in kidneys from normo glycemic control mice as reported by others[398].

In agreement with the *in vitro* results, DMOG treatment normalizes HNE levels to the levels seen in normoglycemic mice, underscoring the relevance of HIF induction for normalizing ROS levels in the presence of hyperglycemia (Figure 8 and Figure 9).

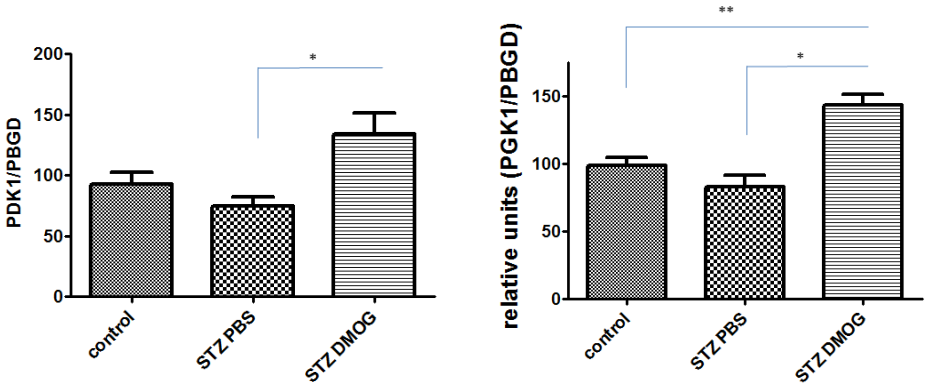


Figure 8**: Effect of systemic DMOG treatment on the mRNA of HIF target genes in kidneys of STZ induced diabetic and control mice.**

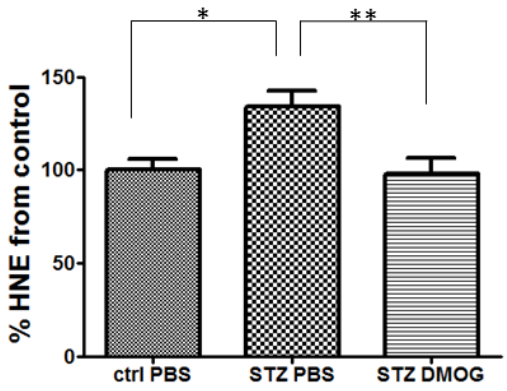


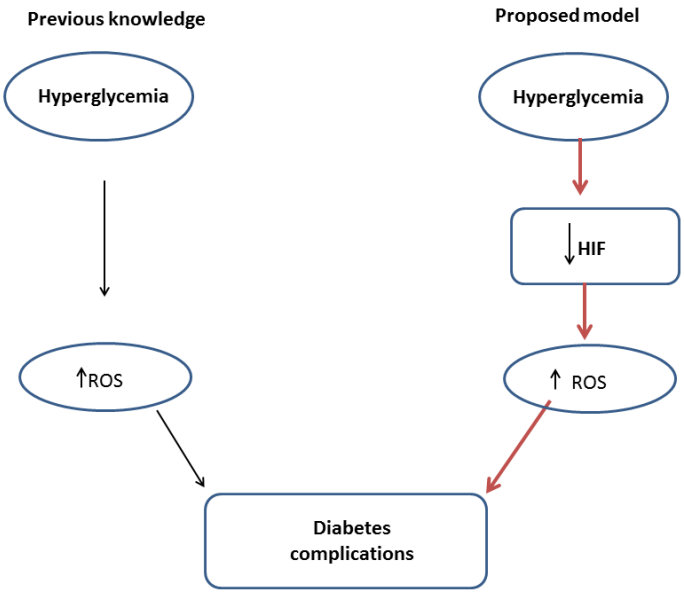
Figure 9**: ROS production is normalised in the kidneys of STZ induced diabetic mice after systemic treatment with DMOG.**

Since the excess ROS production was proposed as the unifying pathogenic mechanism, which contributes to the development of chronic complications of diabetes, many treatments have been experimentally investigated with the aim of normalizing mitochondrial ROS production[223,399]. One strategy was to stimulate the cellular systems that decrease ROS as UCP-1 overexpression or SOD /catalase overexpression[400]. Another strategy targeted PARP formation which is the immediate effector activated by superoxide thus involved in the chronic complications of diabetes[401].

All these approaches have shown that decreasing intracellular superoxide or interfering with its action represents an efficient strategy to correct some of the endothelial dysfunction in diabetes[402] and to ameliorate the progression of chronic complications of diabetes e.g cardiopathy[403,404], nephropathy[405,406], retinopathy[407] and neuropathy[408].

We propose here that the mechanism responsible for the ROS overproduction in diabetes is dependent on HIF repression induced by hyperglycemia. Since HIF regulates many other cellular functions[409] apart from ROS production in hypoxia it is expected that strategies that would aim to stabilize HIF in diabetes will be more efficient therapies.

Proposed model for the contribution of HIF repression in the development of chronic complications of diabetes



Paper III and Paper IV

IGF-I has been associated with the development and progression of both chronic complications of diabetes and neoplasia.

A common denominator for both diseases is dysregulated angiogenesis. An essential pathogenic mechanism behind poor wound healing in diabetes is deficient angiogenesis, whereas Kaposi's Sarcoma (KS) represents a model of neoplasia, where angiogenesis is excessive and again central for the evolution of the disease.

IGF –I is known to have important roles in angiogenesis in addition to hypoxia.

In the next two papers we therefore investigated IGF-I signaling in these two models of dysregulated angiogenesis i.e. diabetic wound healing and Kaposi's sarcoma.

4.7 LIVER SPECIFIC KNOCK- OUT OF IGF-I DOES NOT AFFECT THE WOUND HEALING RATE

IGF-I promotes wound healing by multiple mechanisms e.g. increased chemotactic activity for endothelial cells, enhanced proliferation of keratinocytes and fibroblasts, and augmented wound strength[246]. However, serum IGF-I is reduced both in type 1[279],[280] and insulin-dependent type 2 diabetes[285,286] patients . Additionally, the local IGF-I expression is reduced in diabetic wounds[246,296,302] .

This suggests that poor wound healing in diabetes could be related to IGF-I deficiency and was the basis for using IGF-I as topical therapy for diabetic wound healing[410,411].

The relative contribution of the liver-derived IGF-I (endocrine- acting) versus the locally produced IGF-I (autocrine- paracrine acting) for the wound healing remains however unknown.

We therefore decided in the next study to investigate the contribution of the systemic IGF-I on the wound healing rate of mice under normoglycemic and diabetic conditions. As systemic IGF-I is mainly dependent on the liver secretion we studied wound healing in mice with liver-derived IGF-I deficiency (LI-IGF-I-/- mice) due to complete inactivation of the IGF-I gene in the hepatocytes that consequently results in 75% lower serum levels of IGF-I .

In normoglycemic conditions the wound healing rate in the LI-IGF-I-/- mice is similar to the control mice, despite lower serum IGF-I.

We then studied the wound healing rate after inducing diabetes using streptozotocin (STZ) in 2 months old LI-IGF-I-/- mice and in their controls. Diabetes resulted in decreased serum levels of IGF-I in LI-IGF-I-/-mice versus their controls. However, even though diabetes delayed the wound healing rate compared with normoglycemic animals, there was no difference in wound healing rate between diabetic LI-IGF-I-/- and diabetic control mice.

We have discussed above (Paper I) that the impaired wound healing in diabetes is mainly related to defective angiogenesis and to a reduced function of endothelial precursors cells (EPC)[256] due to a defect in the local expression of stromal derived factor-1 **(**SDF-1α) that binds EPC through the CXR-4 receptors[355]. We investigated

these markers in the wounds of diabetic LI-IGF-I-/- mice versus diabetic control animals and we observed a similar expression of SDF-1 α and CXCR4 mRNA, which is in agreement with the results on the wound healing rate.

In conclusion we show that liver-specific knock-out of IGF-I does not affect the wound healing, in neither normoglycemic conditions nor in diabetes.

This issue is of importance, suggesting that locally delivered IGF-I is sufficient to improve wound healing in diabetes avoiding in this way the potential side effects that are associated with systemic IGF-I therapy[144].

4.8 IGF-I INCREASES HIF-1α AND HIF-2α IN KAPOSI'S SARCOMA

Paper IV

Kaposi's sarcoma (KS) is a highly vascularized tumor which affects predominantly patients with acquired immune deficiency syndrome (AIDS). Previous reports from our group have shown that the biology of the tumor is dependent on IGF-I which stimulates the proliferation of these tumoral cells and increases their survival[412]. The hallmark of KS is the highly angiogenetic phenotype which has been related to VEGF[314]. IGF-I has an additive effectwith VEGF in stimulating the proliferation of KS cells[412].

HIF is a strong signal for VEGF production and IGF-I has been reported to modulate HIF activity[112,117,413]. However, at the time when this study was performed, no information was available about the HIF expression and roles in KS.

We started therefore, by investigating HIF expression in 17 HIV –positive tumor biopsies. Both HIF-α paralogs were expressed throughout the tumor area. The pattern of accumulation for the two paralogs was different underscoring the non-redundant function of the two HIF α subunits. HIF-1α expression increases significantly from the early patch biopsies to the late nodular KS biopsies whereas HIF-2α expression was not significantly modified through different stages.

In order to check the relationship between IGF-I and HIF-α subunits and their significance for the development of KS tumors, we continued our investigation *in vitro*

using KSIMM, an established KS cell line that produces large highly vascularized tumors when injected s.c. in nude mice[414].

IGF-I induces both HIF- α subunits, in a dose dependent manner in these cells. We further investigated whether the accumulation of the HIF-α subunits under IGF –I stimulation has functional consequences. To this end, we transiently transfected the KSIMM cells with an HRE-reporter construct and exposed the cells to IGF-I which increased the luciferase activity. Moreover IGF-I augmented VEGF protein secretion, a classical HIF target gene, highlighting the effects of IGF-I on HIF function.

It is important to emphasise that HIF-2α could be detected in KSIMM cells even in normoxia. This observation is concordant with the expression pattern of HIF-2α in biopsies from patients with AIDS related KS where it was strongly detected from the early phases of tumor evolution and did not change significantly in more advanced lesions.

HIF-2α accumulation at higher oxygen levels has also been shown in HeLa and neuroblastoma cells[415], [416] as opposed to HIF-1α which accumulates only at lower oxygen levels. As HIF-2α has growth promoting effects[30,417] and in other tumor models promotes angiogenesis and invasion via VEGF[418] it might also represent the initiation signal for the neoangiogenesis in KS. Similar, it has been shown that HIF-2α is sufficient to induce angiogenesis in hemangiomas associated with VHL deficiency[419].

The fact that HIF-1α is minimally expressed in the early stages of KS, but increased with the progress of the disease could be a self-limiting reaction of the tumor which in the beginning tries to counteract HIF-2α proangiogenic effects. In mice with tumor xenografts deletion of HIF-1α in vascular endothelial cells reduces tumor expansion by decreasing VEGF signaling and EC proliferation[420] and restricts tumor cell metastasis, whereas HIF-2α has opposing effects[421].

4.9 MECHANISMS OF IGF DEPENDENT HIF ACCUMULATION

We show in this study that IGF-I is able to induce both HIF-1α and HIF-2α isoforms in KSIMM cells but with a lower effect than hypoxia. This suggests that a different regulatory pathway is activated.

Therefore, we next investigated the mechanism by which IGF-I induces HIF α isoforms accumulation. We first investigated the potential modulation of HIF transcription. IGF-I does not modulate the mRNA levels of any of the HIF-α isoforms suggesting a posttranscriptional mechanism for IGF-I action on HIF accumulation.

The main mechanism for HIF protein accumulation induced by hypoxia or hypoxia mimetics is dependent on proteosomal degradation. In order to study whether IGF-I dictates a similar pattern of accumulation, we exposed the KSIMM cells in parallel to IGF-I and $CoCl_2$ (a hypoxia mimetic, which interferes with PHD activities[17,24]). HIF-1α and HIF-2α started to accumulate as early as after 30 minutes after exposure to IGF-I and their levels increased steadily over time in the first 4 hours whereas the two isoforms stabilized from 3 hours after CoCl2 exposure underscoring a different pathway of accumulation.

In order to investigate a potential effect of IGF-I at translation level we exposed KSIMM cells to IGF-I or $CoCl_2$, but blocked the intracellular translation mechanism with cycloheximide (CHX) after 4 hours (when both stimuli induced HIF α–subunits accumulation).

In the absence of CHX, both HIF-α subunits maintained their levels for the next 60 minutes while CHX treatment induced a decline of the 2 HIF-α paralogs induced by IGF-I. The decline started as early as 15 minutes while there was a minimal effect even after 60 minutes, on the α-subunits when they were stabilized by CoCl2. Based on this dynamic we conclude that IGF-I regulates HIF-α subunits by inducing their translation.

4.10 BLOCKING IGF-I SIGNALING PATHWAY DECREASES THE HIF-1α AND HIF-2α ACCUMULATION AND THE EXPRESSION OF THEIR TARGET GENES

IGF-IR has been shown to be present in KSIMM cells[412] and it is important for KS tumor biology. Picropodophyllin (PPP) is a specific IGF-IR tyrosine kinase inhibitor[422] which has been used as a successful therapeutic agent[423-426] in the treatment of different tumors. PPP exerts complex actions on the IGF-IR as it does not function just as an inhibitor of the receptor activity but also down -regulates the expression of IGF-IR[427]. In addition, blocking the IGF-IR signaling by PPP leads to inhibition of VEGF secretion and has antiangiogenic effects[428].

The importance of IGF-IR on HIF-1α and HIF-2α was highlighted by the complete abolishment on their accumulation after blocking the receptor with either αIR3 (a monoclonal blocking antibody) or with PPP. The functional consequences are reflected by the decrease in VEGF mRNA levels to values even lower than in normal cells with potential important effect on KS biology considering the central role played by VEGF for KS.

5 POINTS OF PERSPECTIVES

The aim of this thesis was to characterize new pathogenic mechanisms which lead to dysregulated angiogenesis and are relevant for chronic complications of diabetes and tumors. We focused in this context on two important regulators of angiogenesis: HIF and IGF.

The role of HIF in diabetes is a relatively new field under development. We have studied here the mechanisms by which hyperglycemia impairs HIF stabilization in hypoxia. We showed that the HIF-1alpha destabilization in hyperglycemia is a degradation mechanism dependent on VHL. Moreover we showed that both HIF transactivation domains are regulated by hyperglycemia.

We further showed that hyperglycemia-induced HIF repression is a mechanism relevant for chronic complications of diabetes, by demonstrating its relevance to diabetic ulcers. We proposed that HIF stabilization could be developed in an efficient therapy since we demonstrated that local HIF induction improved the healing rate of the diabetic ulcers.

Given the central role played by ROS in the development of chronic complications of diabetes, we investigated the consequences of hyperglycemia dependent repression of HIF on the production of mitochondrial ROS.

We demonstrated that the repression of HIF during exposure to hyperglycemia plus hypoxia resulted in increased production of mitochondrial ROS with negative functional consequences. However, by restoring the HIF reaction it was possible to normalize the ROS production and reestablish the cell capacity to adapt even in persistent hyperglycemia.

These results might offer the premises for conducting clinical studies on patients who present with chronic complications of diabetes. DFX which efficiently rescued HIF function in hyperglycemia is already in clinical use for other indications. Moreover, intensive research is ongoing to develop HIF- hydroxylases inhibitors for clinical use .

Important future directions of our research are to establish which mechanisms are activated by hyperglycemia and that lead to repressed HIF function in diabetes.

IGF-I has been associated with the development and progression of chronic complications of diabetes as well as neoplasia.

We investigated the contribution of systemic IGF-I to wound healing rate showing that liver-derived IGF-I does not affect wound healing in mice, in neither normoglycemic conditions nor in diabetes. This study suggests that local therapy with IGF-I is sufficient for improving wound healing in diabetes thereby avoiding side effects that would be associated with systemic IGF-I therapy.

We also demonstrated that the highly vascularized phenotype characteristic for Kaposi's Sarcoma is highly dependent on IGF-I and HIF. We further described that accumulation of HIF IGF-I induced was by increasing HIF translation. We demonstrated that IGF-IR inhibitors block the HIF accumulation and function. This makes them potential candidates for therapy since both HIF-1α and HIF-2α are highly expressed in the biopsies of the patients with Kaposi's Sarcoma and play a central role in KS biology.

In conclusion, we identified new mechanisms of dysregulated angiogenesis in diabetes and tumors which can be the basis for new therapeutic strategies.

6 CONCLUDING REMARKS

HIF represents a potential therapeutic target for management of chronic complications of diabetes:

- High glucose impairs the stability and function of HIF-1α
- The repression of HIF-1α induced by hyperglycemia is dependent on VHL mediated proteosomal degradation
- Hyperglycemia dependent repression of HIF-1α is pathogenic for diabetic wounds since local HIF stabilization by hydroxylase inhibitors or by direct adenoviral transfer improves the diabetic wound healing rate
- Glucose dependent HIF repression is responsible for the increased mitochondrial ROS production with deleterious effect on cell survival
- HIF stabilization normalizes the mitochondrial ROS production in hyperglycemia plus hypoxia

Liver specific knock-out of IGF-I does not affect the wound healing rate neither in normoglycemia nor in diabetes

IGF-I represents a potential therapeutic target in Kaposi Sarcoma:

- Kaposi sarcoma expresses both HIF-1α and HIF-2α
- IGF-I induces HIF-1α and HIF-2α accumulation in KSIMM cells by increasing the translation of the paralogues
- Blocking the IGF-IR signaling decreases HIF-α accumulation and blunts the VEGF expression, offering a promising therapeutic strategy for Kaposi's Sarcoma

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It is not when truth is dirty, but when it is shallow, that the lover of knowledge is reluctant to step into its waters.
Friedrich Nietzsche

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