RESPONSES TO HYPOXIA VIA mTOR

Role in Endothelial Cell Proliferation and HIF-1α Stabilization

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SUMMARY

Ischemic cardiovascular disease and cancer are life-threatening disorders in human mortality. Intensive studies on the etiology of and therapeutics for the diseases are ongoing. One of the most intriguing and promising fields for studies in both disorders is angiogenesis. Ischemic myocardium and cancer are closely associated with hypoxia and require de novo blood vessels for tissue survival. However, therapeutically, angiogenesis needs to be induced in the ischemic myocardium whereas it ought to be suppressed in cancer. For both purposes, a thorough understanding of the mechanisms of hypoxia-induced angiogenesis is indispensable.

Angiogenesis, in response to ischemia, requires factors that sense hypoxia and that relay the signals to effectors. Mammalian target of rapamycin (mTOR), a key energy-sensor for cell survival, has recently been shown to be involved in hypoxic signaling. It remains unclear whether mTOR acts as part of the oxygen-sensing machinery and how mTOR regulates the hypoxia-induced signaling transducers. On the other hand, transcription factor hypoxia-inducible factor-1α (HIF-1α) is critical for hypoxic-driven induction of angiogenic molecules. Again, it is unclear how mTOR affects HIF-1α function. To unravel these questions, we have assessed mTOR activity as well as its relationship to HIF-1α in rat aortic endothelial cells (RAECs) in response to hypoxia.

Previous studies in the lab had found that hypoxia potentiates angiogenesis of explants from rat aorta in an mTOR dependent way. In this study, we have extended this observation to proliferation of RAEC *in vitro* and a RAEC-spheroid sprouting assay (angiogenesis *in vitro*). Rapamycin, an inhibitor of mTOR, inhibited proliferation of RAEC and sprouting of endothelial cells in vitro under hypoxia. Interestingly, upon hypoxic stimulation, mTOR is highly phosphorylated; both mTOR and phospho-mTOR accumulate in cell nucleus, as does HIF-1α. However, S6k and 4E-BP1, two downstream targets of mTOR that are involved in translational control are hypophosphorylated at the same time. In low O_2 tension (1% O_2), increased nuclear HIF-1α levels are observed over time as well as with decreased O_2 saturation. Similarly, the growth factor PDGF-BB induces HIF-1α nuclear accumulation under normoxic conditions. Hypoxia and PDGF-BB synergistically enhance HIF-1α nuclear levels. mTOR inhibition strongly reduces nuclear HIF-1α levels under hypoxia or/and PDGF-BB stimulation while MEK1/2 blockage only

reduces PDGF-BB-induced nuclear HIF-1α accumulation in normoxia. Neither JNK nor p38 inhibition alters nuclear HIF-1α protein levels. HIF-1α mRNA levels remain stable under different oxygen saturations and upon mTOR or MEK1/2 inhibition. Notably, rapamycin-decreased HIF-1α nuclear accumulation can be rescued by proteasomal inhibition under hypoxia. Finally, mouse embryonic fibroblasts lacking HIF-1α significantly decreased proliferation rates under hypoxia when compared to wild type cells. However, mTOR over expression restores and further augments hypoxia-triggered proliferation both in HIF-1α wild type and in HIF-1α deficient cells.

Taken together, hypoxia activates both HIF-1α-dependent and HIF-1α-independent regulation of cell proliferation and angiogenesis. Hypoxia-induced mTOR activation reduces S6K1 and 4E-BP1 phosphorylation. mTOR activity is required for protecting HIF-1α from proteasomal degradation. Further investigations on mTOR and HIF-1α during hypoxia in RAEC proliferation, spheroid sprouting assays, and angiogenesis *in vivo* are required. Thus, targeting mTOR to enhance or reduce angiogenesis in response to hypoxia may be clinically relevant.

1. INTRODUCTION

1.1. Growth of new blood vessels

Three distinct forms of vessel formation have been described so far. Vasculogenesis refers to the formation of blood vessels by endothelial progenitors (angioblasts) arising from various embryonic regions or the adult bone marrow (BM). This process involves differentiation, proliferation, migration and association of primitive endothelial cells. Angiogenesis refers to the generation of new capillary blood vessels by sprouting or longitudinal division from pre-existing ones, whilst arteriogenesis contributes to the stabilization of these sprouts by mural cells for the transformation of a small arteriole into much larger conductance artery. Angiogenesis involves the enlargement of venules, which sprout or become divided by pillars of periendothelial cells (intussusceptions) or by transendothelial cell bridges, which then split into individual capillaries. Collateral growth stands for the formation of collateral bridges between arterial networks by expansive growth and remodeling of pre-existing vessels upon occlusion of a supply vessel for example by thrombosis. Recruitment of monocytes to the shear stress-activated endothelium plays a critical role in this process [4-6] (Figure 1).

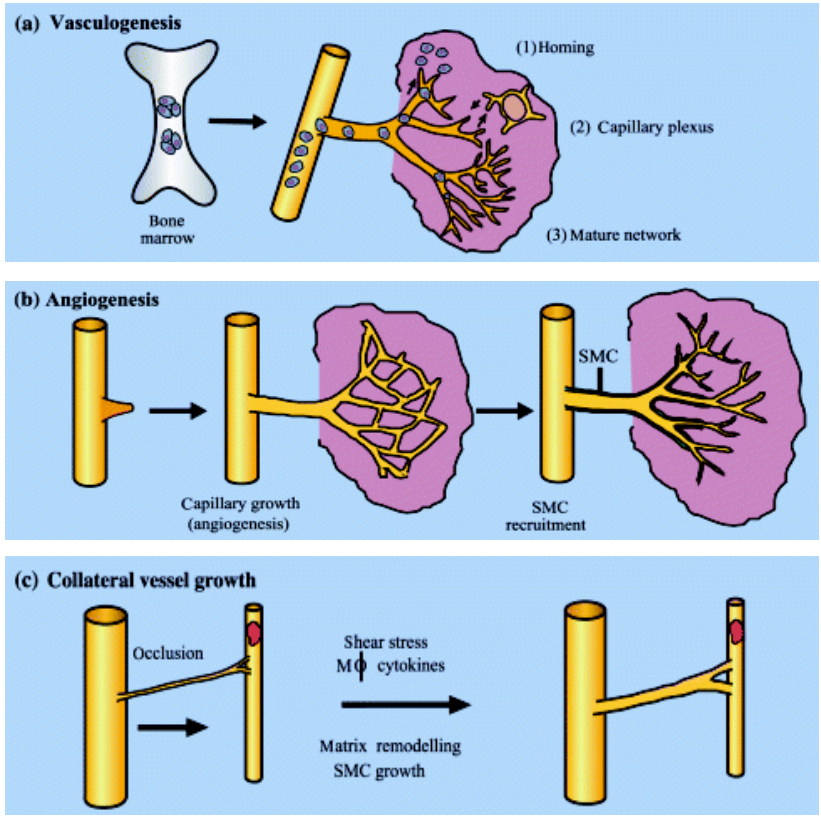


Figure 1. Mechanisms of vessel growth in adult. (a) Vasculogenesis refers to the recruitment of bone marrow-derived endothelial progenitors, which become incorporated into nascent vessels or stimulate new vessel growth by releasing pro-angiogenic factors. (b) Angiogenesis is characterized by the sprouting of new endothelial cell-lined vessels from preexisting vessels; arteriogenesis refers to the subsequent stabilization of these nascent vessels via recruitment of smooth muscle cells. (c) Collateral growth denotes the expansive growth of pre-existing collateral vessels upon occlusion of a supply vessel. Adapted from [4].

1.1.1. Angiogenesis

Angiogenesis can be initiated in response to stimuli like hypoxia, ischemia, inflammation, and blood coagulation. These factors trigger the release of angiogenic molecules (activators and inhibitors) that activate endothelial cells to induce a set of transcriptional events. Consequently, the activities cause conformational changes of the microvasculature.

Angiogenesis is preceded by vasodilation, which is partially induced by nitric oxide (NO). NO and vascular endothelial growth factor (VEGF) induced in response to angiogenic stimuli cause an increase in vascular permeability. This together with the inhibition of endothelial cell receptor Tie-2 (tyrosine kinase with immunoglobulin and EGF homology domains) by Angiopoietin-2 (Ang-2) loosens the extracellular matrix (ECM) surrounding microvessels. In turn, proteases degrade the subendothelial basement membrane and the surrounding ECM which process facilitates endothelial cell migration and liberates activators for angiogenesis. These processes concurrently allow endothelial cells that constitute the inner layer of vessels to migrate and to proliferate. Following migration into the ECM, endothelial cells proliferate, assemble into solid cords, and acquire lumens. The primitive vascular networks (or plexa) are remodeled through branching, sprouting, and pruning of the vascular endothelium. As new vessels form, Ang-2 is withdrawn and Ang-1 is induced from mesenchymal cells so as to recruit pericytes that form single cell layers around endothelial capillaries and to recruit vascular smooth muscle cells (VSMCs) that coat veins and arteries [6-8] (Figure 2).

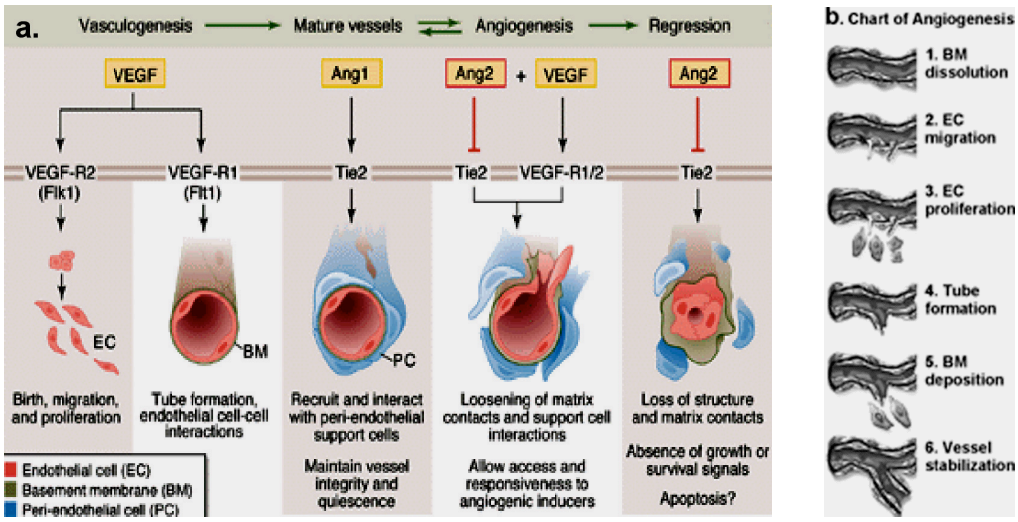


Figure 2. a) Sequential events of angiogenesis in comparison with vascular genesis. The processes have been explained in the text. Adapted from [6]. **b)** Sequential events in angiogenesis. (1) Basement membrane disintegration opens the way for (2) endothelial cell migration. (3) Cords of cells proliferate and (4) define a new vascular channel. (5) Cessation of cell migration and proliferation coincides with the recruitment of perivascular support cells (6) with the formation of a new basement membrane and vessel maturation and stabilization. Adapted from [8].

Angiogenesis happens in both physiological and pathological conditions. It plays essential roles during embryogenesis, normal tissue repair in adult, and the remodeling of the female reproductive organs (menstrual cycle, ovulation and placental development). In an in vitro model, filopodia at the tips of vascular sprouts were shown to build intervascular bridges in the growing vascular plexus of the area vasculosa at embryonic day 3 [9]. Examination of wound drainage fluid from postoperative patients was shown to contain both promoters and inhibitors of angiogenesis. This suggests that the local balance between them may control angiogenesis during healing [10]. Moreover, wound healing is probably regulated by growth factors like FGF-2 and VEGF, and macrophages participate in the process by releasing angiogenic factors [11-13]. Angiogenesis is cyclically regulated in the ovary [14]. A recent study shows that estrogen induces villous placental angiogenesis via stimulating trophoblast VEGF expression for fetal growth and development in early primate pregnancy [15]. Although angiogenesis is the principal process in neovascularization, effective neovascularization may further require mobilization and *in situ* differentiation of bone marrow-derived endothelial progenitor cells [16,17].

Angiogenesis is also turned on in inflammatory, ischemic and cancerous diseases. The rate of angiogenesis is increased in human synovium with rheumatoid arthritis (RA) which may cause subsequent destruction of the articular cartilage [18]. Synovial inflammation and angiogenesis are enhanced in a substantial proportion of patients with osteoarthritis (OA) [19]. Synovial fluids from patients with OA may stimulate endothelial tube formation in vitro [20]. The angiogenic pathway VEGF/flk-1 (KDR) may play an important role in the pathogenesis of RA and OA [21]. Moreover, insufficient angiogenesis presents in peri-infarct area in ischemic coronary heart disease or in ischemic skeletal muscle in peripheral artery disease [22-24]. The contribution of angiogenesis to tumor development has been intensively studied for years. The investigations cover mechanistic studies of tumor growth and metastases as well as therapeutic studies against cancer[25]. Defects in angiogenesis have been considered as etiological components in the inheritance of high blood pressure. The relationships between angiogenesis and hypertension, cardiovascular diseases and tumor will be addressed subsequently in more details.

1.1.2. Angiogenesis and hypertension

Hypertension causes characteristic microvascular arteriolosclerosis, which may impair coronary haemodynamics and facilitate development of adaptive myocardial hypertrophy [26]. In addition, reduced microvascular density, namely rarefaction of arterioles and capillaries has been identified in both rodent hypertensive models and in human hypertensives [22,27]. Furthermore, late-onset hypertension is associated with a lack of coronary angiogenesis and also with a decrease in dilator reserve despite of the absence of myocardial hypertrophy [28]. Therefore, inadequate angiogenesis is closely related to hypertension per se and hypertension-dependent target organ damage [29,30]. Studies in this respect suggest that impaired angiogenesis in hypertensives, besides high blood pressure itself, may be due to reduced nitric oxide (NO) biosynthesis, activation of the Renin-Angiotensin-Aldosterone System (RAAS), and other factors [31,32].

Local delivery of human tissue kallikrein gene rescued rat endothelial dysfunction caused by arterial hypertension and microvascular rarefaction caused by diabetes mellitus [33]. 17β-estradiol, a steroid hormone, has been shown to upregulate the production of endothelial nitric oxide (EDNO), which in turn may induce endothelium-dependent relaxation of coronary arteries as well as anti-atherosclerotic effect [34].

Similarly, 17β-estradiol increased EDNO-mediated vasodilation both in female hypertensive rats and in large coronary conductance arteries as well as coronary microvascular resistance arteries of postmenopausal women [35,36]. In concordance with these findings, 17β-estradiol and other ovarian hormones may stimulate angiogenesis [34].

Angiotensin II (Ang II) is a key regulator of blood pressure, body fluid homeostasis and the final active messenger of the RAAS. Maintenance of Ang II at normal levels during periods of hypertension or high-salt diet completely eliminates rarefaction of vessels in hypertensive rats [37,38]. This process is thought to be mediated via the AT1 and AT2 receptors [37,38]. In contrast, AT receptor antagonist, losartan, did not affect VEGF and sFlt-1 serum levels in hypertensive patients [39]. Moreover, a decrease of circulating Ang II by enalapril, an angiotensin-converting enzyme (ACE) inhibitor, or losartan induces in vivo angiogenesis in mice implying an inhibitory effect of ANG II [40]. Thus, the angiotensin II pathway can both stimulate and inhibit angiogenesis depending on the tissue and the receptors activated [41].

1.1.3. Angiogenesis and cardiovascular diseases

In cardiovascular diseases, angiogenesis is closely related to ischemic heart disease. Myocardial ischemia, a condition of reduced oxygen supply to heart tissue, is a common consequence of narrowing or occlusion of coronary arteries due to atherosclerosis, and may lead to myocardial infarction. To overcome ischemia, the heart develops collateral circulation so as to provide a bypass for blood supply to the ischemic myocardium [42].

Formation of collateral passes to the ischemic tissue in the heart is supposed to involve angiogenesis and collateral recruitment [43,44]. Unfortunately, natural neovascularization does not fully restore blood flow into the ischemic region of the heart [45]. In this process, ischemia-induced decrease in the partial pressure of cellular oxygen is a potent stimulator of neovascularization [42]. Both myocardial ischemia and hypoxia upregulate expression of angiogenic growth factors [45].

Therapeutic angiogenesis can be induced by administration of proteins of angiogenic factors, viruses expressing genes for angiogenic factors, undifferentiated or differentiated embryonic stem cells, and finally endothelial progenitors derived from

BM or umbilical cord blood. The clinical use of growth factors to enhance or promote collateral blood vessels in ischemic tissue has been considered a promising possibility for therapeutic angiogenesis [46]. Angiogenic growth factors in the form of recombinant proteins or of the genes encoding these proteins are often administered through a variety of delivery methods including intravenous, selective intracoronary, intramyocardial (transendocardial and transepicardial) and intrapericardial application [47,48]. The earliest clinical reports assessing therapeutic angiogenesis in myocardium have used recombinant FGF-1 protein and naked plasmid DNA encoding VEGF, respectively [49,50]. Recombinant protein delivery has been associated with side effects such as hypotension, edema, anemia, thrombocytopenia and renal toxicity. Gene transfer provides a more precise way of growth factor administration with the disadvantage of vector-induced cytotoxicity [45,48].

Hypoxia-inducible factor-1α (HIF-1α), a transcription factor for a set of genes involved in angiogenesis, has been defined as a marker of early response of myocardium to ischemia and hypoxia. An alternative strategy of using transgenes encoding angiogenic growth factors is targeting HIF-1α to regulate the expression of multiple angiogenic genes (see below) [42,47].

1.1.4. Angiogenesis and tumor

Angiogenesis is critical for tumorigenesis. Tumor growth and metastasis require new blood vessel growth, namely angiogenesis [51]. Though tumor type-dependent, a tumor surpassing the size of 0.2-2 mm hardly grows without an adequate vasculature [52,53]. Therefore, angiogenesis is associated with prognosis and the risk of metastasis [54,55]. Therapeutically, inhibition of angiogenesis may retard tumor growth, metastasis and may cause tumor regression [56,57].

Angiogenesis is switched on during preneoplastic stages in the development of a tumor. Tumor-induced angiogenesis is switched on due to the production of pro-angiogenic growth factors such as VEGF by the tumor cells, which initiate angiogenesis (Figure 3).

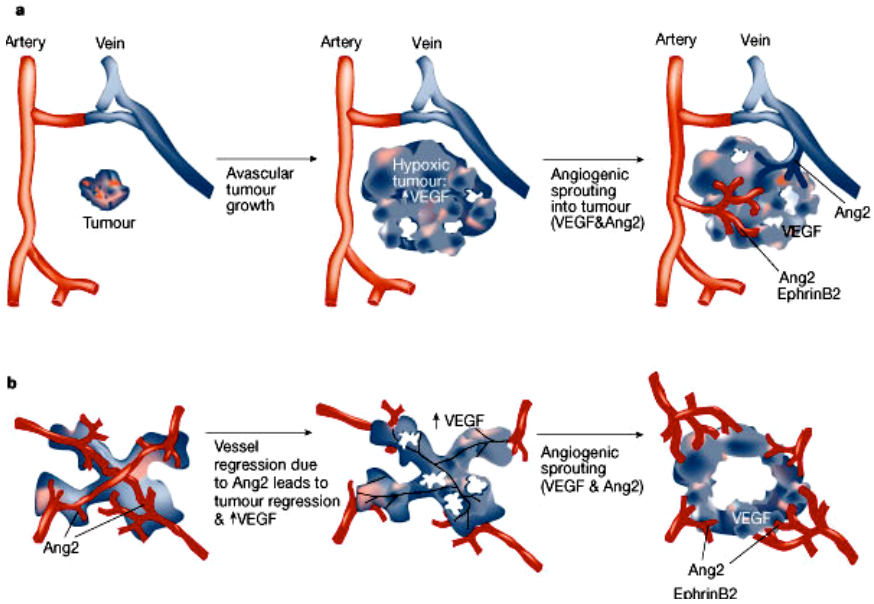


Figure 3. Models of tumor angiogenesis. a) Model of avascular tumor initiation **b)** tumor initiation involving host vessel co-option. Involvement of some angiogenic factors has been displayed in the sequential processes. Adapted from [25].

Understanding mechanisms of tumor angiogenesis greatly facilitates the development of anti-tumor strategies. These include interfering with angiogenic ligands, their receptors and downstream signaling transduction, upregulating endogenous or delivering exogenous inhibitors for angiogenesis, and direct targeting tumor vasculature [58]. Hexapeptides designed to inhibit the interaction of VEGF (165) with VEGF receptor abrogate proliferation of endothelial cells and block angiogenesis, tumor cell growth and metastasis in animal models [59]. This is supported by using antibodies against VEGF to block tumor angiogenesis in clinical trials [60-62]. Targeting endothelial-specific receptor, Flk-1 and Tie2, to impair tumor vascularization also elicit promising results [63,64]. Intrinsic angiogenesis inhibitors such as angiostatin and endostatin as well as administration of extrinsic anti-angiogenic agents offer new insights in anti-tumor therapy [56,57,65]. Ombretastatin A-4 prodrug, a compound designed to target tumor vasculature, decreases tumor blood flow and tumor perfusion [66].

Although promising, anti-angiogenic therapy against tumors has problems. First, the frequently used subcutaneous model of tumor growth does not accurately predict responses in human tumors. Secondly, tumor regression does not rule out relapse.

Third, tumor models used for preclinical studies often use highly proliferative cells. These may be different from slowly growing tumors found in humans [58]. Finally, given the diversity of angiogenic molecules secreted from tumors and the heterogeneity of microvascular endothelial cells, anti-angiogenic treatment of tumors should probably be carried out with mixtures of agents [58,62].

1.2. Hypoxia

Hypoxia potently influences the expression of about 1% of the genes in the genome [67]. However, there is no defined line between hypoxia and normoxia within a mammalian body and tissues. Under normoxic conditions (1 ATA-atmosphere absolute, or 158 mmHg), with the air one breathes into the lung containing about 21% O_2, cells in our body live in an O_2 concentration ranging from 12% to 0.5%. The extents of the two extremes varies in different organs or tissues. For example, O_2 is about 14% in circulating arterial blood, and less than 10% in the myocardium [68]. Organs like liver or tissues as skin, cartilage, bone marrow and lens of eyes, amniotic cavity stay within the O_2 range of 2% to 8% [67]. The heterogeneity of oxygen concentrations in a healthy individual creats difficulties in defining hypoxia.

A practical definition, which is close to biophysiological and clinical settings, is that hypoxia stands for a state when oxygen delivery does not meet the demand of an organ, tissue or cell [67]. Hypoxia appears when 1) O_2 partial pressure is low in arterial blood due to pulmonary diseases or high altitude (hypoxemic hypoxia); 2) the capacity of O_2 in blood is reduced as a result of anemia, methemoglobin formation, or carbon monoxide poisoning (anemic hypoxia); 3) insufficient blood perfuses to tissues (circulatory or ischemic hypoxia); 4) the diffusion geometry is altered such as increased diffusion distances, concurrent versus countercurrent blood flow within microvessels (diffusional hypoxia); 5) intoxication causes cells unable to use O_2 as in cyanide poisoning (histotoxic or cytotoxic hypoxia) [69,70].

Hypoxic areas with O_2 saturation close to zero often occur in ischemic heart tissue. Bordering this hypoxic core, the region short of O_2 extends up to several millimeters with increasing O_2 concentration to the biophysiological normoxic domain [71]. Under this circumstances, mild hypoxia is associated with a 30% to 60% decrease (1% to 3% O_2) in PO_2 [68]. As in the ischemic myocardium, the O_2 gradient exists as well in solid tumors. Tumors thrive on about 1% to 3% O_2 though this range is somehow

tumor type-dependent [69,70]. For experimental cell culture, hypoxia is routinely defined as 0.5-2.0% O_2 (usually complemented with 5% CO_2, the rest nitrogen). At this level toxicity and growth-inhibition to cells are not present, and cellular responses to hypoxia such as induction of HIF-1α can be triggered [67].

1.2.1. Hypoxia and angiogenesis

Once an organism or a single cell is exposed to hypoxia, it may react with switching on mechanisms that regulate responses to hypoxia and adapt to changes in oxygen tension. The switch from aerobic to anaerobic metabolism is mediated by the induction of glycolytic enzymes and glucose transporters. Hypoxia and other yet poorly defined stimuli drive tumoral, inflammatory, and connective tissue cells to generate angiogenic molecules [30]. These adaptive alterations may lead to the improvement of oxygen delivery [72]. No matter how the regulatory mechanisms differ among various organisms or cells, the fact that they work through hypoxia-induced molecules is obvious. Interestingly, the hypoxia-dependent generation and regulation of these molecules, to a large extent, are managed at the transcriptional level by hypoxia-inducible factors (HIFs).

As described in the last chapter, numerous disorders are in close correlation to either an excess or insufficient number of vessels. The angiogenic effect mediated by HIF is particularly important in certain cell types such as endothelial and smooth muscle cells because of their role in building up the neovasculature. An increase in the level of HIF-1/HIF-1 alpha subunit (HIF-1α) is an early response to myocardial ischemia or tumor growth. Thus, HIF-1α is a useful temporal marker of jeopardized myocardium and solid tumors [42,73]. More importantly, understanding of HIF-1 regulation opens promising opportunities for clinical applications.

1.2.2. Hypoxia-inducible factor (HIF) family

Hypoxia-inducible factors are mainly responsible for cellular adaptation to oxygen deprivation. The family of HIF has been growing since the first oxygen-regulated transcriptional factor, HIF-1, was identified more than a decade ago [74]. In general, HIFs function as heterodimeric transcription factor consisting of one of the alpha (α) subunits and one of the beta (β) subunits, which is more precisely called aryl hydrocarbon receptor nuclear translocator (ARNT). Both α and β groups of the

subunits belong to a protein superfamily called basic helix-loop-helix-Per/Arnt/Sim (bHLH-PAS). When oxygen tension drops below physiological level, HIF-α subunits translocate to the nucleus and dimerize with HIF-β subunits [75,76]. In turn, the dimers combine with other cofactors to form functional transcriptional complexes, which bind to hypoxia response elements (HREs, 5'-RCGTG-3') located in the promoter or enhancer regions of hypoxia-inducible genes and thereby activate transcription of target genes [77]. The basic characteristics of HIF family members defined so far have been summarized in Table 1.

Protein name	Aliases	Gene name	Chromosome	Main domain	Function
HIF-1α	hypoxia-inducible factor-1 alpha, HIF-1 alpha, ARNT interacting protein, member of PAS protein 1 (MOP-1)	HIF1A, MOP1, PASD8, HIF-1 alpha, HIF1-ALPHA	14 (human), 6 (rat), 12 (mouse)	DNA binding, HLH, PAS1, PAS2, PAC, ODD, NTAD, ID, CTAD, Nuclear translocator	heterodimerizes with HIF-1β or/and HIF-2β for active transcription mainly in responses to hypoxia [75,78];DNA enhancer binding for active transcription [79]; histone acetyltransferase binding for transcriptional activity [80].
HIF-2α	endothelial PAS domain protein 1 (EPAS-1), member of PAS protein 2 (MOP-2), hypoxia-inducible factor-2 alpha (HIF-2 alpha), HIF2 alpha, HIF-1 alpha-like factor (HLF), HIF-related factor (HRF)	EPAS 1, HIF2A, MOP2, PASD2	2 (human), 6 (rat), 17 (mouse)	DNA binding, HLH, PAS1, PAS2, PAC, ODD, NTAD, CTAD, Nuclear translocator	heterodimerizes with HIF-1β or/and HIF-2β for active transcription mainly in responses to hypoxia [79,81,82]; DNA enhancer binding for active transcription [79]; histone acetyltransferase binding for transcriptional activity [80].
HIF-3α	hypoxia-inducible factor-3 alpha, Inhibitory PAS domain protein, IPAS	HIF3A, HIF-3A IPAS, MOP7, PASD7, HGNC: 15825	19 (human), 1 (rat), 7 (mouse)	HLH, PAS1, PAS2, PAC, Nuclear translocator, ODD, NTAD	Heterodimerizes with HIF-1β for transcriptional activity in response to hypoxia [83]; inhibitory or complementary effects on other HIF family transcriptional factors [84-86]
HIF-1β	ARNT protein, aryl hydrocarbon receptor nulcear translocator, Dioxin receptor nuclear translocator, hypoxia-inducible factor 1 beta (HIF-1 beta)	ARNT, HIF1B, TANGO, HIF1BETA, HIF-1 beta	1 (human), 2 (rat), 3 (mouse)	DNA binding, HLH, PAS1, PAS2, PAC, Nuclear translocator	required for activity of the Ah (dioxin) receptor [87,88]; herterodimerizes with AHR, HIF-1α, HIF-2α or/and HIF-3α for transcriptional activity [75,79,81,83,88]; coactivator for transcription [88].
HIF-2β*	ARNT protein 2, aryl hydrocarbon receptor nuclear translocator 2	ARNT2, KIAA0307	15 (human), 8/1 (rat), 7 (mouse)	DNA binding, HLH, PAS1, PAS2, PAC, Nuclear translocator	has ARNT activity; able to heterodimerize with HIF-1α, HIF-2α for transcriptional activity [78,82]
*** nomenclature is unclear**	HLH: helix-loop-helix, AHR: aryl hydrocarbon receptor	PAS: Period (Per)-Aryl hydrocarbon receptor nuclear translocator (Arnt)-Single-minded protein (Sim),	PAC: PAS-associated C-terminal domain	ODD: oxygen-dependent degradation domain	N(C)TAD: N(C)-terminal transactivation domain, ID: inhibitory domain

Table 1: Basic characteristics of HIF family members.

The β subunits of HIF are constitutively present while the α subunits are induced especially in response to hypoxia although other stimuli such as growth factors, cytokines or chemical compounds may contribute to their induction as well [75,82,89-91]. Among the HIF-α subunits, HIF-1α is the most ubiquitously and abundantly induced in tissues and cells under hypoxic conditions compared to HIF-2α and HIF-3α. Still, the existence of the splice variants of HIF-α subunits enriches the complexity of the HIF family as well as the combination of the functional subunits [86,92-96].

Structurally, most of the variant HIF-α subunits are similar in their bHLH and PAS domains but differ in their Tran activation domains which may explain the presence of overlapping yet distinct transcriptional target genes [86]. Besides, most of the HIF-α variants share a high degree of homology in their oxygen-dependent degradation domains (ODD), including the two critical proline residues (Pro402 and Pro564) that account for O_2 tension-associated stabilization of the proteins. This provides theoretical support for the degradational regulation of the proteins and their responsiveness to hypoxia [86,93]. In addition, the highly conserved 50 amino acids located at the C-termini of some HIF-1α forms and HIF-2α are important for O_2-regulated interaction with the transcriptional coactivator p300 [76].

Functionally, less is known about HIF-2α and HIF-3α than that for HIF-1α. Recognition of the similar core HREs in the hypoxia responsive genes elicits common readouts for HIF-1α, -2α and –3α activation. The N-terminal transactivation domain (NTAD) and the C-terminal transactivation domain (CTAD) synergistically mediate the transcriptional activity of HIF-1α and –2α. Though it might be cell type-dependent, the existence of the inhibitory domain (ID) between NTAD and CTAD potentially counteracts the tansactivational activity of CTAD [97]. However, HIF-3α variants lack the CTAD domain, hence may substantially reduce transcriptional activities in response to hypoxia. IPAS (inhibitory PAS domain protein), a splicing variants of HIF-3α, isolates the dimerizational interaction between HIF-1α and HIF-1β implying the antagonizing role of HIF-3α in the HIF system [84,85]. Despite of this, HIF-3α transcription is induced by hypoxia in multiple organs while mRNA levels of HIF-1α, -1β, and -2α remain unaffected evidencing a reactive aspect of HIF-3α in protection against hypoxic damage [98]. Still, HIF-1α knockout mice exhibit mid-

gestation lethality with severe blood vessel defects indicating a critical role for HIF-1α in embryonic development, hypoxic responses and vascular formation [99].

1.2.2.1. HIF-1alpha (HIF-1α)

Being one of the most important hypoxia-driven factors, HIF-1α (~120 kDa) is often coupled with HIF-1β (91-94 kDa) to form a HIF-1 dimer [75]. This action together with the selective coactivators targeting the different domains in the protein and the target DNA binding of the complex may trigger a series of transcriptional activities for a set of genes involved in the regulation of erythropoiesis, angiogenesis, vasomotor control, and energy metabolism, etc (Table 2).

Function	Genes
Amino acid metabolism	Transglutaminase 2
Angiogenesis	EG-VEGF, ENG, LEP, LRP1, TGF-β3, VEGF
Apoptosis	NIP3, NIX, RTP801
Cell proliferation	Cyclin G2, IGF2, IGF-BP1, IGF-BP-2, IGF-BP3, WAF-1, TGF-α, TGF-β3, ET1
Cell adhesion	MIC2
Cell survival	ADM, EPO, IGF2, IGF-BP1, IGF-BP2, IGF-BP3, NOS2, TGF-α, VEGF
Cytoskeletal structure	KRT14, KRT18, KRT19, VIM
Drug resistance	MDR1
Energy metabolism	LEP
Erythropoiesis	EPO
Extracellular matrix metabolism	CATHD, Collagen type V (α1), FN1, MMP2, PAI1, 4-PH alpha-1, UPAR
Epithelial homeostasis	Intestinal trefoil factor
Glucose metabolism	HK1, HK2, AMF/GPI, ENO1, GLUT1, GAPDH, LDHA, PFKBF3, PFKL, PGK1, PKM, TPI, ALDA, ALDC
Iron metabolism	Ceruloplasmin, Transferrin, Transferrin receptor
Motility	ANF/GPI, c-MET, LRP1, TGF-α
Nucleotide metabolism	Adenylate kinase 3, Ecto-5'-nucleotidase
PH regulation	Carbonic anhydrase 9
Regulation of HIF-1 activity	P35srj
Transcriptional regulation	EC1, DEC2, ETS-1, NUR77
Vascular tone	α_{1B}-adrenergic receptor, ADM, ET1, HO1, NOS2

ADM, adrenomedullin; ALDA, aldolase A; ALDC, aldolase C; AMF, autocrine motility factor; CATHD, cathepsin D; EG-VEGF, endocrine gland-derived VEGF; ENG, endoglin; ET1, endothelin 1; ENO1, enolase 1; EPO, erythropoietin; FN1, fibronectin 1; GLUT1, glucose transporter 1; GLUT3, glucose transporter 3; GAPDH, glyceraldehyde-3-p-dehydrogenase; HK1, hexokinase 1; HK2, hexokinase 2; HO1, haem oxygenase 1; IGF2, insulin-like growth factor 2; IGF-BP1, IGF-binding protein 1; IGF-BP2, IGF-binding protein 2; IGF-BP3, IGF-binding protein 3; KRT14, keratin 14; KRT18, keratin 18; KRT19, keratin 19; LDHA, lactate dehydrogenase A; LEP, leptin; LRP1, LDL receptor-related protein 1; MDR1, multidrug resistance 1; MMP2, matrix metalloproteinase 2; NOS2, nitric oxide synthesis 2; PAI1, plasminogen-activator inhibitor 1; PFKBF3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PFKL, phosphofructokinase L; PGK1, phosphoglycerate kinase 1; 4-PH alpha-1, prolyl 4-hydroxylase alpha-1; PKM, pyruvate kinase M; TGF-α, transforming growth factor, alpha; TGF-b3, transforming growth factor, beta3; TPI, triosephosphate isomerase; VEGF, vascular endothelial growth factor; UPAR, urokinase plasminogen activator receptor; VEGFR2, VEGF receptor 2; VIM, vimentin.

Table 2. HIF-1 target genes. Adapted from [100].

Involvement of HIF-1α in hypoxic response is an instantaneous event. Jewell and colleagues were able to show that HIF-1α protein accumulates in HeLaS3 cells nucleus within 2 minutes of hypoxic exposure, and HIF-1 DNA-binding activity appears earlier in this time range as well [101]. Though the bHLH domain is an indispensable participant in these process, the PAS domains may serve as sensors of oxygen and perform as transducers of signals by protein–protein interactions [102]. In addition, the oxygen-dependent degradation (ODD) domain is fully required for the stabilization and function of HIF-1α since it contains two prolyl residues (Pro402 and Pro564 in human) for hydroxylation, a lysine 532 residue for acetylation and is overlapping with the N-terminal tansactivation domain. The NTAD and CTAD are very important for HIF-1α to exert transcriptional activity since they provide docks for co-activators as p300/CBP, SRC-1 and Ref-1. The ID domain is suggested to repress the activity of CTAD under non-hypoxic conditions. However, other ID-independent but hypoxia-dependent mechanisms may modulate NTAD and CTAD as well [97,103,104]. Besides, the undefined region for phosphorylation and the hydroxylation of the asparagine 803 within the CTAD provide more possibilities for promoting or abolishing the transactivation of HIF-1 [97] (Figure 4).

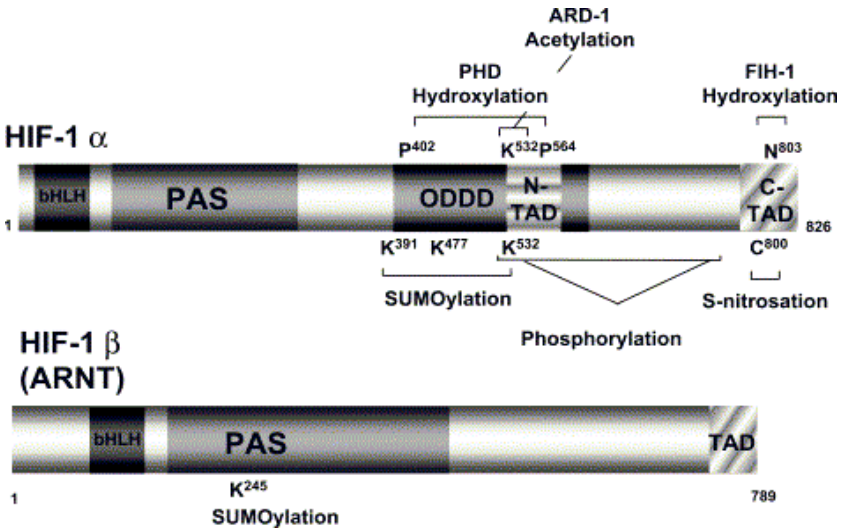


Figure 4. Domain structure and structural location of the posttranslational modifications of HIF-1α and β
bHLH, basic helix-loop-helix; PAS, PER-ARNT-SIM; ODDD, oxygen-dependent degradation domain; PHD, prolyl hydroxylase domain; ARD1, ARrest Defective-1 protein; N-TAD and C-TAD, N- and C-terminal activation domains; FIH-1, factor inhibiting HIF-1. Adapted from [105].

1.2.2.2. Biological regulation of HIF-1α

Many post-translational modifications regulate the functions of HIF-1α. HIF-1α is degraded in normoxia, and stabilized under hypoxia when no other forces are involved. Stimuli other than O_2 such as growth factors, oncogenic mutations or chemicals are able to increase HIF-1α cellular levels.

1.2.2.2.1. Oxygen-dependent regulation of HIF-1α

Alterations between normoxia and hypoxia may switch on or off the modification processes to HIF-1α including phosphorylation, hydroxylation, ubiquitination, acetylation, sumoylation, and S-nitrosation (summarized in Figure 6).

Phosphorylation of HIF-1α or its coactivators has been reported in several studies and is related to enhanced transcriptional activities [106-109]. However, the specific residues within HIF-1α for phosphorylation have yet to be identified. Still, MAPK has been shown to phosphorylate HIF-1α at the residue(s) of 531-826, 796, and 530-744 respectively [107-109]. Though HIF-1β may bind preferentially to the phosphorylated form of HIF-1α, it is controversial whether the enhanced transcriptional activity is directly caused by the phosphorylation of HIF-1α or due to the phosphorylation of its coactivator p300 [106,109]. There is no evidence for c-Jun N-terminal kinase (JNK) in the phosphorylation of HIF-1α, and data are contradictive about the involvement of p38 kinase [106,107].

Hydroxylation of HIF-1α involves two similar yet distinct subsets of 2-oxoglutarate dioxygenases from the iron (II)- and 2-oxoglutarate-dependent oxygenases superfamily. The prolyl-targeting set of dioxygenase in humans are termed Prolyl Hydroxylase Domain (PHD) proteins and contain three members, PHD1, PHD2, and PHD3 [110,111], which hydroxylate specific prolyl residues (Pro402 and/or Pro564) in the ODD domain of HIF-1α with differential efficacy in the presence of O_2. PHD1 and PHD2 modify both of the prolyl sites while PHD3 preferentially hydroxylates Pro564 [111]. Interestingly, siRNA silencing of PHD2 but not PHD1 or PHD3 gene is sufficient to stabilize HIF-1α under normoxic conditions illustrating the critical role of PHD2 in the process of HIF-1α hydroxylation [112]. Prolyl hydroxylation leads to binding of von Hippel-Lindau protein (pVHL) containing E3 ligase complex and subsequent ubiquitination and proteasomal degradation of HIF-1α [113-115] (Figure 6). In addition,

another dioxygenase called Factor Inhibiting HIF-1 (FIH-1) is known to hydroxylate asparagine residue 803 in the C-terminal transactivation domain of HIF-1α in normoxia. This modification attenuates the interaction of HIF-1α with its co-activator CBP/p300 therefore abolishing the transcriptional activity of HIF-1 [116-118] (Figure 5).

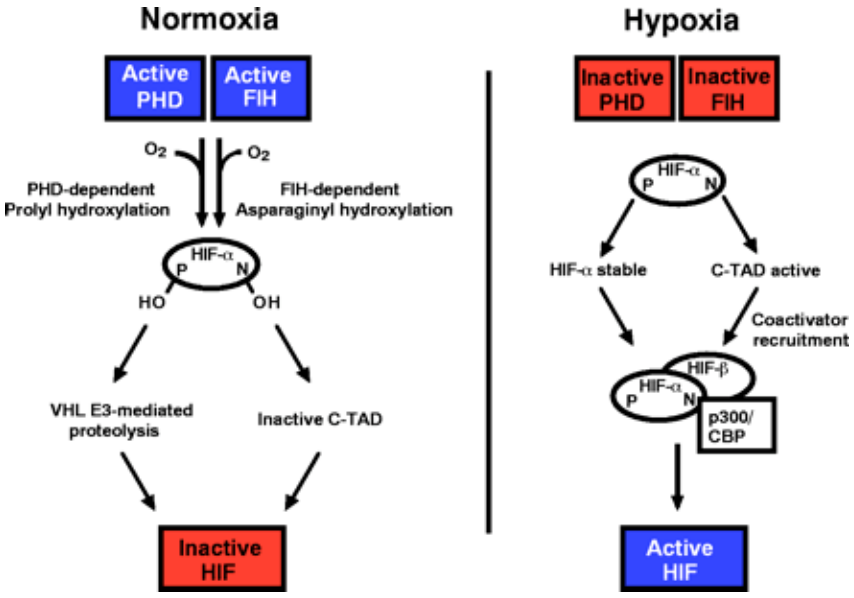


Figure 5. Two independent hydroxylation pathways regulate HIF activity in response to cellular oxygen level. In normoxia, oxygen availability enables PHD-dependent prolyl hydroxylation of the HIF-α ODD. This hydroxylation allows binding of the VHL E3 ligase leading to ubiquitylation and degradation of HIF-α subunits. Oxygen availability also enables FIH-dependent asparaginyl hydroxylation of the C-TAD, blocking interaction with the p300/CBP co-activator. In hypoxia, the PHD and FIH enzymes are inactive and the lack of hydroxylation results in stable HIF-α able to form a DNA-binding heterodimer with HIF-β and recruit p300/CBP at the C-TAD. Adapted from [119].

Acetylation and deacetylation histones have been linked to the regulation of transcriptional activity [120]. Recently, HIF-1α was also reported to be acetylated. This modification favours its interaction with pVHL and thus destabilizes HIF-1α[105]. A putative acetyltransferase, ARest Defective-1 protein (ARD1), is able to acetylate lysine 532 in the ODD domain of HIF-1α[121]. The proteasomal inhibitor MG132 increases levels of acetylated HIF-1α in normoxia than that in hypoxic conditions [121]. In accordance with this, ARD1-acetylated wild type ODD protein showed increased interaction with pVHL whereas ARD1-acetylated ODD-K532R mutant protein did not, indicating that the acetylation of Lys532 by ARD1 is potentially involved in the pVHL-ubiquitin complex mediated proteasomal degradation of HIF-1α [121]. Moreover, a

global inhibitor of deacetylases, butyric acid, is capable of inducing HIF-1α degradation in a way causing an anti-angiogenic effect [122].

Ubiquitination of a target protein is a stepwise process consisting of activation of the E1 enzyme, conjugation of E2, and the consecutive ubiquitin transfer to the target and leading to protein degradation. Normoxic degradation and hypoxic stabilization of HIF-1α have been closely linked to the protein ubiquitination and proteasomal destruction processes. After prolyl hydroxylation and acetylation, HIF-1α is recognized and bound to the β domain of pVHL, which is in a E3 ligase complex [123]. Moreover, another E3 ubiquitin ligase, Murine Double Minute 2 (MDM2), was proposed to ubiquitinate HIF-1α upon p53 and HIF-1α interaction under hypoxia evidencing the role of p53 as a tumor suppressor [124]. Furthermore, Jab1 (Jun activation domain-binding protein-1) is able to compete with p53 and interact with the ODD domain of HIF-1α leading to the stabilization and upregulation of the activity of HIF-1α under hypoxic conditions [125].

In mammalian cells, **sumoylation** post-translationally modifies certain proteins by attaching SUMO (small ubiquitin-like modifier) covalently to specific lysine residues within the proteins, a process involving three SUMO proteases, E1, E2 and E3 [126]. Four SUMO gene-encoded proteins, SUMO-1, -2, -3, -4 have been identified so far [127,128]. In some circumstances, the SUMOs may work in concert to make the sumoylation forming polySUMO chains [129]. Sumoylation has been shown to be engaged in nucleocytoplasmic trafficking and signaling of some proteins as well as negative regulation of transcription in most of the cases [126]. Though sumoylation imposes direct modification on some transcriptional factors, the process could also affect transcription by modulating the coactivators or corepressors for assembling the transactivating complex [130]. For example, sumoylation represses the activity of p300 by enabling the recruitment of the HDAC6 histone deacetylase which, to some extent, may explain the negative role of sumoylation on HIF-1a transactivational function [131]. Furthermore, sumoylation of HIF-1β abrogates its transcriptional activity by preventing it from interacting with its coactivators such as PML (promyelocytic leukemia protein). Despite the negative roles of sumoylation in these regards, covalently modifying of two lysine residues 391, 477 within HIF-1α ODD domain by sumoylation is capable of enhancing the stability of HIF-1α thereby upregulating its transcriptional activity under both normoxic and hypoxic conditions [132].

S-nitrosation has recently been described as an additional post-translational modification to HIF-1α. The action is fulfilled via exogenously and endogenously produced nitric oxide (NO) [133]. S-nitrosation of cysteine 800 in the C-terminal transactivation domain of HIF-1α augments the interaction between HIF-1α and p300 and promotes HIF-1α transcriptional activity [134]. Therefore, S-nitrosation appears as a positive regulator of HIF-1α stability and transactivating activity.

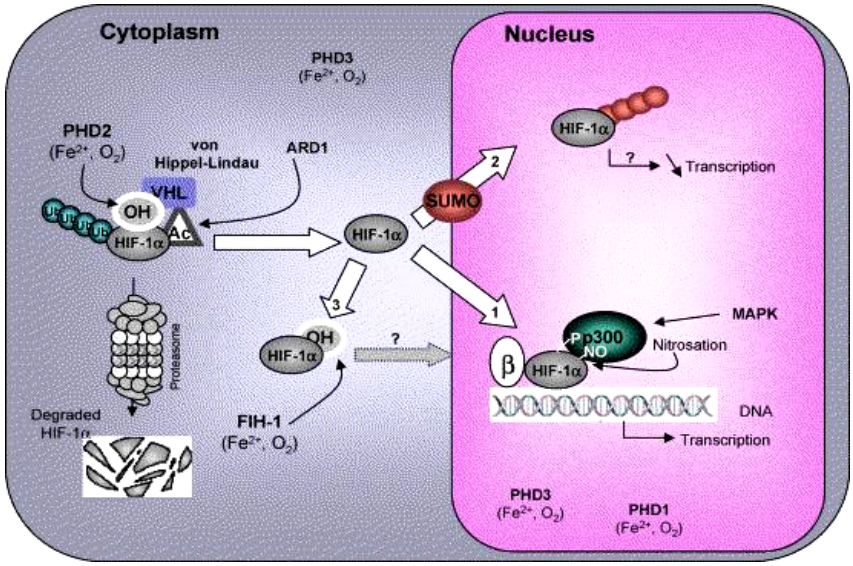


Figure 6. Intracellular localization and functional consequences of the different posttranslational modifications of HIF-1α. Refer the details of the explanations to the context. Adapted from [105].

Apart from the post-translational modifications mentioned above, other alternative ways could also contribute to O_2-dependent mediation of HIF-1α. The inhibitory PAS domain protein (IPAS) as a product of hypoxia-induced splice variant of HIF-3α may interact with HIF-1α and negatively regulate the expression of its target genes [84]. P35srj (also called MRG1, CITED2), a CBP/p300 interacting protein, is markedly upregulated in response to hypoxia or deferoxamine, and binds to the CH1 motif of p300 hence blocks the access of HIF-1α to CBP/p300 for the transcriptional activity [135]. Also, hypoxia-triggered increase of HIF-1α could eventually trigger autocrine signaling to reinforce the transcriptional efficacy of HIF-1α [100].

1.2.2.2.2. Oxygen-independent regulation of HIF-1α

Insulin and insulin-like growth factor-1 (IGF-1) were reported to induce upregulation of HIF-1α in a way different from the hypoxic pathway. Besides, growth factors as PDGF, EGF, FGF-2, IGF-2, TGF-1β, HGF, TNFα, IL-1β have been found to increase HIF-1α levels through receptor-mediated pathways [136,137]. Moreover, Angiotensin II (ANG II) and Thrombin enhance HIF-1α levels and its transcriptional activity [138,139]. Coincidently, these exogenous factors seem to stimulate HIF-1α synthesis via activating similar signaling transduction cascades, i.e., the phosphatidylinositol 3-kinase (PI3K) and/or the mitogen-activitated protein kinase (MAPK) pathways [100]. Mammalian target of rapamycin (mTOR), a known energy sensor as well as a downstream target of PI3K for growth control, has been implicated in hypoxic signaling and HIF-1α regulation [140-143]. However, by which ways mTOR regulates HIF-1α is not known yet. Interestingly, ANG II could increase HIF-1α by a post-transcriptional mechanism via AT(2) receptors, and the increase is likely caused by a downregulation of PHD3 [144]. Thus, growth factors may upregulate HIF-1α at different levels.

Some oncogenic mutations influence HIF-1α expression and function too. Gain of function of ERBB2 (HER2/neu), a gene encoding a member of epidermal growth factor (EGF) receptor family, leads to increasing HIF-1α synthesis and expression of its downstream angiogenesis-inducing genes. The process was suggested to be PI3K/AKT/mTOR signaling dependent [145]. Loss of function of the tumor suppressor gene von Hippel-Lindau (VHL) brings about a remarkable increase in HIF-1 activity due to impaired VHL-mediated ubiquitination and proteasomal degradation of HIF-1α and HIF-2α [100]. Reintroduction of pVHL into $VHL^{-/-}$ cell lines rescues oxygen-dependent regulation of hypoxia-inducible genes and suppresses tumor formation in mouse xenograft assay [146]. PTEN, another tumor suppressor gene, is known to abrogate hypoxia-induced HIF-1α stabilization in a PI3K/AKT signaling-dependent way. PTEN loss and regain function mediates HIF-1α-regulated gene expression [147].

Finally, some of the transition cation-containing chemicals (e.g. Co^{2+}, Ni^{2+}) or iron chelators (e.g. DFX – desferrioxamine) are capable of increasing HIF-1α levels in normoxia. This is though to occur by displacing the Fe^{2+} from the Fe^{2+} binding site in

the oxygenases [148,149]. Besides, direct binding of Co^{2+} to the ODD domain of HIF-1α was reported to modulate of HIF-1α regulation [150]. Similarly, by chelation of Fe^{2+}, the antimycotic compound ciclopirox olamine (CPX) is able to stabilize HIF-1α in normoxia resulting in enhanced angiogenic gene expression and angiogenesis [91,151]. Recently, arsenite was shown able to increase HIF-1α levels in a mTOR-dependent pattern [152].

1.2.2.3. HIF-1α targeting and clinical implications

The important role of HIF-1α in adaptation to cellular oxygen alterations makes it a good candidate for therapeutic targeting. A growing body of evidence has tied hypoxia and HIF-1α to a magnitude of human diseases and the therapeutics against them. Different strategies targeting pathways regulating HIF-1α, HIF-1α transcriptional effectors, and co-activators of HIF-1α are ongoing.

Transgenic expression of ODD-defective HIF-1α in the skin of mice results in a phenotype with markedly induced VEGF isoforms and massively increased blood vessels, which are not associated with leaking and tissue edema [153]. Similarly, a transcriptional active protein produced by hybridized HIF-1α key elements and herpes simplex virus VP16 profoundly improved angiogenic indices of ischemic hindlimbs in a rabbit model [154]. Targeting HIF-1α degradation by using a macrophage-derived peptide PR39 successfully improved the myocardial vasculature in mice [155]. Increased levels and transcriptional activity of HIF-1α as well as enhanced vascularization were observed after using prolyl 4-hydroxylase inhibitors in a rat sponge model [156]. Chetomin, a synthetic compound originally from fungus metabolite, attenuates hypoxia-inducible gene expression and inhibits tumor growth by interrupting the interaction between HIF-1α and CBP/p300 in mouse model [80]. Intratumoral transfer of an anti-sense HIF-1α plasmid downregulates HIF-1α levels, VEGF expression and decreases tumor microvessel density [157]. Furthermore, clinically relevant agents aiming at inhibiting either HIF-1α transduction pathways or HIF-1α activity have also been under development (Table 3).

Agent(s)	Molecular target(s)	Current status
Inhibitors of signal-transduction pathways		
BAY 43-9006	RAF kinase	Clinical trials
CCI-779	mTOR	Clinical trials
Celebrex	COX2	Clinical trials
PD98059	MEK	Not in clinical use
Trastuzumab (Herceptin)	ERBB2 receptor tyrosine kinase	Approved agent
ZD-1839 (Iressa), OSI-774	EGFR tyrosine kinase	Clinical trials
Imatinib (Glivec)	BCR–ABL, PDGFR tyrosine kinases	Approved agent
Small-molecule inhibitors of HIF-1 activity		
2ME2	Microtubule polymerization	Clinical trials
17-AAG	HSP90	Clinical trials
Camptothecin, Topotecan	Topoisomerase I	Approved agents
Pleurotin, 1-methylpropyl 2-imidazolyl disulphide	Thioredoxin 1	Not in clinical use
YC-1	Not determined	Not in clinical use

Table 3. Novel therapeutic agents that inhibit HIF-1α activity. RAF, MAP3K (a v-raf-1 murine leukemia viral oncogene homolog); mTOR, mammalian target of rapamycin; COX2, cyclooxygenase 2; MEK, MAPK/ERK kinase; EGFR, epidermal growth factor receptor; BCR-ABL, breakpoint cluster region-Abelson leukemia; PDGFR, platelet-derived growth factor receptor; HSP90, heat shock protein 90. Adapted from [100].

1.3. Growth factors and angiogenesis

Growth factors are pivotal for the formation of functional blood vessels. Therefore, modulating angiogenesis by targeting growth factors and their receptors is extensively studied. Among more than 20 known angiogenic growth factors, Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), Fibroblast growth factors (aFGF, bFGF), and transforming growth factor-beta (TGF-β) are the most common and well-studied ones [158-163]. Certainly, the reciprocal roles of Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), the pro- and anti-angiogenic function of TGF-β as well as other angiogenic growth factors and inhibitors should not be overlooked in the process of angiogenesis. Here, we will focus on the characteristics of VEGF and PDGF.

1.3.1. Vascular endothelial growth factor (VEGF)

VEGF, also known as vascular permeability factor (VPF), is a heparin-binding glycoprotein specific for vascular endothelial cells. It is secreted from hypoxic, ischemic or malignant cells as a homodimer, and is able to induce angiogenesis [164-167].

Belonging to the vascular endothelium-specific growth factor super family, the VEGF family has five members, i.e., VEGF-A, -B, -C, -D, and placental growth factor (PIGF) [165,166,168-171]. An *orf* virus-encoded VEGF isoform, VEGF-E was also found [172,173]. The most abundant and biologically active form of VEGF is VEGF165, which is a VEGF-A splice variant (others are VEGF121, 145, 189, 206). VEGF expression is generally low in adults with the exception of the female reproductive organ [174]. Expression is increased in some pathological situations such as certain inflammatory diseases, myocardial ischemia, atherosclerosis, and tumor growth [175-177].

To be functional, the various VEGF forms need to bind to their receptor tyrosine-kinases (RTKs) or non-RTKs, Flt1 or VEGFR1, KDR or VEGFR2 (mouse equivalent, Flk-1), Flt4 or VEGFR3. Though Flt1 and KDR regulate blood vessel formation by different means, they are a prerequisite for vasculogenesis and angiogenesis whereas VEGFR3 specifically mediates lymphangiogenesis [178-180]. The different forms of VEGF, VEGFR as well as the activating relationships are shown in Figure 7.

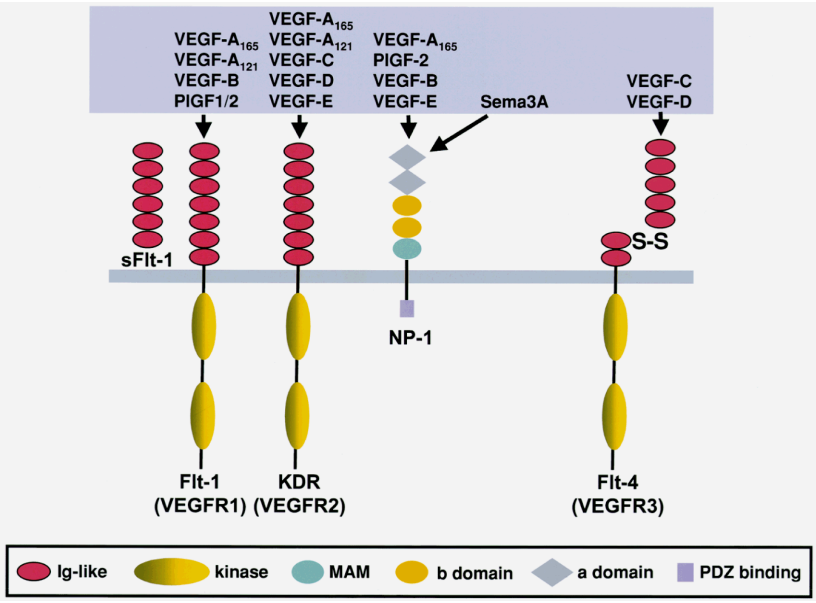


Figure 7. VEGF family ligands and receptors. The RTKs that specifically recognize VEGF-A, Flt1 (VEGFR1) and KDR (VEGFR2), possess an extracellular domain containing seven Ig-like loops, a single hydrophobic membrane-spanning domain, and a cytoplasmic domain comprising a single kinase domain that is interrupted by a non-catalytic region, called the kinase insert. VEGF C, D and E also bind to KDR, while PlGF and VEGF-B bind to Flt1 only. Flt4 (VEGFR3) is a related receptor for VEGFs C and D. NP-1 is a non-RTK receptor for $VEGF_{165}$, the PlGF-2 isoform, VEGF-B and VEGF-E. NP-1 (neuropilin-1) comprises an extracellular region with MAM (meprin, AS, m tyrosine phosphatase), a and b domains, a transmembrane region, and a short cytoplasmic domain. Adapted from [181].

VEGF stimulates angiogenesis by binding to its specific receptors on nearby blood vessels. This is in concert with actions of other vascular-specific growth factors (Figure 2). Thus, VEGF and the corresponding receptors are targeted therapeutically. Dominant-negative expression of Flk-1 inhibits the growth of a range of solid tumors in rodents [182]. Clinical intra-arterial gene transfer of human plasmid phVEGF165 can improve blood supply to the ischemic limb [183]. A designed single-chain antibody was shown to inhibit VEGF-induced KDR phosphorylation and VEGF-stimulated DNA synthesis in human umbilical vein endothelial cells [184]. Bevacizumab (Avastin), a monoclonal antibody against VEGF has been proven clinically effective to metastatic colorectal cancer [62]. The importance of VEGF receptors and other endothelial-specific receptors is also exemplified in receptor knock out studies as shown in Figure 8.

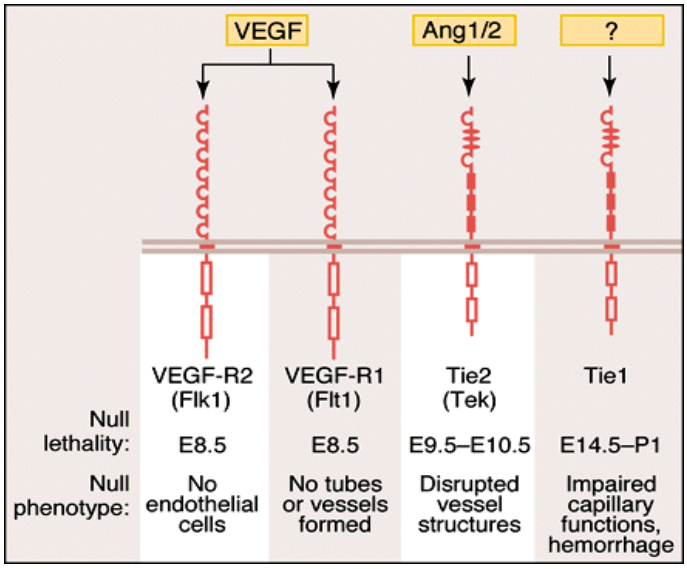


Figure 8. Lessons from gene-knockout mice. The endothelial cell-selective RTKs VEGF-R1, VEGF-R2, Tie1, and Tie2 have all been ablated in gene-knockout mice. Each RTK knockout produced embryonic lethality with vascular defects. However, their distinctive phenotypes indicate that each of these tyrosine kinases controls a specific, complementary function in endothelial cells that collectively can account for a significant part of endothelial cell morphogenesis into functional vessels. Adapted from [6].

VEGF is a classical hypoxia-inducible gene. Transactivation of VEGF by HIF and its stabilization mechanisms in hypoxia as well as the normoxic activation via many oncogenes, epidermal growth factor receptor and erbB2 account for marked upregulation of VEGF in tumor tissues and are of prognostic importance [185-189]. Several studies have shown the close relationship of high serum levels of VEGF with

poor prognosis in cancer patients [190-192]. Therapeutic methods directly targeting VEGF cause vascular hyperpermeability and inflammation. In contrast, regulating VEGF upstream target HIF-1α can improve tissue ischemia with less side effects [153,193,194]. This has been exemplified in other studies, for example the HIF-1α/VP16 hybrid gene transfer in vivo significantly improved functional angiogenesis and perfusion of the hindlimb ischemia in a rabbit model [154].

1.3.2. Platelet-derived growth factor (PDGF)

PDGF was initially purified as a factor from platelets that promotes the proliferation of mesenchymal cells [195,196]. Five biological isoforms of PDGF, PDGF-AA, -BB, -AB, -CC, and -DD, have been found so far [196-203]. Two types of receptors, type A and type B PDGF receptor for one or more of the known PDGF isoforms, were also discovered [2,204-206] (Figure 9). As familial members of the tyrosine kinase receptors, PDGF receptors undergo homo- or/and heterodimerization and autophosphorylation for activation upon binding of the dimeric ligands to the two subunits (α and β) of the receptors. The activation of the receptors further allows binding, phosphorylation and activation of downstream signaling transduction molecules that are associated with the receptors [207].

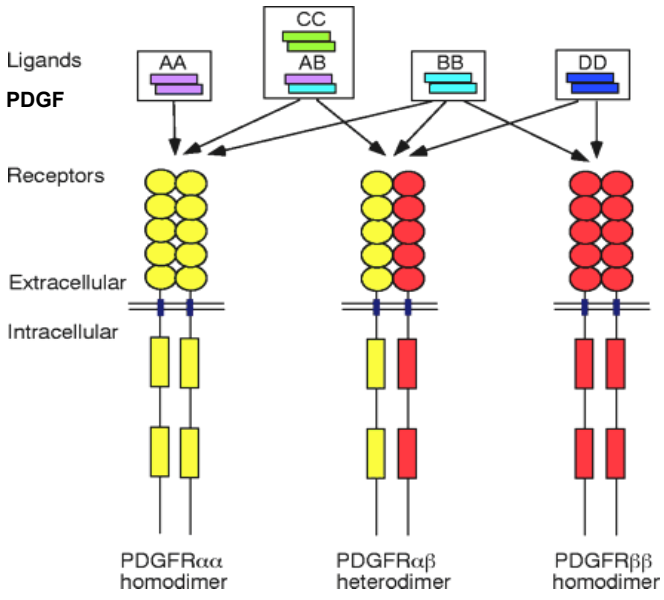


Figure 9. Mammalian PDGF/PDGFR binding interactions. Homo- and heterodimeric PDGF ligands differ in their ability to bind and activate the different receptor dimers in vitro. Adapted from [2].

PDGFA and PDGFRα are broadly required during embryogenesis, whereas PDGFB and PDGFRß are essential for the development of support cells in the vasculature (Figure 2). PDGF-CC and PDGF-DD are secreted in inactive forms, and are activated upon cleavage of the N-terminal CUB domains [2]. They are implicated in vascular smooth muscle cell proliferation, vascularization, wound healing, and tumorigenesis [208]. Besides the function of inducing proliferation of fibroblasts, microglia, and smooth muscle cells, PDGF drives cell survival, migration, and the deposition of extracellular matrix (ECM) and tissue remodeling factors [2] (Table 4). PDGF is synthesized in different cell types with the principle sources from platelets, endothelial cells and placenta [209]. Its synthesis is often increased in response to external stimuli, e.g. growth factors and cytokines [210], thrombin [211,212], low oxygen tension [213].

PDGFR-β	**PDGFR-α**
turnover of PI and IP*	turnover of PI and IP*
Ca2+ fluxes	Ca2+ fluxes
membrane ruffles	membrane ruffles
cytoskeletal rearrangements	cytoskeletal rearrangements
migration	migration
proliferation	proliferation
angiogenesis	Timing of differentiation
*IP, inositol phosphatases	

Table 4. Biological responses transduced by PDGF receptors. Adapted from [214]

A growing body of data supports the role of PDGF and its receptors angiogenesis and as targets for pro- or anti-angiogenic therapies [160,215-220]. In a murine limb ischemia model, PDGFRalpha-p70S6K pathway was shown to be essential for FGF-2-mediated therapeutic neovascularization [221]. PDGF B-chain deficient mouse embryos developed numerous capillary microaneurysms that ruptured at late gestation because the endothelial cells of the sprouting capillaries in the mutant mice were unable to attract PDGFRβ-positive pericyte progenitor cells to form part of the capillary wall [222]. A polyclonal antibody against PDGF is capable of blocking the induced development of an intimal lesion in the carotid artery of athymic nude rats by inhibiting neointimal smooth muscle accumulation [218]. Inhibitors of PDGFRβ

enhanced the antitumor effect of Taxol on s.c. KAT-4 tumors in SCID mice [220]. However, less is known about PDGF induced hypoxic signaling with regards to endothelial cell proliferation and angiogenesis.

1.4. Rapamycin and Target of rapamycin (TOR)

Rapamycin (RPM) is a lipophilic macrolide originally produced by a strain of *Streptomyces hygroscopicus* isolated from a soil sample obtained from the Vai Atore region of Easter Island (Rapa Nui). Firstly identified during antibiotic screening at Ayerst Research Laboratories, RAP ($C_{51}H_{79}NO_{13}$) was subsequently found bearing immunosuppressive and tumoricidal activities besides the growth-inhibitory effect on yeast and filamentous fungi [223]. Rapamycin and analogs such as CCI-779 (Wyeth Ayerst), RAD001 (Novartis Pharmaceutical Inc; Basel, Switzerland), and AP25373 (Ariad Pharmaceuticals Inc; Cambridge, MA) have shown remarkable efficacies in preclinical and clinical trials treating organ transplant rejection, autoimmune diseases as well as tumors [224,225].

To be effective, RPM needs to bind to its intracellular receptors, the FK506 binding proteins (FKBPs). RPM binds to FKBP12 two-fold more tightly than FK506. The coupled RPM-FKBP12 complex then targets the FKBP12-rapamycin binding region (FRB) in the C-terminus of target of rapamycin (TOR) protein and thereby decreases their activities and inhibits the downstream signaling events [223,224,226].

Genetic, structural and functional understanding of TOR has increased after the discovery of the TOR1 and TOR2 genes in yeast during a screen for mutants resistant to rapamycin [226]. Genetically, TOR is conserved among species ranging from yeast (TOR1 and TOR2), through fungi (TOR1), plants (AtTOR), worms (CeTOR), flies (dTOR), to mammals (mTOR) [227]. In general, the TORs (from the N-terminal to the C-terminal) contain 20 tandem HEAT (for amino-acid sequence motif identified in Huntingtin, Elongation factor 3, A subunit of PP2A and TOR1) repeats, a FAT (for FRAP, ATM, TRRAP) domain, a FRB (for FKBP12-RPM binding) site, a kinase domain, and a FATC (for FAT carboxy-terminal domain) [227,228] (Figure 10). The HEAT domain (1200 amino acids) provides a large hydrophobic surface for protein-protein interactions. Moreover, it is responsible for localization of yeast TOR2 to the plasma membrane by interacting with membrane-associated proteins [229]. The FAT domain may act as a structural scaffold or a protein binding site whereas the

FATC domain is key to TOR activity, and the two domains may interact to modulate the kinase function of TOR [230]. The FRB domain provides a docking site for the FKBP12-rapamycin complex [231]. The kinase domain of TOR shares significant homology with lipid kinases, and belongs to the phosphoinositol kinase-related protein kinase (PIKK) family [228]. Structural analysis of TOR1 and TOR2 in yeast indicates that their carboxy-terminal kinase motifs are functionally interchangeable while the other parts within the respective protein are not [232]. To be fully functional *in vivo*, the TORs form complexes with other partner-proteins. So far, two TOR complexes, TORC1 and TORC2, have been identified in yeast and mammals, respectively. The yeast TORC1 is composed of TOR1 or TOR2, kontroller of growth 1 (KOG1), and lethal with sec thirteen (LST8); TORC2 contains TOR2, Adheres Voraciously to TOR2-1 (AVO1), AVO2, AVO3, and LST8 (Figure 10) [227,233]. The mammalian counterpart of TORC1 (mTORC1) consists of mTOR, mKOG1 (raptor, regulatory associated protein of TOR), and mLST8 (GβL, G protein β–subunit-like protein) whereas mTOR, mAVO3 (rictor, rapamycin-insensitive companion of mTOR) and mLST8 form the mTORC2 [234-238]. Deletion studies of the TOR partners, KOG1, AVO1, AVO3, LST8 within the TOR complexes in yeast showed phenotypes mimicking TOR deficiency indicating the indispensable roles the TOR partners play. AVO2, however, seems to act as a chaperone for supporting roles in the TORC2 [233].

The existence of the two distinct yet compatible TOR complexes reflects the diversity of TOR signaling. The fact that TOR2 appears in both TORC1 and TOR2 while TOR1 is only in TORC1 may imply a notion that TOR2 plays a more broader role in TOR signaling than TOR1 does [233]. A description of kinase signaling cascades centered on TOR and the related biological significance are described in the next chapter.

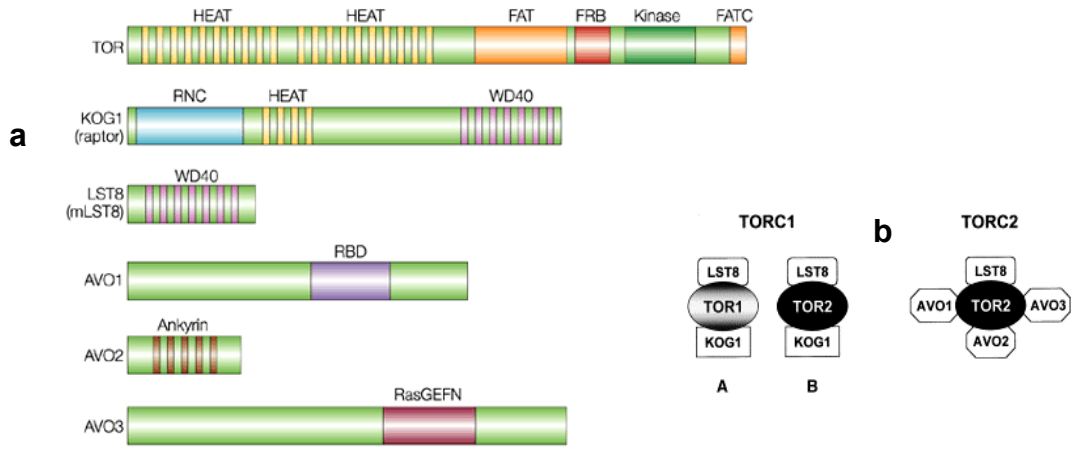


Figure 10. a) Structural domains of TOR and its binding partners. HEAT, an amino-acid sequence motif that was first identified in huntingtin, elongation factor 3, regulatory A subunit of PP2A and TOR1; FAT, a domain that was found in the FRAP (mTOR), ATM and TRRAP proteins; FRB, the binding site for FKBP12–rapamycin; FATC, FAT carboxy-terminal domain; Raptor is the mammalian ortholog of KOG1. The RNC (raptor N-terminal conserved) domain consists of highly conserved residues that are found in all KOG1/raptor orthologues. The structural domain of LST8 consists of seven WD40 repeats (a sequence of about 40 amino acids with a central Trp-Asp motif). AVO, adheres voraciously to TOR2; Regions in AVO1 and AVO3 have homology to the Ras-binding domain (RBD) and RasGEFN domain, respectively. AVO2 contains five ankyrin repeats. **b) Composition of two distinct TOR complexes:** TORC1 and TORC2 models do not reflect the stoichiometries and interactions of subunits within each complex. Adapted from [227,233].

1.4.1. Mammalian target of rapamycin (mTOR)

mTOR is the mammalian intracellular target of the FKBP12 and rapamycin complex, and was identified independently with initially different names besides mTOR [239], FKBP12-rapamycin associated protein (FRAP), rapamycin and FKBP12 target (RAFT), rapamycin target (RAPT), Sirolimus effector protein (SEP), respectively [240-243]. mTOR is a 289 kDa (2549 amino acids) serine/threonine protein kinase belonging to the PIKK family with the gene mapping to chromosome 1p36.2 in human, 5q36 in rat, and 4E1 in mouse [225,244]. Human, rat, and mouse mTOR proteins share 95% identity at the amino acid level, but only 45% identical to yeast TOR2, and 42% identical to yeast TOR1 [239].

The FKBP12-rapamycin complex binding site spanning the residues 2025-2114 in mTOR has been identified, and a point Ser^{2035} within the range (homologous to the Ser^{1972} and Ser^{1975} in yeast TOR1 and TOR2, respectively) is critical for the binding [245]. The targeting of the drug-receptor complex to mTOR accounts for the inhibition of

mTOR mediated cell cycle transition from G1 to S phase [240]. Moreover, mTOR modulates the signaling from nutrients and growth factors in mammalian cell growth. Recent data suggest that rapamycin inhibits mTOR signaling by competing with the mTORC1 partner raptor (mKOG1), which is either in 'constitutive' or in 'nutrient-sensitive' interaction with mTOR. The constitutive interaction is required for mTOR to function. The nutrient-sensitive interaction results in inhibition of mTOR kinase activity upon nutrient-depletion [235]. To some extent in consistence with this mTOR-raptor interaction model, raptor has been shown to bind to the TOR signaling motifs (TOS) within 4E-BP1 and p70 S6K, and the bindings present the two targets in a more effective way for mTOR to phosphorylate.

1.4.2. mTOR and cellular sensing for survival

To survive and develop, mammalian cells need both nutrients and growth factors. mTOR has a pivotal role in sensing these stimuli. Correspondingly, mTOR signaling is mediated through a big variety of upstream inputs and downstream outputs.

1.4.2.1. Common triggers for mTOR signaling

The stimulatory inputs include nutrients such as amino acids, glucose and fatty acids, energy such as ATP, growth factors such as insulin, IGF and PDGF, oxidative stress, hypoxia, and DNA damage caused by chemical compounds or radiation.

As will be discussed later, mTOR positively regulate ribosome biogenesis and mRNA translation, the processes that require a large amount of **amino acids**. The observations that some neutral amino acids contribute to the phosphorylation state of S6K1 and eIF-4E binding protein 1 (4E-BP1), two downstream targets of mTOR, provide a link between cellular demand of amino acids and mTOR signaling [246-248]. Moreover, experiments suggest a basal requirement of essential amino acids for mTOR to respond to growth factors. Thus, different amino acids in culture medium may influence mTOR signaling. Among the essential branched-chain amino acids, leucine appears to be most effective to induce mTOR signaling, and may function independent of insulin or growth factors [247,249]. Interestingly, leucine-induced phosphorylation of S6K1 could be attenuated by interfering an mTOR partner raptor [235]. Thus, the interaction between mTOR and raptor is of great importance for the regulation of the downstream targets in response to amino acids.

Insulin triggers the phosphorylation of 4E-BP1 and S6K1 in a rapamycin sensitive way. This provides evidence for a link between mTOR and **glucose** modulation [250]. Moreover, activation of mTOR by glucose needs support from amino acids, and glucose increases DNA synthesis, a process that can be blocked by rapamycin [251]. Phosphorylation of mTOR downstream targets triggered by insulin may be mediated by metabolites or energy generated from glucose and not glucose per se [250]. Interestingly, mice genetically deficient in S6K1 are mildly intolerant to glucose on normal (low fat) rodent diet mainly due to insufficient production of insulin resulting from decreased pancreatic beta-cell mass [252]. On the other hand, glucagon, an opposite regulator to insulin on glucose metabolism, repressed both basal and amino acid-induced signaling through mTOR. However, the process may involve activation of other kinases such as AMPK [253]. More recently, it has been shown that mTOR inhibits the transcription of insulin receptor substrates (IRS), a family of adaptor proteins that are essential both for PI3K activation and for mediating the pleiotropic effects of insulin thereby imposing a negative feedback to insulin stimulated signaling [254].

The impact of **fatty acids** on mTOR signaling is proposed to be tissue-dependent. Free fatty acid (FFA)-modulated phosphorylation of mTOR downstream targets S6K1 and 4E-BP1 appears in tissues that depend on oxidative metabolism, but not in the ones that rely on glycolytic metabolism [255]. Interestingly, phosphatidic acid (PA), which harbors fatty acid chains, has been implicated in mitogenic activation of mTOR by interacting with the FRB domain within mTOR [256].

Ribosome biogenesis and mRNA translation not only require amino acids as addressed before, but also need large levels of energy. Cellular energy, in the form of **ATP**, is a prerequisite for mTOR signaling [257]. Though mTOR was proposed as an energy (ATP) sensor, the link between ATP availability and mTOR activation is not fully clear yet [257]. Despite of this, 5'AMP-activated protein kinase (AMPK), which is more sensitive to alterations of cellular ATP levels than mTOR, was found to be upstream of mTOR, and the activity of AMPK is negatively related to mTOR activation and S6K1 phosphorylation [258]. Therefore, it is plausible that a decrease of intrinsic ATP levels in cells is sensed by AMPK, which in turn activates other protein kinases that de-active mTOR. The recent finding that tuberous sclerosis complex 2 gene product TSC2 can be phosphorylated by AMPK and has inhibitory effect on mTOR activity, fills the gap between AMPK and mTOR [259,260]. Though mTOR itself

contains phosphorylation sites for AMPK as well, and phosphorylation of Thr^{2446} may cause the inability of Akt phosphorylation of Ser^{2448} in mTOR, direct ties between AMPK and mTOR are still vague [261,262].

Besides insulin, other **growth factors** may activate mTOR downstream targets. However, though growth factors could mildly cause mTOR phosphorylation at Ser^{2448} [263], mutation of this site does not affect mTOR signaling to S6K1 and 4E-BP1 [264]. It is possible that the *in vitro* studies of mTOR activities may not correctly reflect the *in vivo* situations [265]. The mitogen-activated phospholipase D (PLD) is able to mediate mTOR activity by enzymatic induction of PA, which competes with rapamycin for the FRB site in mTOR. This provides a potential link between growth factor and mTOR signaling [256]. In addition, Cdc42, a small Rho family G-protein, is capable of activating S6K1 and 4E-BP1 by upregulating PLD induced PA, a process involves mTOR activation [266]. Moreover, rapamycin resistance is observed in breast cancer cell lines expressing higher levels of PLD and PA, and the sensitivity to rapamycin is restored upon PLD inhibition [267]. Furthermore, growth factors often affect mTOR via complex signaling pathways. For example, mutations in PDGFR and IRS-1 abrogate S6K1 phosphorylation by inactivation of PI3K cascades to mTOR [268]. Our data show that PDGF-BB induces HIF-1α expression via mTOR, and will be discussed later.

Effects of **hypoxia**, to some extent, are analogous to those caused by ATP deficiency since oxidative metabolic function is closely related to ATP levels in cells. Hypoxia potentiates vascular wall cell proliferation and angiogenesis, which can be attenuated by rapamycin implying a role of mTOR in hypoxic signaling [140]. In line with this, HIF-1α activation by hypoxia was blocked by rapamycin in prostate cancer cells (PC-3) suggesting a positive role of mTOR on HIF-1α activation [141,143]. It is worth to note that the PC-3 cells are highly sensitive to rapamycin possibly due to the enhanced proliferative potency [269]. However, it remains unclear how hypoxia triggers mTOR, and how mTOR regulates HIF-1α.

Links between **DNA damage** and mTOR activity have been shown by several studies. DNA-damaging agents like etoposide, cisplatin and mitomycin-C may impair mTOR signaling as dephosphorylation of p70 S6K and 4E-BP1 was observed preceding apoptosis in certain cell lines [270]. In murine erythroleukemic cells, a constitutively active form of p53 selectively impedes the phosphorylation of 4E-BP1 and p70 S6K suggesting an inhibitory effect of p53 on mTOR signaling [271]. Tyrosine

kinase c-Abl, which can be activated by ionizing radiation, was shown to be able to phosphorylate mTOR and inhibit its kinase activity [272]. Radiation increased 4E-BP1 binding to eIF-4E in wild type MEF cells but not in c-Abl-lacking cells [272]. UV radiation on DNA damage is known. Though mTOR may play a role in UV induction of collagenase and stromelysin-1 (a matrix-degrading enzyme) [273], UV could activate p70 S6K1 via JNKs [274]. Still, the function of mTOR in DNA damage caused cellular changes remains an open question.

1.4.2.2. Upstream transduction regulators of mTOR

The potential roles of AMPK, PLD and p53 in upstream activation of mTOR have been described above. Besides, there are other defined transduction mediators such as PI3K/PTEN (phosphatidylinositol 3-kinase/phosphatase and tensin homolog deleted from chromosome 10), Akt (also known as protein kinase B, PKB), TSC1/TSC2 (tuberous sclerosis complex 1 and 2, also called hamartin and tuberin), Rheb (Ras homolog enriched in brain), and REDD1 (for regulated in development and DNA damage responses) involving in mTOR upstream transduction.

PI3K and PTEN: As mentioned before, growth factor-triggered mTOR signaling involves PI3K activation. On one hand, this is substantiated by studies using PI3K inhibitors such as LY294002 and wortmannin, which are able to attenuate phosphorylation of S6K1 and 4E-BP1 [268,275]. Though the inhibitors could directly inhibit mTOR *in vitro*, high concentrations of the drugs are needed for the effects, and much lower concentrations are required for blocking the phosphorylation of S6K1 and 4E-BP *in vivo* [261]. Overexpression of an activated catalytic subunit of PI3K, p110, induces rapamycin-sensitive phosphorylation of 4E-BP1 in the absence of growth factors [276], and overexpression of a dominant-negative form of the regulatory subunit of PI3K, p85, inhibits the phosphorylation of S6K1 induced by insulin [277]. On the other hand, the PI3K activity counteractor, PTEN (also known as mutated in multiple advanced cancers, MMAC1 or TGFβ-regulated and epithelial cell-enriched phosphatase, TEP1) negatively regulates PI3K pathways by dephosphorylating PI3K lipid products phosphatidylinositol 3,4,5-trisphosphate (PIP3) [278]. PTEN-deficient cells exhibit enhanced phosphorylation of S6K1 and 4E-BP1 implying an active mTOR signaling in these cells [269].

Akt (PKB): Recruitment of Akt to the plasma membrane by the PI3K product PIP3 is required for full activation of Akt. Thus, Akt is a downstream effector of PI3K [261]. Several experimental data place Akt upstream of mTOR. Activated Akt augments phosphorylation of 4E-BP1 in a wortmannin-resistant and rapamycin-sensitive manner [276]. Overexpression of a dominant-negative form of Akt abrogates growth factor-induced 4E-BP1 phosphorylation [276]. In contrast, conditional activation of recombinant Akt fusion protein results in mTOR phosphorylation and activation [279]. Besides, Akt has been shown to directly phosphorylate mTOR at its Thr^{2446} and Ser^{2448} sites [263,264]. However, the two phosphorylation points for Akt reside in the 'negative regulatory domain (NRD)' of mTOR [264]. Moreover, transfection of HEK 293 cells with mutated mTOR at Thr^{2446} or/and Ser^{2448} does not affect insulin- or active Akt-stimulated S6K1 activation and 4E-BP1 phosphorylation [264]. Furthermore, these Akt phosphorylation sites are not conserved in dTOR [261]. These data challenge the concept of an upstream positive role of Akt upon mTOR activation.

TSC1/TSC2: A link between Akt and mTOR is the TSC1/TSC2 complex. Tuberous sclerosis (TSC), an autosomal dominant disorder characterized by hamartomas with very large cells in many organs, is caused by mutations of the putative tumor suppressor genes, TSC1 and TSC2 [280]. Coexpression of TSC1 and TSC2 represses 4E-BP1 phosphorylation and S6K1 activity. [280]. Both mammalian and *Drosophila* TSC2 are directly phosphorylated and inactivated by Akt [259,281,282]. TSC2 regulates S6K1 and 4E-BP1 activities by negatively mediating mTOR activation [259]. These results render TSC2 as one of the upstream regulators of mTOR. Interestingly, in Akt1/Akt2-deficient cells, mTOR activity towards 4E-BP1 is dramatically decreased although TSC2 phosphorylation is not impaired, suggesting a TSC2-independent Akt regulation of mTOR activity [283].

Rheb: The search for TSC2 downstream targets for mTOR regulation revealed selective GAP activity of TSC2 on Rheb. RNA interference (RNAi) of Rheb inhibits (but not 17 other GTPases) S6K1 phosphorylation [261]. *Drosophila* cells losing Tsc2 resulted in persistent phosphorylation of S6K whereas loss of Rheb abolished S6K phosphorylation regardless of the presence of amino acids. Besides, S6K remained dephosphorylated in the absence of both Tsc2 and Rheb indicating that Rheb is epistatic to Tsc2 for S6K phosphorylation [284]. Moreover, Over-expression of Rheb promotes the activation of S6 kinase in a rapamycin-dependent manner suggesting

that mTOR is downstream of Rheb [285]. Furthermore, based on knock-out studies, the TSC1/2 complex blocks mTOR by inhibiting Rheb activity [286].

1.4.2.3. Downstream signaling effectors of mTOR

mTOR executes several functions through its downstream target proteins. eIF4E-binding proteins (4E-BPs), Ribosomal protein S6 kinase 1/2 (S6K1 and S6K2), type 2A phosphatase (PP2A), Kip1 (cyclin-dependent kinase inhibitor 1, also called p27 or $p27^{Kip1}$), and RNA polymerases are common downstream effectors for mTOR signaling.

4E-BP: 4E-BP (also known as phosphorylated heat and acid-stable protein regulated by insulin, PHAS-I) is a family of repressor proteins for translation initiation. It targets and impedes eIF4G from binding to eIF4E and hence the dissociation of the eIF4F complex [223,225,261]. Among the mammalian 4E-BPs, 4E-BP1 appears to be the most active and best studied in signaling cascades [261]. Thr^{37} and Thr^{46} in 4E-BP1 are directly phosphorylated by mTOR followed by sequential phosphorylation of the Thr^{70} and Ser^{65} sites both in vitro and in vivo [287]. These consecutive phosphorylation events are considered to work in concert to dislodge 4E-BP1 from eIF4E [261]. Moreover, phosphorylation of the Ser/Thr sites in 4E-BP1 is sensitive to rapamycin [288], and PI3K, Akt, mTOR may interact upstream to induce 4E-BP1 phosphorylation in the presence of serum or growth factors [276].

S6K: The ribosomal protein S6 kinases (S6Ks) are pivotal for ribosomal S6 protein phosphorylation, which is required for ribosome assembly and for recruiting 5'TOP mRNAs to ribosomes [289]. However, amino acids and growth factors may activate the 5'TOP mRNA translation in the absence of S6K [290]. So far, two S6Ks termed S6K1 (p70 S6K) and S6K2 have been identified [291,292]. S6K1 activity is inhibited by wortmannin, LY294002, and rapamycin indicating that PI3K and mTOR are involved in its activation [293]. Interestingly, a truncated mutant S6K1 is rapamycin resistant while it remains sensitive to wortmannin displaying the bifurcate signaling cascades from mTOR and PI3K to S6K1 [294]. Multiple phosphorylation sites in S6K1 have been found with Ser371 and Thr389 in the linker region essential for S6K1 activation [295]. The Thr389 site can be directly phosphorylated by mTOR in vitro [295]. Moreover, this site is potentially regulated by Tsc1/Tsc2, which links S6K1 and mTOR to the far upstream mediators [259].

Phosphatase: The observation that rapamycin causes dramatic dephosphorylation of all sites in S6K1 including those not being phosphorylated by mTOR implicates that rapamycin may activate phosphatases [296]. Rapamycin treated cells increase the calyculin A-sensitive PP2A activity towards 4E-BP1, and PP2A can bind to and inactivate S6K1 [296]. Moreover, mTOR phosphorylates PP2A in vitro and thereby prevents downstream dephosphorylation of 4E-BP1 and S6K1 [296].

Cyclin/cdk: Rapamycin strongly interferes with the cyclin D2- or cyclin D1-cdk complexes in lymphoma cells or osteosarcoma cells. Although cyclin E-cdk2 complexes assembles in the rapamycin treated cells, the complexes bear no protein kinase activity [223]. Also, IL-2-induced cdk activation can be abolished by rapamycin [297]. This suggests that rapamycin induces Kip1, a G1 cyclin cdk inhibitor, activation or alternatively, prevents it from being targeted for destruction. The persistent expression of Kip1 in rapamycin-treated T cells suggests that mTOR serves to mark Kip1 for ubiquitin-dependent proteasomal degradation. mTOR might not execute the function by itself, instead, it may control the translation of certain proteins that allow the ubiquitin-proteasome system to recognize and degrade Kip1 [223].

RNA polymerase: In accordance with the critical role of mTOR in translational and cell cycle control, cellular transcriptional activities have been proved to be regulated indirectly by mTOR [261]. These activities include transcription of rRNA genes by RNA polymerase I (Pol I), ribosomal protein genes by Pol II, and tRNA and 5S genes by Pol III [261]. For example, inhibition of rDNA transcription by rapamycin can be rescued by TIF1A (a Pol I-specific transcription factor), and rapamycin can inhibit UBF (an rDNA transcription factor) phosphorylation, which is required for its activity [261].

1.4.3. mTOR, cell growth and proliferation

Cell growth (an increase in cell mass and size through macromolecular biosynthesis) and cell proliferation (an increase in cell number) are biologically coupled processes critical for embryonal development, maintenance and remodeling of tissues, organs, and neoplastic as well as tumor development. mTOR exhibits a broad range of biological functions that can regulate both cell growth and cell proliferation. However, the two functional readouts of mTOR are not in the same line of signaling control.

They may either bifurcate from an upstream transduction point of mTOR or separate upon mTOR activation by different signaling inputs.

A number of experiments support the role of mTOR in the control of cell size and growth. Phosphorylation of mTOR at Ser^{2448} (possibly by Akt) is associated with nonproliferative skeletal muscle hypertrophy [298]. Overexpression of genetically activated Akt in mice hearts results in enlarged cardiomyocytes and larger hearts, which can be attenuated by rapamycin [299]. In proliferating mammalian cells, rapamycin is able to reduce both proliferation rate and cell size [140,300]. mTOR downstream target S6K-deficient mice are smaller in size when compared to the wild type littermates [301]. Since 4E-BP1, eIF4G, and eIF4B are involved in the regulation of the rate-limiting translation process, mTOR may participate in cell growth and proliferation by signaling to these molecules for translation initiation. eIF4E overexpression increases cell size in human osteosarcoma cells. In addition, coexpression of eIF4E and S6K1 synergistically increases cell size [300]. eIF4E and S6K have been implicated in cell cycle control as well [261]. Activated 4E-BP1 abrogates the cells progressing through the G1 phase [302]. The involvement of TSC1/2 and Rheb in tumor development provides further evidence for mTOR regulation of cell growth and proliferation [261]. The diverse growth-associated readouts regulated by mTOR are shown in Figure 11.

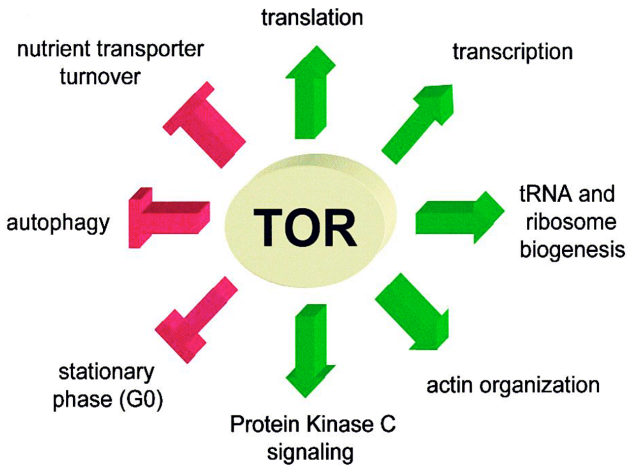


Figure 11. Growth-related readouts controlled by yeast and mammalian TOR. Green arrows indicate activation, and red bars indicate repression. Adapted from [1]

1.4.4. mTOR and angiogenesis

Data from our group have shown that both growth factor and hypoxia could increase vascular wall cell proliferation and *ex-vivo* angiogenesis in a rapamycin sensitive way implying a role of mTOR in the biological processes [140]. Consistent with these observations, rapamycin has been shown to inhibit primary and metastatic tumor growth by suppressing angiogenesis in mouse models, and the antiangiogenic effect was suggested to be due to a blockage of VEGF production [303]. VEGF is a principle angiogenic factor in response to many pathophysiological stimuli including growth factors and hypoxia. Therefore, it is plausible that mTOR is involved in angiogenesis by regulating the angiogenic molecules.

As described before, HIF-1α levels have been considered as a pathological marker for ischemic cardiovascular disease and tumor [42,73]. mTOR has been linked to regulation of HIF-1α and the corresponding transcriptional targets. In human transformed cells, inhibition of mTOR or the upstream mediator PI3K decreases both HIF-1α and VEGF expression induced by growth factors. VEGF gene transcription is abrogated by expression of dominant-negative Akt, PI3K, and wild-type PTEN whereas expression of a constitutively active Akt or dominant-negative PTEN upregulates the transcription [141]. Wild type mTOR transfection of prostate cancer cell lines enhances HIF-1α induction by hypoxia in a rapamycin-sensitive manner [143]. Despite of these interesting findings, it remains unclear at which level(s) mTOR regulates HIF-1α and/or its target genes, how this is modulated, and how hypoxia triggers and mediates mTOR signaling. A recent study in human embryonic kidney cell lines showed that mTOR and its downstream targets S6K1, 4E-BP1, eIF4G as well as ribosomal protein S6 are hypophosphorylated when exposed to hypoxia [304]. This suggests a HIF-independent cellular response to hypoxic stress, which is mediated by mTOR.

1.5. Mitogen-activated protein kinase (MAPK) pathways

Regulation of cellular responses to growth factors, inflammatory cytokines, and other mitogens often occurs via the major mitogen-activated protein kinase (MAPK) pathways. Upon ligand binding to the G-protein-linked or intrinsic protein tyrosine kinase receptors, downstream proteins such as Grb2, SOS, and Ras or Rac are phosphorylated and activated [305]. The active kinases consecutively phosphorylate

other proteins, transmit and amplify signals to modulate a big variety of cellular events including cell cycle progression, regulation of embryonic development, cell movement, apoptosis, cell and neuronal differentiation [3,306].

The evolutionarily conserved MAPK pathways are organized in three levels of regulation, a far upstream activator MAPK kinase kinase (MAP3K, MEKK), a step down effector MAPK kinase (MAP2K, MEK), and a MAPK [3]. This stepwise transduction model contains at least three distinct MAPK signaling cascades including the extracellular signal-regulated kinase 1 and 2 (ERK1/2, also known as p44/p42 MAPK), the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK), and the p38 kinases (called HOG in yeast) [3]. Each of these typical signaling transmitters has several genetic splicing isoforms in mammals [307]. A critical and common feature of the MAPKs is that they are activated upon dual phosphorylation within the threonine-X-tyrosine (TXY) consensus sequence present in the catalytic domain [308-310]. Interestingly, phosphorylation of only one of the two sites does not activate the kinase [311]. The hierarchical activation of the MAPKs leads to a set of cellular events (Figure 12).

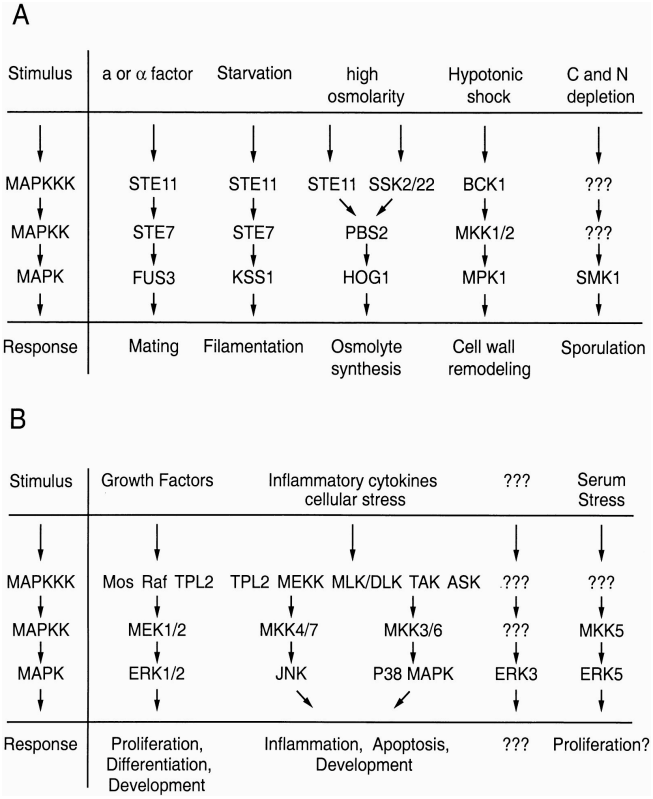


Figure 12. Schematic overview of MAPK modules. **(A)** In *S. cerevisiae*, five MAPK modules regulate mating, filamentation, high-osmolarity responses, cell wall remodeling, and sporulation. **(B)** Mammalian MAPK modules regulate cell growth, differentiation, stress responses, and development. Abbreviations: MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase. Adapted from [3]

The ERK1/2 pathway is activated by growth and mitogenic factors. Downstream targets of ERKs include p90 ribosomal S6 protein kinase ($p90^{rsk}$), ELK1 (member of the ETS oncogene family, a transcription factor), and signal transducer and activator of transcription 3 (STAT3) [307,312]. The JNK/SAPK and p38/HOG cascades can be triggered by lipopolysaccharides, cytokines, UV or ionizing radiation, heat shock, osmotic stress, and tumor promoters. Therefore, the two signaling pathways are often involved in response to inflammation, cellular stress and apoptosis [3,305]. JNK/SAPK downstream targets include the transcription factors c-Jun and ATF-2. p38 targeting proteins include ATF-2, Max, and CREB [313,314].

Opposite to activation of the MAPK pathways, negative regulation of the MAPK superfamily members is mediated by protein phosphatases. The MAPK phosphatases (MKPs) are divided into three major categories depending on their preference for dephosphorylating tyrosine, serine/threonine (mono specificity) or both the tyrosine and threonine (dual specificity). The proactive MAPKs and the mono-and dual-specificity MKPs work in concert to modulate cellular responses to different physiobiological stimuli.

1.5.1. MAPKs and hypoxia

MAPKs can be activated by hypoxia, and trigger activation of certain transcription factors such as c-Jun, c-Fos, ATF-2, elk-1, and nuclear factor-kapaB (NF-κB), and HIF-1α. However, results from different cell types and experimental conditions provide controversial evidence regarding the role of MAPKs in hypoxia.

Hypoxic phosphorylation and activation of ERK1/2, JNK, and p38 was observed in rats exposed to normobaric hypoxia (10% O_2) where JNK showed an early activation pattern, and ERK1/2 as well as p38 were activated in a far later stage. The laggard activation of ERKs and p38 implicates the possible existence of secondary responses of the two kinases, such as reaction to hypoxia-induced growth factors or other mediators, to hypoxia [315]. Increased phospho-MAPK proteins in large and small intrapulmonary arteries during chronic hypoxia may account for hypoxia-induced pulmonary arterial remodeling [315]. However, a bit different from the above findings, moderate hypoxia (5% O_2) has no impact on JNK, p38 β, p38 β2 and p38 δ activities while it activates p38 α, p38 γ, and ERK1/2 in rat pheocytochroma cells [316]. Interestingly, when the same strain of cells are incubated under severe hypoxic

conditions (1% O_2) for 6 hours, ERK1 activity is strongly increased and induces HIF-2α activation [317]. In addition, inhibition of ERK1/2 signaling by PD98059 impairs HIF-1α transactivation activity whereas its stability and DNA binding activity remains unaffected [318,319]. In contrast, hypoxic activation of JNK and p38 and subsequent HIF-1α expression as well as its DNA binding activity are sensitive to the blockage of the kinases in squamous carcinoma cell lines [320]. ERK1/2 may phosphorylate HIF-1α both *in vitro* and *in vivo* [106]. Still, other independent studies could not confirm this effect [321]. MAPKs could also activate other co-activators of HIF-1α, and other signaling cascades may activate HIF-1α.

1.5.2. MAPKs and angiogenesis

As described above, MAPKs pathways are closely related to stress-induced cellular responses including hypoxic induction of HIF-1α. Because of this, not only HIF-1α but also its transcriptional target VEGF, which is involved in angiogenesis, has been studied in relation to MAPK signaling.

Firstly, activation of ERK1/2 pathway rescues fibroblasts and epithelial cells from apoptosis and promotes entry of endothelial cells into cell cycle. In addition, ERK1/2 kinase activity is required for controlling endothelial cell proliferation and growth arrest under confluent conditions. Moreover, ERKs can increase VEGF transcription by recruiting AP-2/Sp1 (activator protein-2) complex to its promoter region and by phosphorylation of HIF-1α [322]. Secondly, JNK and p38 overexpression or activation by hypoxia augments HIF-1α and VEGF induction [320]. ERKs may be required for the initial stages of angiogenesis in addition to other receptor tyrosine kinases whereas p38 is critical for the recruitment of mural cells during later stages of angiogenesis [323]. Inhibition of p38 results in the formation of naked endothelial tubes without mural cells, and the blockage attenuates angiopoietin-1-induced mural cell recruitment [323]. Thus, p38 appears to be important for completing the angiogenic process, and the other MAPKs may function in concert during this event.

1.6. Rationale and aims of the investigations

Ischemic cardiovascular disease and tumors are of intensive concern due to their high incidences and high degree of mortality nowadays. The common existence of tissue hypoxia during progression of the diseases makes angiogenesis a two-bladed sword. On the one hand, hypoxia induces angiogenesis so as to rescue the ischemic myocardium. This natural process needs to be therapeutically enhanced. On the other hand, hypoxia-triggered angiogenesis in tumors contributes to their growth and metastasis and needs to be inhibited.

Hypoxia triggers angiogenesis naturally via HIF-1α. Mechanistic understanding and planned therapeutic intervention have therefore accumulated on HIF-1α. HIF-1α is an important transcription factor that induces a set of key genes driving angiogenesis and that is directly regulated by oxygen saturation. Therapeutic delivery of HIF-1α gene has shown its potency to improve structure and function of the vasculature, especially in ischemic tissues [153,154]. Moreover, interrupting HIF-1α transactivity by different means can be used to retain tumor growth and the associated vascularization [80,157].

Cells involved in angiogenesis respond to hypoxia with an intrinsic oxygen- and energy-sensing system. As a nutrient- and energy-sensing molecule, mTOR may also play an important role in sensing oxygen saturation. Moreover, inhibition of mTOR by rapamycin or other analogs and targeting mTOR-related pathways have been proven to be effective against cell growth, proliferation, and angiogenesis [140,141,225,303].

The major aims of this thesis are to elucidate (**i**) the influence of hypoxia on mTOR activation, i.e. whether mTOR participates in oxygen-sensing; (**ii**) the regulatory relationship between mTOR and HIF-1α and the mechanisms of this interplay, and (**iii**) the contribution of the latter interplay to cell proliferation and angiogenesis *in vitro*. Furthermore, additional goals of this study are to assess other signaling pathways such as mitogen- and stress-induced signaling pathways in the regulation of HIF-1α nuclear accumulation. Data from this study will possibly help to improve our understanding of cellular mechanisms for hypoxia-sensing and –transduction, and may better delineate molecular targeting in diseases associated with ischemia and tumor.

2. MATERIALS AND METHODS

2.1. *In vitro* angiogenesis assay

Fibrin gels were prepared by mixing 3 mg fibrinogen (Sigma-Aldrich, Buchs, Switzerland) per milliliter of serum-free DMEM (Oxoid AG, Basel, Switzerland) with 300 μg/ml of thrombin on ice. 48-well plates were coated with the prepared fibrin gel at a volume of 100 μl/well and allowed to polymerize for about half an hour at 37°C. Aorta excised from adult male Sprague-Dawley rat (around 200 g; Charles River Laboratories, France) was cut into 1 mm^2 squares, and the pieces were placed with endothelium side down onto the coated gel. Then, another portion of fibrin gel was overlaid over the aortic pieces. After gel polymerization at 37°C, 500 μl/well of serum-free DMEM medium was loaded to the wells. Subsequently, different agonists or antagonists were added to the medium every 3 days. 300 μg/ml α–amino caproic acid was used every third day to protect the fibrin gel from degradation. Samples were incubated in normoxia (21% O_2) and hypoxia (1% O_2), respectively. After 10 days of culturing, angiogenesis-like sprouts were photographed digitally on a light microscope (Zeiss, Feldbach, Switzerland) for standardized scoring or for endothelial cell isolation.

2.2. Cells and culture conditions

Rat aortic endothelial cells (RAECs) were derived from *in vitro* angiogenesis assay as described above and submitted to cell culture. They were used for experimental purposes during passages 4 to 10 after characterization with endothelial cell specific immunofluorescent marker PECAM-1 (CD31, LabForce AG, Nunningen, Switzerland) or von Willebrand Factor (VWF, LabForce AG). RAECs were cultured in DMEM complemented with 10% fetal calf serum (FCS, Oxoid AG), 1% sodium pyruvate (Oxoid AG), 1% non-essential amino acids (Oxoid AG), and 1% penicillin-streptomycin (GIBCO™, Invitrogen AG, Basel, Switzerland). For hypoxia, cells were cultured in hypoxia incubator (Ismatec, Basel, Switzerland) with a gas mixture containing 1% O_2 and 5% CO_2, balanced with nitrogen. Normoxic condition was defined as 21% O_2 and 5% CO_2 with nitrogen as complement. Mouse embryo fibroblast with HIF-1α (HIF-$1\alpha^{+/+}$) wild-type cells and knockout HIF-1α (HIF-$1\alpha^{-/-}$) cells

(gifts from Prof. Max Gassmann, Zürich, Switzerland) were cultured in the same condition as described above.

Following inhibitors were used: 5 - 500 nM rapamycin for mTOR, 5 - 80 μM PD98059 for MEK1/2, 5 - 200 nM JNK-Inhibitor II for Jun kinase, 0.1 - 3 μM SB203580 for p38 kinase, 0.1 - 10 μM epoxomicin for proteasome activity (all from Calbiochem, Läufelfingen, Switzerland).

2.3. Endothelial cell spheroid sprouting assay

RAECs were washed and trypsinized. Centrifugation-collected cells were resuspended in 10% FCS- and 0.25% (w/v) carboxymethylcellulose-containing DMEM. After cell counting, cells were seeded in non-treated round-bottom 96-well plates (Fisher Scientific AG, Wohlen, Switzerland) at the density of 1500 cells/well and were subjected to incubation at 37°C for about 24 hours. The cells in each well of the plates were capable of forming a single spheroid [324]. The spheroids were then embedded into bilayers of a fibrin gel and treated with different reagents as described formerly in the *in vitro* angiogenesis assay. Sprouts from the spheroids were analyzed and quantified by using a computerized system affiliated to an invert light microscope (Zeiss, Feldbach, Switzerland).

2.4. Cell proliferation assay

Subconfluent cells in normoxic culture were washed twice in 37°C 1X PBS and trypsinized for cell counting. Cells were then seeded and cultured in duplicate 96-well cell culture plates at an initial density of 4.0 x 10^3 cells/well for 24 h under normoxic condition followed by an additional 30 h normoxic culturing in serum-free DMEM (starvation; FCS was substituted with 0.1% BSA). Then antagonists at different concentrations were added to the medium and duplicate cultures were incubated in normoxia and hypoxia, respectively for 1 h. Then agonists e.g. human recombinant PDGF-BB (10 ng/ml, Bühlmann Laboratories AG, Basel, Switzerland) were added. Each condition under observation in a plate was performed in octuplicate. After another 24 h incubation, Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals, Rotkreuz, Switzerland) was added to the cell-containing wells according to the manufacturer's instructions. Cell numbers were assessed by quantifying the formazan dye produced by metabolically active cells on a microplate

spectrophotometer (SPECTRAmax®190, Molecular Device Corporation, Sunnyvale, California).

2.5. Cytosolic and nuclear protein extraction

Cells were seeded in tissue culture dishes (Becton Dickinson Labware Europe, Le Pont De Claix, France) at an initial density of 4.0 x 10^6 cells/150 mm x 25 mm-dish. Upon 70% confluence, cells were washed, starved, separately incubated in normoxia or hypoxia, and inhibited where required as described before. Ciclopirox Olamine (CPX) was used to induce HIF-1α as a positive control. Subsequently, agonist was added to the cell culture dishes according to the experimental design. Finally, the cultures were put back to the nomoxic and hypoxic conditions, respectively. After culturing for 4.5 hours, extraction of nuclear proteins from the cell was performed. Cells were rinsed twice in ice-cold 1X PBS, scraped into 2ml sterilized microcentrifuge tubes and subjected to centrifugation at 1500 rpm and 4°C for 3 minutes. Consecutively, supernatant was aspirated and the pellet was resuspended in 3 times pellet volume of freshly prepared cell lysis buffer (20 mM Hepes PH7.9, 10mM KCl, 0.1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 0.2% NP-40, 10% glycerol, 1 mM DTT, 1 mM PMSF, Protease inhibitor cocktail tablet - Complete Mini from Roche Diagnostics GmbH, Mannheim, Germany), and incubated on ice for 10 minutes with periodical vortexing. After incubation and centrifugation, the cytosolic supernatant was transferred into a new sterilized eppendorf tube, and nuclear proteins were extracted by incubating the nuclei containing pellet in 2 times pellet volume of nuclear extraction buffer (420 mM NaCl, 20 mM Hepes PH7.9, 10 mM KCl, 0.1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM DTT, 1 mM PMSF, Protease inhibitor cocktail tablet) on ice for 15 minutes with interval vortexing. The supernatant containing nuclear proteins was transferred to a new sterilized eppendorf tube. Finally, protein concentration was determined by using BCA protein assay reagent (Socochim, Lausanne, Switzerland).

2.6. Western blotting

Equal amount of protein extracts were subjected to SDS-polyacrylamide gel electrophoresis. Subsequently, proteins were transferred onto Polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Bedford, U.S.A). The membrane was blocked with 4% skim milk powder in TBS-Tween solution and probed stepwise with

antibodies, chicken polyclonal antibody against HIF-1α (gift from Prof. Max Gassmann, University of Zürich), secondary anti-chicken IgY (Catalys AG, Wallisellen, Switzerland); mTOR, Phospho-mTOR (Ser^{2448}), Phospho-p70 S6 Kinase (Thr^{389}), Phospho-4E-BP1 ($Thr^{37/46}$) rabbit polyclonal antibodies (all from Cell Signaling Technology, MA) against the corresponding proteins, and HRP-linked anti-rabbit IgG (Transduction Laboratories, San Diego, CA). After incubation with antibodies and washing in 1X TBS-T solution, the membrane was subjected to ECL western blotting reagents (Amersham, Buckinghamshire, England) for imaging with X-ray films (Kodak, Geroldswil, Switzerland). ImageJ software (Wayne Rasband, NIH, USA) was used for densitometrical quantification and analysis of the protein bands appearing on images.

2.7. Immunofluorescence microscopy

RAECs were seeded on cover-slips at the density of 4.0 x 10^4 /well in 24 well tissue culture testplates and cultured in normoxia. At 70% confluency, cells were starved, exposed to antagonists or/and agonists, and incubated in 21% O_2 or 1% O_2, respectively. After 4.5 hours of incubation, cells were rinsed and fixed with 4% paraformaldehyde for 15 min at room temperature. The samples were blocked with goat serum (Fluka, Buchs, Switzerland) in PBS with 0.25% BSA. The fixed cells were probed consecutively with primary goat polyclonal IgG against HIF-1α, secondary anti-goat Cy2-conjugated IgG (Santa Cruz Biotechnology, CA). TOTO-3 (Molecular Probes, Eugene, USA) or Hoechst dye (Polysciences Europe GmbH, Eppelheim, Germany) was used for nuclear staining. Finally, the cover-slips with cells were fixed on slides and observed as well as analyzed under confocal Laser Scanning Microscope (LSM 510, Axiovert 100M, Plan-Neofluar objectives; Carl Zeiss AG, Feldbach, Switzerland).

2.8. Cellular total RNA isolation and cDNA synthesis

After 4.5 h conditioned culturing, RAECs were scraped off from culture dishes and were collected into 1.5 ml sterilized Eppendorf tubes. The cells were pelleted by centrifugation and resuspended with 1 ml TRIzol® Reagent (Invitrogen AG). After repeatedly pipetting up and down, the samples were incubated for 5 minutes at room temperature (15 – 30°C). 0.2 ml of chloroform and 0.5 ml Isopropyl alcohol were

used in turn to phase-separate and precipitate cellular RNA. Proceeding centrifugation, the gel-like RNA pellet was washed with 75% ethanol and re-dissolved in 30 μl Rnase-free water followed by 10 minutes of incubation at 55 – 60°C. For first-strand cDNA synthesis, the solution-mixture, 1 μg of newly synthesized RNA, 0.5 μg random primers, 5 μl of M-MLV RT buffer, 1 μl of M-MLV reverse transcriptase, 5 μl of dNTP mix, 0.625 μl of Rnasin (Catalys AG) complemented with DEPC-treated water to 25 μl, was incubated at 37°C for 1 h. Reverse transcriptase was deactivated at 95°C for 5 minutes.

2.9. RT-PCR

Primers for HIF-1α RT-PCR (hot-start) analysis were designed as follows: forward primer, 5'-CGTGTGAGGAAACTTCTAGGTG-3'; and reverse primer, 5'-GGCTCATAACCCATCAACTCA-3' for HIF-1α to amplify a sequence of 599-bp. Forward primer, 5'-ATGGTGGGTATGGGTCAGAA-3'; reverse primer, 5'-ACCCTCATAGATGGGCACAG-3' for β-actin (as internal control) to amplify a sequence of 375-bp. 2 μl of the synthesized cDNA samples were denatured at 94°C for 5 minutes in PCR tubes, proceeded with adding reaction solution A (Mg-free 10X buffer 4 μl, 25 mM $MgCl_2$ 2.4 μl, primers 0.5 μl, dNTPs 6.1 μl, made up to 40 μl by ddH2O). After covering with mineral oil and equilibrating the samples at 75°C for 2 minutes, solution B (Mg-free 10X buffer 1 μl, 25 mM $MgCl_2$ 0.6 μl, Taq polymerase 0.2 μl, made up to 10 μl by ddH2O) was added to the reaction solution mixture. RT-PCR for both reactions were run as follows: 30 amplification cycles with each consisting of denaturing at 94°C for 1 minute 10 seconds, annealing at 54°C for 1 minute 10 seconds, elongating at 72°C for 2 minutes. PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining under a ChemiImager (5500-Rev D, Alpha Innotech, CA, USA). Finally, images were taken and analyzed.

2.10. Real-Time RT-PCR

HIF-1α primers were designed by using Primer Express® version 2.0 software (Applied Biosystems). Sequences of primers used for HIF-1α cDNA were 5'-GGCGAAGCAAAGAGTCTGAAGT-3' for sense strand and 5'-AGCTTTATCAAGATGGGAGCTCA-3' for antisense strand. The concentrations of

the primers were optimized for the lowest threshold cycle (C_T) and maximum ΔR_n. SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) had been used to complement reactions. β-actin was used as an endogenous control for the relative quantitations. Sequences of primers used for β-actin cDNA were 5'-TTCAACACCCCAGCCATGT-3' (sense) and 5'-GGAGCGCGTAACCCTCATAG-3' (antisense). With the cDNA samples ready, Real-time PCR was performed on GeneAmp® 5700 (AME Bioscience) to check up the changes of HIF-1α mRNA level upon different stimuli in the studied settings. Relative standard curve method was used to analyze the data derived from the PCR reactions.

2.11. Statistical analysis

Data were analyzed for normal distribution (one-way ANOVA), followed by Bonferroni t test and the Student-Newman-Keuls test using the program SPSS (SPSS Inc. Chicago, USA), and given as mean ± SEM. The number of samples examined is indicated by n. A value of $p < 0.05$ was considered as significant.

3. RESULTS

3.1. Hypoxia-enhanced endothelial sprout-formation and cell proliferation is rapamycin sensitive

To date, most studies concerning the influence of hypoxia or/and mTOR on angiogenesis and cell proliferation are done in highly proliferative cells or transformed cells. In order to characterize the impact of low oxygen tension as well as mTOR on these two activities in non-cancerous and non-transformed vascular cells, we performed endothelial spheroids angiogenesis assays and endothelial proliferation assays using RAECs in normoxia (21% O_2) or severe hypoxia (1% O_2).

Angiogenic-like sprouts from the endothelial spheroids were observed in both normoxia and hypoxia. Hypoxia strongly enhanced endothelial sprout formation (Figure 13A, top panels). Interestingly, the spheroids cultured in the rapamycin (RPM)-containing media showed a strong decrease in the degree of sprouting regardless of the different O_2 saturation during incubation. However, RPM reduced the sprouting from endothelial spheroids more profoundly under hypoxia than under normoxia (Figure 13A). To quantify the differences of the sprouting in these conditions, we used a computerized morphometric system after collecting data from three individual experiments (Figure 13B). Endothelial sprout-formation in hypoxia was more than doubled when compared to the response in normoxia, and the spheroids under both conditions are significantly sensitive to RPM ($p < 0.05$).

A similar response was observed in RAEC proliferation assay. Hypoxia alone increased RAEC proliferation to about 1.5 fold when compared to diluent normoxic control and PDGF-BB enhanced proliferation about 1.7 fold under normoxia (Figure 14). Hypoxia together with PDGF-BB increased RAEC proliferation 2.4 fold. These augmented proliferation rates could be effectively reduced by mTOR inhibition with RPM. Low concentrations of rapamycin (2 nM) effectively inhibited proliferation under hypoxia (with or without growth factor), whereas a higher concentration (20 nM RPM) was required to completely inhibit PDGF-BB-induced proliferation under normoxia (Figure 14). These data suggest that mTOR-signaling plays an important role in angiogenesis *in vitro* and endothelial cell proliferation in response to hypoxia.

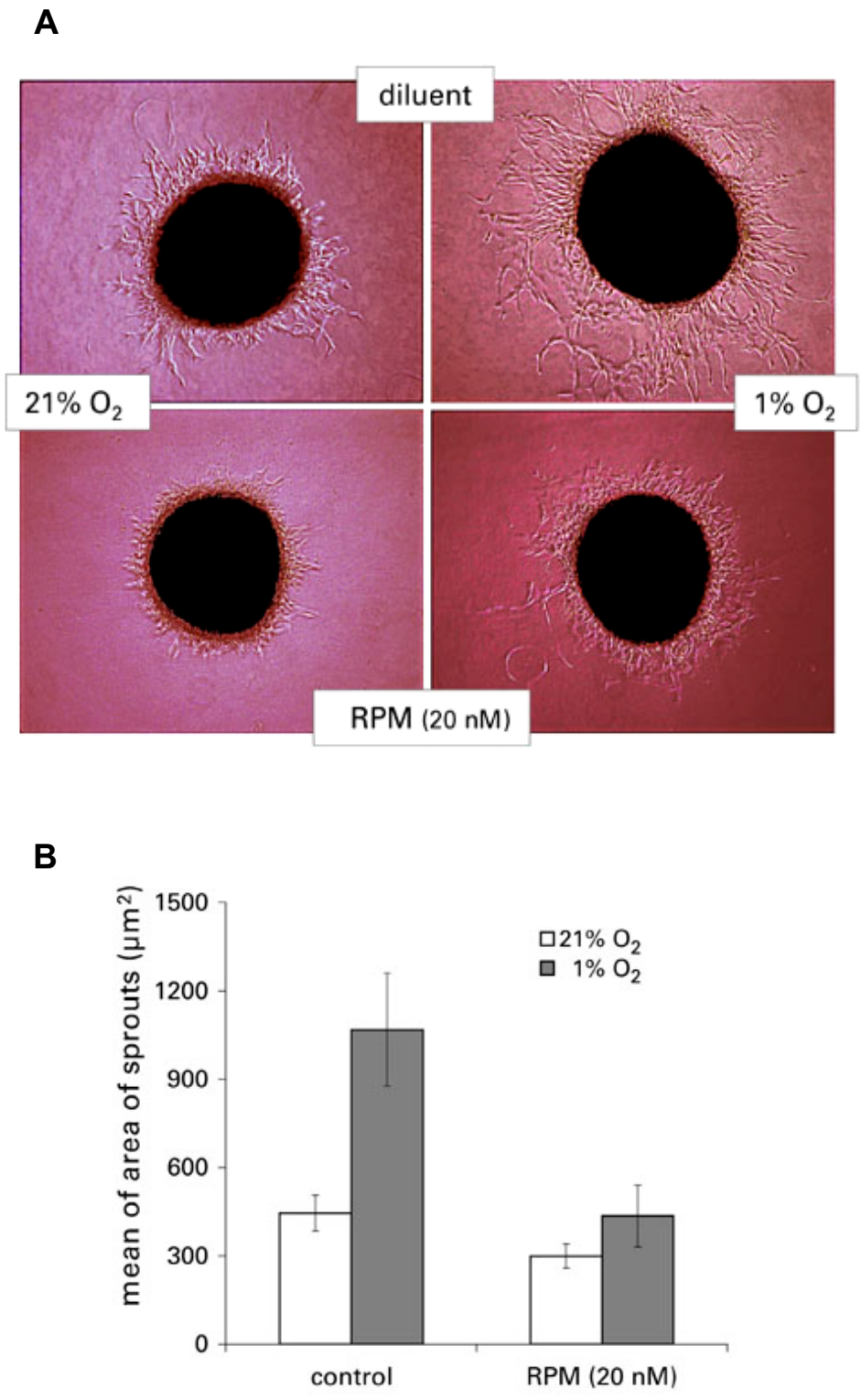


Figure 13. Rapamycin effectively inhibits RAEC spheroid sprout-formation especially under hypoxia. A) Typical micrographs of rat aortic endothelial spheroids after a 24 h incubation under normoxia (21% O_2) or hypoxia (1% O_2) without (control) or with RPM. **B**) Quantification of endothelial sprouts from three independent experiments (each containing about 30 spheroids per condition). Data are given as mean ± SEM, n = 3.

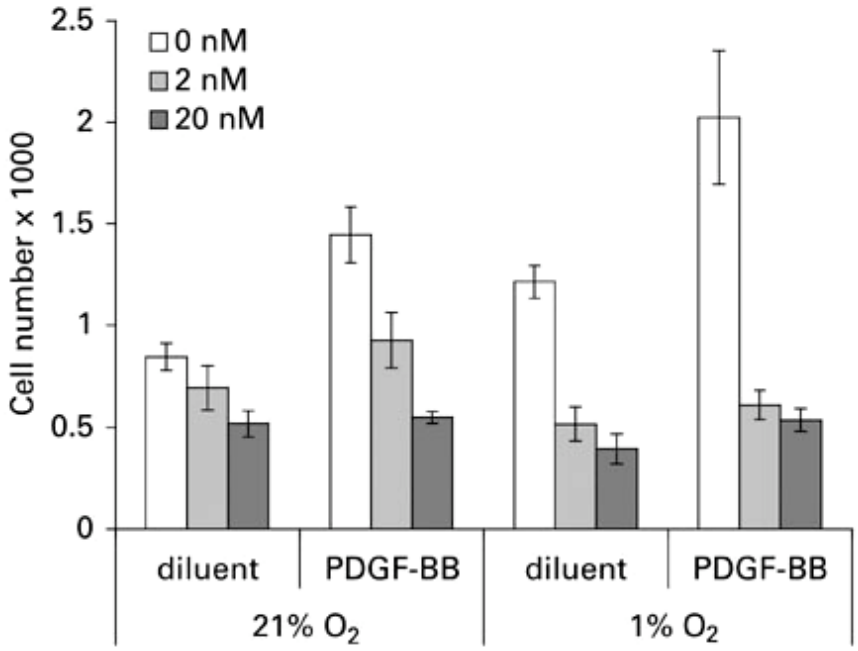


Figure 14. Hypoxia or/and PDGF-BB increases RAEC proliferation in a rapamycin-sensitive way. Cell numbers of RAEC were determined after 24 h culturing under normoxic (21% O_2) or hypoxia (1% O_2) with or without PDGF-BB (10 ng/ml), in the absence (open bars) or presence (filled bars) of the indicated concentrations of rapamycin (RPM). Y-axis represents the mean of cell number, compiled from three individual experiments with octuplicate samples. Data are given as mean ± SEM, n = 3.

3.2. Hypoxia induces mTOR phosphorylation, nuclear accumulation, and S6K1, 4E-BP1 hypophosphorylation

Next, we determined whether decreasing oxygen saturation modulates the activity of mTOR, i.e., affects mTOR phosphorylation (Ser^{2448}) and phosphorylation of mTOR downstream targets p70/p85 S6K (S6K1) and 4E-BP1. Quiescent RAEC were incubated under decreasing oxygen saturations (21%, 11%, 6%, 3% and 1% O_2) for 12 h. This time period was used because HIF-1α was maximally induced in the studied cell type after 12 h of hypoxia exposure (see data below). Western blot analysis showed that under normoxia (21% O_2), weak phosphorylation of mTOR at Ser^{2448} and distinct phosphorylation of S6K1 and 4E-BP1 were detected in cytoplasmic compartment of RAEC. mTOR-Ser^{2448} phosphorylation increased under 11% O_2 and augmented further with lowering O_2 saturations with a maximum at 1% O_2. In contrast, mTOR downstream targets S6K1 and 4E-BP1 were almost totally dephosphorylated under any O_2 saturation from 11% down to 1% (Figure 15). mTOR

protein as well as β-actin protein levels were not affected by oxygen saturation. These results illustrate a different phosphorylation as well as activation pattern of mTOR and its two target effectors S6K1 and 4E-BP1.

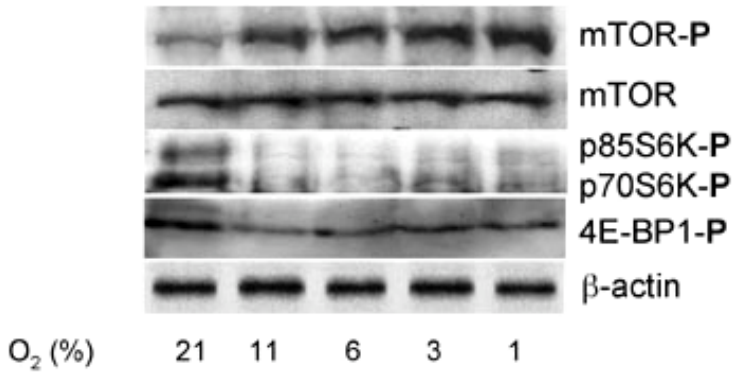


Figure 15. Distinct phosphorylation pattern of mTOR on Ser^{2448} and of S6K1 as well as 4E-BP1 in hypoxia. Decreasing O_2 tension increases phosphorylation of mTOR on Ser^{2448}, abrogates phosphorylation of mTOR downstream targets S6K1 and 4E-BP1. β-actin was detected as a protein loading control. mTOR-P (mTOR-Ser^{2448} phosphorylation); p85S6K-P/p70S6K-P (S6K1-Thr^{389} phosphorylation); 4E-BP1-P (4E-BP1-Thr$^{37/46}$ phosphorylation).

Hypoxia-induced cytoplasmic Ser^{2448} phosphorylation of mTOR peaked at 1% O_2. Similarly, HIF-1α expression peaked at 1% O_2. We therefore examined whether hypoxia influences mTOR nuclear distribution under 1% O_2 over time (0 – 24 h). Whereas mTOR and phospho-mTOR levels were almost undetectable at time point 0, the two proteins already appeared in the nuclear fraction after 2 h of incubation. The levels of both mTOR and phospho-mTOR remained stable with further incubation under hypoxia (Figure 16). Thus, mTOR protein accumulates in the nuclear compartment of RAEC after exposure to hypoxia.

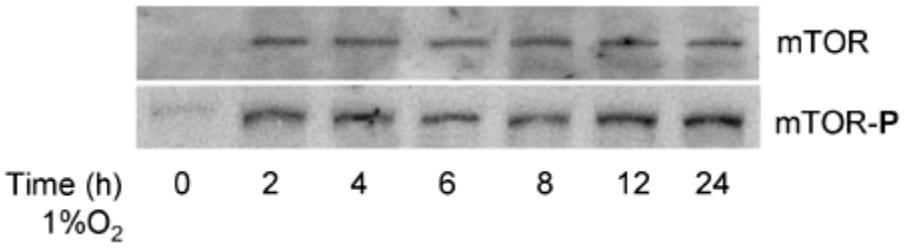


Figure 16. Nuclear mTOR and phospho-mTOR (Ser2448) levels in hypoxia over time. Quiescent RAEC were cultured for increasing periods of time (2 - 24 h) under conditions of 1% O_2, or left in normoxia (time point = 0). Nuclear extracts were prepared, and proteins were resolved by SDS-PAGE followed by immunoblotting using anti-HIF-1α, -mTOR and -phospho-mTOR-Ser^{2448} (mTOR-P) antibodies.

3.3. HIF-1α nuclear accumulation is oxygen saturation- and time-dependent

As known, HIF-1α is a key element for cells to adapt to hypoxic conditions, and plays an important role in angiogenesis. To be functional, HIF-1α needs to be translocated into the nucleus in order to transactivate hypoxia-adaptive genes [76]. We therefore characterized HIF-1α nuclear levels in severe hypoxia (1% O_2) over time (0 – 24 h). In normoxia (Time point = 0), basal levels of HIF-1α were detected in nuclear extracts. After 2 h of hypoxic exposure, HIF-1α protein levels consistently increased and peaked after incubation between 12 and 24 h (Figure 17 top panel). We then incubated RAECs in different oxygen saturation environments for 12 h to check for nuclear HIF-1α levels. As shown, HIF-1α protein amount in RAEC nuclear extracts enhanced almost linearly with decreasing oxygen saturation and peaked at oxygen concentrations of 1% - 3% O_2 (Figure 17 bottom panel). Thus, the optimal condition for HIF-1α induction in RAEC is exposure to 1% O_2 for 12 h. Most of the HIF-1α protein levels were measured after 4.5 h of hypoxic/nomoxic incubation since HIF-1α is already clearly detectable at this time point.

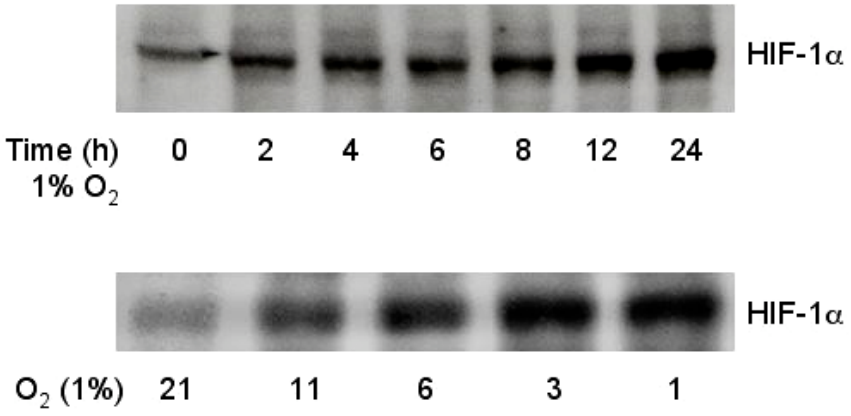


Figure 17. HIF-1α nuclear levels over time in hypoxia and over decreasing oxygen saturation. RAECs were cultured in 1% O_2 for time-dependent observation of HIF-1α levels in nuclear compartment by western-blotting analysis (upper panel). Characterization of HIF-1α levels in RAEC nuclei over changes of oxygen tension from 21% to 1% O_2 (bottom panel).

3.4. Hypoxia synergistically increases PDGF-BB-induced HIF-1α nuclear accumulation

To further confirm HIF-1α distribution in the cells under distinct oxygen condition, i.e. normoxia or hypoxia, and to assess how growth factors might interfere with this process, quiescent RAEC seeded on cover-slips, were incubated for 4.5 h with or without PDGF-BB under conditions of normoxia (21% O_2) or hypoxia (1% O_2). Cells were fixed, immunostained for HIF-1α and nuclear compartment and analyzed by confocal microscopy.

HIF-1α was barely detectable under conditions of normoxia (Figure 18A top-left panel). However, the protein accumulated in the nuclei under conditions of hypoxia or normoxia plus PDGF-BB (Figure 18A top-right and bottom-left panel). The highest HIF-1α concentration in the nuclei was detected when RAEC were stimulated with PDGF-BB (10 ng/ml) under conditions of hypoxia (1% O_2) (Figure 18A bottom-right panel).

To quantify HIF-1α nuclear accumulation under these conditions, nuclear protein extracts were analyzed by SDS-PAGE and HIF-1α protein was detected by western blotting. Densitometric quantifications of three individual experiments illustrate that either hypoxia (1% O_2) or PDGF-BB (10 ng/ml) alone induced considerable accumulation of HIF-1α (4.2 fold and 5.2 fold, respectively, compared to normoxic control) in RAECs' nuclei. Nuclear HIF-1α protein accumulation was maximal (11.9 fold compared to normoxic control) when PDGF-BB stimulation was carried out under 1% O_2 (Figure 18B). Thus, results from immunoblotting are in accordance with the data from immunofluorescent staining both of which suggest an independent induction of HIF-1α in RAEC nuclei by hypoxia or PDGF-BB. Furthermore, a synergistic effect of HIF-1α nuclear accumulation is observed under co-stimulation of hypoxia and PDGF-BB.

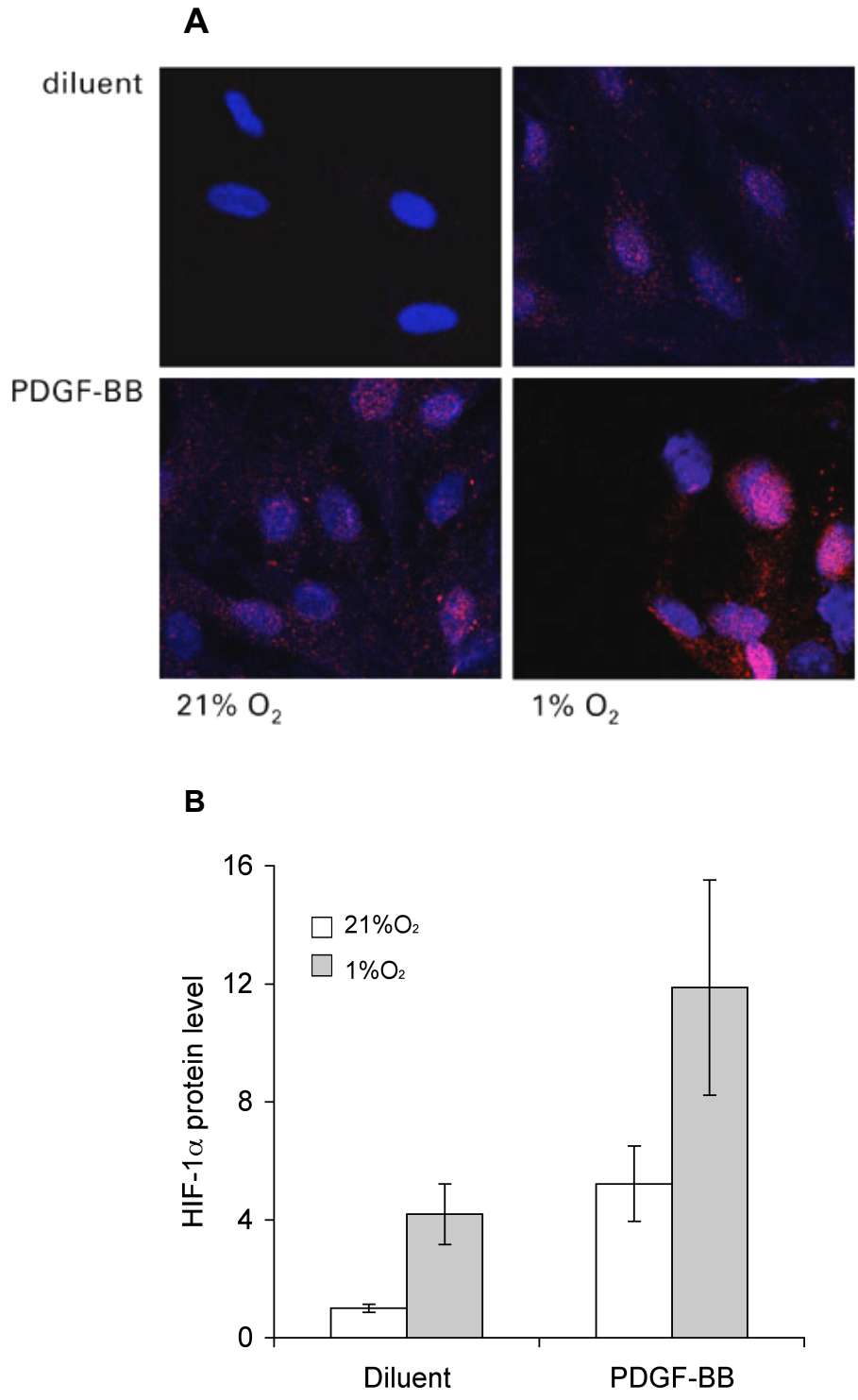


Figure 18. Hypoxia or PDGF-BB independently and the both synergistically induce HIF-1α nuclear accumulation in RAEC. A) Confocal microscopy results: immunofluorescent staining of HIF-1α (red staining) and nuclear compartment (blue staining) of RAEC stimulated with or without PDGF-BB (10 ng/ml) under conditions of normoxia (21% O_2) or hypoxia (1% O_2). **B**) Densitometric quantification of Western blots of RAEC nuclear fractions for HIF-1α. The inductions of nuclear HIF-1α by hypoxia, PDGF-BB as well as hypoxia plus PDGF-BB are significantly higher than that of normoxic control group ($P < 0.001$, n = 3).

3.5. mTOR inhibition abrogates hypoxia- and/or PDGF-BB-stimulated HIF-1α nuclear accumulation

To assess an involvement of mTOR in HIF-1α regulation, we examined the effect of mTOR inhibition on HIF-1α nuclear accumulation triggered by hypoxia or/and PDGF. We treated RAECs with increasing doses of rapamycin. Under all tested conditions, rapamycin considerably lowered HIF-1α nuclear protein levels under hypoxia alone (64±3%), PDGF-BB stimulation under normoxia (86±4%) and hypoxia (76±4%). The synergistic effect from hypoxia and PDGF-BB on HIF-1α nuclear accumulation observed in this experiment was sensitive to rapamycin, however, rapamycin failed to fully block HIF-1α nuclear accumulation to a basal level (Figure 19). These readouts suggest a general role of mTOR in the modulation of HIF-1α nuclear accumulation.

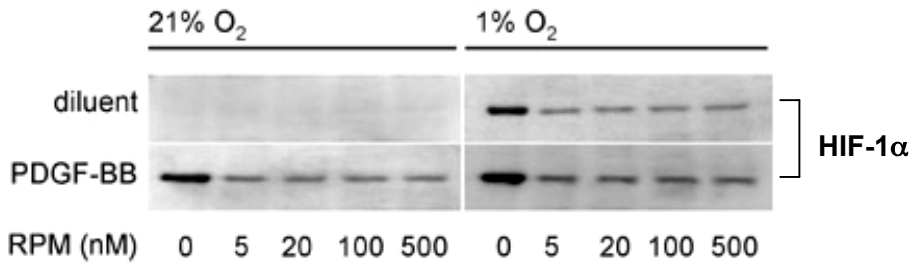


Figure19. Rapamycin attenuates hypoxia- or/and PDGF-BB-induced nuclear accumulation of HIF-1α. Quiescent RAECs were treated with mTOR inhibitor rapamycin (RPM), and nuclear protein fractions were separated by SDS-PAGE and HIF-1α was detected at 120 kDa. Trace amount of HIF-1a was detectable on western blots (top-left panel). Rapamycin markedly but not completely reduced the amount of nuclear HIF-1α induced by hypoxia (top-right panel) or PDGF-BB (10 ng/ml) alone (bottom-left panel) as well as the two factors together (bottom-right panel).

3.6. MEK1/2 but not JNK or p38 inhibition impairs PDGF-BB-induced HIF-1α nuclear accumulation only under normoxia

As common mitogen- or/and stress- activated protein kinase pathways, MAPK signaling cascades have been implicated in cellular responses to hypoxia [315,318,319]. In order to test the involvement of these pathways in HIF-1α regulation, we used PD98059, SB203580 and JNK-I-II to inhibit MEK1/2-, p38- and Jun kinase, respectively, and detected nuclear HIF-1α by immunoblotting. MEK1/2 inhibition did not reduce hypoxia- or hypoxia and PDGF-BB-induced HIF-1α nuclear accumulation. However, under normoxic condition, the enhancement of nuclear HIF-1α induced by

PDGF-BB stimulation was dramatically reduced (by 86±3%) (Figure 20 top panels). In contrast, neither blockage of JNK nor p38 was able to attenuate hypoxia- or/and PDGF-BB-induced HIF-1α nuclear accumulation (Figure 20 middle and bottom panels). Thus, MEK1/2 pathway is reactive to PDGF-BB but not to hypoxia for HIF-1α nuclear accumulation in RAEC.

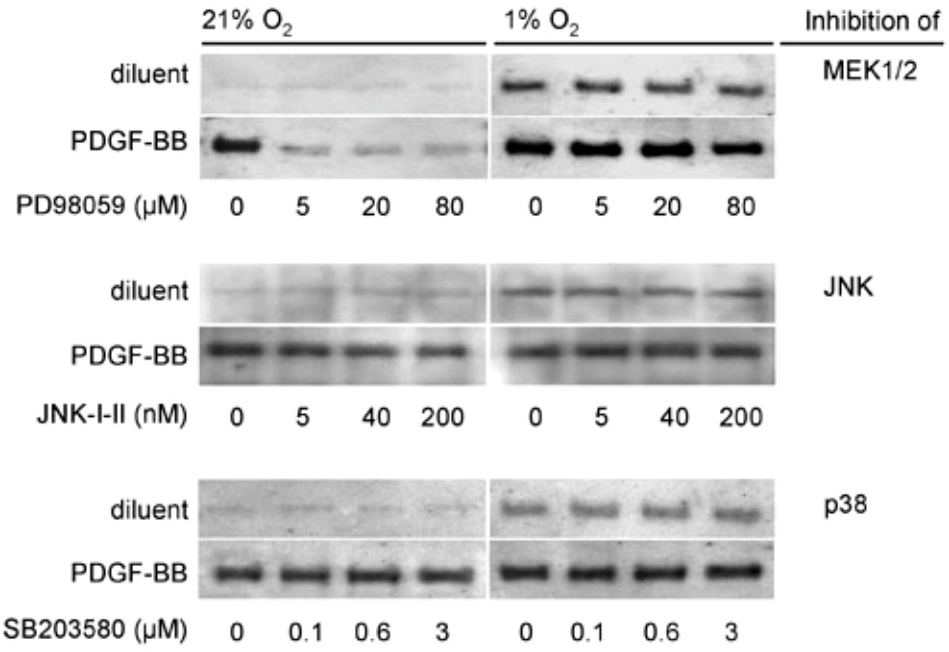
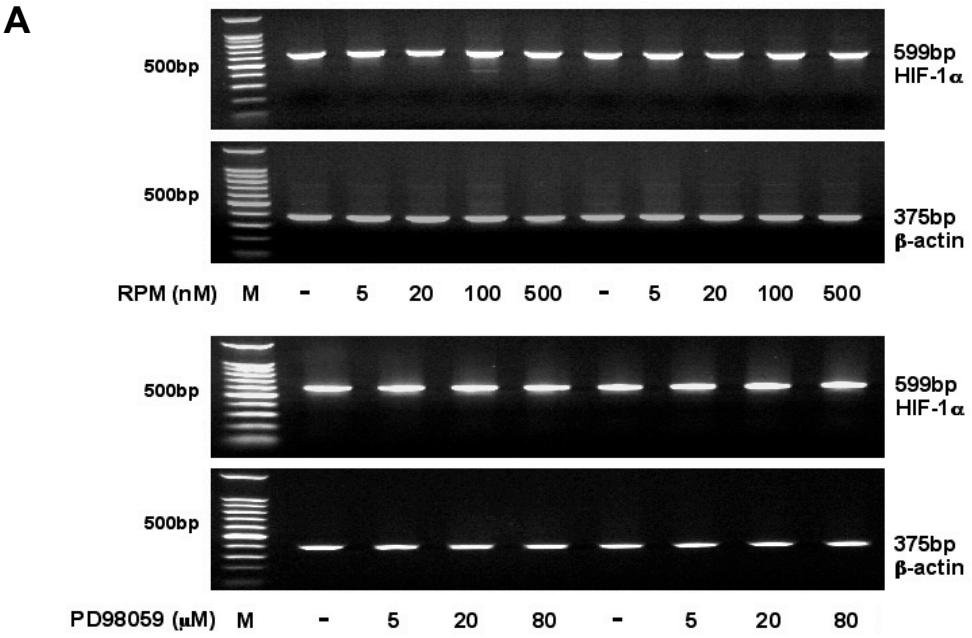


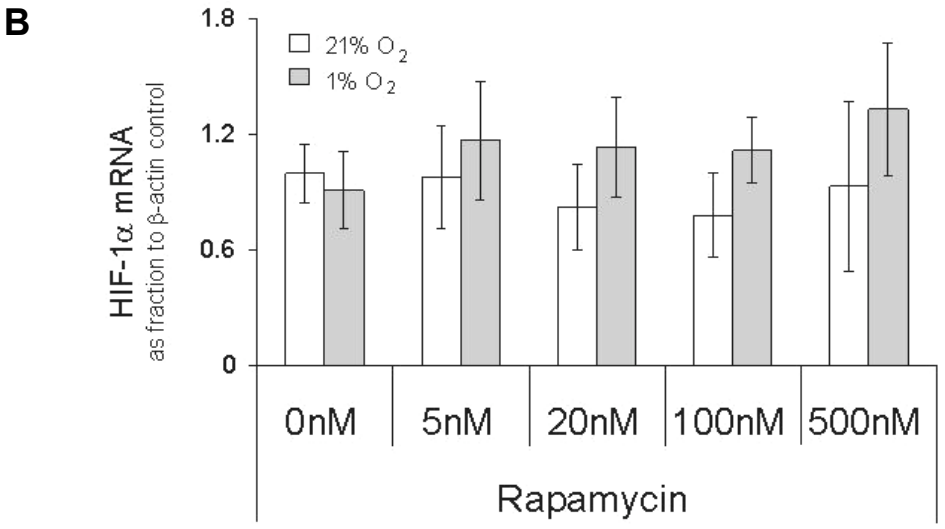
Figure 20. Impact of inhibition of MAPK pathways on HIF-1α nuclear accumulation. Serum-starved RAECs were treated with different inhibitors of MAPK transduction cascades and proceedingly cultured in normoxia or hypoxia as described formerly for 4.5 h before subjecting to western blotting. MEK1/2 inhibitor (PD98059, 5-80 μM, upper four panels); Jun kinase inhibitor (JNK-I-II, 5-200 nM, middle four panels); p38 kinase inhibitor (SB203580, 0.1-3 μM, bottom four panels). HIF-1α was detected at 120 kDa. Only under normoxia, inhibition of MEK1/2 pathway strongly reduces PDGF-BB-induced HIF-1α nuclear accumulation whereas JNK and p38 signaling do not play a role at all.

3.7. HIF-1α mRNA levels remain stable over changes of oxygen tension and upon mTOR or MEK1/2 inhibition

The data above have shown an involvement of mTOR or ERK1/2 signaling pathway in the regulation of HIF-1α nuclear accumulation. We therefore assessed at which levels the two pathways regulate HIF-1α. The increase of cellular HIF-1α may result from transcriptional or translational upregulation as well as post-translational stabilization. We therefore investigated the transcriptional activity of HIF-1α by measuring its mRNA levels. PDGF-BB stimulation proceeding mTOR or MEK1/2

inhibition of RAEC in normoxia or hypoxia was used because high HIF-1α nuclear induction rates were observed in the former experiments. However, when normal RT-PCR was primarily used for analysis, no differences in HIF-1α levels in the tested settings were observed as shown in Figure 21A. Also, the Real-Time RT-PCR followed by β-actin normalized quantification showed that HIF-1α mRNA levels were constitutive after, i.e., were not significantly influenced by oxygen saturation or inhibition of mTOR and MEK1/2 signaling, respectively (Figure 21B and C).





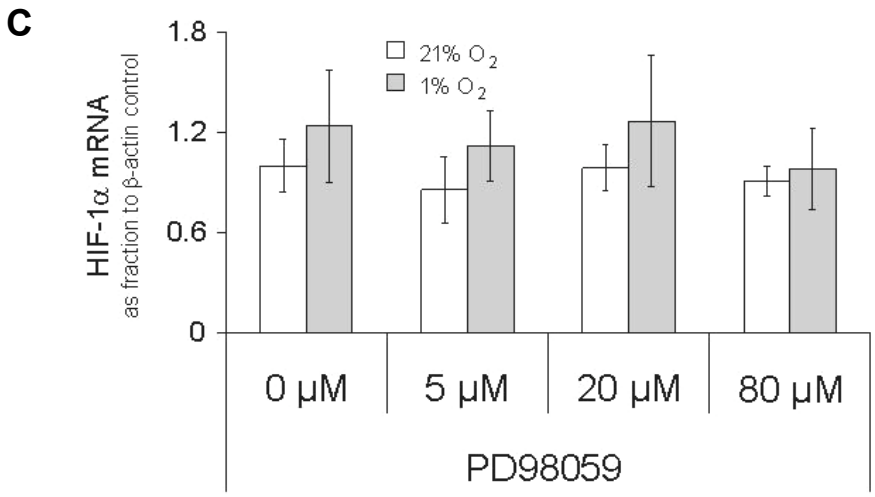


Figure 21. Comparison of HIF-1α mRNA levels in normoxia and hypoxia under the conditions of mTOR or MEK1/2 inhibition and growth factor stimulation. RAECs treated with rapamycin (5 – 500 nM) or PD98059 (5 – 80 μM) and stimulated with PDGF-BB (10 ng/ml) were cultured in normoxia or hypoxia for 4.5 h. cDNAs were subsequently synthesized proceeding cellular total mRNA extraction. **A)** RT-PCR of a 599 bp fragment of HIF-1α and a 375 bp fragment of b-actin (control) from the synthesized cDNAs of the designated cultures. **B)** Real-time RT-PCR (using the same batch of cDNAs as described above) of HIF-1α mRNA levels depicted as fraction of internal β-actin control.

3.8. Proteasomal inhibition rescues HIF-1α cellular accumulation attenuated by mTOR blockage

In order to assess the proximal impact of rapamycin on HIF-1α proteasomal degradation, RAECs were treated with the specific proteasome-inhibitor epoxomicin (EPX, 1 μM) for different time points, and the cellular levels of HIF-1α were measured under normoxic conditions. Cytosolic mTOR and phospho-mTOR-Ser^{2448} were used as internal controls. Treatment with the proteasome inhibitor alone resulted in a progressive increase in cellular HIF-1α levels with time (2-8 h, the cells showed an unhealthy phenotype after 8 hours) presumably due to a congestion of ubiquitinated HIF-1α intermediates. mTOR and phospho-mTOR levels were not affected by EPX (Figure 22A). We then induced HIF-1α by exposing RAEC cells for 4.5 h to hypoxia (1% O_2) in the absence or presence of rapamycin (RPM, 100 nM). As expected, HIF-1α levels of both nuclear and cytosolic compartments decreased by 60% to 70% in the RPM treated samples. Phospho-mTOR but not mTOR showed a slight decrease in cytosol. Notably, administration of increasing concentrations of EPX (0.1 – 10 μM) to the rapamycin treated cultures abrogated the suppressive effect of rapamycin on

hypoxia-induced HIF-1α nuclear accumulation. Again, there were no changes in mTOR and phospho-mTOR levels (Figure 22B).

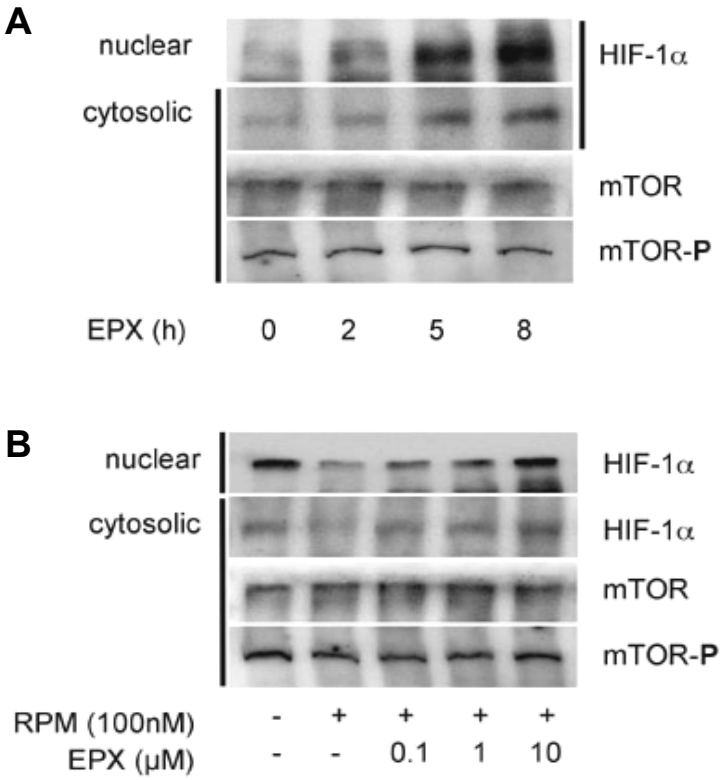
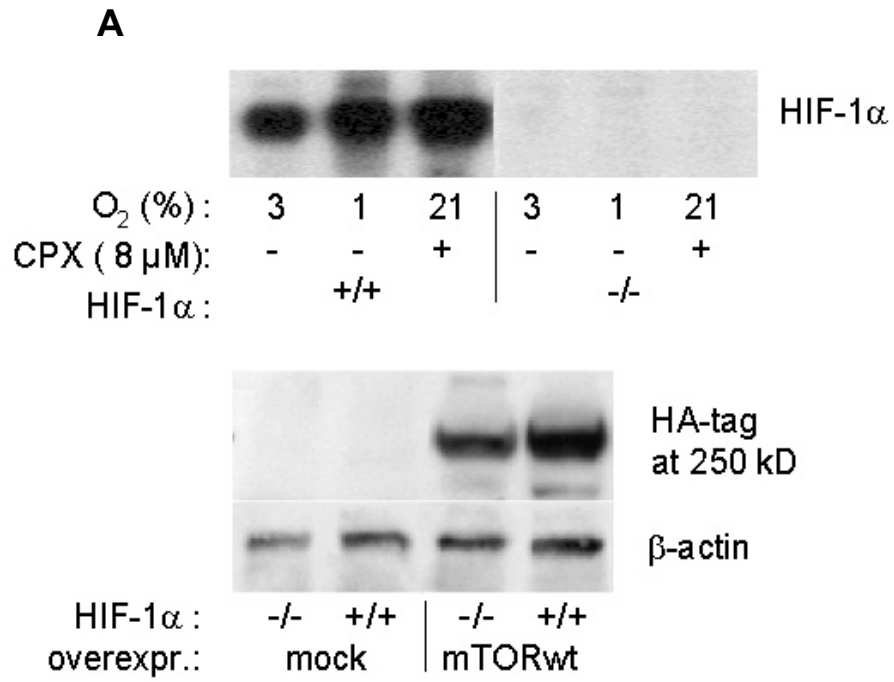


Figure 22. Proteasome inhibitor can rescue the decrease of HIF-1α by rapamycin in hypoxia. A) Proteasome inhibition increases cellular HIF-1α but not mTOR and phospho-mTOR-Ser^{2448} (mTOR-P) levels in normoxia. Epoxomicin (EPX, 1 μM) effectively blocked HIF-1α proteasomal degradation. **B)** EPX rescues both nuclear and cytosolic HIF-1α accumulation attenuated by mTOR inhibition under conditions of hypoxia in RAEC.

3.9. mTOR enhances both HIF-1α-dependent and -independent cell proliferation under hypoxia

Our data have shown that hypoxia and/or PDGF-BB stimulate both RAEC proliferation (Figure 14) and HIF-1α nuclear accumulation (Figure 19) in an mTOR-dependent manner. To assess the role of HIF-1α in hypoxia-triggered and mTOR-dependent proliferation we used mouse embryonic fibroblasts (MEFs) that lack the HIF-1α gene (HIF-1α$^{-/-}$ MEFs) and wild type MEFs (HIF-1α$^{+/+}$ MEFs) (Figure 23A upper panels). Mice defective in HIF-1α are embryonically lethal at day E10.5 due to severe cardiac and vascular malformations [325]. Quiescent MEFs were incubated under 21% and 1% O_2, respectively. After 24 h cell numbers were measured as

described. Proliferation under hypoxia was significantly more enhanced in HIF-1$\alpha^{+/+}$ compared to HIF-1$\alpha^{-/-}$ cells (n = 3; P < 0.001), suggesting that HIF-1α is required for hypoxia-induced proliferation (Figure 23B). We then assessed proliferation in HIF-1$\alpha^{+/+}$ and HIF-1$\alpha^{-/-}$ MEFs overexpressing mTOR wild type protein. mTOR overexpression compared to mock transfection significantly increased the capacity of proliferation of both cell types to respond to hypoxia (p < 0.001, n = 3) (Figure 23B). In HIF-1$\alpha^{+/+}$ cells, mTOR overexpression increased proliferation 3.0 fold under hypoxia, and to a similar degree also under normoxia (2.8 fold) compared to the respective mock values under hypoxia or normoxia (Figure 23B). Surprisingly, in HIF-1$\alpha^{-/-}$ cells, mTOR overexpression increased proliferation 4 fold compared to mock-transfected HIF-1$\alpha^{-/-}$ cells under hypoxia with no effect under normoxia (Figure 23B). These findings indicate that HIF-1α is less required for the response to hypoxia. However, mTOR is prerequisite for cell proliferation, and that mTOR overexpression can potentiate proliferation of HIF-1α-deficient cells.



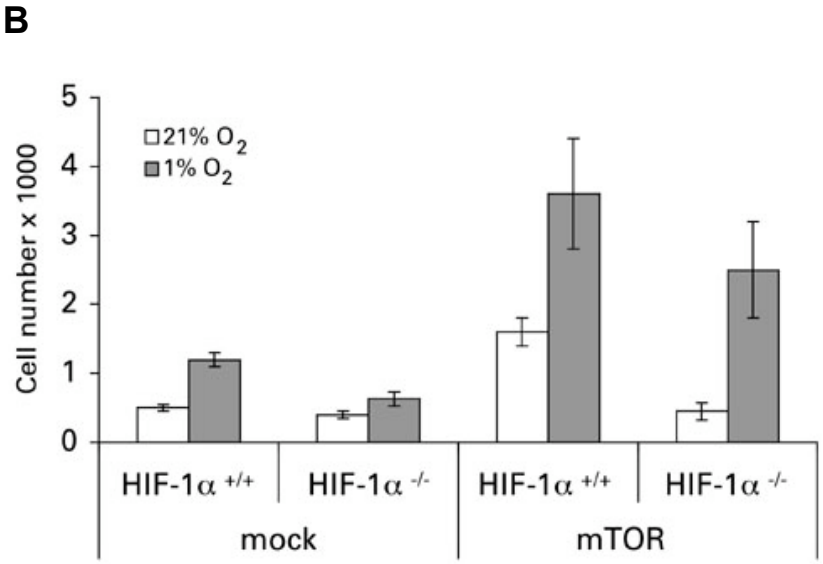


Figure 23. mTOR wild type overexpression potentiates proliferation of HIF-1$\alpha^{+/+}$ and HIF-1$\alpha^{-/-}$ MEFs under hypoxia. A) HIF-1$\alpha^{+/+}$ and HIF-1$\alpha^{-/-}$ MEFs were stimulated with CPX and incubated in 1, 3 or 21% O_2 to test for HIF-1α protein expression (upper panel). These cells were transfected with pBABE retroviruses harboring no insert (mock) or HA-tagged mTOR wild type (mTORwt). Immunoblotting shows expressed HA-tag at mTOR protein size of about 250 kDa. **B)** Serum-deprived genetically modified and mTOR-non- or –overexpressing MEFs were incubated under conditions of 21% or 1% O_2 for 24 h and cell numbers were determined. Y-axis represents the mean of cell number, compiled from three individual experiments with octuplicate samples. Data are given as mean ± SEM, n = 3.

4. DISCUSSION

Hypoxia is a pathophysiological constituent of the major diseases causing mortality in Western societies: heart attack, stroke, and cancer. The ability to sense and respond to changes in oxygen availability is thus critical for many developmental, physiological and pathophysiological processes including angiogenesis, cerebral and myocardial ischemia and tumorigenesis. Many molecular mechanisms of hypoxia sensing are conserved from unicellular prokaryotes to humans. In order to adapt to low oxygen it is first necessary to be able to detect hypoxia, then to initiate the appropriate defense mechanisms. In mammalian cells, exposure to hypoxia triggers an evolutionarily conserved hypoxic response pathway centered on the regulated expression of the hypoxia-inducible transcription factor (HIF). Accordingly, rat and human HIF-1α mRNA sequences alignments are of 100% identical. HIF promotes transcription of genes that mediate cellular adaptation to hypoxia through their roles in glycolytic metabolism, cellular proliferation and survival, and oxygen delivery.

Angiogenesis is one of the major responses regulated by HIF. Proliferation of the endothelial cells lining the pre-existing vessels is an important stage for the angiogenic process [6,7]. The study therefore was designed to use primary endothelial cells so as to be close to biological relevance. According to the prior works in the lab, rat aortic endothelial cells (RAECs) derived from *in vitro* angiogenesis assays were selected for the experiments [140,326].

mTOR regulates both cell growth and proliferation in many cell types, and is associated with *in vitro* and *in vivo* angiogenesis [140,298-300,303]. However, related data from primary endothelial cell studies are still rare, and the mechanisms about how mTOR modulates hypoxic cell proliferation are unclear. We therefore conducted experiments in order to improve the understanding of the role that mTOR plays in hypoxia-stimulated endothelial cell proliferation and angiogenesis and to uncover the regulating mechanisms.

This thesis demonstrates that mTOR signaling is an important component of the cellular response to hypoxia. It describes, that both activating and inhibitory modulations of mTOR occur in response to hypoxia simultaneously in primary endothelial cells. At the same time we observed an increase of mTOR-dependent

modulations that facilitate the formation of new microvessels in response to hypoxia, including the stabilization of the conserved transcription factor HIF-1α.

4.1. mTOR is involved in regulating hypoxia-triggered endothelial sprout-formation and cell proliferation

Endothelial spheroid sprouting provides an accurate method for *in vitro* observation of endothelial cell angiogenesis. In accordance with earlier findings in another *in vitro* angiogenesis assay in the lab [140], hypoxia markedly enhances endothelial spheroid sprouting. This implies a direct and tight link between the hypoxia-input and the angiogenisis-like-output. This link is possibly due to a metabolically adaptive switch from aerobic to anaerobic conditions, and the change provides for example glycolysis-related factors which in turn stimulate angiogenesis to occur [30]. In addition, hypoxia-induced angiogenic molecules produced by the autocrine and paracrine mechanisms may concomitantly contribute to the angiogenic effect [30].

Augmented spheroid sprouting under hypoxia is associated with an increased number and elongated shape of endothelial cells spreading outwards from the spheroids, suggesting an improved proliferation rate of the primary endothelial cells. Moreover, the sprouting can be reduced by rapamycin with a far higher effect under hypoxic than normoxic conditions. But rapamycin in both cases is not able to fully inhibit the sprout-formation. In line, the RAEC proliferation assay confirmed that hypoxia potentiates cell proliferation. Interestingly, hypoxia and PDGF-BB are capable of synergistically inducing RAEC proliferation to an even higher extent than those induced by the respective stimulus. Furthermore, all these increases can be brought down markedly but not fully by rapamycin. The inhibitory effect of rapamycin on angiogenesis is also observed *in vivo* where tumor growth is abrogated, and the abrogation was suggested to be due to an impaired VEGF production [303]. These observations implicate prominent involvement of mTOR in the regulation of endothelial cell angiogenic and proliferative effects during hypoxia. These effects of mTOR might be mediated through its upstream TSC1/2 or/and Rheb [261]. It is also possible that hypoxia or/and PDGF-BB triggers other pathways/factors such as PI3K or Akt overlapping with mTOR signaling for the common readouts [1,268,279].

4.2. mTOR and downstream targets S6K1 and 4E-BP1 are differentially phosphorylated under hypoxia

We have observed a direct activating effect of hypoxia on mTOR: Decreasing oxygen saturation lead to a sustained increase in mTOR-Ser^{2448} phosphorylation with a maximum at the lowest oxygen saturation examined. The PKB consensus phosphorylation site Ser^{2448} of mTOR has been proposed to increase mTOR kinase activity [279]. At the same time, we observed hypo-phosphorylation of mTOR downstream targets and translational regulators S6K1 and 4E-BP1. Another independent study in HEK293 cells showed that hypoxia led to mTOR-dependent hypo-phosphorylation of p70/p85 S6 kinase and 4E-BP1 but no change in mTOR-Ser^{2448} phosphorylation [304]. A recent report in primary pulmonary artery adventitial fibroblasts shows that hypoxia significantly increases mTOR-Ser^{2448} phosphorylation [327]. In this study and our own experiments, cells were exposed to hypoxia under serum-deprived conditions whereas experiments on HEK293 cells were carried out in the presence of insulin [304]. Insulin induces phosphorylation on mTOR Ser^{2448} via Akt [263,279], and might therefore have masked the effect of hypoxia on mTOR-Ser^{2448}.

Although it is still controversial whether mTOR negative regulator TSC2 is indispensable for Akt activation of mTOR [259,283], the TSC1/TSC2 complex seems to be required for hypoxic inhibition of mTOR downstream targets S6K1 and 4E-BP1 [292]. Results from others and our own data show that hypoxia decreases phosphorylation of p70 S6K and 4E-BP1 reflecting§ downregulated mTOR activity towards the two downstream targets [304]. The mechanisms about how hypoxia could modulate mTOR signaling remain unclear. Recent studies using Tsc2 knockout and knockdown cell types demonstrate that Tsc2 is required for downregulation of mTOR by hypoxia and a de novo hypoxia-inducible gene REDD1 (RTP801) [292]. In addition, Redd1 knockout MEF cells failed to decrease S6K phosphorylation under conditions of hypoxia indicating an indispensable role of REDD1 in hypoxic regulation of mTOR [292]. However, though REDD1 appears as a upstream regulator of mTOR, its mRNA is significantly downregulated by inhibitors of PI3K and mTOR, LY294002 and Rapamycin, respectively in human prostate cancer cell lines [328]. In accordance with this, hypoxia-dependent REDD1 mRNA induction is controlled by HIF-1α since the HIF-1α knockout mouse ES cells failed to express REDD1 [329]. These latter data complicate the mTOR transduction network with potential feedback loops for

adaptive signaling. In addition, the existence of two mTOR complexes (mTORC1 and 2) with mTORC2 insensitive to rapamycin, amino acids or serum depletion and physiologically irrelevant to S6K1 and 4E-BP1 phosphorylation may explain the ambivalent activation and inactivation of mTOR by hypoxia [234].

4.3. mTOR and phospho-mTOR accumulate in endothelial cell nuclei in response to hypoxia

We show for the first time that mTOR and phospho-mTOR rapidly (within 2 h) accumulate in the nucleus of RAEC in response to hypoxia under serum-free conditions. Previous reports addressed that mTOR nuclear translocation was promoted by serum [330]. It has been suggested that mTOR cytoplasmic-nuclear shuttling is required for its ability to activate cytoplasmic effectors (S6K1 and 4E-BP1) as well as a nuclear effector (S6K2) [330,331]. It is still unclear what the exact function of mTOR in the nucleus is. It is worth noting that HIF-1α accumulates in the nucleus at a similar time range upon hypoxic exposure. This coincidence may not be by chance only. Cytoplasmic-nuclear shuttling may imply a coordination of multiple functions that mTOR supports in various cellular compartments [330], and may contribute to HIF-1α function. These results support the notion that the activities of mTOR, S6K1, and 4E-BP1 are changing over alterations of cellular oxygen tension, and therefore may participate in the hypoxia-sensing process.

4.4. Hypoxia and PDGF-BB synergistically induce HIF-1α nuclear accumulation

Nuclear residence is a prerequisite for HIF-1α to transactivate certain angiogenesis-associated genes. It has been shown in transformed cells that HIF-1α accumulates in nuclei and its DNA-binding activity appears within a very short time upon hypoxic exposure [101]. These characteristics of HIF-1α make it a good candidate for diagnostic as well as prognostic purposes in ischemic heart diseases and solid tumors [42,73].

The current and other studies could show that HIF-1α is detectable in a very little amount even under conditions of normoxia (Figure 17, 18). This is probably because of the existence of pericellularly hypoxic microenvironment [332]. Our immunoblotting

and immunocytochemistry study illustrated that HIF-1α protein was already highly stabilized and accumulated in RAECs' nuclei after 4.5h exposure to hypoxia or/and PDGF-BB stimulation, implicating a quick response of the RAECs to hypoxic conditions and mitogens (Figure 17, 18). Furthermore, when hypoxia and PDGF-BB acted as co-inducers, the amount of nuclear HIF-1α was not purely additive from the value induced by each factor separately, indicating a synergistic effect of the factors on HIF-1α accumulation (Figure 18). Growth factors including PDGF and its receptors have been proven to be actively involved in angiogenesis [218,222], and PDGF synthesis could be triggered by hypoxia [213]. Thus, additional secretion of PDGF may account for the synergistic effect on HIF-1α induction under hypoxia. Similarly, that hypoxia and PDGF-BB acted in concert to upregulate the expression of HIF-1α transcriptional target gene VEGF in other cell lines [333,334]. The mechanisms hidden behind these phenomena need to be investigated further.

4.5. mTOR is required for hypoxia- and/or PDGF-BB-induced HIF-1α nuclear accumulation

As shown and discussed before, mTOR is involved in hypoxia-sensing, regulation of hypoxia- as well as PDGF-BB-induced endothelial sprout-formation and cell proliferation. On the other hand, both hypoxia and PDGF-BB induce HIF-1α nuclear accumulation that ultimately leads to target gene transactivation. It is therefore reasonable to infer a link between mTOR and HIF-1α nuclear accumulation.

Since HIF-1α is in a distal position for transactivating target genes, it is most likely that mTOR imposes a regulatory function on HIF-1α. However, an opposite effect, i.e., HIF-1α influences mTOR activity, cannot be excluded. The experimental results from this study clearly show that mTOR inhibition by rapamycin strongly reduces hypoxia- and PDGF-BB-induced HIF-1α nuclear accumulation indicating an upstream requirement of mTOR in HIF-1α regulation. This is supported by evidence showing that inhibition of mTOR or the upstream mediator PI3K decreased both HIF-1α and VEGF expression induced by growth factors [141]. Moreover, rapamycin attenuated wild-type mTOR transfection-enhanced HIF-1α induction by hypoxia or $CoCl_2$ in prostate cancer cell lines while the rapamycin-resistant mutant mTOR transfected cells have unaffected HIF-1α levels as well as transactivities [143]. Thus,

mTOR seems to play an indispensable role in protecting hypoxia- and/or PDGF-BB-induced HIF-1α function.

4.6. MEK1/2 but not JNK or p38 regulates PDGF-BB-induced HIF-1α nuclear accumulation only under normoxia

MAPKs have been implicated in cell signaling triggered by mitogens, growth factors/cytokines or hypoxia. However, the sorts of MAPKs involved in the process induced by hypoxia remain uncertain [307,315-317]. Therefore, the involvement of MAPK pathways in RAECs stimulated by hypoxia and/or growth factor has been investigated in the study.

As shown in the results, inhibition of MEK1/2 by PD98059 attenuated PDGF-BB induced HIF-1α nuclear accumulation only under normoxia, suggesting that MEK1/2 activity is not necessary for HIF-1α nuclear accumulation under hypoxia. This result is in line with another observation showing that inhibition of ERK1/2 signaling impairs HIF-1α transactivation activity with no impact on its stability and DNA binding activity [318,319]. An explanation for the unaffected HIF-1α levels but the increased transactivity upon ERK1/2 inhibition might be that ERK1/2 interacts with HIF-1α coactivators or the factors that regulate them. On the other hand, similar to rapamycin, PD98059 was not able to completely inhibit HIF-1α nuclear accumulation. Thus, molecules in addition to MEK1/2 may contribute to HIF-1α nuclear accumulation. It is possible that mTOR and ERK1/2 pathways or even others interfere, overlap and cooperate during growth factor induction of HIF-1α upregulation and nuclear accumulation. In the study, both JNK and p38 inhibition showed no influence on enhanced HIF-1α nuclear levels induced by hypoxia or/and PDGF-BB. Thus, JNK and p38 signaling do not contribute to HIF-1α accumulation in rat aortic endothelial cells. Our observation fails to support the results from other studies showing a JNK- and p38-dependent HIF-1α regulation under hypoxia [315,320]. It is possible that cell type and the type of genetic transformation, and the intracellular signaling milieu may direct hypoxic signals differentially to signal relays.

4.7. HIF-1α cellular accumulation is regulated neither at transcriptional nor at translational level

HIF-1α has been shown to be regulated at transcriptional, translational and post-translational levels [105,139,145]. So far, the regulation of HIF-1α in endothelial cells has not been well characterized. In this study, we examined the potential modulation levels for HIF-1α in RAEC.

Both R-T and Real-Time R-T PCR data demonstrate comparably stable HIF-1α mRNA levels in normoxia and hypoxia, respectively. Also, rapamycin has no effect on HIF-1α mRNA levels in growth factor-stimulated RAECs. These results suggest a non-involvement of transcriptional regulation in HIF-1α cellular accumulation. Meanwhile, the immunoblotting showed a decreased activation of the translation regulators S6K1 and 4E-BP1 under hypoxia, implicating a reduced protein synthesis in this circumstances and a non-involvement of translational modulation in HIF-1α accumulation in RAEC. Therefore, unlike angiotensin II [139], hypoxia-triggered HIF-1α stabilization and cellular accumulation, which are rapamycin sensitive, are not mediated via transcriptional and translational mechanisms.

4.8. mTOR activity protects HIF-1α from proteasomal degradation under hypoxia

HIF-1α protein is modified in multiple ways that are thought to allow precise regulation of its levels as well as its functions [105]. We show that inhibition of proteasomal activity rescued rapamycin-induced HIF-1α destabilization. Thus, mTOR activity protects HIF-1α from proteasomal degradation. Data from another independent study showed that mTOR activity increases the steady-state level of HIF-1α in both normoxic and hypoxic prostate cancer cells. Furthermore, this study showed that the ODD domain of HIF-1α is involved in mTOR-dependent stabilization of the HIF-1α protein during cellular exposure to hypoxia-mimetic agents [143].

Hypothetically, mTOR-dependent effects on HIF-1α stabilization may include one or more post-translational modification(s) such as phosphorylation and sumoylation that

protects HIF-1α from degradation [105]. So far, there are no direct links between mTOR and HIF-1α phosphorylation. In mammalian cells, sumoylation is involved in post-translational modification of certain proteins by covalently attaching SUMO to specific lysine residues within the proteins [126]. By this way, sumoylation regulates various cellular processes like nuclear transport, signal transduction, stress response and cell cycle progression [126]. But, in contrast to ubiquitination, sumoylation does not tag proteins for degradation, but seems to enhance their stability or modulate their subcellular compartmentalization [132]. It has been shown that protein-sumoylation is significantly increased in bovine aortic endothelial cells after exposure to hypoxia for 48 h [335]. Furthermore, increased levels of SUMO-1 were shown to participate in the modulation of HIF-1α function through sumoylation in hypoxic brain and heart. There, SUMO-1 co-localizes in cell nuclei in vivo with HIF-1α in response to hypoxia [336]. Interestingly, we found that mTOR localized in endothelial nuclei after exposure to hypoxia. Therefore, sumoylation might be a good candidate mechanism to protect HIF-1α from proteasomal degradation. Possibly, mTOR has a function in sumoylation and nuclear localization of HIF-1α under hypoxia.

4.9. mTOR enhances both HIF-1α-dependent and -independent cell proliferation under hypoxia

As described earlier, mTOR is required for hypoxia-triggered endothelial cell proliferation. Moreover, mTOR, acting upstream of HIF-1α, is involved in hypoxia sensing. Still, it remains unclear how mTOR could interact with HIF-1α to regulate cell proliferation. To study whether inhibition of HIF-1α function impairs mTOR-dependent cell proliferation under hypoxia, we used mouse embryonic fibroblasts (MEFs) derived from mice that are deficient in HIF-1α. The HIF-1α-deficient mice are embryonically lethal at day E10.5 due to severe cardiac and vascular malformations [99].

The results demonstrate that HIF-1α is not the unique effector of mTOR in the response to hypoxia. HIF-1α$^{-/-}$ MEFs proliferate less than HIF-1α$^{+/+}$ cells under hypoxia. Therefore, HIF-1α certainly confers a growth advantage to cells under low oxygen saturation. However, HIF-1α$^{-/-}$ MEFs overexpressing mTOR acquire the capacity to proliferate under hypoxia but not under normoxia. We conclude that

hypoxia-triggered signaling via mTOR involves downstream interactions with both HIF-1α-dependent and HIF-1α-independent mechanisms.

HIF-1α-independent hypoxia effectors still need to be defined and may involve other HIFs (HIF-2, HIF-3). With HIF-3α expression the most restricted, HIF-2α is more widely expressed in vascular endothelial cells during embryo development and in kidney fibroblasts, liver hepatocytes, epithelial cells of intestinal lumen, pancreatic interstitial cells, heart myocytes and interstitial cells, lung type II pneumocytes, tumor vascular cells as well as in a vast variety of tumor cells [76,337,338]. Interestingly, it has been shown recently that HIF-2α knockout mice are able to survive postnatally but eventually die with defects in vascular system, lung maturation, and catecholamine production. Moreover, bearing abnormalities, the genetically engineered HIF-2α knockout mice is compatible with life [67,339]. Though overlapping transcriptional targets exist, HIF-1α and HIF-2α exert differential regulatory functions when low oxygen tension appears [76]. For example, HIF-1α but not HIF-2α is critical for glycol sis during O_2 deprivation. In renal carcinoma cells exclusively expressing HIF-2α, a number of hypoxia-inducible genes can be induced without transactivation from HIF-1α [76].

Alternatively, mTOR may transduce hypoxic signals also via modulating cell cycle regulators. In NIH 3T3 cells, degradation of newly synthesized cyclin D1 protein was accelerated by rapamycin, a process prevented by inclusion of a proteasome inhibitor [340]. In transformed mouse 3T3 fibroblasts (BP-A31) rapamycin reduced the level of total p21 (WAF1/CIP1) as well as that of p21 (WAF1/CIP1) associated with the cyclin D1/cdk4 complexes [341]. Besides its inhibitory activity toward cdk, p21 (WAF1/CIP1) has been recently found to participate in the formation, stabilization and nuclear translocation of cyclin D1/cdk4 complexes [341]. Thus, cyclin D1 and p21 appear to be regulated similarly to HIF-1α with respect to protein stability and nuclear translocation. However, it is not known, whether cyclin D1 or p21 protein levels increase under conditions of hypoxia, and if so, whether this increase might be rapamycin (mTOR) sensitive. Possibly, these effectors are also regulated by mTOR-mediated, protective protein modifications.

4.10. Limitations

Most of the experiments were done in RAECs. It is difficult to predict whether the effects of the studied molecules, e.g. mTOR and HIF-1α, are conserved in other species, e.g. humans. It is unclear whether our data can be extrapolated to other cell types and tissues. Besides, the readouts of the *in vitro* studies performed currently may not reflect the *in vivo* situation. Therefore, caution should be taken when the data are utilized to interpret *in vivo* or clinical phenomena.

A potential pitfall in this study is that the experiments conducted in the MEFs do not reflect the situation in RAECs and angiogenesis *in vitro*, where mTOR and HIF-1α may interplay differently for cell proliferation. In addition, phosphorylation of mTOR may not be really in accordance with its signaling activity, and its downstream targets S6K1 and 4E-BP1 could be mediated by upstream molecules other than mTOR. It is likely that other pathways triggered by hypoxia interfere with the mTOR pathway to induce the common readouts of HIF-1α.

The inefficiency of transfecting RAECs confined the process and depth of mTOR- and HIF-1α-associated studies. This is in part due to the extensive size of the mTOR gene. However, the findings of the upstream regulators of mTOR, TSC1/2, Rheb, and REDD1, and the studies of the differential mTOR complexes as well as the modifiers for HIF-1α in other cell types will certainly facilitate to elucidate the regulatory relationships between mTOR and HIF-1α.

5. CONCLUSIONS, KEY POINTS

In conclusion, we have observed an increase of mTOR-dependent modulation that facilitates endothelial cell angiogenic effect and proliferation in response to hypoxia. Hypoxia imposes both activating and inhibitory modulations differentially but concurrently on mTOR in rat aortic endothelial cells. On one hand, mTOR is activated by phosphorylation and translocated into the nucleus. On the other hand, translational regulators S6K and 4E-BP1 lying at least in part downstream of mTOR are hypo-phosphorylated. Furthermore, mTOR augments nuclear HIF-1α levels by protecting the protein from proteasomal degradation under hypoxia, and thereby potentially reinsures oxygen supply by induction of neovascularization. mTOR may also induce hypoxia-adaptive responses by effectors other than HIF-1α. Finally, mTOR is fully required for cell proliferation, which can be reinforced by HIF-1α. Thus, as a dominant and important transducer of the hypoxic response, mTOR acts via downstream interaction with both HIF-1α-dependent and -independent mechanisms.

The main findings in this study are summarized as following key points:

1. Hypoxia is an active and positive trigger for endothelial sprout-formation and cell proliferation, and mTOR is required for the process.
2. Hypoxia causes mTOR phosphorylation, phospho-mTOR-Ser^{2448}/mTOR nuclear accumulation, and hypo-phosphorylation of S6K1 and 4E-BP1.
3. Hypoxia and PDGF-BB could interact to enhance endothelial cell proliferation and HIF-1α nuclear protein levels.
4. mTOR is strongly but not fully required for hypoxia- and PDGF-induced HIF-1α nuclear accumulation.
5. ERK1/2 participates specifically in the regulation of growth factor-stimulated HIF-1α nuclear accumulation.
6. mTOR modulates HIF-1α nuclear levels under hypoxia neither at transcriptional nor at translational level.
7. HIF-1α is not critical for cell proliferation but can reinforce induction of pro-proliferation via mTOR.

6. OUTLOOK

Hypoxia induces activation of a set of distinct signaling pathways for cells to survive in an altered environment. Studies highlighting the role of TSC1/TSC2 complex in hypoxic regulation of mTOR, S6K1 and 4E-BP1 are emerging [292]. Thus, it will be important to determine what role the TSC1/TSC2 complex plays in hypoxia-mediated mTOR-phosphorylation on Ser^{2448} or even other phosphorylation sites in mTOR, -nuclear translocation, -stabilization of HIF-1α and cell proliferation.

mTOR catalytic or kinase function is influenced by a growing list of directly interacting binding proteins such as mKOG1 (raptor), mAVO3 (rictor) and mLST8 (GβL) that determine two differently composed mTOR complexes (mTORC1 and mTORC2) [233-235,237]. The latter complexes may be differentially activated by hypoxia generating differential responses to hypoxia. Therefore, insights into the mTOR-interacting proteins within the mTORCs in relation to HIF-1α under hypoxia will shed light on activation and inhibition of mTOR by hypoxia. Also, one of the two mTORCs may mediate the regulation of HIF-1α.

Closely related to HIF-1α, HIF-2α and HIF-3α, may play important roles in cells under hypoxia. Thus, it is interesting to know how the latter two HIF alpha isoforms respond to hypoxia and how these factors co-operate to produce the coordinated responses for cell to adapt to the low oxygen environment. At the same time, it is possible that these hypoxia-inducible factors are also regulated by mTOR-mediated, protective protein modifications.

A promising utility of the *in vitro* assay of endothelial cell (EC) spheroid sprouting is to investigate the angiogenic potentials of transfected ECs with different target genes. The assay per se may be useful for high throughput screening for agonists and antagonists engaged in angiogenesis.

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8. ABBREVIATIONS

ACE	angiotensin-converting enzyme
Akt	v-akt murine thymoma viral oncogene homolog
AMPK	5'AMP-activated protein kinase
Ang	Angiopoietin
Ang II	angiotensin II
ARD1	arrest defective-1 protein
ARNT	aryl hydrocarbon receptor nuclear translocator
AT receptor	angiotensin receptor
(m)AVO	(mammalian) adheres voraciously to TOR2
BM	basement membrane
BSA	bovine serum albumin
CBP	CREB binding protein
cdk	cyclin-dependent kinase
CPX	ciclopirox olamine
CREB	cAMP responsive element binding protein
DFX	desferrioxamine
DMEM	Dulbecco's Modified Eagle's Medium
4E-BP	eIF4E-binding protein
EC	endothelial cell
ECM	extracellular matrix
EDNO	endothelial nitric oxide
EGF	epidermal growth factor
eIF	Eukaryotic translation initiation factor
EPO	erythropoietin
EPX	epoxomicin
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, HER2/neu
ERK	extracellular signal-regulated kinase
FAT	FRAP, AMT, TRRAP domain
FATC	FAT carboxy-terminal domain
FCS	fetal calf serum
FFA	free fatty acid
FGF	fibroblast growth factor
FIH-1	factor inhibiting HIF-1

FKBP	FK506 binding protein
Flk-1	Fetal liver kinase-1, VEGFR2, KDR
Flt-1	FMS-like tyrosine kinase-1, VEGFR1
FRAP	FKBP12-rapamycin associated protein
FRB	FKBP12-rapamycin binding
GAP	GTPase-activating protein
GβL	G protein β-subunit-like protein
HDAC	histone deacetylase
HGF	Hepatocyte growth factor
HIF	hypoxia-inducible factor
HIF-1α	hypoxia-inducible factor-1 alpha subunit
HLH	helix-loop-helix
HOG	high osmolarity glycerol
HRE	hypoxia response element
HSP90	heat shock protein 90
ID	inhibitory domain
IL	Interleukin
IPAS	inhibitory PAS domain protein
IRS	insulin receptor substrates
Jab	Jun activation domain-binding protein
JNK	c-Jun N-terminal kinase
kDa/kD	kiloDalton
KDR	kinase insert domain receptor, VEGFR2, Flk-1
Kip1	cyclin-dependent kinase inhibitor 1
(m)KOG	(mammalian) kontroller of growth
(m)LST	(mammalian) lethal with sec thirteen
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MEK	MAPK kinase (MAP2K)
MEKK	MAPK kinase kinase (MAP3K)
NO	nitric oxide
OA	osteoarthritis
ODD	oxygen-dependent degradation domain
P38	p38 MAP kinase
PA	phosphatidic acid

PAC	PAS-associated C-terminal domain
PAS	period(Per)-Aryl hydrocarbon receptor nuclear translocator (Arnt)-single-minded protein (Sim)
PC	peri-endothelial cell
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PECAM-1	Platelet-Endothelial Cell Adhesion Molecule-1, CD31
PHAS	phosohorylated heat and acid-stable protein regulated by insulin
PHD	prolyl hydroxylase domain protein
PI3K	phosphatidylinositol 3-kinase
PIKK	phosphoinositol kinase-related protein kinase
PKB	protein kinase B, Akt
PLD	phospholipase D
PML	promyelocytic leukemia protein
PP2A	type 2A phosphatase
PTEN	phosphatase and tensin homolog
pVHL	von Hippel-Lindau protein
RA	rheumatoid arthritis
RAAS	renin-angiotensin-aldosterone system
RAEC	rat aortic endothelial cell
RAFT	rapamycin and FKBP12 target
RAPT	rapamycin target
raptor	regulatory associated protein of TOR, mKOG1
REDD	regulated in development and DNA damage responses
Ref	redox factor
Rheb	Ras homolog enriched in brain
rictor	rapamycin-insensitive companion of mTOR
RNAi	RNA interference
RPM	rapamycin
RTK	receptor tyrosine kinase
S6K	ribosomal protein S6 kinase
SAPK	stress-activated protein kinase
SEP	Sirolimus effector protein
SRC	steroid receptor co-activator
STAT	signal transducer and activator of transcription

SUMO	small ubiquitin-like modifier
TAD-N(C)	N (C)-terminal transactivation domain
TGF	transforming growth factor
Tie	tyrosine kinase with immunoglobulin and EGF homology domains
TNF	tumor necrosis factor
TOR	target of rapamycin
TOS	TOR signaling motifs
TSC	tuberous sclerosis complex
(m)TOR	(mammalian) target of rapamycin
(m)TORC	(mammalian) target of rapamycin complex
VEGF	vascular endothelial cell growth factor
VEGFR	vascular endothelial cell growth factor receptor
VSMC	vascular smooth muscle cell