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## MOLECULAR ASPECTS OF ENDOTHELIAL CELL FUNCTION AND HYPOXIA-DEPENDENT TUMOR ANGIOGENESIS

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# MOLECULAR ASPECTS OF ENDOTHELIAL CELL FUNCTION AND HYPOXIA-DEPENDENT TUMOR ANGIOGENESIS

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*To my family*  
*Dla mojej kochanej rodziny*

*“Nothing in life is to be feared,  
it is only to be understood.”*

*Maria Skłodowska-Curie*



# Abstract

Hypoxia, or deficient oxygen levels, is an inherent feature of rapidly expanding tumor tissue. In order to survive in the hostile, hypoxic environment and to restore the supply of oxygen and nutrients tumor cells activate a variety of adaptive mechanisms. Among them, hypoxia-induced angiogenesis, *i.e.* the formation of tumor blood vessels from pre-existing vasculature, has proved to be of critical importance, and has led to the development of anti-angiogenic therapy in the treatment of cancer patients. Anti-angiogenic drugs targeting the vascular endothelial growth factor (VEGF) pathway may prolong progression-free survival and overall survival in some patients. However, the results are more modest than predicted from pre-clinical studies, reflecting the complexity of tumor angiogenesis, and underlining the requirement of further studies aiming at the identification of the inherent limitations of this treatment as well as new targets of anti-angiogenic therapy.

This thesis aims at investigating molecular aspects of hypoxia-dependent regulation of endothelial cell functions, and to identify new, potential targets of anti-angiogenic therapy. In the first part of the thesis, it is demonstrated that polyamines are potent modulators of endothelial cell biology. Accordingly, polyamines regulate both hypoxia-induced apoptosis in endothelial cells, through PI3K/AKT and MCL-1 dependent pathways, and sprouting angiogenesis via endothelial cell migration, partially explaining the anti-angiogenic effects of polyamine depletion in animal tumor models. During the initial steps of hypoxia-driven angiogenesis, endothelial cells respond to hypoxia by induction of a variety of adaptive mechanisms. Results presented in this thesis show that microRNAs seem to be involved in the endothelial cell adaptation to low oxygen levels, since hypoxia profoundly regulates the expression of a subset of miRNAs, and one of them, miRNA-424\*, has a functional role in hypoxia-dependent pro-angiogenic activity of endothelial cells. Finally, exosomes derived from aggressive brain tumor cells (glioblastoma) seem to be a novel, intercellular communication route eliciting a hypoxic, pro-angiogenic response in endothelial cells; a defined, hypoxic molecular profile of circulating, plasma-derived exosomes may serve as a non-invasive biomarker to assess the oxygenation status and aggressiveness of glioblastoma tumors.

In conclusion, data presented in this thesis identify new players of hypoxia-mediated tumor angiogenesis and implicates them as potential targets of anti-angiogenic therapeutic intervention as well as biomarkers of cancer.



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# List of papers

This thesis is based on the following original papers and manuscripts, referred to in the text by their Roman numerals:

- I. **Kucharzewska P**, Welch JE, Svensson KJ, Belting M. *The polyamines regulate endothelial cell survival during hypoxic stress through PI3K/AKT and MCL-1*. Biochem Biophys Res Commun. 2009;380(2):413-418.
  
- II. **Kucharzewska P**, Welch JE, Svensson KJ, Belting M. *Ornithine decarboxylase and extracellular polyamines regulate microvascular sprouting and actin cytoskeleton dynamics in endothelial cells*. Exp Cell Res. 2010;316(16):2683-2691.
  
- III. **Kucharzewska P**, Christianson HC, Rignér M, Belting M. *Hypoxia upregulates microRNA-424\* to modulate the pro-angiogenic response of endothelial cells*. Manuscript.
  
- IV. **Kucharzewska P**, Christianson HC, Welch JE, Svensson KJ, Fredlund E, Mörgelin M, Rignér M, Bengzon J, Belting M. *Exosome vesicles constitute a novel mediator of hypoxic signaling in glioblastoma*. Manuscript.

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## Papers not included in the thesis:

- Svensson KJ, Welch JE, **Kucharzewska P**, Bengtson P, Bjurberg M, Pålman S, Ten Dam GB, Persson L, Belting M. *Hypoxia-mediated induction of the polyamine system provides opportunities for tumor growth inhibition by combined targeting of vascular endothelial growth factor and ornithine decarboxylase*. *Cancer Res*. 2008;68(22):9291-9301.
- **Kucharzewska P**, Welch JE, Birgersson J, Belting M. *Establishment of heparan sulphate deficient primary endothelial cells from EXT-1(flox/flox) mouse lungs and sprouting aortas*. *In Vitro Cell Dev Biol Anim*. 2010;46(7):577-584.
- Wittrup A, Zhang SH, Svensson KJ, **Kucharzewska P**, Johansson MC, Mörgelin M, Belting M. *Magnetic nanoparticle-based isolation of endocytic vesicles reveals a role of the heat shock protein GRP75 in macromolecular delivery*. *Proc Natl Acad Sci U S A*. 2010;107(30):13342-13347.
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- Larsson A, Johansson ME, Wangefjord S, Gaber A, Nodin B, **Kucharzewska P**, Welinder C, Belting M, Eberhard J, Johnsson A, Uhlén M, Jirström K. *Overexpression of podocalyxin-like protein is an independent factor of poor prognosis in colorectal cancer*. *Br J Cancer*. 2011;105(5):666-672.
- Svensson KJ, Christianson HC, **Kucharzewska P**, Fagerström V, Lundstedt L, Borgquist S, Jirström K, Belting M. *Chondroitin sulfate expression predicts poor outcome in breast cancer*. *Int J Oncol*. 2011;39(6):1421-1428.

# Abbreviations

ANGPT	Angiopoietin
BMDCs	Bone marrow-derived cells
CAIX	Carbonic anhydrase 9
CDC42	Cell division control protein 42 homolog
DFMO	2-difluoromethylornithine
DLL4	Delta like ligand 4
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMVs	Extracellular membrane vesicles
EPCs	Endothelial progenitor cells
ERK1/2	Extracellular-signal-regulated kinase 1/2
FGF	Fibroblast growth factor
GBM	Glioblastoma multiforme
GLUT	Glucose transporter
GSK-3	Glycogen synthase kinase 3
HIF	Hypoxia inducible factor
HSPGs	Heparan sulfate proteoglycans
ILVs	Intraluminal vesicles
LOX	Lysyl oxidase
MCL-1	Myeloid cell leukemia sequence 1
miRISC	miRNA-induced silencing complex
miRNAs	MicroRNAs
MMPs	Matrix metalloproteinases
mTOR	Mammalian target of rapamycin
MVBs	Multivesicular bodies
ODC	Ornithine decarboxylase
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet derived growth factor
PDGFR- $\beta$	Platelet derived growth factor receptor $\beta$
PE	Phosphatidylethanolamine
PHD	Prolyl hydroxylase domain
PI3K	Phosphatidylinositol 3 kinase
PIGF	Placenta growth factor
pre-miRNAs	Precursor-miRNAs
pri-miRNAs	Primary miRNAs
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
RTKs	Receptor tyrosine kinases
SDF-1	Stromal derived factor
SMVs	Shedding microvesicles
TGF $\beta$	Transforming growth factor $\beta$
THBS1	Thrombospondin 1
TIMP	Tissue inhibitor of metalloproteinases
TKIs	Tyrosine kinase inhibitors
UTRs	Untranslated regions
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	Von Hippel–Lindau

# Background

## Hallmarks of cancer

Current state of knowledge describes cancer as a genetic disease developing through a multistep process analogous to Darwinian evolution. Inherited or somatic alterations in key regulatory genes (oncogenes and/or tumor suppressors) lead to cellular transformation of normal cells, which subsequently modulated by genetic and epigenetic changes acquire a full-blown malignant tumor cell phenotype [1].

In spite of the diversity, most if not all tumor types share some essential properties that enable tumor growth and metastatic dissemination. These common traits, proposed as ‘hallmarks of cancer’ by Douglas Hanahan and Robert Weinberg [2,3], include: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of cell death, replicative immortality, induction of angiogenesis, tissue invasion and metastasis, tumor promoting inflammation, genome instability and mutations, deregulation of cellular energetics, and avoidance of immune destruction.

Normal tissue architecture and function are maintained by highly controlled balance between growth-promoting and growth-inhibitory signals. Entry of normal cells into an active proliferative state requires external growth signals transmitted from growth factors, extracellular matrix components and cell adhesion molecules through transmembrane receptors. Conversely, excessive growth is halted by the action of anti-growth signals, inducing cell quiescence or terminal differentiation. Deregulated cancer cell proliferation results from defects in these regulatory circuits, such as endogenous amplification and/or overexpression of growth factors and/or receptors, expression of mutated receptors and components of the downstream cytoplasmic signaling circuitry, and stimulation of growth factor release from stromal cells. Gain of function mutations in the Ras oncogene in human colon carcinomas [4] and expression of constitutively active epidermal growth factor receptor (EGFRvIII) in aggressive gliomas [5] are two illustrative examples of mechanisms sustaining proliferative signaling in cancer.

Tissue homeostasis is controlled not only by cellular growth but also by cell death. Apoptosis, *i.e.* programmed cell death, is the mechanism by which unwanted or defective cells are removed from the normal tissue [6]. Cancer cells limit or circumvent apoptosis by aberration of proapoptotic and/or antiapoptotic signaling. A commonly occurring loss of proapoptotic regulator through mutation involves the p53 tumor suppressor gene. Functional inactivation of p53 protein is seen in more than 50% of human cancers, and results in loss of a key DNA damage sensor that can induce apoptosis and cell cycle arrest [7]. It is widely accepted that cancer cells require unlimited replicative potential in order to form macroscopic tumors. This is in contrast to most normal cells, which go through

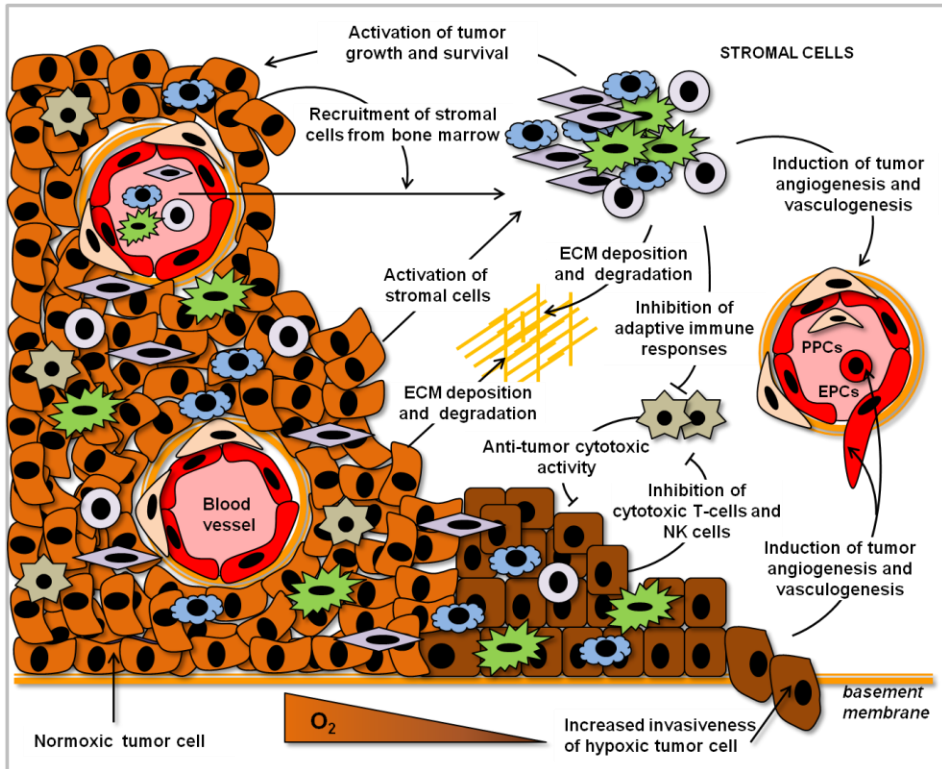
only a certain number of divisions before they reach a stage of senescence or die. It has been shown that shortening of telomeres, the protective ends of chromosomes, with every cell division is partially responsible for this phenomenon [8]. Tumor cells gain endless replicative potential by activating telomerase, *i.e.* an enzyme responsible for elongation of telomere ends [9]. Similarly to normal tissues, tumors require access to oxygen and nutrients. Hence, in order to grow beyond the size of a few millimeters, solid tumors activate an ‘angiogenic switch’ by changing the balance of angiogenic inducers and inhibitors, leading to blood vessel recruitment [10]. Further, malignant tumor cells acquire the ability to invade adjacent tissues and eventually travel to distant sites within the body where they succeed in forming metastases, the cause of most cancer related deaths. This capability enables neoplastic cells to escape the primary tumor mass and colonize new tissues where, in the beginning, nutrients and space are not limiting [11]. An increasing body of research indicates that two additional, emerging hallmarks of cancer are involved in the pathogenesis of cancer. The first involves switch of cellular energy metabolism, replacing metabolic program that operates in most normal tissues with ‘aerobic glycolysis’ (‘Warburg effect’), in order to fuel cell growth and division [12]. The second allows cancer cells to evade immunological destruction mediated by T and B lymphocytes, macrophages and natural killer (NK) cells.

Underlying all hallmarks are two ‘enabling characteristics’. Most prominent is the development of genomic instability in cancer cells, which generates genetic diversity through random mutations, including chromosomal rearrangements, and thus accelerates their genetic evolution towards malignancy [13]. A second enabling characteristic is inflammation, which foster multiple hallmark capabilities by supplying bioactive molecules, such as growth factors, extracellular matrix-modifying enzymes and reactive oxygen species, to the tumor microenvironment [14].

# Tumor microenvironment

Cancer has long been viewed as a disease of transformed cells acquiring cell-autonomous hyperproliferative, invasive and anti-apoptotic capacities. Accordingly, primary anticancer therapeutic strategies have been concentrated on targeting these malignant cells. Modest effectiveness of these drugs has led to reassessment of basic assumptions about the nature of cancer and its treatment. Extensive research over the past decades has recognized tumors as multicellular organs composed of neoplastic cells (parenchyma) surrounded by a variety of extracellular matrix components and non-neoplastic tumor cells, most notably fibroblasts, myofibroblasts, adipocytes, endothelial cells, pericytes, mesenchymal stem cells (MSCs) and immune cells, which collectively form a stroma, often termed the tumor microenvironment [3,15]. The complexity of the tumor microenvironment is depicted in Figure 1 to illustrate the concept that the microenvironment of the developing tumor is a crucial regulator of tumorigenesis, which was originally proposed by Paget in his ‘seed and soil hypothesis’ [16]. Recent advances in cancer research have provided strong support to this idea in many experimental models, *e.g.* tumor-associated stromal cells have been convincingly shown to actively promote tumor initiation and progression [17,18]. In addition, molecular profiling of the stromal compartment of human tumor specimens has shown prognostic correlation [19-21].

The most obvious evidence of a tumor supportive role for the stroma has come from studies of tumor neovascularization. Tumor angiogenesis is initiated early on tumor development through ‘angiogenic switch’ mechanisms [22] that promote tumor growth and facilitate tumor metastasis. Angiogenesis involves endothelial cell recruitment to the tumor mass in response to pro-angiogenic signals, particularly vascular endothelial growth factor (VEGF)-A, released from neoplastic cells and stromal cells, such as Tie-2 expressing monocytes (TEMs) [23], macrophages [24] and fibroblasts [25]. Stabilization of newly formed vasculature is partially mediated by angiopoietin (ANGPT)-1 secreted by tumor-associated pericytes [26-28]. Infiltrating cells of the immune system are well established constituents of tumors. Apart from tumor-antagonizing leukocytes, such as CD8 cytotoxic T lymphocytes (CTLs) and NK cells, which mediate largely ineffective antitumor response in most cancers, neoplastic lesions contain a broad spectrum of tumor-promoting immune cells, including macrophages, mast cells, neutrophils, T and B lymphocytes. These inflammatory cells have been shown to secrete pro-angiogenic growth factors, cytokines and chemokines as well as pro-angiogenic and/or proinvasive matrix-degrading enzymes, such as matrix metalloproteinase (MMP)-9, and thus to induce tumor angiogenesis, cancer cell proliferation, invasion and metastatic dissemination [29,30]. The most abundant mesenchymal cells within most solid tumors - fibroblasts and myofibroblasts - may reinforce tumor phenotypes through secretion of transforming growth factor (TGF)- $\beta$  [31], stromal derived factor (SDF)-1 [32] and lysyl oxidase (LOX) [33].



**Figure 1. Heterotypic cellular interactions in the tumor microenvironment.** Malignant tumors may be viewed as multicellular organs composed of neoplastic cells co-existing with a variety of extracellular matrix components and non-tumoral stromal cells, notably fibroblasts, myofibroblasts, adipocytes, endothelial cells, pericytes, mesenchymal stem cells (MSCs) and immune cells. Tumor cells mold this environment for their own needs via intercellular communication pathways, such as direct cell-to-cell contacts and the release of growth factors, matrix metalloproteinases (MMPs), ECM proteins and extracellular membrane vesicles. Tumor cell-mediated stromal modifications include: suppression of anti-tumoral immune responses, extracellular matrix deposition and degradation, hypoxia-mediated induction of sprouting angiogenesis and postnatal vasculogenesis, which in turn resolve hypoxia and support tumor growth, as well as activation of pre-existing or recruitment of bone-marrow derived stromal cells. Stromal cells in turn promote tumor progression by stimulation of tumor growth and survival, ECM modification-mediated tumor invasion/metastasis, induction of tumor angiogenesis and vasculogenesis as well as inhibition of anti-tumor immune responses. Hypoxia, which is an inherent feature of solid tumors, enhances and modifies intercellular communication pathways in order to support tumor progression. Abbreviations: ECM, extracellular matrix; PPCs, perivascular progenitor cells; EPCs, endothelial progenitor cells.

Moreover, these cells are responsible for the release of connective tissue components in the ECM, including collagens and structural proteoglycans, as well as various classes of proteolytic enzymes [34]. Adipocytes have also been recognized as important components of the tumor microenvironment. Adipose tissue is an endocrine and paracrine organ that secretes several cytokines, including

the adipokines leptin, adiponectin, resistin and visfatin as well as MMPs that promote inflammation and angiogenesis [35]. This profound effect of adipocytes on tumor microenvironment may explain the relationship between cancer and obesity, and inclusion of obesity as an independent risk factor for several types of cancer [36].

Several lines of evidence indicate that stromal cells may be supplied to growing tumors by proliferation of preexisting stromal cells, by differentiation of local stem/progenitor cells recruited from adjacent normal tissues or via recruitment of bone marrow-derived stem/progenitor cells. Among stromal cells of bone marrow origin are endothelial cells and pericytes, however the extent to which tumor vasculature is derived from endothelial progenitor cells is highly variable and debated [37]. Most often, tumor-associated vessels are formed by endothelial cells attracted from adjacent normal tissue.

The successful evolution of invasive cancer requires an active collaboration of neoplastic cells and stromal cells via heterotypic cellular interactions. A number of soluble cytokines and growth factors as well as microvesicles/exosomes carrying proteins and mRNAs/microRNAs are attractive candidates for the signaling molecules mediating this reciprocal communication. Interestingly, one of the most pervasive microenvironmental stresses, hypoxia (low oxygen tension), seems to play an important role in tumor-stroma interactions by modulating these intercellular communication pathways. In paper **IV** of this thesis, we provide new insights into the influence of exosomes on hypoxia-driven angiogenesis and tumor development.



# Tumor hypoxia

Molecular oxygen ( $O_2$ ) is an essential substrate in cellular metabolism and bioenergetics of aerobic organisms. Thus, in the face of hypoxia, here defined as reduced oxygen levels  $< 2\% O_2$ , living cells are exposed to significant stress and activate a number of adaptive responses to match oxygen supply with metabolic, bioenergetic and redox demands. Cells temporally arrest in the cell cycle, reduce energy consumption and secrete survival and pro-angiogenic factors. These events are regulated by various cellular pathways, including unfolded protein response (UPR), mammalian target of rapamycin (mTOR) signaling and most importantly transcriptional response mediated by hypoxia inducible factors (HIFs) [38]. Although paucity of tissue oxygenation accompanies several physiological conditions, hypoxic tissues are most often associated with disease.

Hypoxia is a common feature of tumor microenvironment, albeit with variable incidence and severity in different tumor types and within a single tumor [39]. Rapidly expanding tumor mass outgrows the existing vascular network supplying its cells with oxygen. As a consequence, cells residing near oxygen diffusion limit, defined as a distance of 100-150  $\mu\text{m}$  from the vessel wall [40], are subjected to chronic hypoxia. Additionally, tumor vessels are structurally and functionally abnormal [41], and neoplastic cells residing in close proximity to this microvasculature may encounter acute or fluctuating perfusion-limited hypoxia. Different hypoxic areas combine in a heterogenous pattern throughout the tumor so that severe, intermediate and low levels of hypoxia can be detected. From the clinical standpoint it has been suggested that the intermediate hypoxic cells, which are resistant to therapy and still are able to proliferate, are the most important for prognosis [42]. There is ample evidence suggesting that hypoxia plays an active role in tumor progression. Measurement of hypoxia in many different human cancers has been associated with invasion [43], metastases [44], tumor recurrence [45] and decreased patient survival [46]. Underlying mechanisms of hypoxia mediated tumor progression include genomic instability, altered metabolism, resistance to cell death, invasiveness and metastasis as well as suppression of immune reactivity [47]. Furthermore, there is now evidence that hypoxia promotes spontaneous dedifferentiation and stem cell properties in neuroblastoma and breast cancer [48].

In part due to these profound effects on tumor development, hypoxia is implicated in resistance to both radio- and chemotherapy through multiple mechanisms [49,50]. Ionizing radiation requires oxygen to induce DNA damage, thus hypoxic tumor cells are more resistant to radiation. Hypoxic cells are usually distant from tumor vasculature, and thereby are exposed to lower concentrations of systemic drugs than neoplastic cells residing next to functional vessels. Most cytostatic drugs target rapidly dividing cells, and therefore have proved to be less effective in slowly proliferating hypoxic cells. Moreover, hypoxic cells exhibit decreased sensitivity to a broad spectrum of chemotherapeutic agents through

increased resistance to apoptosis, suppression of DNA repair and expression of ABC transporters, enabling efflux of chemotherapeutic drugs [51].

Different methods have been developed for assessing hypoxic levels in tumors *in vivo*, and polarographic O<sub>2</sub> microelectrode (Eppendorf electrode) has for long been considered state-of-the-art [49]. However, owing to the invasive nature of this technique and its limitation to superficial tumors, it cannot be broadly used in the clinic. Thus, evaluation of hypoxia is increasingly shifting to the monitoring of exogenous probes, such as pimonidazole, fluoromisonidazole (FMISO) and etanidazole pentafluoride (EF5), that bind covalently to SH-containing molecules (thiols) in hypoxic tissue [51], and <sup>18</sup>F-labelled versions of 2-nitroimidazole probes that can be detected with positron-emission tomography (PET) [52]. Alternatively, endogenous markers, which are proteins upregulated in association with hypoxia, can be measured in blood plasma or immunohistochemically in tumor biopsies. No single marker has been validated in clinical practice yet, although a correlation with patient outcome has been found with carbonic anhydrase IX (CAIX), osteopontin and plasminogen activator inhibitor (PAI)-1 [51]. Attempts have been made to combine various markers to create a prognostic hypoxic profile, however with moderate success [53]. There is also great potential for global gene and microRNA expression signatures for the identification of hypoxia [54,55]. In spite of ongoing extensive research, the majority of these approaches have not attained routine use in the clinic, probably due to the substantial heterogeneity of tumor tissue.

## **Hypoxia inducible factor (HIF) system**

One of the main cellular events of hypoxia is activation of hypoxia inducible factors (HIFs), which are the master regulators of oxygen homeostasis [56]. HIFs are a family of basic helix-loop-helix-PAS (bHLH-PAS) transcription factors. In their transcriptionally active state, HIFs are heterodimers each composed of one constitutively expressed HIF- $\beta$  subunit (also known as arylhydrocarbon receptor nuclear translocator, ARNT) and one, highly-regulated HIF- $\alpha$  subunit [57]. There are currently three known HIF- $\alpha$  subunits, including HIF-1 $\alpha$  [58], HIF-2 $\alpha$  (also known as endothelial PAS domain protein or EPAS1) [59] and HIF-3 $\alpha$  (also known as inhibitory PAS domain protein or IPAS) [60]. HIF-1  $\alpha$  and HIF-2 $\alpha$  are relatively well characterized and known to be important for oxygen sensing [56,57]. In contrast, HIF-3 $\alpha$  acts as dominant negative regulator of HIF-1 $\alpha$  and HIF-2 $\alpha$ -mediated transcription [60]. HIF-1 $\alpha$  is ubiquitously expressed whereas HIF-2 $\alpha$  expression is restricted to vascular endothelium, liver parenchyma, lung type II pneumocytes and kidney epithelial cells [61]. HIF-1 $\alpha$  and HIF-2 $\alpha$  have a similar domain organization containing the N-terminal basic domain conferring DNA binding, the HLS and PAS domains responsible for interaction with HIF- $\beta$ , and two transactivation domains (one N-terminal and one C-terminal) interacting with other transcriptional co-regulators. Furthermore, the N-terminal

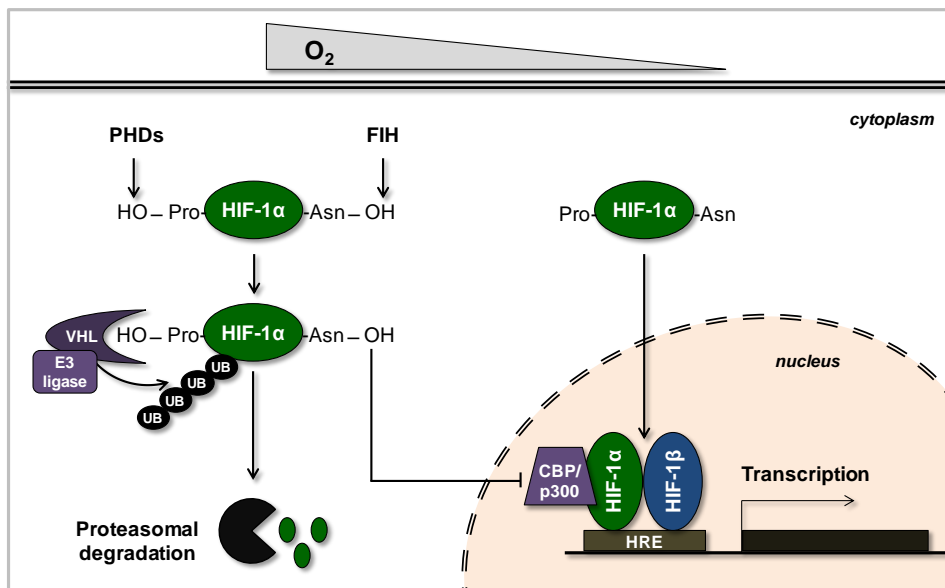
transactivation domain contains the oxygen-dependent degradation domain (ODD) [62].

Although HIF- $\alpha$  subunits have been shown to be regulated by a variety of mechanisms, most oxygen-dependent regulation occurs at the level of post-translational modification and protein stability (Figure 2). At normoxia, HIF- $\alpha$  subunits are hydroxylated at two proline residues located in the ODD. This hydroxylation is mediated by oxygen-, Fe(II)- and 2-oxoglutarate-dependent prolyl hydroxylase-domain (PHD) proteins 1-3, which are true oxygen-sensing molecules controlling the hypoxic response [62]. Hydroxylation of HIF- $\alpha$  subunits in turn mediates interactions with the von Hippel-Lindau (VHL) and E3 ubiquitin ligase complex that targets HIF- $\alpha$  for ubiquitin-dependent proteasomal destruction [63]. Additionally, HIF- $\alpha$  levels are modulated by a second hydroxylation-dependent mechanism, in which  $\beta$ -hydroxylation of an asparaginyl residue in the C-terminal activation domain (C-TAD) is regulated by factor inhibiting HIF (FIH) [64]. This oxygen-dependent process blocks interaction of HIF- $\alpha$  C-TAD with the transcriptional coactivator p300/CBP [64]. Under hypoxic conditions, however, HIF- $\alpha$  hydroxylation by PHDs and FIH are inhibited, leading to stabilisation of HIF-1 $\alpha$  and HIF-2 $\alpha$ . Then HIF- $\alpha$  subunits translocate into the nucleus, where they heterodimerize with HIF- $\beta$  and a number of co-regulators, to form the transcriptional complex within hypoxia-response elements (HREs) in the promoters of HIF target genes and activate transcription [57]. Although HIF-1 $\alpha$  and -2 $\alpha$  share many similarities in structure and regulation, recent lines of evidence indicate that they have overlapping but distinct specificities with regards to physiological inducers and transcriptional targets [38].

In addition to hypoxia, HIFs may be stabilized due to excessive transcription, protein synthesis or stability mediated by growth factor-dependent activation of receptor tyrosine kinases (RTKs) and their downstream signaling pathways, such as phosphatidylinositol 3 kinase (PI3K)/AKT/mTOR and Ras/extracellular-signal-regulated kinase 1/2 (ERK1/2) pathways [65,66]. Interestingly, many components of these pathways are oncogenes (EGFR; PI3K; Ras; Src) or tumor suppressors (phosphatase and tensin homolog or PTEN; tuberous sclerosis complex or TSC; p53) that are frequently deregulated in cancer and may thus lead to activation of a hypoxic transcriptional response in tumor cells residing in well oxygenated areas [67-69]. Such a pseudo-hypoxic phenomenon may also result from genetic alterations of enzymes directly and indirectly involved in the oxygen sensing machinery. The most prominent example is VHL loss-of-function, which results in constitutive overexpression of HIF-1 $\alpha$  and HIF-2 $\alpha$  in familial and sporadic renal cell carcinomas [70]. Furthermore, increased levels of HIFs may result from decreased hydroxylation of HIF- $\alpha$  subunits under normoxic conditions. PHD dependent hydroxylation of HIF- $\alpha$  involves conversion of Krebs cycle intermediate - 2-oxoglutarate and O<sub>2</sub> to succinate and CO<sub>2</sub>, thus high levels of succinate can slow this enzyme, leading to HIF- $\alpha$  stabilisation. The impact of this regulation was recently appreciated when defects in Krebs cycle enzymes,

including succinate dehydrogenase (SDH) and fumarase, were associated with pheochromocytomas and leiomyomata, respectively [71,72]. In addition, recent studies of glioma tumors revealed frequent heterozygous mutation in the gene coding for cytosolic isocitrate dehydrogenase (IDH), which converts isocitrate to 2-oxoglutarate. This mutation results in decreased levels of 2-oxoglutarate and formation of onco-metabolite R(-)-2-hydroxyglutarate inhibiting PHD activity [73].

The net result of these genetic and physiological changes within the tumor is to induce HIF-dependent responses in both hypoxic and oxygenated parts of the tumor in order to stimulate tumor progression. Indeed, immunohistochemical approaches have demonstrated the overexpression and association with poor patient survival of both HIF-1 $\alpha$  [74-76] and HIF-2 $\alpha$  [77-79] in a broad range of human malignancies.



**Figure 2. Oxygen-dependent, post-transcriptional regulation of the HIF system.** The HIF- $\alpha$  protein is constitutively expressed in most cells, but constantly degraded in the presence of oxygen. Under normoxic conditions, newly synthesized cytoplasmic HIF- $\alpha$  is rapidly hydroxylated by PHD enzymes (primarily PHD2). This modification leads to binding and ubiquitylation by the VHL/E3 ligase complex and subsequent destruction of HIF- $\alpha$  in a ubiquitin dependent manner in the 26S proteasome. Additionally, HIF- $\alpha$  levels are modulated by second hydroxylation-dependent mechanism regulated by factor inhibiting HIF (FIH). This oxygen-dependent process blocks interaction of HIF- $\alpha$  with the transcriptional coactivator CBP/p300 and inhibits HIF-mediated gene expression. Under hypoxic conditions, however, HIF- $\alpha$  is not hydroxylated and is therefore stabilized. Subsequently, HIF- $\alpha$  subunit translocates into the nucleus, where it heterodimerizes with HIF- $\beta$  and a number of co-regulators, such as CBP/p300, and induces expression of target genes by binding to hypoxia-response elements (HREs) in the promoter regions.

## **Multifaceted role of hypoxia in the hallmarks of cancer**

Hypoxia is one of the most pervasive stresses in the tumor microenvironment and a driving force of a number of adaptive responses in neoplastic cells, which augment hallmarks of cancer and thus facilitate successful tumor progression.

One of the first responses occurring in cancer cells under hypoxic conditions is the metabolic switch from O<sub>2</sub>-dependent oxidative phosphorylation to anaerobic glycolysis. HIF-1 $\alpha$  is a master regulator of this adaptation to hypoxia and mediates it by promoting glycolysis through increased expression of glucose transporters (GLUT1 and GLUT3), glycolytic enzymes and lactate dehydrogenase (LDHA) as well as by decreasing mitochondrial function and biogenesis [80]. These effects reduce the O<sub>2</sub> demand of tumor cells within hypoxic tissue, while still providing sufficient energy to the cell. Additional beneficial effects of this metabolic switch may be explained by recent theories. Increased glycolysis and reduced mitochondrial activity result in accumulation of intermediates of glycolytic pathway and tricarboxylic acid cycle, respectively, which in turn may be used in biosynthetic pathways generating nucleosides and amino acids [81]. Furthermore, decreased function of mitochondria reduces production of toxic reactive oxygen species (ROS) and conserves O<sub>2</sub> for non-energy producing cellular functions, *e.g.* sterol synthesis [80]. One of the consequences of glycolytic switch is increased production of lactic and carbonic acids, leading to intracellular acidification. To survive and proliferate, tumor cells must extrude these acids through activity of several pumps, *e.g.* HIF-regulated membrane-bound ectoenzyme CAIX, to counteract local acidosis [82]. This adaptation induces acute and chronic acidification of the tumor extracellular milieu, which may facilitate tumor invasion through, *e.g.* destruction of adjacent normal cells and enzyme-dependent degradation of ECM [82,83]. In addition, acidosis has been shown to trigger mutagenesis and induce transformation of stromal fibroblasts [83].

To continue growing under hypoxic conditions, tumor cells induce expression of various growth factors, such as EGF, insulin, insulin growth factor (IGF)-1 and -2, and platelet derived growth factor (PDGF), which are known to promote cell proliferation [56]. Sustained proliferation may also result from HIF-dependent enhancement of c-Myc transcriptional activity leading to repression of genes encoding p21 and p27 as well as activation of cyclin D2. While HIF-2 $\alpha$  appears to stimulate cancer cell proliferation via this pathway, HIF-1 $\alpha$  has been shown to induce cell cycle arrest by functionally counteracting c-Myc [38,61]. Interestingly, several lines of evidence indicate that hypoxia and HIFs may induce limitless replicative potential by increasing telomerase activity in cancer cells [84,85].

Hypoxia is a potent inducer of apoptosis and mediates this effect through HIF-1 $\alpha$ . HIF-1 $\alpha$  has been shown to induce p53-dependent apoptosis [86] as well as BNIP3/NIX-mediated mitochondrial-pore permeability transition and cell death [87]. Conversely to normal cells, cancer cells may undertake genetic and adaptive changes in response to hypoxia that increase their evasion of apoptosis under hypoxia. Tumor cells have developed several mechanisms for escaping HIF-1 $\alpha$ -

mediated apoptosis in a hypoxic microenvironment, including upregulation of negative regulator of p53 (MDM2) [88], increased expression of anti-apoptotic proteins [89] or polyamines [90], activation of the PI3K/AKT survival pathway [91] and promotion of autophagy [92]. Additionally, hypoxia may confer selection pressure during tumor growth for expansion of neoplastic cells that lost their apoptotic potential by, *e.g.* p53 mutations [93].

Hypoxia-mediated angiogenesis involves increased expression of pro-angiogenic proteins from tumor cells and stromal cells that stimulate vascularization and re-establish access to oxygen and nutrients [10]. Hypoxia-dependent angiogenesis and its role in tumor development will be described in more detail further below.

Invasion and metastasis involve increased capacity of tumor cells to promote ECM remodeling, increased migration and digestion of the basement membrane. Animal and clinical studies have shown that tumor hypoxia is one of the determinants for tumor cell dissemination [38,61]. Hypoxia and HIFs have been shown to induce expression of proteins conferring proinvasive properties, including fibronectin, MMP-2, cathepsin D, urokinase plasminogen activator receptor (uPAR), the receptor tyrosine kinase c-Met and the cytokine receptor CXCR4 [82]. Epithelial-to-mesenchymal transition (EMT) is a key feature of invasive cells. Interestingly, hypoxia and HIF-1 $\alpha$  overexpression have been reported to promote the expression of EMT activators [38]. Hypoxia-mediated HIF-1 $\alpha$  activation is associated with a concomitant loss of E-cadherin, a crucial feature of EMT, through increased expression of LOX/Snail pathway [38,47]. Notch signaling and WNT pathways are also required to convert the hypoxic stimulus into EMT [94,95]. Moreover, it has been shown that LOX secreted by the primary tumor remodels distant premetastatic niches to recruit tumor and stromal cells [96].

As mentioned above, the tumor microenvironment is a multicellular organ composed of neoplastic cells and a variety of stromal cells. Thus, similarly to cancer cells, surrounding normal cells are often exposed to low oxygen tensions modulating their activity. Macrophages are recruited to tumor areas primarily by HIF-target proteins, such as colony stimulating factor (CSF)-1 and VEGF, released by hypoxic tumor and stromal cells [97]. In addition, apoptotic cells from hypoxic regions of a tumor appear to produce soluble factors, *e.g.* TGF- $\beta$ , to attract monocytes and macrophages [98]. Once recruited, these cells accumulate in hypoxic areas of tumors and respond to decreased oxygen levels by upregulating HIFs, the activation of which leads to increased transcription of genes that regulate cell proliferation, metabolism, angiogenesis and invasion [97]. Hypoxia may also protect tumor cells from immune attack through the inhibition of anti-tumor T-cells [99].

Emerging evidence indicates that hypoxia as a tumor microenvironmental stress can drive genomic instability through increased chromosomal rearrangement, gene amplification and induction of intrachromosomal fragile sites

[100]. Moreover, cancer cells grown under severe hypoxic conditions show increased frequency of point mutation than normally oxygenated cells, and this is partially mediated by hypoxia-dependent decreased expression of DNA mismatch repair (MMR) genes [100]. The loss of MMR genes has been reported to render human colon carcinoma cells hypersensitive to hypoxia-dependent microsatellite instability, generating highly drug-resistant clones in the surviving population [101].

Collectively, these data depict hypoxia as a key tumor microenvironmental stress selecting for more aggressive tumor phenotype, which is associated with treatment failure and poor outcome for a wide variety of human malignancies. Thus, hypoxia needs to be considered as a prognostic and therapeutic option for cancer patients. Indeed, several therapeutic approaches targeting hypoxia have been tested, including prodrugs activated by hypoxia as well as inhibitors of HIFs, RTKs, mTOR and UPR pathway [51].

# Tumor angiogenesis

## Molecular mechanisms of angiogenesis

The cardiovascular system constitutes the largest network in our body ensuring the delivery of nutrients, oxygen and immune cells as well as disposal of waste metabolites. Its formation occurs early during embryo development and results from two tightly regulated processes, namely vasculogenesis and angiogenesis. Embryonic vasculogenesis leads to the formation of a primitive vascular plexus via aggregation of angioblast-derived endothelial cells, which then undergoes further remodeling, including sprouting, microvascular growth and fusion into mature and functional vessels, in a process known as angiogenesis. During adulthood, the majority of vessels are quiescent, and physiological angiogenesis occurs only in order to maintain homeostasis and tissue integrity during skeletal growth, the menstrual cycle, pregnancy, inflammation and wound healing [102]. Angiogenesis can also occur in pathological conditions. Insufficient vessel growth and abnormal vessel regression underlie many disorders, such as brain and heart ischemia, neurodegeneration, hypertension and pre-eclampsia, whereas excessive angiogenesis fuels *e.g.* inflammatory disorders, blinding eye disease, obesity and cancer [102].

## VEGF and its receptors

One of the major hypoxia-induced genes is VEGF-A, which encodes a protein with great angiogenic potency. VEGF-A is a member of a gene family that also includes VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF) [103]. VEGF-B has a role in coronary artery development [104], while VEGF-C and VEGF-D are lymphangiogenic cytokines that can also induce angiogenesis under appropriate circumstances [105]. PlGF may be dispensable for development and specifically relevant in pathological angiogenesis, where it is thought to supplement and potentiate VEGF-A activity [106]. According to current knowledge, VEGF-A has the most important role in vascular development. Its importance in early vasculogenesis/angiogenesis is underscored by phenotypic lethality of mice lacking a single VEGF-A allele at approximately embryonic day (E)9.5 [107]. Multiple variants of VEGF-A that have different affinities for heparan sulfate proteoglycans (HSPGs) at the cell-surface and in ECM are generated by alternative splicing or proteolytic processing. Among them, VEGF<sup>165</sup>, which exerts intermediate binding to HSPGs, elicits the most potent pro-angiogenic activities [103]. VEGF family members mediate their activity through three tyrosine kinase receptors: VEGFR1, VEGFR2 and VEGFR3 [103,108]. Whereas VEGFR2 primarily mediates VEGF-A-stimulated endothelial cell mitogenesis and vascular permeability, the role of VEGFR1 is complex and context dependent. PlGF and VEGF-B selectively bind to VEGFR1, whereas



VEGF-C and VEGF-D activate VEGFR2 and VEGFR3 leading to stimulation of angiogenesis and lymphangiogenesis, respectively [103,108]. In addition to the VEGF receptor tyrosine kinases, two non-kinase receptors, neuropilin-1 and -2 (NRP-1, NRP-2), also interact with members of the VEGF family, resulting in the potentiation of VEGFR2 signaling [109].

### **Steps of angiogenesis**

The first step in vessel sprouting towards avascular regions of the tissue is the activation of endothelial cells in response to a gradient of heparin-binding VEGF-A isoforms that are released from hypoxic cells. This activation leads to the conversion of a quiescent endothelial cell into a highly motile tip cell, guiding the developing capillary sprout through the ECM toward an angiogenic stimulus [110]. Tip cells begin to secrete proteolytic enzymes, MMPs as well as ANGPT-2 antagonizing ANGPT-1 and Tie-2 signaling, enabling basement membrane degradation and pericyte detachment, respectively [111,112]. Subsequently, endothelial cells loosen intercellular junctions and the nascent vessel dilates. Increased permeability of the endothelial cell layer causes plasma proteins to extravasate and form provisional ECM scaffold. In response to integrin signaling, endothelial cells migrate onto this matrix. Meanwhile, VEGF-A mediated activation of VEGFR2 induces delta like ligand 4 (DLL4) in tip cells, which leads to activation of Notch receptors in the neighboring stalk cells [113]. Notch receptor activation suppresses VEGFR2 production in stalk cells, which dampens migratory behavior compared with that of tip cells. As a consequence, endothelial stalk cells proliferate in response to many pro-angiogenic molecules (WNT, PlGF, FGFs) as they follow behind a tip cell causing the capillary sprout to elongate. These stalk cells become the trunk of the newly formed capillary. When the tip cells of two or more capillary sprouts converge at the source of VEGF-A secretion, they fuse together and create a continuous lumen. Blood flow to the newly vascularized areas raises local oxygen levels leading to a decrease in VEGF-A levels. This resolution phase leads to the impairment of endothelial cell proliferation as well as recruitment of mural cells, such as pericytes and vascular smooth muscle cells, to nascent vessels, which provide stabilization, remodeling and maturation signals [114]. PDGF-BB and its receptor, PDGFR- $\beta$ , have essential role in the stabilization process. PDGF-BB is secreted by endothelial cells and retain close to these cells through its carboxy-terminal retention motif that binds HSPGs, in order to form a guidance cue for pericytes [115]. Attracted mural cells cover newly formed vessels and induce their maturation, partly by secreting ANGPT-1 that in turn binds to Tie-2 receptors abundantly expressed on the surface of endothelial cells. This signaling suppresses endothelial cell apoptosis, tightens endothelial cell junctions mediated by VE-cadherin, claudins and occludins as well as facilitates endothelial cell-matrix and endothelial cell-mural cell interactions [116]. Re-established contact between endothelial cells and

mural cells also induces the expression of TGF $\beta$  promoting ECM production [117,118] as well as tissue inhibitor of metalloproteinases (TIMP)-2, TIMP-3 and PAI-1 impairing the proteolytic phenotype in endothelial cells [119]. As a result, endothelial cells and surrounding cells deposit basement membrane, by secreting collagen IV, laminins, nidogen, fibronectin and HSPGs, that provide structural support and maintain endothelial cell quiescence [120].

## **Mechanisms underlying tumor angiogenesis**

The first observation that angiogenesis occurs around tumors, was made almost 80 years ago [121], and in 1971 Judah Folkman proposed currently accepted concept that tumor angiogenesis is a rate limiting process in tumor growth, *i.e.* without new blood vessels, tumor cells can not sustain proliferation and thus are likely to remain dormant [122].

Tumor angiogenesis shares many events with physiological angiogenesis, however, some of these steps are flawed. A major difference is that tumor vessel formation does not reach resolution upon the establishment of vascular perfusion due to unbalanced and continuous secretion of a subset of pro-angiogenic cytokines, particularly VEGF-A. Within tumor microenvironment, these factors are abundantly expressed by hypoxia stimulated tumor cells as well as tumor cells with pseudo-hypoxic phenotypes, as described above. Moreover, cancer stroma contains tumor accessory cells, such as fibroblasts and inflammatory cells, both of which play important roles in amplifying pathological angiogenesis. In response to this extremely pro-angiogenic environment, tumor blood vessels are disorganized, tortuous and their spatial distribution is heterogenous, resulting in uneven drug distribution in tumors. In addition, tumor vessels do not follow the hierarchy of arterioles, capillaries and venules and are hyperpermeable, because endothelial cells are loosely connected and poorly covered by mural cells.

Studies over the last decade have established that angiogenic tumor blood vessels can be classified into at least four structurally and functionally distinct types, including mother vessels, capillaries, glomeruloid microvascular proliferations (GMPs) and vascular malformations (VMs) [123]. Mother vessels are large, thin-walled, serpentine, pericyte-poor and strongly VEGFR-positive sinusoids originating from pre-existing normal venules [124]. They have sluggish blood flow and are subject to thrombosis. Mother vessels are transient structures that evolve into daughter vessels. Many split into smaller capillary-like channels by a process of internal division [124]. GMPs are another mother vessel derivative, which begin as focal collections of CD31 positive cells arising in the endothelial lining of mother vessels from either preexisting endothelial cells or circulating bone marrow precursors. These cells proliferate rapidly and extend both into the mother vessel lumen and outward into the extravascular connective tissue. They are accompanied by replicating pericytes and form microvessels with tiny lumens [124,125]. Like mother vessels, GMPs require a continuous supply of

exogenous VEGF-A. VMs are a third type of mother vessel progeny. They form as mother vessels acquire a coating of smooth muscle cells. Similarly to capillaries, VMs are not leaky and are independent of exogenous VEGF-A supplies [123,124].

In addition to local capillary ingrowth from surrounding vasculature, tumour vessel formation has been shown to involve the mobilization of bone marrow-derived cells (BMDCs) and their incorporation into functional vasculature, in a process of postnatal vasculogenesis [126,127]. Among the variety of BMDCs with pro-angiogenic properties, the population of circulating endothelial progenitor cells (EPCs) is the most investigated. However, the extent of BM-derived EPCs incorporation in tumor vessels varies greatly with tumor type from 50% [128] to very few [129]. The chemokine SDF-1 $\alpha$  and its receptor CXCR4 have been reported to be important in the recruitment of these circulating EPCs to sites of neoangiogenesis [130]. Consistent with their role in tissue regeneration, EPCs are also recruited when the tumor vasculature is destroyed by chemo/radiation therapy, both in animal models and in clinical studies [131-133]. To complicate matters further, tumors may become independent of endothelial cell vasculature. Tumor cells of certain melanomas may undergo a dedifferentiation program called vasculogenic mimicry and ultimately construct fluid-conducting channels [134]. Furthermore, recent studies have shown that glioblastoma stem-like cells may transdifferentiate into functional vascular endothelium and participate in neovascularization [135]. These findings emphasize the complexity of tumor vasculature and the challenges in achieving effective therapeutic targeting of tumor-angiogenesis.

## **Anti-angiogenic therapy**

Because of the key role of VEGF-A and its receptors in physiological and tumor vascularization, antiangiogenesis research has been largely focused on this signaling pathway. This has resulted in Food and Drug Administration (FDA) approval of clinical use of first generation VEGF inhibitors, such as the monoclonal anti-VEGF antibody (bevacizumab) in combination with chemotherapy for metastatic colorectal cancer, metastatic non-squamous non-small-cell lung cancer and metastatic breast cancer, and in combination with interferon- $\alpha$  in metastatic renal cell carcinoma [136]. Recurrent glioblastoma multiforme (GBM or grade IV astrocytoma) is so far the only type of cancer treated with bevacizumab as monotherapy [137]. GBM is the most common and aggressive primary tumor of the central nervous system. In spite of advances in detection, surgery, radiotherapy and pharmacological therapies, the median survival after initial diagnosis is still only 15 months [138]. The rapid growth of GBM and its defective tumor vasculature, characterized by excessive leakiness and frequent thrombosis, cause formation of necrotic regions surrounded by hypoxic pseudopalisading cells. These regions of low oxygen levels in turn activate hypoxia-dependent angiogenesis and sustain hypervascular state of the

tumor. Indeed, GBM is among the most vascular tumors and thereby anti-angiogenic therapy is an attractive strategy to improve GBM patient survival [139]. In addition to anti-VEGF antibody, a variety of small molecule tyrosine kinase inhibitors (TKIs) targeting the VEGF pathway are undergoing clinical development, and some have been approved by FDA for cancer therapy, including sorafenib and sunitinib [136]. Sorafenib is a Raf kinase inhibitor that also inhibits VEGFR2 and -3, PDGFR- $\beta$ , Flt-3 and c-kit. Sorafenib has been approved for metastatic RCC and unresectable hepatocellular carcinoma [140]. Similarly, sunitinib inhibits several RTKs, including VEGFRs, PDGFR, c-kit and Flt-3, and has shown efficacy in metastatic RCC [140]. Conversely to bevacizumab, TKIs are capable of suppressing tumor growth as monotherapy, likely because they target a variety of kinases, of which some may confer additional therapeutic benefit (such as inhibition of PDGFR). Thalidomide, which inhibits the activity of bFGF, is another anti-angiogenic agent used in combination with dexamethasone as a treatment mode of patients newly diagnosed with multiple myeloma [136]. In addition to blockers of angiogenic pathways, some drugs may elicit their therapeutic effects via indirect inhibition of angiogenesis. Indeed, two mTOR inhibitors, *i.e.* everolimus and temsirolimus, approved by the FDA for use in advanced RCC, may inhibit tumor angiogenesis by impairing mTOR-mediated HIF activation [51].

### **Modes of resistance to anti-angiogenic therapy**

The clinical effects with VEGF blockers have shown that translating the preclinical successes of anti-angiogenic therapy into clinical settings is more challenging than anticipated. The greatest challenge today is that many cancer patients are intrinsically resistant to anti-angiogenic drugs and do not respond at all or minimally, *e.g.* pancreatic cancer patients [141]. Other patients, who initially respond, acquire resistance to VEGF inhibitors after a several month period of clinical benefit and inevitably experience tumor progression. Growing body of research reveals mechanisms underlying intrinsic refractoriness and evasive escape (acquired resistance) from anti-VEGF agents, which seem to be attributable to changes in tumor cells and their microenvironment [142-144]. Tumor vessels can become less sensitive to VEGF blockers, and sustained tumor angiogenesis may occur via VEGF-independent mechanisms. Vessel pruning by anti-angiogenic agents can induce profound hypoxia, which results in increased expression of tumor cell-derived angiogenic factors, such as PlGF, FGFs, ANGPTs and ephrins and thereby maintained tumor neovascularization [144]. Some tumor endothelial cells have cytogenetical abnormalities, including aneuploidy and multiple chromosomes, raising the possibility that such instability may contribute to resistance to anti-angiogenic therapies [145]. Furthermore, GBM stem-like cells can differentiate into tumor endothelial cells, and VEGF blockers can only partially impair this process [135]. Hypoxia induced by vessel regression due to

anti-angiogenic treatment can also turn on more aggressive phenotype of tumor cell conferring them independence from tumor vasculature. Accordingly, the GBM cells were seen to co-opt normal blood vessel and migrate along them (perivascular invasion) into normal brain tissue [146]. In other cases, tumor cells can acquire extra mutations and become hypoxia-tolerant. For example, lack of p53 in the tumor cells reduced their response to anti-angiogenic therapy while p53<sup>+/+</sup> cells were quite sensitive to the same treatment [147]. In addition to tumor cells, stromal cells appear to have an important contribution to the resistance to VEGF blockade. Hypoxia resulting from anti-angiogenic therapy may lead to recruitment of various BMDCs, which are capable of supporting tumor growth by inducing tumor vascularization [143,144,148].

One of the major concerns, regarding the use of VEGF blockers as anti-angiogenic treatment in cancer patients, is disease progression leading to increased invasiveness and metastasis. Some preclinical models show that VEGF blockage aggravates hypoxia, which promotes invasiveness and metastasis, despite inhibition of primary tumor growth and prolongation of survival [146,149]. Clinical results that seem consistent with these preclinical findings have emerged. Increases in local foci and/or distant metastasis were observed in retrospective analyses of patients with RCC who discontinued either sunitinib or sorafenib [150], and in isolated case reports [151]. However, a meta-analysis of advanced cancers shows that bevacizumab does not trigger metastatic disease and shortened survival of various tumor types [152]. Due to contradictory and largely limited data, the concept of VEGF-blockade-mediated tumor progression still remains speculative. The exception seems to be GBM, as 30–50% of patients treated with bevacizumab develop progressive disease accompanied by a high rate of diffuse infiltrative lesions, and this finding has been noted in several studies [153,154]. In preclinical mouse models of GBM, where VEGF or HIF-1 $\alpha$  was genetically or pharmacologically blocked, initial tumor stabilization and/or shrinkage was followed by tumor re-growth as well as increases in new microsatellite lesions in adjacent sites with infiltrative behavior [146,148,155,156]. The results of the fully included phase-III trial AVAGLIO that randomized GBM patients to receive standard radiochemotherapy with placebo or bevacizumab will clarify these issues.

In conclusion, anti-angiogenic strategies have undoubtedly enabled significant advances in cancer therapy and in our understanding of tumor biology. However, the lack of substantial benefits for the majority of patients, in terms of increased long-term overall survival times, still remains a challenge, and indicates the need of further studies aiming at identification of the basis of this treatment limitations as well as new targets of anti-angiogenic therapy in the treatment of cancer patients.

# Polyamines

## Polyamine metabolism

The original discovery of polyamines dates back to 1678, when Van Luewenhoek observed polyamine crystals in semen. Polyamines are low molecular weight, polycationic alkylamines found in prokaryotes and eukaryotes. Putrescine, spermidine and spermine are the major polyamine species, however, other amines have been identified, *e.g.* in extreme thermophiles [157]. Due to their chemical properties, polyamine amino groups become ionized at physiological pH, and as a consequence, positively charged polyamines interact with numerous anions in the cell, including DNA, RNA, proteins and phospholipids [158]. The polyamine fraction ionically bound to RNA and DNA account for more than 90% of total intracellular polyamine content, and rapid changes in the free pool of polyamines, resulting from increased synthesis/uptake/efflux, may have critical effects on cellular physiology [159]. Given their promiscuity in binding other molecules, polyamines are involved in many functions, such as cell growth, proliferation and survival, which are essential for optimal growth of unicellular organisms, such as bacteria and yeast, as well as development of mammals. Indeed, mouse knockouts of genes encoding key enzymes involved in polyamine synthesis, such as ornithine decarboxylase (ODC) or S-adenosylmethionine decarboxylase (AMD), are lethal at very early embryonic stages, and these phenotypes seem to result from increased apoptosis and decreased proliferation in developing mouse embryo, respectively [160,161]. Furthermore, polyamines have been implicated in the growth and development of a variety of mammalian tissues and in remodeling processes associated with tissue repair [162,163]. The tight relationship between polyamines and cell growth makes them highly interesting in the context of pathological conditions, and it is now well established that dysregulated levels of polyamines may accompany Parkinson's disease, inflammation and cancer development.

Given the importance of unperturbed polyamine levels in regulating physiological responses, cellular polyamine content is precisely controlled by changes in the rate of synthesis, catabolism and transport. A summary of these different processes are depicted in Figure 3.

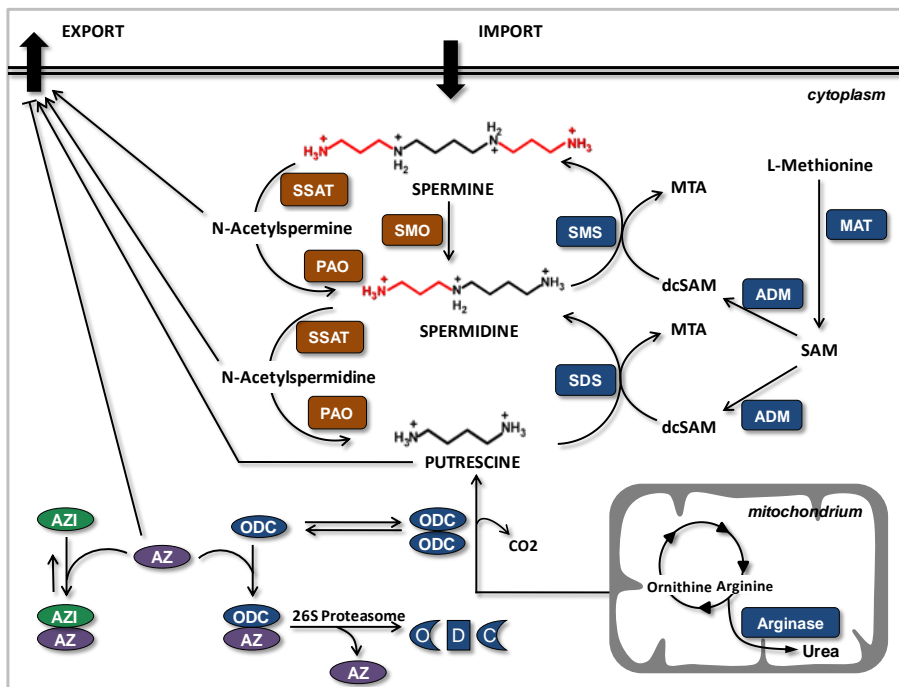
## Polyamine synthesis and catabolism

Polyamines are synthesized in mammalian cells from the amino acids arginine, ornithine and methionine. The first step of synthesis pathway involves the production of ornithine from arginine by the mitochondrial enzyme arginase [164]. Ornithine is then decarboxylated to the diamine putrescine by ODC [165]. Meanwhile, methionine is converted into S-adenosyl-L-methionine (SAM) by methionine adenosyltransferase (MAT), which is then decarboxylated by AMD to

produce decarboxylated SAM (dcSAM) [166]. The higher polyamines, triamine spermidine and tetramine spermine, are formed from putrescine by successive attachment of aminopropyl groups, donated by dcSAM, by the action of aminopropyl transferases, namely spermidine- and spermine synthase, respectively [167]. The synthesis pathway is highly regulated by the two enzymes, *i.e.* ODC and AMD, which are rate-limiting factors in the production of putrescine and the supply of adenopropyl donor, respectively. ODC is a cytosolic enzyme, active as a homodimer with an extremely short half-life of 10-30 minutes [165]. Enzymatic activity of ODC is regulated by the changes in the protein levels, resulting from modulation of transcription, mRNA stability, translation and protein degradation. ODC transcription has been shown to be induced by many factors, including hormones, growth factors and tumor promoters (*e.g.* c-Myc), linking polyamine biosynthesis with tumorigenesis [165,168]. Translational regulation of ODC may result from internal ribosome entry site (IRES)-mediated translation in response to Ras oncogene [169]. One of the most important regulatory mechanisms of ODC activity is based on protein degradation, mediated by polyamine content. High intracellular levels of polyamines induce expression of antizyme (AZ), *i.e.* ODC specific inhibitor, via a unique +1 frameshifting mechanism on AZ mRNA, enabling the translation of the complete AZ protein [170]. Once translated, AZ binds to the ODC monomer, thus inactivating and targeting it for ubiquitin-independent degradation by the 26S proteasome [171]. Additionally, AZ has been found to downregulate the polyamine transport system [172,173], making AZ a master regulator of intracellular polyamine pool by controlling polyamine synthesis and uptake. AZ activity is in turn regulated by antizyme inhibitor (AZI), which binds to AZ more tightly than ODC and can displace it from ODC/AZ complex and thereby prevent ODC degradation [174]. Disruption of the gene encoding AZI has been shown to be lethal in mice, confirming the critical role of this protein in regulating polyamine levels [175]. Similarly to ODC, AZ and AZI turn over rapidly but require ubiquitination for proteasomal degradation. Degradation of AZ is inhibited by high polyamine levels, whereas AZI is stabilized when bound to AZ [176].

Conversion of higher polyamines back into putrescine and their degradation are mediated by a different group of enzymes, due to the irreversible nature of decarboxylation and aminopropyl transferase reactions. The rate-limiting enzyme of polyamine catabolism is the cytosolic spermidine/spermine N<sup>1</sup>-acetyltransferase (SSAT), which acetylates spermidine and spermine by the transfer of the acetyl group from acetyl-coenzyme A to the primary amino groups [177]. Acetylated spermidine and spermine then move into the peroxisome where they are oxidized by N<sup>1</sup>-acetylpolyamine oxidase (PAO), and thereby converted into putrescine and spermidine, respectively [178]. Interestingly, k-Ras oncogene negatively regulates SSAT, contributing to the increase in polyamines by k-Ras activation [179]. Spermine can also be back-converted into spermidine by recently discovered spermine oxidase (SMO) [180]. In contrast with PAO, the preferred substrate of

SMO is spermine itself, and not its acetylated derivative. Importantly, tumor necrosis factor (TNF) induces SMO and the resulting by-product  $H_2O_2$  has been implicated as a common pathway of inflammation-driven mutagenic changes in epithelial cells [181].



**Figure 3. Polyamine metabolism in mammals.** Arginine metabolism, which is mediated by arginase in the urea cycle, results in the production of ornithine. Ornithine is then decarboxylated by ornithine decarboxylase (ODC) to produce putrescine. Simultaneously, L-methionine is converted into S-adenosyl-L-methionine (SAM) by methionine adenosyltransferase (MAT), which is then decarboxylated by SAM decarboxylase (ADM) to produce decarboxylated SAM (dcSAM). The higher polyamines, spermidine and spermine, are formed from putrescine by successive attachment of aminopropyl groups, which are donated by dcSAM, by the action of spermidine- and spermine synthase, respectively. ODC activity is mostly regulated by protein degradation. High intracellular levels of polyamines induce expression of antizyme (AZ), which binds to the ODC monomer, thus inactivating and targeting it for ubiquitin-independent degradation by the 26S proteasome. Additionally, AZ has been found to downregulate the polyamine transport system. AZ activity is regulated by antizyme inhibitor (AZI), which binds to AZ more tightly than ODC and can displace it from the ODC/AZ complex and thus prevent ODC degradation. Conversion of higher polyamines back into putrescine involves acetylation of spermine and spermidine, which is mediated by spermidine/spermine  $N^1$ -acetyltransferase (SSAT). Acetylated spermine and spermidine can be removed from the cell by the efflux mechanism or become oxidized by  $N^1$ -acetylpolyamine oxidase (PAO) and converted to spermidine and putrescine, respectively. Spermine can also be back-converted into spermidine by spermine oxidase (SMO). Putrescine, spermidine and spermine can be imported from extracellular compartments through a polyamine transport system, facilitated by heparan sulfate proteoglycans (HSPGs).



## **Polyamine transport**

Uptake of exogenous polyamines, derived from dietary intake [182] and the intestinal bacterial flora [183], support cellular synthesis in increasing polyamine levels, and inhibition of endogenous biosynthesis results in compensatory upregulation of uptake [184]. Whereas transport systems for polyamine uptake in bacterium, yeast and plant cells have been characterized [185], there is to date no mammalian polyamine transporter cloned, and the mechanism by which polyamines enter mammalian cells is still not fully understood. Growing evidence suggest that the polyamine transport system is not highly specific and can transport a number of molecules with structures resembling the natural polyamines [186]. Furthermore, the polyamine transport system is blocked by AZ, but the mechanism of this effect is unknown [172,173]. As for now, the best characterized mechanism suggests that polyamine uptake could be performed by endocytosis [187], which at least for the higher polyamines spermidine and spermine, is dependent on cell-surface HSPGs [188]. In support of this notion, cell surface associated, recycling glypican-1 (GPC-1) HSPG has been shown to work as a membrane carrier of polyamines and to trigger their uptake via endocytic pathway [189]. Recently, it was suggested that polyamines associate with lipid rafts and that they are internalized by a clathrin-independent, caveolin-1 regulated endocytic pathway. In addition, phosphorylation of caveolin-1 at Tyr<sup>14</sup> increased the activity of this system and oncogenes like k-Ras has been shown to stimulate this phosphorylation, contributing to increased cellular polyamine levels [190]. In conclusion, polyamine uptake can follow a caveolin-1-dependent endocytic pathway, facilitated by cell surface HSPGs bound to polyamines. The extent to which this accounts for all polyamine transport is unclear.

Recently, a diamine exporter was identified that mediates putrescine and acetylpolyamine efflux. This efflux has been demonstrated to be coupled to arginine uptake, suggesting an arginine/putrescine exchange reaction [191]. Moreover, the exporter was found to be associated with SSAT, indicating that the export and acetylation systems might be linked, and k-Ras seems to negatively regulate its expression [191].

## **Functions of polyamines**

The cellular functions of natural polyamines are still poorly characterized, although majority of them seem to result from readily reversible ionic interactions with other molecules. To regulate cellular growth, viability, migration and many other cellular functions, polyamines mediate gene expression and ion-channel functioning, free-radical scavenging, activation/inactivation of signaling cascades, reorganization of the actin cytoskeleton and control of cell-to-cell interactions [158]. Spermidine and spermine have been shown to interact with DNA and change its conformation from B- to Z-DNA, and thus likely to affect the function of DNA [192]. Moreover, polyamines are known to bend DNA and thereby

increase the interactions between transcription factors, such as estrogen receptors and their response elements (EREs) [193]. Polyamine response elements have been identified in some genes, such as SSAT, and transcription of many genes seems to be influenced through the polyamine status [194]. Recent studies have shown that polyamines alter histone acetylation by changing the activity of histone acetylases (HATs) and histone deacetylases (HDACs), strengthening the role of polyamines in gene transcription [195]. Indirect effects of polyamines on gene expression may result from polyamine-mediated expression of transcription factors, such as c-Myc and c-Jun, which in turn can induce expression of other effector molecules [196,197]. Similarly, polyamines selectively influence the translation of many mRNAs through several mechanisms, including stimulation of the assembly of 30S ribosomal subunits, stimulation of Ile-tRNA formation, an increase in the fidelity of protein synthesis, a markedly elevated +1 ribosomal frameshifting efficiency at the AZ frameshift site and modulation of HuR-mediated mRNA stability [193,198]. Another function of the polyamines, specific for spermidine and related to cellular growth, is the formation of the amino acid hypusine. Hypusine is an essential modification of a specific lysine residue in the eukaryotic initiation factor 5A (eIF-5A), which stimulates its activity, required for eIF-5A-dependent protein synthesis, RNA transport and mRNA stability [199]. Polyamines also evoke cellular effects not explained by changes in protein expression. Several lines of evidence indicate that polyamines can affect phosphorylation by protein kinases, such as protein kinase A, through formation of ternary complex ATP-Mg<sup>2+</sup>-spermine [200]. There is also a report that the activity of the serine/threonine protein kinase casein kinase 2 (CK2) is strongly enhanced by polyamines, through spermine binding to the  $\beta$ -subunit of CK2 [201]. Moreover, spermidine and spermine may bind and thereby inhibit plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), which is a substrate for PI3K and phospholipase C signaling molecules as well as an essential participant in membrane trafficking, including endocytosis and exocytosis, receptor and G-protein events as well as integrin signaling [202]. Furthermore, polyamines may regulate ion channels, including glutamate receptor ion channel, *e.g.* N-methyl-D-aspartate (NMDA), mediating excitatory synaptic transmission in the mammalian brain; inwardly rectifying potassium channels (Kir), which control membrane potential and potassium homeostasis in many cells; voltage-activated Ca<sup>2+</sup> channels and certain connexin-linked gap junctions [203,204]. Polyamines have also been implicated in cell-to-cell interactions mediated via cadherins [205] and Toll-like receptors [206], regulation of the actin cytoskeleton by changes in the activity of G-proteins such as Rac1 and RhoA [207,208], and alterations in the microtubule network [209].

## Polyamines and cancer

The tight relationship between polyamines and cell growth makes them highly interesting in the context of cancer development. The association of increased polyamine synthesis with cancer was first demonstrated in the late 1960s, when Russell and Snyder reported high levels of ODC in regenerating rat liver and in several human cancers [210]. This observation spurred an intensive research, which strengthened this link showing increased levels of polyamines in the uterine of cancer patients [211], increased expression of ODC and high polyamine levels in a large number of tumors [212] as well as the induction of ODC activity in cells transformed with oncogenes or treated with carcinogens [179]. In addition, ODC appears, at least under certain conditions, to act as a protooncogene, as its overexpression was sufficient to induce transformation in fibroblasts [213] and keratinocytes [214]. In other circumstances, ODC deregulation is not sufficient to initiate the transformation process but rather supports it, as ODC-overexpressing mice more readily developed skin papillomas in response to a two-stage chemical tumorigenesis [215] or UV radiation [216].

Increased polyamine levels trigger tumor cell proliferation, partially due to increased expression of c-Myc [217] and decreased levels of JunD [218]. Other hallmarks of cancer, such as evasion of apoptosis and increased invasion and metastasis are also supported by higher levels of polyamines in tumor cells [90,219,220]. In addition to regulating tumor cell behavior, polyamines are necessary for tumor angiogenesis. Accordingly, ODC overexpressing NIH 3T3 cells induce rapidly growing and highly vascularized tumors in nude mice [221], whereas polyamine depletion-mediated inhibition of growth of many tumors results partially from anti-angiogenic effects [219,222]. Despite the profound effect of polyamines on tumor angiogenesis, the mechanisms underlying polyamine functions in endothelial cells are still ill-defined. In papers **I** and **II** we provide new insights into the role of polyamines in this process.

The recognition that polyamines are frequently dysregulated in cancer led to the development of 2-difluoromethylornithine (DFMO), *i.e.* an enzyme-activated irreversible inhibitor of ODC [223]. DFMO has proven to be an effective inhibitor of polyamine synthesis and tumor-promoting polyamine effects in pre-clinical studies [224,225]. This resulted in clinical trials with DFMO both as a single agent and in combination with other agents against several tumors, including lung, gliomas, melanomas, breast, prostate and cervical cancers [179]. In spite of some benefit of DFMO in patients with glioma as a post-irradiation adjuvant in combination with other treatment regimen [226], results in other cancer treatment trials demonstrated no effect. Disappointing results with DFMO in clinical settings probably relate to its cytostatic rather than cytotoxic effects in tumor cells as well as to the development of compensatory mechanisms in polyamine-depleted tumor cells, such as upregulation of AMD or ODC and increased uptake of circulating polyamines [223]. Thus, combination strategies with DFMO and inhibitors of polyamine uptake may offer more rational targeting of the polyamine system [227-

229]. Owing to the fact that DFMO is well tolerated and polyamines have been implicated in the carcinogenic process, DFMO is now clinically tested as a chemopreventive agent in multiple epithelial cancers, both as a single agent and in combination with cyclooxygenase inhibitors [230], and preliminary results from clinical trials support the preventive role of DFMO in the development of colorectal and skin cancers [179].

# MicroRNAs

MicroRNAs (miRNAs) comprise a large family of endogenous, small ~22-nucleotide-long non-coding RNAs that have emerged as essential post-transcriptional regulators of gene expression in animals and plants. The first miRNAs to be discovered, *lin-4* and *let-7*, were recognized as regulators of temporal control of development in *C. elegans* and this form of regulation was considered a fascinating but likely worm-specific phenomenon [231-233]. This all changed upon the discovery that *let-7* homologs exist in many vertebrate species including humans, suggesting a more general role for miRNAs in gene regulation [234]. The importance of these findings was fully appreciated when hundreds of similar small RNAs were discovered in *C. elegans*, *Drosophila* and mammals by molecular cloning and bioinformatics analyses [235-237]. Since these initial descriptions, more than 900 miRNAs have been discovered in human and the estimated number of miRNA genes is as high as 3000 in the human genome. Considering the fact that each miRNA can regulate hundreds of targets, it is not surprising that miRNAs are predicted to control the activity of most protein-coding genes [238], and consequently participate in the coordination of a wide variety of cellular processes, including differentiation, proliferation, death and metabolism. In addition to providing critical functions during cellular homeostasis, abnormal expression of miRNAs has been increasingly shown to contribute to many pathological conditions, including cardiovascular diseases, obesity and cancer.

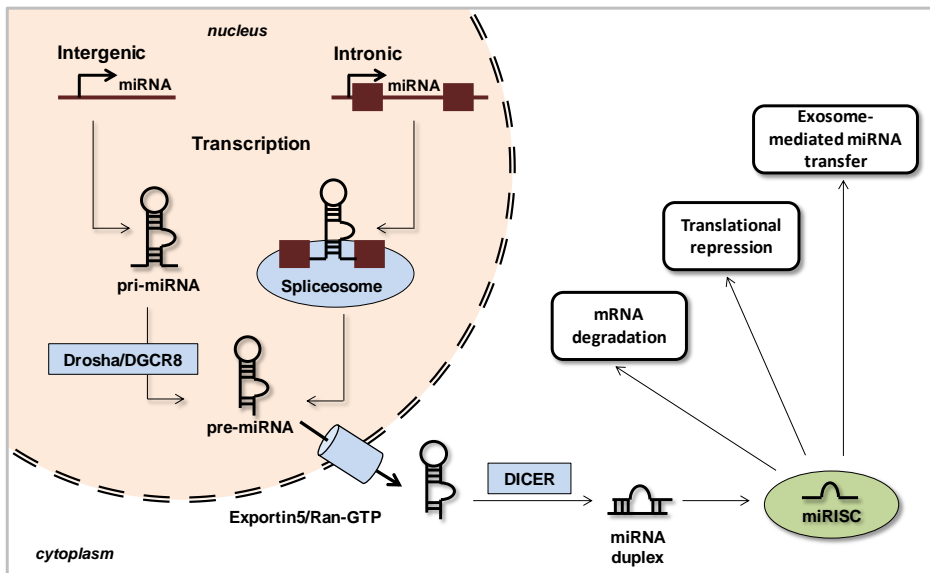
## MicroRNA biogenesis

MiRNA biogenesis is a complex process comprising several steps as depicted in Figure 4. Many miRNA genes are found in the intergenic regions or in anti-sense orientation to genes and contain their own miRNA gene promoter and regulatory units [235-237]. Alternatively, 40% of miRNA genes may lie in the introns of protein and non-protein coding genes or even in exons of long nonprotein-coding transcripts. These are usually, though not exclusively, found in a sense orientation, and thus regulated together with their host genes [239]. Additionally, some miRNA genes are clustered in the genome, implying transcription as a multicistronic primary transcript containing multiple discrete loops, from which two or more miRNAs are processed [235,236,240]. MiRNA genes are usually transcribed by RNA polymerase II as long primary transcripts known as primary miRNAs (pri-miRNAs) [241]. Similarly to other polymerase II transcripts, pri-miRNAs are capped with a specially-modified nucleotide at the 5' end, polyadenylated, and are usually several kb in length [242]. Within the pri-miRNA, the miRNA itself is contained within ~ 60-80 nucleotide sequence, which can fold back on itself to form a stem-loop hairpin structure. MiRNA hairpins are recognized and excised from pri-miRNAs in the nucleus by the Microprocessor complex, containing among others RNase III enzyme Drosha and its binding nuclear protein known as

DiGeorge Syndrome Critical Region 8 (DGCR8) [243]. The excised miRNA hairpins, referred to as pre-miRNAs (precursor-miRNAs), have a two-nucleotide overhang characteristic of RNase III cleavage products. Some pre-miRNAs are produced from very short introns (mirtrons) as a result of splicing and debranching, thereby bypassing the Drosha-DGCR8 step [244]. Pre-miRNAs are rapidly exported from the nuclei to the cytoplasm by the nuclear export factor exportin-5, which uses Ran-GTP as a cofactor [245,246]. In the cytoplasm, pre-miRNAs act as substrates for a second RNase III enzyme called Dicer that further processes them into 20-24-bp imperfect miRNA/miRNA\* duplexes [247]. This fully processed duplex is loaded into a large protein complex known as the miRNA-induced silencing complex (miRISC), which includes as core components the argonaute proteins (Arg-1-4 in humans) and glycine-tryptophan (GW) repeat-containing protein of 182 kDa (GW182) [248,249]. Association of miRNA duplex with an Ago protein results in rapid miRNA unwinding after which the strand with the weakest 5'-end base pairing is selected as the mature miRNA and is retained in the complex, whereas the other strand, the so-called miRNA\*, is either degraded or recognizes a different group of target transcripts for regulation of gene expression [250]. miRNAs act as guides that direct miRISC to target mRNAs and mediate post-transcriptional regulation of gene expression.

## **MicroRNA mechanisms of action**

In animals, most miRNAs target mRNA transcript via imperfect base pairing to multiple sites in 3' untranslated regions (UTRs), and miRNA 5'-proximal 'seed' region (positions 2-8) provides most of the pairing specificity [249]. Interestingly, animal miRNAs may also target 5'UTRs and coding regions of mRNAs [251,252]. The gene expression regulation by miRNAs is achieved in two ways, which are thought to depend on the degree of complementarity between the miRNA and its target mRNA. Perfect complementary binding of a miRNA will result in the cleavage of the target mRNA, while partial complementary binding primarily causes repression of translation. The mRNA degradation occurs by 5'→3' exonucleolytic activity and is preceded by mRNA deadenylation and decapping [253]. Much less consensus exists about the mechanisms of translational repression, and miRISC has been demonstrated to inhibit translation through a variety of mechanisms, including inhibition of translation initiation, impairment of elongation and premature termination [253]. The complexity of miRNA-dependent gene expression is further extended by the fact that, in addition to repressing gene expression, miRNAs have been recently shown to activate translation of several genes, and binding of miRNAs to complementary sequences in 5'UTR of target mRNAs may partially evoke this mechanism [251]. Furthermore, it has been shown that human miRNA-29b is predominantly localized to the nucleus as a result of its terminal hexanucleotide motif, suggesting that miRNAs may also regulate transcription or splicing of transcripts within the nucleus [254].



**Figure 4. Biogenesis of miRNAs.** MiRNAs are processed from RNA polymerase II (RNAPII)-transcripts of independent genes or from introns of other genes (mirtrons). In the canonical pathway, primary precursor (pri-miRNA) is processed by the nuclear complex composed of Drosha and DGCR8 into approximately 60-70 nucleotides long stem-loop precursor miRNA (pre-miRNA). In case of mirtrons, Drosha/DGCR8 step is omitted and pre-miRNAs are processed from introns in a process involving splicing and debranching. Pre-miRNAs are rapidly exported from the nuclei to the cytoplasm by the nuclear export factor exportin-5 in a Ran-GTP dependent manner. In the cytoplasm, pre-miRNAs are cleaved by DICER into 20-24-bp imperfect miRNA/miRNA\* duplexes. Following processing one strand of the duplex is incorporated into a miRNA-induced silencing complex (miRISC), which after binding to the complementary 3'UTR leads to the translational repression or degradation of the target mRNA. MiRNAs are also localized in exosomes, which mediate their transfer to neighbouring cells.

## Regulation of microRNA activity

Due to recent advances in miRNA research, it has become apparent that miRNA regulators themselves are subject to sophisticated and complex control. The first level of regulation includes the miRNA transcription, which similarly to transcription of protein-coding genes, is highly modulated by many transcription factors, including c-Myc and p53 transcription factors as well as epigenetics events, such as chromatin modifications. This regulation is a major level of control responsible for tissue-specific or development-specific expression of miRNAs [255]. Control of miRNA biogenesis has emerged as another important mechanism in regulation of miRNA expression, and includes many processes regulating Drosha and Dicer activity as well as pri- and pre-miRNA stability. The miRNA pathway is also extensively controlled at steps downstream of miRNA biogenesis. This type of control has been shown to involve profound regulation of RISC

proteins, such as Ago and GW182, interplay between miRISCs and many proteins binding to the same mRNAs as well as intracellular localization of miRISCs within particular cellular structures, such as P-bodies, stress granules and multivesicular bodies (MVBs) [255].

## Involvement of microRNAs in angiogenesis

Although multiple growth factors have been shown to regulate angiogenesis and vascular development, little is known about the complex regulation of gene expression during these processes. Recent studies have demonstrated that miRNAs are highly expressed in vasculature, where they may play important roles in vascular cell proliferation, migration and apoptosis, and when deregulated contribute to vascular diseases. The importance of miRNAs in angiogenesis and endothelial function was firstly revealed in Dicer knockout mice and zebrafish [256,257]; Dicer-deficient mice died between days 12.5 and 14.5 of gestation showing an impaired blood vessel formation and yolk sac vascularization [256]. Consistently, zebrafish Dicer mutant embryos displayed disrupted blood circulation [257]. Dicer is constitutively expressed in endothelial cells and its silencing via RNAi approach has been shown to reduce the formation of capillary-like structures, matrigel tube formation, proliferation and migration [258-260]. Additional *in vivo* studies were performed with endothelial cell-specific Dicer knockout mice models, a conditional Tie2-Cre;dicer<sup>f/f</sup> line and tamoxifen-inducible VECad-Cre-ER<sup>T2</sup>;dicer<sup>f/f</sup> line. While these animals were viable and displayed no apparent vascular phenotype, the postnatal angiogenesis response to many pro-angiogenic stimuli, such as exogenous VEGF, limb ischemia, wound healing and tumor growth, appeared to be hampered, suggesting essential role of endothelial miRNAs for postnatal angiogenesis [261]. Gene expression analyses indicated that downregulation of Dicer evoked changes in the expression of a number of angiogenesis-related genes, including thrombospondin-1 (THBS1), TEK/Tie-2, KDR/VEGFR2, Tie-1, eNOS and IL-8 [258,259]. Further studies are required to determine what mechanisms account for anti-angiogenic phenotypes observed in Dicer knockdown models.

In contrast to Dicer, little is known about the role of Drosha in vascular biology. *In vitro* experiments have demonstrated that knockdown of Drosha in human endothelial cells results in impaired angiogenesis, although the effect of Drosha on endothelial function is less profound than that of Dicer [259]. Surprisingly, Drosha seems not to be involved in angiogenic processes *in vivo*, and the discrepancy between effects of knockdown of Dicer and Drosha on angiogenesis might be due to the involvement of Dicer in other processes and Drosha-independent miRNA processing, compensating for loss of Drosha [259].

The above phenotypic changes observed in Dicer-deficient animals and endothelial cells have led to explosion of research aiming at unraveling the biological roles of individual miRNAs in endothelial cell biology and



angiogenesis. MiRNA profiles of endothelial cells have revealed that specific miRNAs are enriched in endothelial cells, including among others the let-7 family, miR-21, miR-29, miR-100, miR-221, miR-222, miR-23~24 cluster, miR-17~92 cluster and miR-126 [258,259,262]. Most of these miRNAs were also found to be highly expressed in normal rat carotid arteries, suggesting that they constitute a specific miRNA signature of the vasculature [263]. To date, miR-126 is the only miRNA known to be expressed specifically in the endothelial lineage and hematopoietic progenitor cells, and miR-126 knockout zebrafish and mice models revealed an essential function of this miRNA in governing vascular integrity and angiogenesis [264,265]. Growing body of evidence indicates that miRNAs expressed on a lower level in endothelial cells under physiological conditions are also of scientific interest, because their expression may be highly increased in response to angiogenic stimuli, such as hypoxia, inflammation and shear stress, and in turn be involved in the modulation of vascular development [266]. So far, approximately forty miRNAs have been demonstrated to be part of the gene expression machinery, profoundly modulating biological activities of endothelial cells. These angiogenesis-related miRNAs, angiomiRs, can be divided into two groups: pro-angiogenic miRNAs and anti-angiogenic miRNAs. Pro-angiogenic miRNAs promote angiogenesis by targeting negative regulators in angiogenic signaling pathways, whereas anti-angiogenic miRNAs inhibit vascular development by targeting positive regulators of angiogenesis. Recent progress toward understanding the functions of specific pro- and anti-angiogenic miRNAs in endothelial cell biology is summarized in Table 1.

Noteworthy is the fact that among angiomiRs there is a considerably big group of miRNAs, which so far have been demonstrated to regulate angiogenesis in a non-endothelial cell-autonomous manner; non-endothelial cells, such as tumor cells, express miRNAs which can regulate the expression of angiogenic factors or inhibitors, and thereby modulate angiogenesis in a paracrine fashion. One of the best examples is the miR-17~92 cluster, also named oncomiR-1, due to its tumor-promoting properties [267]. This gene cluster, consisting of miR-17-(3p/5p), miR-18a, miR-19a, miR-20a, miR-19b and miR-92a, is significantly upregulated in c-Myc-induced tumors and its overexpression in Ras-positive tumor cells enhances tumor vessel growth in a paracrine manner [268]. Reinforced neovascularization correlates with downregulation of anti-angiogenic THBS-1 and connective tissue growth factor (CTGF), which are predicated and experimentally validated targets for repression by miR-17~92 cluster [268]. Interestingly, members of miR-17 ~ 92 clusters exhibit a cell-intrinsic anti-angiogenic function in endothelial cells, indicating that they can act in a context-dependent manner to either promote or block angiogenesis [269].

**Table 1.** List of angiomiRs

Pro-angiogenic miRNAs	Biological function	Target	Ref.	
miR-126	Required for vascular integrity and angiogenesis <i>in vivo</i>	SPRED-1, PIK3R2, VCAM-1	264, 265, 284	
miR-130a	Antagonizes the inhibitory effects of homeobox genes GAX and HOXA5 on endothelial cell tube formation <i>in vitro</i>	GAX, HOXA5	275	
miR-296	Promotes tumor angiogenesis <i>in vivo</i> by HGS-mediated degradation of the growth factor receptors VEGFR2 and PDGFR $\beta$	HGS	274	
let-7f	Promotes angiogenesis <i>in vitro</i>	THBS1	259	
let-7b	Promotes angiogenesis <i>in vivo</i>	TIMP-1	285	
miR-27b	Promotes angiogenesis <i>in vitro</i>	ND	259	
miR-21	Promotes viral protein-induced endothelial cell migration	ND	277	
	Promotes tumor angiogenesis <i>in vivo</i> by AKT and ERK1/2-mediated activation of the HIF-1/ VEGF axis <sup>&amp;</sup>	PTEN	286	
miR-31	Promotes viral protein-induced endothelial cell migration	FAT4	277, 287	
miR-132	Promotes tumor angiogenesis <i>in vivo</i> by Ras-activation	p120RasGAP	288	
miR-210	Promotes angiogenesis and survival under hypoxic conditions <i>in vitro</i>	Ephrin-A3	271	
miR-424	Promotes angiogenesis under hypoxic conditions both <i>in vitro</i> and <i>in vivo</i>	CUL2	272	
miR-200a	Induces endothelial cell viability and migration <i>in vitro</i>	THBS-1	289	
miR-10b	Induces endothelial cell migration and tube formation <i>in vitro</i>	HOXD10	290	
miR-23~24~27 cluster	miR-23/ miR-27	Promotes angiogenesis and choroidal neovascularization <i>in vivo</i>	SPRY2, SEMA6A, SEMA6D	291
	miR-93	Promotes tumor angiogenesis <sup>&amp;</sup>	Integrin- $\beta$ 8	292
	miR-378	Promotes tumor angiogenesis <sup>&amp;</sup>	SUFU, TUSC2	293
miR-17~92 cluster	miR-17-5p	Promotes tumor angiogenesis <sup>&amp;</sup>	TIMP-1 TGF $\beta$ RII	285, 294
	miR-18a	Promotes tumor angiogenesis <sup>&amp;</sup>	CTGF, SMAD4	268, 294
	miR-19a	Promotes tumor angiogenesis <sup>&amp;</sup>	THBS1	268
	miR-20a	Promotes tumor angiogenesis <sup>&amp;</sup>	TGF $\beta$ RII	294

Anti-angiogenic miRNAs	Biological function	Target	Ref.	
miR-221	Impairs angiogenesis and endothelial cell migration <i>in vitro</i> Impairs viral protein-induced endothelial cell migration	c-Kit, ZEB2, ETS-2	262, 287, 295	
miR-222	Impairs angiogenesis and endothelial cell migration <i>in vitro</i> Impairs viral protein-induced endothelial cell migration	c-Kit, ETS-1	262, 287,	
miR-320	Inhibits proliferation and migration of endothelial cells derived from diabetic rats	IGF-1	278	
miR-34a	Impairs EPC-mediated angiogenesis <i>in vitro</i> by inducing EPC senescence and apoptosis	SIRT1	296	
miR-214	Inhibits endothelial cell migration and tube formation <i>in vitro</i>	eNOS	297	
miR-200b	Impairs angiogenesis under hypoxic conditions <i>in vitro</i> Impairs VEGF-mediated angiogenesis in diabetic retinopathy	ETS-1, VEGF	273, 298	
miR-100	Impairs endothelial cell proliferation and its inhibition stimulates angiogenesis <i>in vivo</i>	mTOR	299	
miR-101	Inhibits endothelial cell migration and tube formation <i>in vitro</i> Impairs tumor angiogenesis <i>in vitro</i> <sup>&amp;</sup>	EZH2	300, 301	
miR-503	Contributes to diabetes mellitus-induced impairment of endothelial function and reparative angiogenesis after limb ischemia	CCNE1, CDC25A	302	
miR-483-5p	Impairs angiogenesis under hypoxic conditions <i>in vitro</i>	SRF	303	
miR-15b/miR-16	Inhibit VEGF expression <sup>&amp;</sup>	VEGF	304	
miR-20b	Impairs HIF-dependent VEGF expression <sup>&amp;</sup>	HIF-1 $\alpha$	305	
miR-107	Impairs HIF-mediated tumor angiogenesis <sup>&amp;</sup>	HIF-1 $\beta$	306	
miR-519c	Impairs HIF-dependent tumor angiogenesis <sup>&amp;</sup>	HIF-1 $\alpha$	307	
miR-145	Impairs HIF-dependent tumor angiogenesis <sup>&amp;</sup>	p70S6K1	308	
miR-29b	Impairs tumor angiogenesis <sup>&amp;</sup>	MMP-2	309	
miR-125b	Impairs tumor angiogenesis <sup>&amp;</sup>	PIGF	310	
miR-17~92 cluster	miR-20a	Impairs VEGF expression <sup>&amp;</sup>	VEGF, HIF-1 $\alpha$	304, 311
	miR-92a	Impairs ischemia induced angiogenesis <i>in vivo</i>	ITGA5	312

Abbreviations: SPRED-1, Sprouty-related, EVH1 domain-containing protein 1; PIK3R2, Phosphatidylinositol 3-kinase regulatory subunit beta; VCAM-1, Vascular cell adhesion protein 1; GAX, Growth arrest-specific; HOXA5, homeobox A5; HGS, Hepatocyte growth factor-regulated tyrosine kinase substrate; THBS-1, Thrombospondin 1; TIMP-1, Tissue inhibitor of metalloproteinases 1; PTEN, Phosphatase and tensin homolog; FAT4, FAT tumor suppressor homolog 4; p120RasGAP, RAS p21 protein activator 1; CUL2, Cullin-2; HOXD10, Homeobox D10; SPRY2, Sprouty homolog 2; SEMA6A, Semaphorin-6A; SEMA6D, Semaphorin-6D; TGF $\beta$ RII, Transforming growth factor, beta receptor II; CTGF, Connective tissue growth factor; SMAD4, SMAD family member 4; SuFu, Suppressor of fused homolog; TUSC2, Tumor suppressor candidate 2; ZEB2, Zinc finger E-box-binding homeobox 2; ETS-2, v-ets erythroblastosis virus E26 oncogene homolog 2; ETS-1, v-ets erythroblastosis virus E26 oncogene homolog 1; IGF-1, insulin-like growth factor 1; SIRT1, Sirtuin 1; eNOS, Endothelial nitric oxide synthase; VEGF, Vascular endothelial growth factor; mTOR, mammalian target of rapamycin; EZH2, Histone-lysine N-methyltransferase; CCNE1, Cyclin E1; CDC25A, Cell division cycle 25 homolog A; SRF, serum response factor; HIF-1 $\alpha$ , hypoxia inducible factor 1, alpha subunit; HIF-1 $\beta$ , hypoxia inducible factor 1, beta subunit; p70S6K1, p70S6 kinase; MMP-2, matrix metalloproteinase 2; PIGF, placental growth factor; ITGA5, Integrin alpha-5  
ND - not determined

& - non-endothelial cell-autonomous effects of miRNAs

The elucidation of the regulation of miRNAs during pathophysiological processes is another important field of research, which may uncover further insights into the role of miRNAs in vascular biology. So far, little is known about the impact of a variety of factors on the expression profile of miRNAs in vascular cells. Hypoxia is one of the strongest inducers of vascular remodeling, and miRNA profiles of cancer cells revealed that at least a subgroup of miRNAs is induced by hypoxia, and HIF transcription factor seems to play a key role in the expression of hypoxia-regulated miRNAs (HRMs) [270]. Recent studies have extended this observation to endothelial cells showing that hypoxia triggers the expression of pro-angiogenic miR-210 [271] and miR-424 [272] as well as decreased levels of anti-angiogenic miR-200b [273]. In paper **III** of this thesis, we provide new insights into the influence of miRNAs on hypoxia-driven angiogenic activity of endothelial cells. Suárez *et al.* found that VEGF induces time-dependent expression of miR-191, -155, -31, -17-5p, -18a and miR-20a with little change in miR-126 and miR-222 expression [261]. Expression of miR-296 is elevated in human brain tumor-derived endothelial cells compared to normal brain endothelial cells. VEGF, EGF and conditioned medium from tumor cells are sufficient to upregulate miR-296 expression in endothelial cells [274]. Furthermore, miR-130a can be strongly upregulated by serum [275], the miR-17~92 cluster is directly activated by c-Myc [276], miR-21 and miR-31 are induced in endothelial cells exposed to viral protein K15 [277], and miR-320 is upregulated in myocardial microvascular endothelial cells from diabetic rats [278]. Shear stress is another important regulator of endothelial cell biology and has been shown to induce expression of miR-21 [279]. Additional level of miRNA regulation in endothelial cells may involve the shuttling of miRNAs from other cells, such as tumor cells, through secretion inside exosomes/microvesicles.

Indeed, several lines of evidence indicate that exosome/microvesicle-mediated intercellular transfer of functionally active miRNAs exists and operates between T cells and antigen-presenting cells [280], hepatocellular cancer cells and stromal cells [281], monocytes and endothelial cells [282], thereby modulating activities of acceptor cells. Moreover, miRNAs can be transferred between cells through other intercellular communication routes, such as gap-junctions, which have been recently shown to mediate import of microRNA from bone marrow stromal cells eliciting cell cycle quiescence in breast cancer cells [283].

Although more than 900 miRNAs have been identified in the human genome, a very small subset of miRNAs that regulate endothelial cell function and angiogenesis has been validated. Given the fact that angiomiRs elicit profound effects in endothelial cells, and when deregulated promote tumor angiogenesis and vascular diseases, *e.g.* atherosclerosis, it is of great importance to identify new angiomiRs and characterize their mechanisms of action as well as to recognize miRNA signatures of vascular diseases. These studies will undoubtedly characterize some miRNAs as important targets of therapeutic strategies to enhance neovascularization of ischemic tissue or to interfere with dysregulated vascular remodeling during tumor progression.

## Extracellular membrane vesicles

The development of multicellular organisms required the ability of cells to communicate with neighboring cells and cells residing in more distant parts within the organism. Over the course of evolution, several forms of intercellular communication have appeared, including (a) secretion of signaling molecules like hormones, cytokines, growth factors and lipid mediators that activate other cells locally or distantly through receptors or non-receptor mechanisms; (b) cell-cell contacts mediated by tight junctions, desmosomes, adherens junctions and gap junctions that are involved in intracellular signaling and the exchange of small molecules between cells; and (c) cell-ECM contacts through which cell surface receptors, integrins, regulate intracellular signal transduction pathways by binding to matrix components. This consensus on the modes of intercellular communication has been recently challenged by findings demonstrating membrane transfer as a new route of intercellular communication. The transfer of membrane components between donor and acceptor cells was first described in the study from 1973, in which it was observed that donor thymocytes could acquire host-derived MHC molecules [313]. Since then, many reports have established that portions of cell membranes can be transferred between cells either via direct cell-cell contacts, called tunneling nanotubes, or through long-range membrane exchange involving secreted membrane vesicles [314,315].

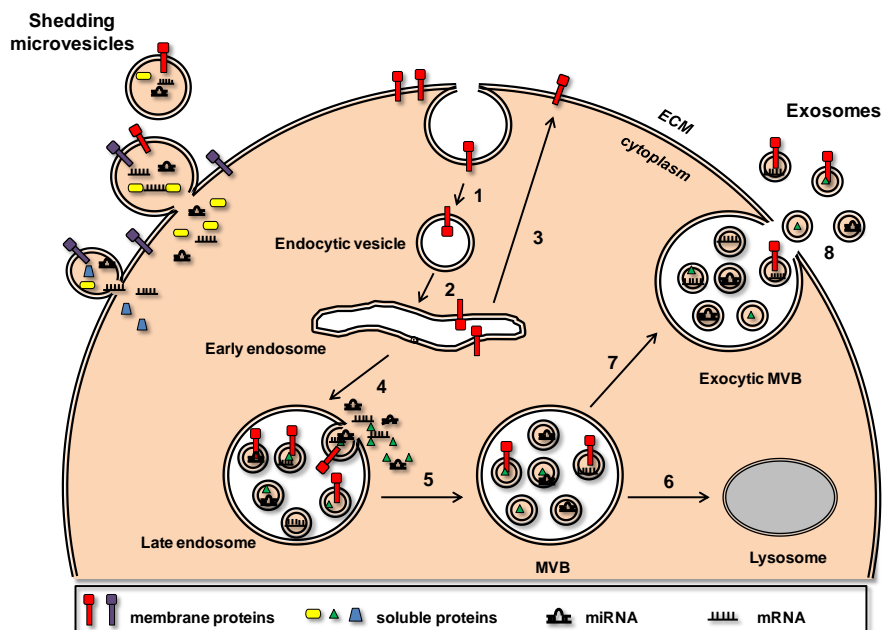
Vesicle secretion is a physiological phenomenon associated with cell growth and activation, and thereby most types of cells within the body are thought to utilize this mechanism [315]. Extracellular membrane vesicles (EMVs) are spherical structures that are composed of hydrophilic soluble components enclosed by a lipid bilayer of similar composition to that of cell membranes. Based on robust and detailed analyses, various types of EMVs, with distinct structural and biochemical properties depending on their intracellular site of origin, have been identified [315,316]. Current research interest in the field focuses primarily on two major types of extracellular vesicles, namely exosomes and shedding microvesicles (SMVs). Membrane vesicles are also released by dying and/or late apoptotic cells and are known as large apoptotic bodies [315]. Owing to the fact that these vesicles have different features to those derived from live cells, they are not a subject of further discussion in this introduction.

### Biogenesis of membrane vesicles

Exosomes are small vesicles (30-100 nm in diameter) of endocytic origin. First discovered in maturing mammalian reticulocytes, they were shown to be a mechanism for selective removal of many plasma membrane proteins, *e.g.* transferrin receptor [317]. Their formation starts with the endocytosis of proteins at the cell surface (see Figure 5). The endocytic vesicles are delivered to early endosomes, in which proteins can be recycled to the plasma membrane or

delivered to late endosomes. In late endosomes, proteins are sorted into intraluminal vesicles (ILVs) by inward budding of the limiting membrane into the endosomal lumen, forming MVBs. MVBs can either evolve into lysosomes for protein degradation or fuse with the plasma membrane, resulting in the release of the ILVs as exosomes [318]. Since exosome formation includes two inward budding processes, exosomes maintain the same topological orientation as the cell, with membrane proteins on the outside and cytosol in their lumen. The mechanisms underlying sorting of proteins and lipids into ILVs at the endosomal membrane are largely unknown. However, based on recent findings both monoubiquitinated and non-ubiquitinated proteins are recognized by the Endosomal Sorting Complex Required for Transport (ESCRT) machinery that together with some additional proteins mediates protein inclusion into MVBs [319,320]. Alternatively, some proteins have been shown to be sorted via ESCRT-independent and sphingolipid ceramide-mediated mechanism [321]. The secretion of exosomes can be spontaneous or induced depending on the cell type. Epstein-Barr virus (EBV)-transformed B cells, dendritic cells (DCs) and macrophages constitutively secrete exosomes *in vitro*, as do most tumor cell lines [322]. By contrast, reticulocytes, T cells, mastocytes and resting B cells secrete detectable levels of exosomes only following the activation of a cell surface receptor [322]. Additionally, deleterious treatments, such as radiation, increase the secretion of exosomes via p53 transcription factor-dependent expression of the transmembrane protein tumor suppressor-activated pathway 6 (TSAP6) [323].

SMVs constitute a heterogeneous population of vesicles larger than exosomes (100-1000 nm in diameter) that are shed from the plasma membrane (see Figure 5). SMVs were first described by Chargaff and West in 1946 as a precipitable factor in platelet free plasma with ability to generate thrombin [324]. Conversely to exosomes, the rate of steady state release of SMVs is generally low, except for tumor cells that release them constitutively [322]. Regulated release of vesicles is efficiently induced upon activation of cell surface receptors or apoptosis. In accordance, SMV release was observed in platelets following thrombin receptor activation [325], in monocytes and neutrophils following ATP-mediated activation of P2X<sub>7</sub> receptor [326] and in DCs upon lipopolysaccharide (LPS) stimulation of Toll-like receptor 4 [327]. In response to cell activation, intracellular levels of Ca<sup>2+</sup> increase and trigger changes in asymmetric phospholipid distribution of the plasma membrane, characterized by increased levels of phosphatidylserine (PS) and phosphatidylethanolamine (PE) on the outer surface of plasma membrane [316,328,329]. Additionally, Ca<sup>2+</sup> activates cytosolic proteinases, such as calpain and gelsolin, leading to disruption of cortical actin cytoskeleton, which in turn allows membrane blebbing [316,330,331]. Following blebbing of the plasma membrane, fission of the plasma membrane stalk detaches the cytoplasmic protrusions, resulting in the formation of SMVs [332]. As in the case for exosomes, the sorting mechanisms for inclusion of cargos into SMVs remain obscure.



**Figure 5. Biogenesis and release of extracellular membrane vesicles into the extracellular space.** The formation of exosomes starts with the endocytosis of membrane proteins at the cell surface (1), which are then delivered to early endosomes (2). In early endosomes, proteins are either recycled to the plasma membrane (3) or delivered to late endosomes (4). In late endosomes, the endocytosed membrane-bound cargo as well as cytoplasmic proteins and mRNAs/miRNAs are sequestered in intraluminal vesicles (ILVs), formed by budding of endosome membrane into the lumen of endosome. As a consequence, multivesicular body (MVB) occurs (5), which depending on biophysical properties can either evolve into lysosome for protein degradation (6) or the exocytic MVB, fusing its membrane with the plasma membrane (7) and thereby releasing ILVs into extracellular space as exosomes (8). Secreted membrane vesicles may also form at the plasma membrane by direct budding off into the extracellular space, giving rise to shedding microvesicles with the same topology as exosomes, however with different composition. Abbreviations: ECM, extracellular matrix; miRNA, microRNA.

## Composition of membrane vesicles

Owing to extensive analyses by various techniques, the molecular composition of EMVs has become partially decoded. Both the membrane molecular pattern and the internal content of the vesicle seem to depend on the mechanism of EMV release. In case of exosomes, all proteins analyzed so far originate from the endosomes, plasma membrane and the cytosol. Consistent with their endosomal origin, exosomes do not contain any nuclear, mitochondrial, Golgi or endoplasmic reticulum proteins. Exosomes contain common protein families such as chaperones (Hsp70 and Hsp90), cytoskeletal proteins (tubulin, actin), ESCRT proteins (Tsg101 and Alix), annexins, Rab proteins as well as tetraspanins (CD9, CD63, CD81 and CD82) [315,318,322]. The composition of SMVs is not as



thoroughly analyzed as for exosomes, however, this population of vesicles seems to be enriched with ADP-ribosylation factor 6 (ARF6), vesicle associated membrane protein 3 (VAMP3), integrin  $\beta$ 1, MMPs, selectins and CD40 ligand [322,333]. Besides the wide spectrum of commonly displayed proteins, both exosomes and SMVs carry some cell-specific proteins which reflect to some extent the proteome of the cells from which they originate. Accordingly, exosomes secreted from antigen presenting cells, such as B lymphocytes and dendritic cells, contain MHC-I, MHC-II and co-stimulatory molecules [322], tumor cell-derived vesicles carry mutant EGFRvIII [334], while exosomes released from reticulocytes harbor transferrin receptor [317]. In case of lipid composition, both exosomes and SMVs have been shown to expose negatively charged PS and PE molecules on the outer surface of the membrane as well as to contain cholesterol-rich lipid rafts [315,322]. Interestingly, in addition to proteins and lipids, nucleic acids, such as mRNAs and miRNAs, have been found to be present in EMVs [316].

## Functions of membrane vesicles

Functions of EMVs appear to depend on the cell type from which they originate and on conditions under which they are released, as this confers them specific molecular composition. Exosome release was initially characterized as a mechanism to eliminate proteins during reticulocyte maturation and differentiation [317]. Reticulocytes do not contain lysosomes, and it seems as if exosome release is an alternative to lysosome degradation. Moreover, a recent study by Ohshima *et al.* demonstrated that tumor cells may discard tumor-suppressor let-7 miRNA via exosomes into the extracellular environment to maintain their oncogenesis [335]. In addition to removal of obsolete proteins and nucleic acids, exosomes and SMVs have been shown to function as a secretory pathway for numerous molecules, such as tumor necrosis factor (TNF) receptor, interleukin (IL)-1 $\beta$  and CD44 [315,336]. The release of EMVs also provides another mechanism of intercellular communication. Upon release from their cells of origin, EMVs may circulate in the extracellular space adjacent to the site of the discharge, where they can target neighboring cells. Alternatively, some of them can diffuse over longer distances and appear in biological fluids, such as cerebrospinal fluid, blood and urine, indicating systemic effects of EMVs. EMVs may influence the biology of target cells in several ways. Many vesicles do not remain intact in the extracellular space for long, but are instead broken, releasing their cargo, such as MMPs and growth factors. Alternatively, they can interact with cell membrane receptors, initiating a signaling cascade. Furthermore, EMVs can deliver surface receptors, cytosolic proteins and nucleic acids by ligand-receptor mediated internalization of whole vesicles into endocytic compartments or by fusion of vesicles with the plasma membrane [322,333].

Based on current knowledge, EMVs play essential roles as mediators of intercellular communication under many physiological conditions. Pattern

formation in developing *Drosophila* tissues occurs in response to gradient of morphogens, such as Wingless and Hedgehog. Interestingly, Wingless is associated with exosomes, which released from producing cells as argosomes function to spread Wingless protein gradient by multiple transcytosis events [337]. Postnatal processes are also highly modulated by the activity of EMVs. Pioneering studies by Raposo *et al.* showed that exosomes secreted by EBV-transformed B cells can stimulate human CD4+ T-cell clones in an antigen specific manner [338]. This discovery has spurred intensive research, which established the role of EMVs in the regulation of immune response. Vesicles secreted from various cellular sources have numerous activating effects on immune cells, including direct peptide-MHC complex presentation to T-cells, antigen and/or peptide-MHC complex transfer to DCs leading to indirect antigen presentation, maturation of DCs as well as activation of NK, B cells and macrophages [322]. Vesicles derived from different cells have also immunosuppressive properties. In support of this notion, trophoblast cells, in order to protect the fetus, secrete membrane vesicles, which carrying FasL and HLA-G in their membrane induce apoptosis of the maternal cytotoxic T-cells and the immunosuppressive effect against NK cells, respectively [316]. It has been estimated that platelet derived EMVs constitute approximately 80% of the EMV population in the plasma of humans [316]. These EMVs have been shown to be released by activated platelets and to evoke pro-coagulant activities in several ways. Most importantly, platelet-derived EMVs carry tissue factor (TF), *i.e.* an initiator of coagulation, and provide negatively charged phospholipids, efficiently binding coagulation factors and thus enabling their further activity [316,339]. SMVs released by various cell types are also known to participate in inflammation. Accordingly, vesicles shed by neutrophils may either stimulate the release of anti-inflammatory factors, such as TGF $\beta$ -1 and IL-10, from macrophages or stimulate the pro-inflammatory response by mediating the transfer of chemokine receptors, such as CCR4 and CCR5 [316,333]. Based on recent studies, exosomes and SMVs may be considered as shuttles for nucleic acids, such as mRNAs and miRNAs, which delivered to target cells may be translated and sequestered to complementary sequences within 3'UTR regions, respectively, and thereby provide the recipient cells with new functions [334,340,341]. Finally, in accordance with their shuttling properties, exosomes have been demonstrated to act as vehicles spreading infectious agents, including viruses and prion proteins, among different cells [342,343].

## **Membrane vesicles in cancer progression**

Solid tumors are regarded as multicellular, invasive organisms highly dependent on effective communication between neoplastic cells and numerous stromal cells infiltrating the interstitium. In this context, EMVs may provide intercellular communication pathway, which successfully synchronizes the activities of numerous cells within the tumor microenvironment, and thereby supports tumor

progression. The association of EMVs with cancer was already noticed in the late 1970s, when vesicles were identified in cultures of spleen nodules and lymph nodes of a patient with Hodgkin disease [344]. Since then, numerous studies have shown elevated levels of circulating vesicles in peripheral blood of cancer patients [345,346]. Interestingly, in case of gastric cancer, increased numbers of circulating vesicles have been correlated with increased metastasis and worse prognosis [347]. The underlying mechanism leading to increased secretion of EMVs from tumor cells is still ill-defined. A recent report suggests that prostate cancer cells release exosomes as a response to radiation-induced senescence through p53-dependent mechanisms [348]. In addition, cell transformation [349] and hypoxia treatment [350] have been shown to increase EMV secretion. Based on current knowledge, EMVs are implicated in most hallmarks of cancer, including evasion of apoptosis and immune surveillance, angiogenesis, invasion and metastasis.

Growing body of evidence clearly indicates that EMVs may evoke acquisition of aggressive cancerous phenotypes and even transformation in recipient cells, through horizontal transfer of oncogenic receptors and other molecules. Accordingly, SMV-mediated transfer of a truncated, constitutively active form of EGFR, EGFRvIII, from aggressive glioma cells to non-aggressive population of glioma cells resulted in morphological transformation and an increase in anchorage-independent growth [349]. Furthermore, a recent study by Antonyak *et al.* demonstrated that SMVs shed from breast cancer and glioma cells are capable of conferring onto normal fibroblasts and epithelial cells the transformed characteristics of cancer cells, *e.g.* anchorage-independent growth and enhanced survival capability. Detailed analysis revealed that this effect requires SMV-mediated transfer of the protein cross-linking enzyme tissue transglutaminase (tTG) together with its binding partner and cross-linking substrate fibronectin (FN) [351].

In the context of the present thesis work, it is most notable that tumor and stromal cells may utilize membrane vesicles in order to communicate with endothelial cells and induce their pro-angiogenic activities. Al-Nedawi and colleagues showed that SMVs produced by human glioma cells, harboring activated EGFR, can be taken up by cultured endothelial cells, in which they elicit EGFR-dependent responses, including activation of MAPK and AKT pathways. This transfer was also accompanied by increased expression of VEGF and its receptor VEGFR2 [352]. Tumor-derived EMVs have also been shown to stimulate angiogenesis by delivering DLL4 into endothelial cells [353] or by inducing expression of matrix-degrading enzymes, MMPs, in endothelial cells [354]. Tissue factor (TF)-bearing vesicles, which are released by platelets and tumor cells, play important role in tumor angiogenesis. It has been recently shown that glioma cell-derived TF-bearing SMVs may bind to and thus activate protease-activated receptor (PAR)-2 on hypoxic endothelial cells, leading to expression of the potent angiogenic molecule, heparin binding-EGF (HB-EGF), in endothelial cells [355]. TF-bearing vesicles may also evoke pro-angiogenic activities by inducing

coagulation and fibrin formation. Fibrin matrix not only supports the outgrowth of new blood vessels but also activates cells via cleavage of PARs and thus induces expression of VEGF [356]. Lipids from EMVs can impact endothelial cell migration and angiogenesis. In this regard, vesicle-associated sphingomyelin shed from fibrosarcoma cell line, together with VEGF, was shown to confer migratory properties to endothelial cells [357]. Besides proteins and lipids, vesicle-mediated transfer of mRNAs and miRNAs has also been demonstrated to modulate the translational profile of endothelial cells and thereby promote acquisition of an angiogenic phenotype [334,358].

Degradation of ECM is essential for tumor invasion and metastasis, and is mediated by a variety of matrix-degrading enzymes. Tumor-derived EMVs have been demonstrated to mediate tumor cell invasiveness by harboring MMPs, such as MMP-2 and MMP-9, their zymogens, EMMPRIN (an inducer of MMPs) and urokinase-type plasminogen activator (uPA) [354,356]. Upon lysis of EMVs in acidic tumor environment, all these proteins become released and evoke matrix remodeling. MMPs degrade basement collagens, whereas uPA catalyzes the conversion of plasminogen into plasmin, a protease that facilitates the activation of MMP zymogens as well as degradation of matrix components, such as fibrin. Furthermore, it has been recently shown that macrophages regulate the invasiveness of breast cancer cells through exosome-mediated delivery of oncogenic miRNA [359]. The procoagulant properties of cancer cell-derived EMVs may contribute to haematological spread and metastasis of cancer cells by activation of platelets and intravascular fibrin formation, which collectively facilitate adherence of cancer cells to the vessel wall [356]. Finally, several lines of evidence indicate that EMVs contribute to dissemination and metastasis by molding the pre-metastatic niches in lymph nodes and distant organs [358,360].

## **Membrane vesicles as biomarkers**

As EMVs can be detected in biological fluids, such as blood, urine and ascites, they could potentially serve as easily accessible biomarkers in the management of cancer patients. Tumor-specific markers that are exposed on circulating EMVs might be useful as prognostic biomarkers. Indeed, a pilot study by Smalley *et al.* identified eight proteins that were expressed at higher levels in urine-derived EMVs from cancer patients compared with controls, indicating that EMVs can potentially be used for the early detection of bladder cancer [361]. Similarly, cancer-specific mRNAs and miRNAs can be applied as markers for recognition of cancer. Cancer specific mRNA, encoding mutant EGFRvIII, was observed in EMVs purified from serum samples of GBM patients [334], whereas cancer specific miRNAs were identified in EMVs isolated from plasma samples of ovarian cancer patients [346]. In addition, the protein composition of EMVs seem to reflect molecular changes in tumor cells from which they are derived, and therefore can potentially serve as a predictor of disease stage, survival and efficacy

of treatment. In support of this notion, miRNA profile of EMVs isolated from patients with malignant ovarian cancer was significantly different from the profile observed in patients with benign disease [346]. Different studies have also evaluated the association between the levels of EMVs and disease stage or survival of cancer patients. In a study by Kim *et al.* levels of platelet-derived EMVs in plasma of gastric cancer patients correlated with the stages of the disease and predicted distant metastasis [347].

# Present investigation

## Aims of study

The overall aim of this thesis was to unravel basic mechanisms of endothelial cell biology and angiogenesis, and thereby identify potential new targets of anti-angiogenic therapies in the fight against cancer.

Specific aims of the included papers were:

- I.** To elucidate the role of polyamines in the signaling machinery underlying endothelial cell survival under hypoxic conditions.
- II.** To reveal the role of polyamines in the mechanisms governing endothelial cell migration.
- III.** To investigate the effects of acute and chronic hypoxic conditions on the microRNA profile of endothelial cells, and the involvement of miRNA-424\* in endothelial cell function.
- IV.** To study the role of GBM cell-derived exosomes in hypoxia-mediated tumor angiogenesis and to evaluate the possibility of using plasma-derived EMVs as biomarkers of hypoxic signaling activation in GBM tumors.

## Methods

Below is given the list of methods used in papers I-IV of the present thesis. For a detailed description, please see the corresponding ‘Materials and Methods’ section of the respective paper.

- Differential centrifugation for the isolation of EMVs from cell culture media and mouse and human blood plasma (paper IV)
- Electron microscopy (paper IV)
- *Ex vivo* mouse aortic ring angiogenesis model (papers II and IV)
- Flow cytometry (paper IV)
- Fluorescence and confocal microscopy imaging (papers II and IV)
- Immunoblot assay (papers I, II, III and IV)
- *In vitro* cell adhesion and migration assay (papers II and III)
- *In vitro* endothelial cell tube formation on Matrigel (papers I, III and IV)
- *In vitro* proliferation assays (papers III and IV)
- *In vitro* survival assays (papers I, III and IV)
- *In vivo* xenograft tumor model (paper IV)
- Laser capture microdissection (LCD) (paper IV)
- Measurement of polyamine levels (paper II)
- MicroRNA and gene expression arrays (papers III and IV)
- Quantitative real-time polymerase chain reaction (qRT-PCR) (papers III and IV)
- RNA interference (papers I and III)

# Results

## **PAPER I - The polyamines regulate endothelial cell survival during hypoxic stress through PI3K/AKT and MCL-1.**

**Introduction** - Hypoxia, *i.e.* decreased oxygen tension, is an inherent feature of solid tumors and is the driving force of the angiogenic process. Initial steps of hypoxia-triggered angiogenesis include endothelial cell survival, migration and proliferation, which have been shown to be regulated by many cytokines and growth factors. Endothelial cell apoptosis seems to be necessary during early stages of neovascularization and several mechanisms of hypoxia-induced apoptosis have been proposed [362,363]. Polyamines are essential for cell proliferation and transformation with implications in cancer and inflammatory disease. Depending on the cytotoxic stimulus and cell type studied, polyamines have been shown to both promote and prevent apoptosis, through several signaling pathways, *e.g.* AKT, Src, ERK1/2 and Jun-N-terminal kinase [364-366]. However, the role of polyamines in the regulation of endothelial cell survival is still poorly characterized. In this study, we decided to explore how polyamines may modulate the effects of hypoxia on endothelial cell survival.

**Results** - Polyamine depletion in primary endothelial cells by specific inhibition of ODC with DFMO was, somewhat unexpectedly, shown to promote endothelial cell survival during hypoxic stress. Depletion of endogenous polyamines resulted in a significant induction of the PI3K/AKT pathway and its downstream target MCL-1, *i.e.* an anti-apoptotic member of the BCL-2 family. Specific inhibitor of PI3K reversed the decrease of hypoxia induced apoptosis in DFMO-treated cells by preventing the increased levels of MCL-1. Moreover, siRNA-mediated downregulation of MCL-1 counteracted the protective effect of polyamine inhibition.

We provide new insights into the mechanisms of hypoxia-mediated cell-death in endothelial cells and show that the polyamines regulate hypoxia-induced apoptosis in these cells through PI3K/AKT and MCL-1 dependent pathways.

## **PAPER II - Ornithine decarboxylase and extracellular polyamines regulate microvascular sprouting and actin cytoskeleton dynamics in endothelial cells.**

**Introduction** - As growth promoting polycations, polyamines are indispensable for neoplastic transformation and are associated with increased proliferation, decreased apoptosis and upregulated expression of genes involved in tumor invasion and metastasis. As such, the polyamine system is an attractive target for anti-cancer therapy. Intracellular depletion of polyamines by DFMO has been shown to inhibit tumor growth in numerous animal models and this activity has largely been attributed to its direct inhibitory effect on the tumor cell proliferation.



However, *in vivo* studies have suggested that DFMO may cause retardation of tumor-associated angiogenesis [219,222]. This indicates that polyamines may have an important role in neovascularization. The purpose of this study was to investigate how polyamines may be involved in microvascular sprouting and endothelial cell migration, *i.e.* early and crucial steps of angiogenesis.

**Results** - DFMO-mediated polyamine depletion was shown to almost completely inhibit microvascular sprouting angiogenesis in the *ex vivo* mouse aortic ring assay, and this phenomenon occurred to be partially evoked by the attenuation of endothelial cell migration. DFMO inhibition was reversed by extracellular polyamine supplementation, showing that anti-angiogenic effects of DFMO were specifically related to polyamine levels. Confocal microscopy of phalloidin-stained cells revealed that polyamine depletion was associated with an abnormal morphology of the actin cytoskeleton in migrating endothelial cells. In accordance with the data from paper I, DFMO-treated endothelial cells were shown to exhibit upregulation of the PI3K/AKT pathway and increased phosphorylation of its downstream target GSK-3 $\beta$ . However, studies with specific inhibitors of PI3K and GSK-3 did not reverse the inhibitory effect of DFMO on migration in endothelial cells. Defects in migration of polyamine-depleted endothelial cells may instead be related to constitutive activation of the small GTPase CDC42, *i.e.* a well-known regulator of cell motility and actin cytoskeleton remodeling.

Our results indicate the importance of the polyamine system in sprouting angiogenesis and provide some mechanistic insights into polyamine-mediated endothelial cell migration.

### **PAPER III - Hypoxia upregulates microRNA-424\* to modulate the pro-angiogenic response of endothelial cells.**

**Introduction** - Hypoxia-driven angiogenesis is a crucial biological response that accompanies various physiological and pathological conditions, such as embryonic development, wound healing and tumor progression [102]. During the initial steps of this process, endothelial cells respond to low oxygen levels by the induction of a complex program of signaling pathways, which encompass HIF-dependent transcriptional activation of many genes, leading to modulation of endothelial cell biology. Although this response has been extensively characterized, little is known about the role of microRNAs in the modulation of endothelial cell response to hypoxia. In this study, we have investigated how miRNAs may respond to hypoxia and regulate the angiogenic activities of endothelial cells.

**Results** - Culture of endothelial cells under conditions of both acute (6 hours) and chronic (48 hours) hypoxia resulted in significant changes in the expression of a subset of miRNAs. Specifically, miR-424\* levels occurred to progressively increase up to 24 h in a HIF1 $\alpha$ -dependent manner upon exposure to hypoxia, as a mechanism supporting endothelial cell adaptation to hypoxia. Endothelial cells

overexpressing miR-424\* exhibited increased angiogenic activity, whereas miR-424\*-depletion led to inhibition of endothelial cell function, as evidenced by the formation of capillary-like structures, migration and survival. Using an *in silico* approach, we found that miR-424\* potentially downregulates the expression of molecules known to negatively regulate cell survival, migration and pro-angiogenic signaling pathways, such as Ras/Raf-1/ERK and Wnt. Interestingly, PTX-3, *i.e.* an inhibitor of FGF2-driven angiogenesis, was validated at the protein level as a target regulated by miR-424\*.

In conclusion, preliminary data suggest that hypoxia is a potent modulator of miRNAs in endothelial cells, and one of them, miRNA-424\*, seems to have a functional role in hypoxia-dependent pro-angiogenic activity of endothelial cells.

#### **PAPER IV - Exosome vesicles constitute a novel mediator of hypoxic signalling in glioblastoma.**

**Introduction** - The progression of cancer is highly dependent on hypoxia-driven angiogenesis. Extensive studies over the last decades have contributed to the understanding of the angiogenic process, and have led to the introduction of inhibitors of angiogenesis in the treatment of cancer [136,137]. Although clinical data show significant effects on survival and disease relapse in certain groups of cancer patients, the great expectations from preclinical studies have not been fulfilled. This reveals the complexity of the angiogenic process but also the lack of predictive markers of anti-angiogenic treatment allowing pre-selection of responsive patients at the start of therapy or at an early stage of treatment. Recent discovery of extracellular membrane vesicles as mediators of intercellular communication has spurred research aiming at unravelling the role of these vesicles in tumor progression. Several types of tumors have been shown to secrete exosomes, and it has been suggested that exosomes can harbor complex biological information consisting of mRNAs, miRNAs as well as soluble and trans-membrane proteins that, altogether, may elicit a multifaceted, biological response in adjacent cells and potentially also at the systemic level [315,316].

We hypothesized that exosomes constitute a novel player in hypoxic signaling activation of cancer cells, and that intercellular transfer of EMVs may elicit a hypoxic, pro-angiogenic response in these cells.

**Results** - Extensive analyses of GBM cells and exosomes from normoxic and hypoxic conditions revealed that hypoxia substantially modulates the molecular composition and activity of GBM cell-derived exosomes. Accordingly, exosomes derived from hypoxic GBM cells contained higher levels of several hypoxia-regulated mRNAs (ADM, LOX, IGFBP-3, BNIP3, NDRG-1, PLOD-2, and SERPINE-1) and proteins (*e.g.* IL-8), which collectively made them more potent at stimulating tumor growth, microvessel sprouting, as well as the proliferation and survival of primary endothelial cells than exosomes derived from normoxic

cancer cells. Interestingly, hypoxic GBM cell-derived exosomes may modulate the tumor microenvironment through several mechanisms, as supported by increased pericyte coverage of GBM tumor vessels. Furthermore, several hypoxia-induced exosomal mRNAs were upregulated in hypoxic GBM tumor regions, and over-expression of these mRNAs significantly correlated with poor GBM patient outcome. Given the fact that the molecular profile of GBM cell-derived exosomes showed a close overlap with that of donor cells, we decided to explore the possibility of isolating circulating vesicles from plasma of GBM tumor-bearing mice and pre-operative GBM patients. Could exosomes be a non-invasive source of circulating biomarker of the hypoxic signalling status of the tumor? Interestingly, plasma-derived exosomes were successfully isolated from both sources, and preliminary data, showing increased levels of IL-8, NDRG-1 and ADM proteins in vesicles from tumor-bearing patients compared to vesicles from healthy control specimens, support the possibility of using circulating exosomes as markers of tumor hypoxia and aggressiveness.

In conclusion, exosomes may constitute a novel, potentially targetable mediator of hypoxia-driven tumor development, and the hypoxic exosomal signature may serve as a non-invasive biomarker to assess the oxygenation status and aggressiveness of GBM tumors.

## Conclusions and discussion

In this thesis, the role of polyamines, miRNAs and tumor cell-derived exosomes in hypoxia-dependent endothelial cell biology has been studied and partially revealed. Below is given a brief summary of major conclusions and their implications in therapeutic intervention of cancer.

### **Polyamines are true modulators of endothelial cell functions (papers I and II)**

The anti-tumor activity of polyamine depletion secondary to DFMO treatment has generally been attributed to its anti-proliferative effects on malignant cells. However, a few studies in tumor animal models presented the idea that this effect may also result from retardation of tumor-associated angiogenesis [219,222], reflecting an essential role of polyamines in the function of non-malignant cells, such as endothelial cells. In spite of the effects of DFMO treatment on tumor angiogenesis, very limited data on the role of polyamines in endothelial cell biology have been presented. So far, it has only been shown that polyamines are necessary for endothelial cell proliferation [367]. In papers **I** and **II** we provide data broadening the spectrum of angiogenic activities of polyamines, and demonstrate for the first time that the polyamine system is a modulator of endothelial cell survival and migration, respectively. Whereas the anti-migratory effect of polyamine-depletion in endothelial cells (paper **II**) may partially explain the anti-angiogenic effects of DFMO treatment in animal tumor models obtained by other research groups, the pro-survival effect of polyamine depletion in hypoxic endothelial cells (paper **I**) seems not to be in line with these data. This could be explained in several ways. According to previous studies, polyamines can either promote or prevent apoptosis in the same type of cells depending on the nature of the apoptotic trigger [366]. Hence, it is fair to speculate that polyamine depletion in endothelial cells may actually elicit pro-apoptotic effects in response to other stimuli within the tumor microenvironment, such as tumor cell and stromal cell-derived factors as well as acidosis, *i.e.* a condition resulting from hypoxic metabolism. Alternatively, tumor associated endothelial cells, which are known to differ from normal counterparts, may become sensitized to hypoxia-mediated apoptosis in the absence of intracellular polyamines. Moreover, it is possible that the anti-migratory effect of DFMO on endothelial cells may be more potent and overcome the anti-apoptotic effect of DFMO on tumor angiogenesis. Finally, it is well established that blood vessel development is accompanied by vessel regression, which is essential for the removal of redundant and harmful vessels and highly dependent on apoptosis. As such, decreased pro-apoptotic potential of polyamine-depleted endothelial cells might actually result in aberrant angiogenesis explaining the anti-angiogenic effects of DFMO in animal tumor models. In support of this notion, studies by Segura I *et al.* [368] and Masri F *et al.* [369] have shown that endothelial cells with diminished pro-apoptotic properties

were impaired in tube formation activity. Further studies are required to unravel the specific role of polyamines in endothelial cell survival, depending on the nature of the angiogenic process (physiological vs pathological), the type of endothelial cell (endothelial cell of physiological or pathological vessel or even endothelial progenitor cells) and the type of apoptotic stimuli. From a more general cell biology point of view, it is interesting to conclude that the induction of PI3K/AKT signaling as a result of polyamine depletion can feed into both pro-survival and anti-migratory pathways. Whether this is a general phenomenon of PI3K/AKT induction in endothelial cells, or a more specific effect in the context of polyamine depletion, remains to be investigated.

In spite of the disappointing anti-tumor effects of DFMO in clinical trials, this compound is now clinically tested as a chemopreventive agent in multiple epithelial cancers [230], and preliminary results support the preventive role of DFMO in the development of colorectal and skin cancers [179]. Similarly to anti-angiogenic effects of polyamine depletion in animal tumor models, the chemopreventive effects of DFMO may result not only from direct effect of DFMO on premalignant cells but also may involve the anti-proliferative and anti-migratory effects of DFMO in endothelial cells (paper II), eliciting inhibition of the angiogenic switch, an early event in carcinogenesis.

Taken together our results motivate further *in vivo* studies testing the anti-vascular effects of polyamine inhibition in combination with established anti-angiogenic therapies for both chemopreventive and anti-tumor purposes. Moreover, mounting evidence strongly suggests that vascular endothelial cells are impaired through apoptosis, contributing to the overall endothelial dysfunction in a range of clinic settings, including ischemic heart diseases. Therefore, preventing endothelial cell apoptosis under hypoxic conditions, as shown in paper II, could result in improved angiogenesis and endothelial function in patients with vascular diseases.

### **Hypoxia is a potent modulator of miRNAs in endothelial cells with potential roles in hypoxia-driven tumor angiogenesis (paper III)**

Hypoxia is a driving force of physiological and pathological angiogenesis, *e.g.* in tumor development, during which endothelial cells are exposed to low oxygen levels. In spite of growing body of research elucidating the role of HIF-activated genes in the modulation of endothelial cell biology, there is very limited knowledge about the role of miRNAs as regulators of endothelial cell response to hypoxia. In paper III of this thesis, we demonstrate that hypoxia is a potent modulator of miRNAs in endothelial cells, and depending on the duration of hypoxia, different groups of miRNAs are up- and downregulated in comparison to normoxic endothelial cells. This data is in agreement with the study by Kulshreshtha *et al.* [270], which shows profound modulation of miRNAs by hypoxia in different cancer cell lines. Interestingly, miRNA-210 is the only

common hypoxia-target of cancer cells and endothelial cells. This would indicate that miRNA-210 may be a master regulator of the hypoxic response in many types of cells, whereas other miRNAs may confer cell type specific responses to hypoxia. Alternatively, given the fact that different miRNAs may target the same mRNA 3'UTR, different types of cells expressing tissue specific miRNAs may respond to hypoxia in a similar manner. Further studies are required to unravel the role of individual hypoxia-regulated miRNAs in endothelial cell biology. Of note, the microarray-based strategy leaves open the possibility that other microRNAs may respond to hypoxia and were not detected by this screen. Therefore other strategies, such as deep sequencing, should be considered in further search of miRNAs with a substantial role in the hypoxic response of endothelial cells.

In paper **III** of this thesis, we demonstrate that hypoxia-regulated miRNAs seem to be of important value for endothelial cells under hypoxic conditions. One of them, miRNA-424\*, appears to have a functional role in hypoxia-dependent pro-angiogenic activity of endothelial cells, as revealed by increased formation of capillary-like structures, migration and survival of endothelial cells overexpressing miRNA-424\*. Moreover, using an *in silico* approach, we found that miR-424\* potentially downregulates the expression of molecules known to negatively regulate cell survival, migration and pro-angiogenic signaling pathways. However, further studies are necessary to validate these targets and unravel their role in miRNA-424\*-mediated pro-angiogenic activity of endothelial cells exposed to hypoxia. This data together with a recently published study by Ghosh *et al.* [272] showing hypoxia-mediated induction of miR-424 specifically in endothelial cells, indicate that the miR-424 gene, encoding both mature and star (\*) sequence, is an endothelial cell specific modulator of hypoxia-induced angiogenesis. More importantly, this data implicates miRNA-424\* and its mature counterpart as potential targets of anti-angiogenic therapeutical interventions in the management of cancer patients. The advantage of using miRNA-based strategies over the current anti-angiogenic therapies stems from the fact that miRNA therapeutics provide a natural means of normalizing the expression of several genes simultaneously, which may potentially avoid the toxicity or drug resistance caused by switching a single target on/off. The use of miRNA-based therapies as anti-angiogenic strategy for cancer treatment has been recently supported by a study by Anand *et al.* [288], in which the *in vivo* delivery of anti-miRNA-132-containing nanoparticles targeted to integrin  $\alpha_v\beta_3$ , resulted in substantial reduction of miRNA-132 levels in activated tumor vessels, followed by decreased tumor angiogenesis and tumor growth in mice. In spite of this encouraging pre-clinical data, there are potential challenges for angiomiR therapeutics, which include the efficiency and specificity of the delivery system, and the currently incomplete understanding of the biology of individual miRNAs. The latter can be illustrated by mir-17~92 cluster that is known to exert pro-angiogenic activities when overexpressed in tumor cells, and evoke cell-intrinsic anti-angiogenic effects in endothelial cells [268,269]. This underlines the complexity of miRNA-mediated

regulation of angiogenesis and the necessity of further studies unraveling the role of miR-424\* and its mature sequence in tumor cell biology as well as in *in vivo* models of tumor angiogenesis.

### **Tumor-cell derived exosomes as mediators of hypoxia-induced pro-angiogenic responses (paper IV)**

In paper **IV** of this thesis, a novel role of glioma-cell derived exosomes in the adaptive response to hypoxic stress is demonstrated. Tumor angiogenesis is essential for primary tumor development and metastasis, and is to a large extent driven by hypoxia-mediated signaling. According to the generally accepted view, hypoxic tumor cells secrete a plethora of soluble factors, *e.g.* VEGF-A, into the extracellular space, which collectively activate endothelial cells and thereby induce neovascularization. Based on the data presented in paper **IV**, this picture becomes enriched with hypoxic tumor-cell derived exosomes, which contain high levels of hypoxia-induced, pro-angiogenic proteins and mRNAs, and thus evoke stronger pro-angiogenic activity compared to exosomes from normoxic cells. Due to the fact that all the components of secreted vesicles, such as lipids, proteins, mRNAs and miRNAs [315,316,322], have been shown to activate the recipient cells, it is conceivable that the strong pro-angiogenic activity of hypoxic exosomes is the net result of a multifaceted and complex signaling response. Given the fact that stromal cells, such as fibroblasts and bone-marrow derived myeloid cells, constitute an important compartment of the tumor microenvironment mediating tumor angiogenesis [17,29], it would be of great interest to further investigate the impact of hypoxia on the composition of EMVs derived from these cells.

Interestingly, hypoxic GBM cell-derived exosomes may modulate the tumor microenvironment through several mechanisms. Apart from inducing strong pro-angiogenic effects in endothelial cells both *in vitro* and *in vivo*, hypoxic exosomes were demonstrated to stimulate pericyte-mediated tumor vessel coverage (paper **IV**). Ongoing studies in our lab are focused on unraveling the mechanisms underlying this phenomenon. It is well established that pericyte recruitment and attachment to forming vessels is mediated by PDGF released from endothelial cells [114,115]. Therefore, exosomes may mediate recruitment of pericytes via indirect mechanisms, *e.g.* exosome-mediated induction of endothelial cell-derived PDGF that act on pericytes. Additionally, tumor-derived pericytes have been shown to differentiate from PDGFR<sup>+</sup> progenitor perivascular cells recruited to tumor perivascular niches from bone marrow [27]. Exosomes as mediators of intercellular communication may be involved in this mechanism.

Collectively, the data presented in paper **IV** suggests that hypoxia has a great impact on the composition and biological activity of tumor cell-derived exosomes. These findings implicate tumor-derived exosomes as new targets of anti-angiogenic therapies in cancer patients. In support of this notion, the use of Diannexin, *i.e.* a blocker of EMV uptake, was shown to impair EGFR-driven glioma tumor growth and to reduce tumor microvascular density in mice [352].

Metastasis is a multistep process responsible for most cancer deaths, and it is facilitated by ‘premetastatic niches’ formed in destination organs by invading BMDCs. Hypoxia is clinically associated with metastasis and poor patient outcome, and some underlying mechanisms have been recently revealed. According to these findings, hypoxic primary tumor cells secrete LOX into the circulation, and that LOX then accumulates and cross-links collagen IV in distant organs [96]. Adhesion of CD11b+ cells to cross-linked matrix increases BMDC MMP-2 activity, which cleaves collagen, enhancing the invasion and recruitment of BMDCs and metastasizing tumor cells [96]. In this context, hypoxic exosomes, which contain high protein and mRNA levels of LOX (paper IV) might be a potential source of circulating LOX, and thereby mediate tumor invasiveness and metastasis. Furthermore, hypoxic EMVs may promote the formation of a pre-metastatic niche by stimulating angiogenesis, which is known to facilitate the establishment of tumors at distant sites. Further studies are required to investigate in more detail the role of exosomes in hypoxia-driven tumor invasiveness and pre-metastatic niche formation. In this context, several important issues need to be addressed, *e.g.* what is the half-life of exosomes in the circulation? What is the mechanism of their uptake into recipient cells? To what degree is exosomal transfer cell-specific?

#### **Tumor-derived exosomes as a potential biomarker (paper IV)**

Hypoxia represents a key tumor microenvironmental stress selecting for a more aggressive tumor phenotype, which is associated with treatment failure and poor outcome for a wide variety of human malignancies [44-46]. Thus, the identification of markers that reflect the oxygenation status of tumor tissue should provide an important diagnostic and prognostic tool. In this context, the use of membrane vesicles as markers of hypoxia seems to be an attractive idea. In support of this notion, membrane vesicles have been demonstrated to be present in various body fluids, *e.g.* blood, and to be associated with tumor stage and patient survival [346,347]. In paper IV of the present thesis, we demonstrate for the first time that the hypoxic exosomal signature reflects the molecular composition of donor cells, and may serve as a non-invasive biomarker to assess the oxygenation status and aggressiveness of GBM tumors. Additionally, some of the molecular components of exosomes derived from hypoxic glioma cells are also associated with pro-angiogenic signalling (paper IV). Thus the molecular composition of plasma-derived vesicles from cancer patients may serve as predictive markers for the selection of patients for specific regimens of anti-angiogenic therapy as well as biomarkers identifying escape pathways that should be targeted to prevent resistance development to a given anti-angiogenic agent.

Circulating membrane vesicles may offer a great opportunity for the development of prognostic and predictive biomarkers in the management of cancer patients due to their accessibility, possibility of repeated isolation from body fluids as well as heterogeneity and constant release from the tumor, which reflect the



dynamic nature of cancer. However, before they can reach routine use in the clinic, several issues have to be resolved, among which the development of standardized methods for their isolation from the body fluids is of greatest importance. Ongoing studies in our lab are focused on the possibility of using proteins, mRNAs and miRNAs contained within blood-derived membrane vesicles of GBM patients as markers of tumor hypoxia as well as markers of response and resistance to anti-angiogenic therapy.

## Streszczenie (Summary in Polish)

Charakterystyczną cechą nowotworów złośliwych jest szybkie tempo wzrostu, które wynika z bardzo intensywnych podziałów komórkowych. Jednak wraz z rozwojem guza komórki nowotworowe są coraz bardziej narażone na niedobór tlenu i składników odżywczych, co jest skutkiem zbyt słabo rozwiniętej sieci naczyń krwionośnych. W konsekwencji dochodzi do powstania niedotlenienia (hipoksji) w słabo unaczynionych obszarach guza. Aby przetrwać w tym trudnym mikrośrodowisku, niedotlenione komórki nowotworowe uruchamiają różne mechanizmy adaptacyjne. Jednym z nich jest stymulowanie wzrostu nowych naczyń krwionośnych, które ma na celu przywrócenie hipoksyjnym komórkom nowotworowym dostępu do tlenu i składników odżywczych, umożliwiających dalszy ich wzrost. Nowotworowe naczynia krwionośne powstają z istniejących już naczyń w procesie angiogenezy, który jest zainicjowany przez czynniki angiogenne wydzielane z hipoksyjnych komórek guza, wśród których naczyniowo-śródbłonkowy czynnik wzrostu (ang. "vascular endothelial growth factor" VEGF) pełni kluczową rolę. Angiogeneza jest procesem wieloetapowym, na który składają się degradacja błony podstawnej naczynia krwionośnego już istniejącego oraz stymulacja budujących go komórek śródbłonkowych do zwiększonej proliferacji, migracji i tworzenia pączka naczyniowego, z którego formowane jest światło nowego naczynia.

Dzięki badaniom przeprowadzonym na przestrzeni ostatnich kilku dekad stało się oczywiste, iż nowotwory nie mogą się rozwijać bez własnej sieci naczyń krwionośnych, a blokowanie angiogenezy nieuchronnie prowadzi do śmierci komórek nowotworowych. Obserwacje te doprowadziły do rozwoju terapii antyangiogennych w leczeniu pacjentów z chorobami nowotworowymi. Jak dotąd, skutecznymi preparatami antyangiogennymi okazały się leki hamujące aktywność VEGF oraz jego receptorów. Chociaż terapie antyangiogenne przedłużają życie niektórym pacjentom z chorobami nowotworowymi, ich działanie jest znacznie mniej skuteczne, niż wskazywałyby na to badania przedkliniczne. Główną przyczyną tego zjawiska wydają się być liczne mechanizmy nadające komórkom nowotworowym oporność na terapie blokujące angiogenezę. Z tego powodu konieczne są dalsze badania, które pomogą rozwikłać złożoność procesu angiogenezy oraz poznać mechanizmy leżące u podstaw ograniczeniom terapii antyangiogennych.

W początkowych etapach angiogenezy komórki śródbłonkowe ulegają znacznemu niedotlenieniu. W odpowiedzi komórki te aktywują liczne mechanizmy adaptacyjne, umożliwiające im przeżycie oraz tworzenie nowych naczyń krwionośnych. Celem badań, które składają się na niniejszą pracę doktorską było odkrycie mechanizmów regulujących funkcjonowanie komórek śródbłonkowych w warunkach hipoksji oraz zidentyfikowanie nowych, potencjalnych celów terapii antyangiogennej. Pierwsza część pracy ukazuje poliaminy jako czynniki, które w istotny sposób wpływają na aktywność komórek śródbłonkowych. Poliaminy to związki organiczne, obdarzone w warunkach fizjologicznych ładunkiem dodatnim, które poprzez oddziaływanie z negatywnie naładowanymi cząsteczkami, takimi jak DNA, RNA, białka i fosfolipidy, uczestniczą w wielu biochemicznych procesach

komórkowych. Z naszych badań wynika, iż poliaminy w istotny sposób regulują angiogenezę poprzez modulowanie przeżywalności słabo utlenowanych komórek śródbłonkowych oraz stymulowanie ich migracji. Obserwacje te w pewnym stopniu mogą tłumaczyć wyniki innych grup badawczych, ukazujące antyangiogenne i zarazem antynowotworowe działanie  $\alpha$ -difluorometyloornityny (DFMO), która jest specyficznym inhibitorem enzymu odpowiedzialnego za syntezę poliamin. Ponadto nasze badania pokazują, iż hipoksja wpływa na funkcjonowanie komórek śródbłonkowych poprzez modulowanie ekspresji mikroRNA, które są jednoniciowymi cząsteczkami RNA regulującymi aktywność innych genów. Jedno z nich, mikroRNA-424\*, ulega zwiększonej ekspresji w słabo utlenowanych komórkach śródbłonkowych, w celu nasilenia ich aktywności angiogennej.

Aby doszło do utworzenia sieci naczyń krwionośnych w hipoksyjnych obszarach guza, niedotlenione komórki nowotworowe muszą zaktywować komórki śródbłonka do procesu angiogenezy. W tym celu wydzielają one liczne białka angiogenne, które po dotarciu do komórek śródbłonkowych stymulują ich angiogenne właściwości. Według ostatnich osiągnięć naukowych nie jest to jedyny sposób w jaki komórki nowotworowe komunikują się z innymi komórkami w obrębie mikrośrodowiska guza. Okazuje się, iż komórki nowotworowe wydzielają do przestrzeni międzykomórkowej różnego rodzaju pęcherzyki, które niosą na swojej powierzchni lub w swoim wnętrzu aktywne cząsteczki, takie jak białka, fosfolipidy i materiał genetyczny (mRNA i mikroRNA). Pęcherzyki te po dotarciu do komórek śródbłonkowych mogą silnie pobudzać ich aktywność i stymulować proces angiogenezy. Ostatnia część niniejszej pracy doktorskiej jest poświęcona temu rodzajowi komunikacji międzykomórkowej w procesie angiogenezy stymulowanej przez hipoksyjne komórki guza mózgu (glejaka). Z naszych badań wynika, iż hipoksyjne komórki glejaka wydzielają do otaczającego je środowiska pęcherzyki o nieco innym składzie molekularnym niż komórki guza mózgu hodowane w dobrze utlenowanych warunkach. W konsekwencji pęcherzyki pochodzące z hipoksyjnych komórek guza charakteryzują się silną aktywnością angiogenną, dzięki czemu przyczyniają się do znacznego przyspieszenia wzrostu nowotworu. Dodatkowo analiza molekularna pęcherzyków wykazała, że ich skład molekularny odzwierciedla skład molekularny komórek, z których się wywodzą, co sugerowałoby możliwość ich izolowania np. z krwi i wykorzystania jako bezinwazyjnych biomarkerów do oceny stanu niedotlenienia glejaków. Nasze wstępne badania wskazują na taką możliwość.

Podsumowując badania zaprezentowane w tej pracy doktorskiej pozwoliły na identyfikację nowych mechanizmów regulujących aktywność komórek śródbłonkowych w warunkach hipoksji, które w przyszłości mogą posłużyć jako nowe cele terapii antyangiogennej.

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