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On the Role of Polyamines and Microvesicles in Tumour Development. Regulation by Hypoxia and Implications for Therapeutic Intervention of Cancer.

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On the Role of Polyamines and Microvesicles in Tumour Development

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To my family

”Ju mer man tänker, desto mer inser man att det inte finns något enkelt svar”

- Nalle Puh

Abstract

Novel strategies for specific tumour cell targeting are necessary in order to improve survival rates and to reduce side effects of current therapies in cancer patients.

Hypoxia is a hallmark of solid tumours and one of the major driving forces for tumour progression. Targeting of the adaptive responses of cancer cells to hypoxia offers opportunities for tumour specific therapies.

The aim of this thesis was to study the hypoxic regulation of polyamines and tumour cell-derived microvesicles (MVs), both of which have been associated with tumour development. It was initially demonstrated that epitope-specific interference of cell surface heparan sulphate proteoglycans (HSPGs) by anti-HS antibodies inhibits the bioavailability of exogenous polyamines, and that combined targeting of polyamine biosynthesis by α-difluoromethylornithine (DFMO) and HSPG-dependent uptake by a HIV-1 trans-activator of transcription (TAT) peptide, Tat, results in reduced tumour growth. We found a novel role for the polyamine system in the hypoxia-induced adaptive response of tumour cells; depletion of polyamines sensitizes tumour cells to hypoxic stress and enhances the tumour inhibiting effect of anti-angiogenic therapy. Finally, tumour cell-derived MVs are shown to elicit a hypoxic, pro-angiogenic response in endothelial cells (ECs) and the upregulation of a specific subset of microvesicular proteins and mRNAs by hypoxia implicates MVs as novel biomarkers for hypoxic signalling in cancer cells.

This thesis implicates the targeting of hypoxia-induced adaptive responses as a means of obtaining tumour cell specific therapies and suggests the polyamine system and tumour-derived MVs as promising candidates for therapeutic intervention or as biomarkers of cancer disease.

Table of contents

List of publications

This thesis is based on the following papers referred to in the text by the roman numerals indicated below (please note my maiden name, Lilja):

I. Mani, K., Sandgren S., **Lilja, J**., Cheng, F., Svensson, K., Persson, L., Belting, M. HIV-Tat protein transduction domain specifically attenuates growth of polyamine deprived tumor cells. *Molecular Cancer Therapeutics* **6**, 782-788 (2007)

II. **Welch, J.E**., Bengtson, P., Svensson, K., Wittrup, A., Jenniskens, G.J., Ten Dam, G.B., van Kuppevelt, T.H., Belting, M. Single chain fragment anti-heparan sulfate antibody targets the polyamine transport system and attenuates polyamine-dependent cell proliferation. *International Journal of Oncology* **32**, 749-756 (2008)

III. Svensson, K.J*., **Welch, J.E***., Kucharzewska, P., Bengtson, P., Bjurberg, M., Påhlman, S., Ten Dam, G.B., 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 Research* **68**, 9291-9301 (2008)

IV. **Welch, J.E***., Kucharzewska, P*., Fredlund, E., Svensson, K.J., Mörgelin, M., Rigner, M., Belting, M. Microvesicles may constitute a novel marker of hypoxia driven pro-angiogenic signalling in cancer.
Manuscript

* *equal contribution*

Publications not included in the thesis

The following papers are co-authored by the author of this thesis but are not included herein:

Magzoub, M., Sandgren, S., Lundberg, P., Oglecka, K., **Lilja, J**., Wittrup, A., Göran Eriksson, L.E., Langel, Ü., Belting, M., Gräslund, A. N-terminal peptides from unprocessed prion proteins enter cells by micropinocytosis. *Biochemical and Biophysical Research Communication* **348**, 379-385 (2006)

Wittrup, A., Sandgren, S., **Lilja, J**., Bratt, C., Gustavsson, N., Mörgelin, M., Belting, M. Identification of proteins released by mammalian cells that mediate DNA internalization through proteoglycan-dependent macropinocytosis. *The Journal of Biological Chemistry* **282**, 27897-27904 (2007)

Kucharzewska, P., **Welch, J.E**., Svensson, K.J., Belting, M. The polyamines regulate endothelial cell survival during hypoxic stress through PI3K/AKT and MCL-1. *Biochemical and Biophysical Research Communication* **380**, 413-418 (2009)

Wittrup, A., Zhang, S.H., ten Dam, G.B., van Kuppevelt, T.H., Bengtson, P., Johansson, M., **Welch, J**., Mörgelin, M., Belting, M. ScFv antibody-induced translocation of cell-surface heparan sulfate proteoglycan to endocytic vesicles: evidence for heparan sulfate epitope specificity and role of both syndecan and glypican. *The Journal of Biological Chemistry* **284**, 32959-32967 (2009)

Kucharzewska, P., **Welch, J.E**., Birgersson, J., Belting, M. Establishment of heparan sulphate deficient primary endothelial cells from EXT-1 (flox/flox) mouse lungs and sprouting aortas. *In Vitro Cellular & Developmental Biology. Animal* **46**, 577-584 (2010)

Kucharzewska, P., **Welch, J.E**., Svensson, K.J., Belting, M. Ornithine decarboxylase and extracellular polyamines regulate microvascular sprouting and actin cytoskeleton dynamics in endothelial cells. *Experimental Cell Reserach* **316**, 2683-2691 (2010)

Welch, J.E., Svensson, K.J., Kucharzewska, P., Belting, M. Methods for study of heparan sulphate proteoglycan-mediated polyamine uptake mechanism. *Methods in Molecular Biology. In: Polyamines: Methods and Protocols, Humana Press. Edited by Anthony Pegg, in press*

Abbreviations

AZ	Antizyme
AZI	Antizyme inhibitor
bFGF	basic FGF
CS	Chondroitin sulphate
DFMO	α-difluoromethyl-ornithine
EC	Endothelial cell
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EPC	Endothelial progenitor cell
FasL	Fas ligand
FDA	United States Food and Drug administration
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GBM	Glioblastoma multiforme
GlcA	Glucuronic acid
GlcNAc	N-acetyl glucosamine
HIF	Hypoxia inducible factor
HS	Heparan sulphate
IL	Interleukin
IRES	Internal ribosome entry site
mTOR	Mammalian target of rapamycin
MV	Microvesicle
MVB	Multivesicular body
ODC	Ornithine decarboxylase
ODD	Oxygen dependent degradation
PDGF	Platelet derived growth factor
PG	Proteoglycan
PHD	Prolyl hydroxylase domain
PS	Phosphatidylserine
PTD	Protein transduction domain
ScFv	Single chain variable fragment
SDF	Stromal derived factor
SSAT	Spermidine / spermine N^1-acetyltransferase
TAT	Trans-activator of transcription
Tat	TAT protein transduction domain
TF	Tissue factor
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
WT	Wild-type

Introduction to cancer

Hallmarks of cancer

The transformation of normal cells into malignant cells is a multistep process associated with the accumulation of genetic alterations. As a defence against abnormal growth, tissues and cells are normally controlled by a vast number of mechanisms regulating their proliferation and survival. The successive gathering of mutations, chromosomal translocations, amplifications and epigenetic alterations involved in the malignant progression all contribute to the breaching of this defence. Genomic instability is a hallmark of cancer cells and is indeed a prerequisite for these genomic alterations to occur. The dominant gain of function of oncogenes and recessive loss of function of tumour suppressor genes are the fundamental bases for the malignant process. Oncogenes are tumour promoting genes that, when mutated or overexpressed, confer a growth advantage on the tumour cells. In contrast, tumour suppressor genes are growth inhibitory and when inactivated, instead promote tumour growth. During malignant transformation, normal cells acquire certain capabilities characteristic for most, if not all, cancer cells. These capabilities (self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and ability of tissue invasion and metastasis) have been presented and discussed by Hanahan and Weinberg[1] and they are briefly summarized below. Some of them, *e.g.* evasion of apoptosis and sustained angiogenesis, will be discussed in more detail later in this introduction.

Normal cells require growth signals to proliferate. These signals are usually diffusible growth stimuli provided by adjacent cells or transmitted through contact with the extracellular matrix (ECM). Tumour cells are less dependent on stimulation from their microenvironment as they frequently have acquired the ability of *self-sufficiency in growth signals*. Generally, this can be achieved in three ways; synthesis of growth factors resulting in autocrine stimulation, alterations in the components of downstream signalling pathways transmitting the growth signals and finally, overexpression of growth factor receptors or expression of structurally altered receptors. The latter can be exemplified by the frequent mutations in the epidermal growth factor receptor (EGFR) in human gliomas[2].

Within normal tissue, growth promoting stimuli are balanced by anti-proliferative signals in order to maintain tissue homeostasis. These signals regulate the progression through the cell cycle, especially the transit from G_1 phase to S phase. Tumour cells acquire *insensitivity to anti-growth signals* through *e.g.* disruption of the retinoblastoma protein (pRb) pathway which is one of the major transmitters of anti-proliferative signals. In addition, cell multiplicity is constrained by signals instructing cells to enter post-mitotic, differential states but the upregulation of *e.g.* the transcription factor c-myc by tumour cells will favour proliferation instead of differentiation.

Tissue homeostasis is controlled not only by cellular growth, but also by cell death. Apoptosis is a form of programmed cell death that can be regarded as cellular suicide[3]. Briefly, the apoptotic pathway can be divided into sensors and effectors. The sensors monitor the extra- and intracellular environment for conditions of abnormalities and upon pro-apoptotic stimuli, signal to the effectors that execute the apoptotic program, characterized by membrane blebbing, chromosome degradation and engulfment by nearby cells. Extracellular signals of apoptosis can be lack of survival signals from growth factor/growth factor complexes or stimulation of the cell surface, pro-apoptotic Fas receptor by the Fas ligand (FasL). Abnormalities detected by intracellular sensors include DNA damage and hypoxia (lack of oxygen). Many of the apoptotic signals converge on the mitochondria, which respond by releasing cytochrome C, a potent catalyst of apoptosis. The ultimate effectors of apoptosis are intracellular cystein proteases called caspases[4]. These proteases are further sub-classified into initiator (caspase -2, -8, -9 and -10) and effector caspases (caspase -3, -6 and -7). Initiator caspases set off the caspase cascade during apoptosis and can be activated by *e.g.* the Fas receptor or cytochrome C. They subsequently activate the effector caspases that, in turn, execute the death programme through selective degradation of sub-cellular structures and organelles. Tumour cells frequently acquire ways of *evading apoptosis* and one of the most common ways to do so is through inactivation of the P53 tumour suppressor gene. p53 responds to numerous stress stimuli and can induce several cellular responses including apoptosis and cell cycle arrest[5].

Most, if not all, normal cells carry an intrinsic program limiting their replicative ability - after a certain number of replications, the cells will enter senescence. This is due to a shortening of the telomeres, *i.e.* repetitive sequences at the ends of chromosomes, at every replication round. This, in turn, is due to an inability of the DNA polymerase to completely replicate the 3' ends of

chromosomal DNA. The telomeres protect the chromosomes but as they are shortened with every cell division, they are eventually depleted, leaving the free chromosomal ends to take part in end-to-end fusions which ultimately will result in cell death. Tumour cells acquire *limitless replicative potential* by upregulating telomerase, the enzyme that elongates the telomeres[6].

Sufficient oxygen and nutrient supply is crucial for tissue growth and all cells are required to reside no longer than 150-200 μm, the distance of oxygen diffusion, from the vasculature. Hence, in order to grow beyond the size of a few millimetres, tumours need to *sustain angiogenesis*, *i.e.* the formation of new blood vessels. Blood vessel development is regulated by a fine balance of pro- and anti-angiogenic factors and tumours tip this balance in favour of stimulatory signalling via an "angiogenic switch", characterized by upregulation of pro-angiogenic factors like vascular endothelial growth factor (VEGF) and downregulation of inhibitors such as thrombospondin-1[7].

The settlement of tumour cells in distant tissues – metastasis – is the cause of most cancer related deaths and it is the acquired ability of *tissue invasion and metastasis* that distinguishes benign from malignant tumours. Metastasis involves detachment of tumour cells from the primary tumour, degradation of and migration through the ECM and finally settlement at distant sites. This requires altered expression of a large number of proteins involved in cell-cell and cell-matrix adhesion as well as matrix remodelling and include cadherins, integrins, proteoglycans and extracellular proteases, just to mention a few[8].

Tumour hypoxia

Cancer and hypoxia

Areas of reduced oxygen tension, hypoxia, is a common feature of solid tumours[9]. Normal, well-vascularised tissues typically exhibit an oxygen tension of 5-6 % whereas the oxygen levels in hypoxic tumour areas can be < 1 %[10]. The increased proliferation rate of tumour cells causes the tumour to "outgrow" its blood supply, and therefore also its oxygen supply, until a point where the oxygen diffusion becomes the limiting factor. Cells residing 150-200 μm from the vessel wall will hence be subjected to inadequate oxygen levels, which may be referred to as "chronic hypoxia". Alternatively, "acute hypoxia", or perfusion limited hypoxia, occurs as a result of the temporary obstruction or occlusion of aberrant tumour blood vessels[9]. Hypoxia is toxic to both cancer cells and normal cells but malignant cells undergo genetic and adaptive changes (discussed more in detail below) that allow them to survive and even proliferate in this hostile environment[11]. Sustained hypoxia might even give rise to genotypic and phenotypic changes that will result in a clinically more aggressive tumour phenotype. Indeed, high levels of hypoxia in primary tumours have been associated with metastasis and poor survival in *e.g.* patients suffering from primary brain, head and neck, cervical and breast cancers[12-15]. Interestingly, hypoxia has been shown to confer a more immature, dedifferentiated phenotype on human neuroblastoma and breast cancer cells[16] and it has been proposed that hypoxia could contribute to the conversion of differentiated tumour cells into cancer stem cells[17]. In addition, hypoxic tumours are difficult to treat because hypoxic cells are relatively resistant to both radio- and chemotherapy[18]. Ionizing radiation, used in radiotherapy, requires oxygen to promote DNA damage and consequently, hypoxic cells are less sensitive to radiation. Hypoxic cells are usually distant from the vasculature and will therefore be exposed to lower concentrations of systemic drugs than cells adjacent to blood vessels[19]. Moreover, hypoxic cells generally exhibit a reduced proliferation rate compared to normoxic cells and as most cytostatic drugs primarily target rapidly dividing cells, their

effectiveness would be expected to decrease as a function of distance from the blood vessels[19]. Hence, hypoxia has a key negative role in tumour prognosis; it selects for a more aggressive tumour phenotype and causes resistance to standard therapies.

As severe hypoxia (< 1% oxygen) is a tumour-specific phenomenon, the existence of hypoxic regions however, may also provide opportunities for tumour selective therapies. Several approaches have been tested in order to utilize hypoxia as a means to obtain tumour cell-specific targeting; prodrugs activated by hypoxia, hypoxia-selective gene therapy, targeting the transcription factors that orchestrate the cellular hypoxic response, *i.e.* hypoxia inducible factors (HIFs) and the use of recombinant obligate anaerobic bacteria[20]. The constitutive activation of receptor tyrosine kinases (RTKs) and/or downstream signalling pathways in cancer cells converge with the HIF pathway and HIF is activated in many cancers irrespective of hypoxia. Thus, anti-cancer drugs targeting these pathways will also reduce the activity of HIF. A growing number of anti-cancer agents have indeed been shown to inhibit HIF activity[21], an inhibition that most likely contributes to their therapeutic effect.

Hypoxia inducible factors

As mentioned above, the HIF transcription factors are the master regulators of oxygen homeostasis and they facilitate both oxygen delivery and adaptation to oxygen deprivation by activating a large number of genes involved in diverse processes like metabolism, apoptosis and angiogenesis[22]. HIFs are heterodimeric proteins that consist of a constitutively expressed HIF-β subunit (also known as arylhydrocarbon receptor nuclear translocator – ARNT) and a highly regulated HIF-α subunit[23]. To date, HIF-1 and -2 are well characterized and known to be important for oxygen sensing[24]. The role of HIF-3 in hypoxic regulation *in vivo* is still largely unknown[24]. HIF-1 is ubiquitously expressed whereas HIF-2 expression is confined to specific cell types such as ECs, glial cells and neural crest derivates[25]. HIF-1α and -2α are structurally similar and seem to have overlapping but distinct specificities with regards to physiological inducers and transcriptional targets[26]. At normoxic conditions, HIF-1/2α is subjected to ubiquitination and degradation by the proteasome. This is mediated by the tumour suppressor von Hippel-Lindau (VHL) protein. Hydroxylation of two proline residues in the oxygen dependent degradation (ODD) domain of HIF-1/2α by oxygen-, Fe(II) and 2-oxoglutarate-dependent prolyl hydroxylase-domain proteins (PHDs)[27] is

required for this interaction, and hence for HIF degradation[23]. Thus, the PHDs are the true oxygen-sensing molecules controlling the hypoxic response. At hypoxic conditions however, hydroxylation is inhibited and HIF-1/2α subunits are stabilized and translocate into the nucleus where they heterodimerize with HIF-β and bind to hypoxia responsive elements (HREs) within regulatory elements in HIF target genes[23]. For transcriptional activation of target genes, additional transcriptional activators such as p300/CBP are recruited and the interaction between HIF-1/2 and these proteins is further regulated in an oxygen-dependent manner by factor inhibiting HIF-1 (FIH)[28]. FIH is another member of the 2-oxoglutarate and Fe(II) oxygenase superfamily and at normoxic conditions, this enzyme hydroxylates asparaginyl residues in the most carboxy-terminal transactivation domain (C-TAD) of HIF-1/2α, inhibiting binding to p300/CBP[29]. Thus, full activation of HIF at hypoxic conditions requires both protein stabilization and C-TAD activation by suppression of PHD and FIH activities, respectively.

HIF can also be activated in tumours under normoxic conditions, either through genetic alterations of the oxygen-sensing pathway or through activation of RTK signalling pathways. Loss-of-function mutations of VHL are common events in familial and sporadic renal cell carcinomas and result in accumulation of both HIF-1 and -2α and continuous activation of hypoxia-response genes at normoxic conditions[30]. Activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signalling pathway through constitutive active cell-surface receptors[31] or loss of negative regulators such as phosphatase and tensin homolog (PTEN)[32] has been shown to increase HIF-1α expression and activity under normoxic conditions. Whereas hypoxia-regulated HIF expression depends on decreased degradation, this type of upregulation is rather due to increased protein synthesis[31]. In addition, activation of the RAF-MEK-ERK signalling pathway has also been shown to phosphorylate HIF-1α, resulting in stimulation of the transactivation domain[33]. Hence, oncogenic mutations that activate signal-transduction pathways can induce HIF activity through different mechanism, all mimicking the effects of hypoxia.

The hypoxic response

Hypoxia inducible genes regulate diverse biological process, including pH-balance, cell death, genomic stability, proliferation and angiogenesis and they all

aim at adapting cells to the hypoxic stress. Tumour cells evolve to take advantage of some of these adaptive responses and to escape others and an aggressive tumour phenotype is probably achieved through a stepwise selection process, in part driven by hypoxia.

As a consequence of reduced oxygen availability, hypoxic cells switch their mechanism of ATP production from the high energy producing oxidative phosphorylation to the more energy conserving oxygen-independent glycolysis. HIF-1α regulates a large number of genes involved in glycolytic metabolism, including lactate dehydrogenase A (LDHA) and glucose transporters which facilitate the cellular uptake of glucose[9, 24, 26].

This shift in metabolic activity of hypoxic cells, leading to increased production of lactic acid, can generate an acidic microenvironment[29]. The adaptation of tumour cells to lower pH will provide a growth advantage and the upregulation of *e.g.* carbonic anhydrase IX (CA IX), which facilitates cellular alkalinisation, represents one way of adaptation to acidic pH[34].

The role of HIFs in proliferation seems to be bifunctional; HIF-2α plays an important role in promoting tumour cell growth and is both necessary and sufficient to maintain tumour growth in VHL deficient renal cell carcinoma cells[35, 36]. One mechanism by which HIF-2α promotes cellular proliferation is through the enhancement of c-myc transcriptional activity, inhibiting the expression of genes encoding p21 and p27 and augmenting the expression of *e.g.* cyclinD2[37]. On the other hand, HIF-1α has been shown to induce cell cycle arrest by functionally counteracting c-myc[38]. A "stop-and-go" model has been proposed[39] in which HIF-1α modulates tumour growth by inducing both growth arrest and angiogenesis in response to hypoxia. Upon rapid expansion of the tumour cell mass, hypoxia-induced HIF-1α will trigger growth arrest. Subsequently, HIF-1α-induced angiogenesis will relieve the hypoxic stress, HIF-1α will be degraded and growth restored[39]. Depending on the growth stimuli and the genetic make-up of the cells, HIF-1 and -2α probably work in concert, fine-tuning the growth response of tumour cells in order to maintain sustained tumour growth and survival.

Metastasis is a critical step in tumour pathogenesis. HIF-1α was demonstrated to be a critical regulator of metastasis in a transgenic mouse model[40] and HIF activation correlates with metastasis in numerous tumours[25]. The molecular basis for this correlation might, in part, be loss of E-cadherin, one of the landmarks of cellular invasion[41] and the upregulation of lysyl oxidase (LOX), an extracellular matrix protein critical for tumour cell metastatic potential[42].

Hypoxia and HIF-1α are potent inducers of apoptosis, although the exact regulatory mechanisms are not fully understood. HIF-1α activates the pro-apoptotic proteins BNIP3 and NIX which, in turn, induce mitochondrial-mediated cell death[43]. HIF-1α has also been demonstrated to induce p53-dependent apoptosis[44]. Hypoxic regions often appear in the early stages of tumour development and thus confer an early selection pressure on tumour cells to undertake adaptive changes that permit them to be refractory to hypoxia-induced apoptosis[9]. Hypoxia has been shown to upregulate negative regulators of p53, anti-apoptotic proteins as well as the PI3K/Akt survival pathway[45]. In addition, hypoxia acts as a physiological, selective pressure during tumour growth for the preferential expansion of cells with diminished apoptotic potential. This was elegantly shown by Graeber *et al.* in a co-culture experiment in which transformed cells lacking p53 were mixed with similar cells expressing wild-type (WT) p53 at a 1:1000 ratio. After several rounds of hypoxic exposure, the p53 mutants had overtaken the p53 wild-type cells, illustrating that hypoxia can select for apoptosis-resistant cells[46].

The ability to survive under hypoxic conditions is one of the fundamental physiological differences between malignant cells and normal cells. Several important questions remain to be answered in order to fully understand, and to be able to therapeutically exploit, the hypoxia-induced adaptive responses in tumour cells. Depending on the ratio of HIF-induced pro- and anti-apoptotic pathways activated in response to hypoxia, therapeutics targeting HIF will have dramatically different outcomes. Moreover, does hypoxia generate an aggressive tumour phenotype or is it an aggressive tumour phenotype, due to genetic alterations, that causes rapid proliferation and hence hypoxia? Again, these different scenarios would probably give different therapeutic outcomes when targeting HIF or HIF-induced adaptive responses.

Angiogenesis is probably the most important adaptive tumour response to hypoxia and the above questions are linked to the current discussion on whether the demonstrated benefit of anti-angiogenic therapy stems from increased hypoxia-dependent tumour cell death or from normalization of the tumour vasculature. Critics of the concept of anti-angiogenic therapy even argue that failure of this class of anti-cancer agents in the clinic is due to the induction of more severe hypoxia. Hypoxia-induced angiogenesis and anti-angiogenic therapy is further discussed below.

Tumour angiogenesis

Physiological angiogenesis is an essential process during *e.g.* foetal development, would healing, and in the placenta during pregnancy[47]. Angiogenesis is tightly regulated by a fine balance of pro- and anti-angiogenic molecules and during adulthood, the majority of vessels remain quiescent. However, they maintain their ability to rapidly divide in response to various stimuli, *e.g.* hypoxia and inflammation. In several diseases, this stimulus becomes excessive, tilting the balance in favour of deregulated angiogenesis – the angiogenic switch is "on"[48]. Cancer is one of the most well characterized conditions in which angiogenesis strongly contributes to its pathology[47, 49].

The fact that angiogenesis occurs around tumours was first observed almost 100 years ago[50] and in the early 1970's, Judah Folkman postulated that tumour growth and metastasis are angiogenesis dependent and hence, that anti-angiogenesis could be a strategy to arrest tumour growth[51]. Angiogenesis involves a large number of molecules derived from many different cell types, all contributing to an integrated series of events. Consistent with the pivotal role of hypoxia in triggering angiogenesis, numerous genes involved in different parts of the process are responsive to hypoxia. These include, but are not limited to, the growth factors VEGF[52, 53], angiopoietin-1 and -2 (Ang-1 and -2), fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and their receptors, interleukins (ILs) and proteins involved in degradation of ECM such as matrix metalloproteinases (MMPs) and plasminogen activator receptors and inhibitors[54]. In response to hypoxia, tumour vessels develop by sprouting from adjacent pre-existing vessels. Initially, tumour cell-derived VEGF causes the vessels to dilate and Ang-2-mediated antagonism of the Ang-1-promoted vessel stabilization together with MMP-mediated degradation of the basement membrane destabilize vessels and promote endothelial cell (EC) proliferation and migration. VEGF controls angiogenic sprouting through guidance of extended filopodia on specialized ECs at the tip of the protruding vascular sprout, "tip cells". These cells migrate in response to a gradient of VEGF and the proliferative response to VEGF occurs in so called "stalk cells" residing behind the tip cell in the sprout[55]. Other angiogenic growth factors include members of the FGF family that are bound to heparan sulphate (HS) in the ECM and that are released by the action of heparanase that degrades HS in the ECM and on cell surfaces[56].

In addition to local capillary ingrowth from surrounding vasculature, tumour angiogenesis is also supported by the mobilization of bone marrow-derived

endothelial progenitor cells (EPCs)[57]. The chemokine stromal derived factor-1α (SDF-1α) and its receptor CXCR4 have been suggested to be important in the recruitment of these circulating EPCs[58] to sites of neoangiogeneis. SDF-1α is upregulated by hypoxia and HIF-1α[58, 59]. VEGF, basic FGF (bFGF) and macrophage-colony stimulating factor (GM-CSF) have also been implicated in the mobilization of EPCs[48]. Maturation of nascent vessels involves formation of new basement membrane and normally also the recruitment of mural cells such as pericytes. EC-derived PDGF-BB attracts PDGF-Rβ expressing pericytes[60] and the tight association between ECs and pericytes is required for stabilization of the vessels[61]. Unlike normal tissue, tumour stroma contains inflammatory infiltrates, and tumour-associated macrophages (TAMs), monocytes and T-cells all release pro-angiogenic factors, like VEGF and IL-8, that will stimulate tumour angiogenesis[62, 63]. Carcinoma-activated fibroblasts (CAFs) express PDGF-Rβ and are recruited in response to PDGF-BB. CAFs contribute to the angiogenic process not only by secreting angiogenic factors such as VEGF and placental growth factor (PlGF)[64] but also by recruiting EPCs through their release of SDF-1α[65]. Last but not least, numerous recent studies have illustrated the importance of tumour cell-derived microvesicles (MVs) in tumour angiogenesis (described in detail in the last section of this thesis).

As a consequence of the imbalance between pro- and anti-angiogenic factors, tumour vessels are, compared to normal vessels, structurally and functionally abnormal. The tumour vasculature is highly disorganized; the vessels are tortuous with uneven diameter and excessive branching which leads to uneven flow[48]. The vessel wall is not always covered with a homogenous layer of ECs but might instead consist of a mix of malignant cells and ECs[66]. Furthermore, tumour vessels often lack the coverage of protective pericytes[61] and are thus leaky and dilated. This will lead to inadequate oxygenation of distal tissue and a vicious circle of continued hypoxic stimuli, further ingrowth of dysfunctional vessels etc.

Anti-angiogenic therapy

The necessity of angiogenesis for primary tumour and metastasis development has, since Judah Folkman's first proposal, spurred an intensive search for angiogenesis regulators that could represent therapeutic targets. To date, there are four anti-angiogenic drugs (bevacizumab, sorafenib, sunitinib and thalidomide) approved for use in the clinic[67]. They have a different mechanism of action compared to traditional chemotherapy agents and radiotherapy and can be given in combination

with traditional drugs to increase treatment efficiency. The clinical breakthrough of anti-angiogenic therapy came in 2004 from a phase III trial that demonstrated a significantly prolonged survival when bevacizumab, a humanized anti-VEGF antibody, was added to chemotherapy in patients with metastatic colorectal cancer[68]. The same year, bevacizumab was the first angiogenesis inhibitor to be approved for clinical use by the United States Food and Drug Administration (FDA) and the antibody is, to date, approved for use in combination with chemotherapy for metastatic colorectal cancer, HER-2 negative metastatic breast cancer, advanced non-small-cell-lung cancer (NSCLC), in combination with interferon (IFN)-α in metastatic renal cell carcinoma and in recurrent glioblastoma[67]. The development and FDA approval of bevacizumab have paved the way for other novel angiogenesis inhibitors; two small-molecule tyrosine kinase inhibitors that target numerous tyrosine kinases vital for pro-angiogenic signalling have been approved; sorafenib[69] and sunitinib[70]. The forth anti-angiogenic agent to be approved for use by the FDA was thalidomide, a bFGF activity inhibitor. In addition, everolimus[71] and temsirolimus[72], two mTOR inhibitors, are approved by the FDA for use in advanced renal cell carcinoma. mTOR is an upstream activator of *e.g.* HIF and its inactivation can result in inhibition of angiogenesis.

The mechanism of action of angiogenesis inhibitors is under debate. Low-dose continuous chemotherapy (metronomic chemotherapy) is suggested to have inhibitory effects on ECs[73] and the simultaneous inhibition of the survival factor VEGF is thought to amplify the effect, resulting in increased hypoxic stress and thereby enhanced killing of tumour cells. The relatively long half-lives of antibodies in the circulation might also contribute to the effect of *e.g.* bevacizumab as it might allow for continued suppression of neovascularisation between the courses of conventional chemotherapy. Bevacizumab has indeed been shown to induce hypoxia in animal models and the resultant tumour cell death and subsequent tumour shrinkage was determined by the tumour cell's susceptibility to apoptosis[74]. As discussed above, hypoxic stress is a strong inducer of apoptosis but whether tumour cells will succumb or adapt to this stress is determined by their genetic make-up. Hence, increasing tumour hypoxia by anti-angiogenic therapy might have different outcomes depending on tumour type. In this context, the importance of interfering with the ability of tumour cells to evade apoptosis becomes clear. In paper **III** of this thesis, we employ combined anti-angiogenic therapy with polyamine inhibition as a successful strategy to sensitize tumour cells

to hypoxia-induced apoptosis (described in detail under "the present investigation" below).

An alternative mechanism of action of angiogenesis inhibitors was proposed by Jain and collaborators[75]. According to their model, anti-angiogenic therapy leads to a "normalization" of the tumour vasculature which, in turn, results in a decrease of the otherwise high interstitial pressure and thereby improved perfusion and oxygenation. These effects should improve the efficacy of radiotherapy, and the delivery of chemotherapy to tumour cells. The mechanisms of resistance to anti-angiogenic therapy are gradually being elucidated and are thought to be attributed to a) alterations in the expression of pro-angiogenic signalling pathways, b) changes in blood vessel formation - vessel mimicry by tumour cells, c) genetically unstable tumour ECs, d) the recruitment of EPCs from the bone marrow and e) tumour co-option of pre-existing vessel[76, 77]. These reports emphasize the importance of a combined targeting of numerous pro-angiogenic targets in order to achieve successful angiogenesis inhibition. A closely related issue is the one of patient selection. Who is likely to benefit from anti-angiogenic therapy and how is treatment response best measured? Currently, there are no validated biomarkers to monitor the response to anti-angiogenic therapy although the use of circulating levels of ECs and EPCs as surrogate biomarkers for angiogenesis has been proposed[78].

Gliomas represent a class of highly vascularised solid tumours for which hypoxia is an important determinant of aggressiveness and for which anti-angiogenic therapy is currently being evaluated in several clinical trials. Gliomas are the most common primary tumours arising in the central nervous system and despite recent advances in therapeutics, the prognosis for patients with newly diagnosed glioblastoma multiforme (GBM), the most aggressive form, is dismal; median survival from the time of diagnosis is only 15 months[79]. According to the World Health Organization (WHO), human gliomas are classified into pilocytic astrocytomas (grade I), diffuse astrocytomas (grade II), anaplastic astrocytomas (grade III) and GBM (IV). GBM might be primary or develop from grade II and III astrocytomas[80] and are among the most vascular tumours known[81]. Hence, anti-angiogenic therapy is an attractive strategy to improve the prognosis for these patients. The rapid growth of GBM tumours cause regions of local hypoxia which frequently result in a necrotic core. As with tumour vasculature in general, hypoxia-stimulated angiogenesis results in tumour vessels that demonstrate excessive leakiness and frequent thrombosis, further promoting local hypoxia.

Several reports have demonstrated the link between the severity of hypoxia, and the activation of HIF signalling pathways, with tumour grade[12, 82, 83]. Interestingly, it was recently reported that HIF-2α is selectively activated in glioma stem cells in response to hypoxia and that targeting of HIF-2α attenuated the tumour initiating potential of these cells in mouse xenografts[82]. On the basis of two phase II trials reporting relatively high response rates and 6-months progression free survival of patients with recurrent GBM, bevacizumab was approved by the FDA for use in recurrent GBM in 2009[84, 85]. However, due to questions concerning drug activity, the application to the European Medicines Agency was rejected in the late 2009[86].

In summary, anti-angiogenic therapy appears to be a novel and promising, yet challenging, approach to treat cancer disease. The response to anti-angiogenic therapy will likely depend on the adaptability of the tumour vasculature and on the susceptibility of the tumour cells to hypoxia-induced cell death. In this thesis, a novel role for the polyamine system in hypoxia-induced apoptosis is presented. A detailed overview of the polyamine system and its role in tumour development is given below.

Polyamines

Definition and physiological role of polyamines

The polyamines spermidine and spermine and their precursor diamine putrescine are low molecular weight organic cations that, at physiological pH, carry a positive charge on each nitrogen atom[87, 88]. Their positive charge enables them to interact with anionic molecules such as nucleic acids, phospholipids and negatively charged proteins[88]. The polyamines are absolutely essential for cell growth and viability; the gene coding for ornithine decarboxylase (ODC), *i.e.* the enzyme responsible for the first step in polyamine biosynthesis, is indispensable for mammalian development[89]. Polyamines are implicated in gene regulation and changes in polyamine levels have been shown to change chromatin structure[90, 91]. In addition, the polyamines were recently implicated in the regulation of histone acetylation[92]. The polyamines interact with certain membrane-ion channels such as N-methyl-D-aspartate (NMDA) and voltage-activated Ca^{2+} channels[93]. Another function of the polyamines, specific for spermidine and related to cellular growth, is the formation of the amino acid hypusine. Hyposine is an integral part of the eukaryotic initiation factor 5A (eIF-5A)[94] and is required for proliferation of mammalian cells. The roles of polyamines in cell death processes like apoptosis are ambiguous. Depending on cell type and death stimuli, there are reports of both positive and negative effects of polyamine depletion on cell survival[95, 96]. Thus, it appears that the polyamines are bivalent regulators of both cell growth and cell death depending on environmental signals. Marked variations in enzyme activity of several biosynthetic and catabolic enzymes, followed by changes in polyamine levels, have been recorded during the different phases of the cell cycle[97], suggesting an important role for the different polyamines in cell cycle regulation. Accordingly, reduction of polyamine levels results in growth arrest, most often in G_1 phase[88]. This tight relationship between polyamines and cell growth makes them highly interesting in the context of cancer development and it is now more than 30 years ago since the first observation linked increased levels of ODC to cancer[98]. Increased cellular levels of polyamines were soon thereafter reported in

tumour cells[99] and in the urine of patients with cancer[100]. Since then, upregulation of ODC and other biosynthetic enzymes and increased levels of polyamines have been reported in a large number of tumours[101-104] and the association between the polyamine system and carcinogenesis is now well established. However, before going into details about the role of polyamines in tumour development, an introduction to their metabolism and transport will be given below.

Polyamine metabolism

Intracellular levels of polyamines are tightly regulated by anabolic and catabolic pathways (see **Figure 1**) as well as uptake from the extracellular environment and export out of cells. In eukaryotic cells, L-arginine and L-methionine are the primary precursors for polyamines synthesis. Arginine is degraded by arginase to ornithine and methionine is converted to S-adenosylmethionine (AdoMet) by methionine adenosyltransferase (MAT) before taking part in the polyamine biosynthetic machinery[88].

The diamine putrescine is formed by decarboxylation of ornithine by ODC. ODC activity is the rate limiting step in putrescine synthesis but the conversion of putrescine into the higher polyamines requires the availability of an aminopropyl group derived from decarboxylated S-adenosylmethionine (dcAdoMet). Hence, the action of S-adenosylmethionine decarboxylase (AdoMetDC), facilitating the decarboxylation of AdoMet, also influences the rate of polyamine synthesis[105]. The higher polyamines, spermidine and spermine, are formed from putrescine by successive attachment of two aminopropyl groups by the action of aminopropyl transferases, namely spermidine- and spermine synthase, respectively[87, 88, 105]. ODC is a cytosolic, homodimeric and highly inducible protein with an extremely short half-life of 10 min - 1 hour[106]. Enzymatic activity appears to be regulated via changes in protein amount and ODC expression is regulated at the level of transcription, mRNA stability, translation and degradation[106]. Interestingly, transcription of ODC has been shown to be regulated by certain oncogenes, *e.g* c-myc[107], linking polyamine biosynthesis with tumour formation. ODC is subjected to feedback regulation with high intracellular polyamine levels resulting in the induction of antizyme (AZ)[108], an ODC-specific inhibitor. Polyamine-mediated regulation of AZ synthesis is predominantly carried out via a unique +1 frame shifting mechanism on the AZ mRNA required for translation of the complete AZ protein[109]. Once translated, AZ will bind to ODC monomers and thereby

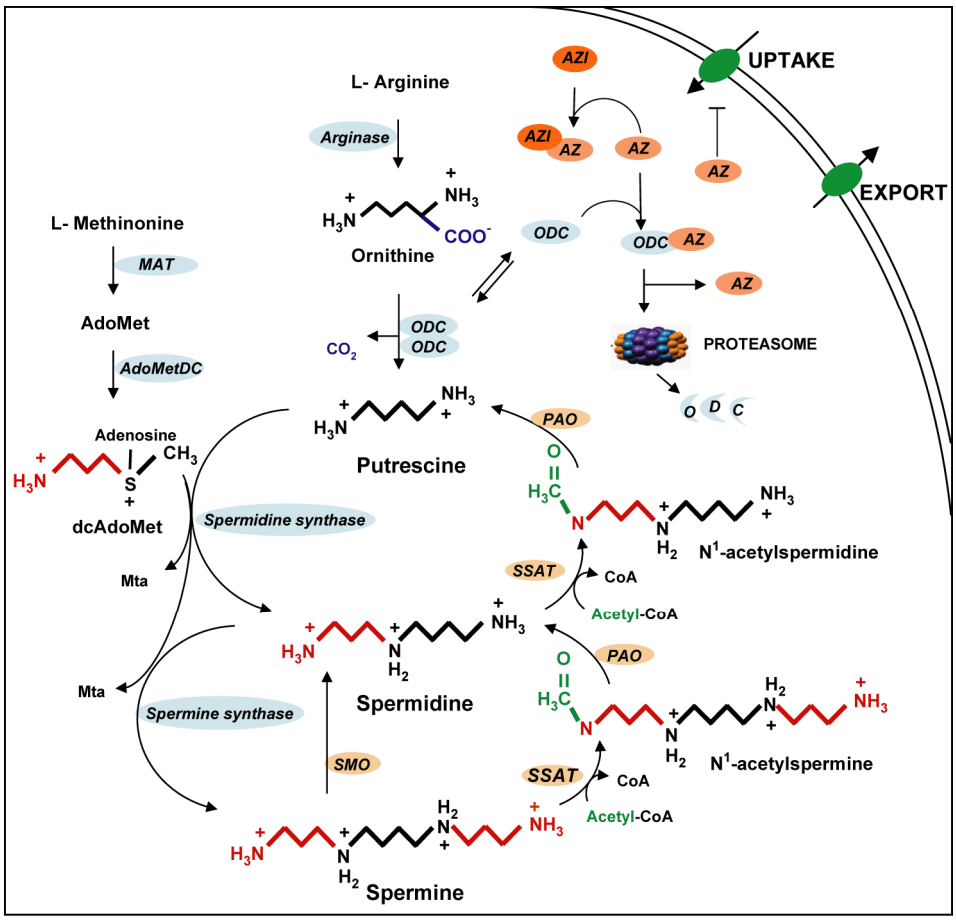

Figure 1. Key features in polyamine metabolism.
MAT, methionine adenosyltransferase; AdoMet, S-adenosylmethionine; AdoMetDC, S-adenosylmethionine decarboxylase; dcAdoMet, decarboxylated S-adenosylmethionine; Mta, methylthioadenosine; ODC, ornithine decarboxylase; PAO, polyamine oxidase; SSAT, spermidine/spermine acetyltransferase; SMO, spermine oxidase; AZI, antizyme inhibitor; AZ, antizyme. Adapted from [88] and [105].

inactivate, and more importantly, target ODC for degradation by the 26 proteasome in an ubiquitin-independent manner[110]. In addition, AZ has been found to downregulate the polyamine transport system[111, 112], making AZ a "master regulator" of polyamine homeostasis by controlling biosynthesis as well as uptake. AZ, in turn, has its own regulator, AZ inhibitor (AZI)[113]. AZI binds AZ with a

higher affinity than ODC, mediating the release of ODC from the ODC/AZ complex[114]. Like ODC, AZI is also rapidly degraded but in contrast to ODC, it is degraded in an AZ-independent, ubiquitin-dependent manner. In fact, binding to AZ stabilizes AZI, protecting it from degradation[115]. siRNA-mediated downregulation of AZI increases AZ activity and downregulates ODC levels and polyamine levels[116]. Conversely, overexpression of AZI results in elevation of ODC activity, increased polyamine uptake and enables tumour formation in nude mice[117], demonstrating the important regulatory role of AZI in polyamine homeostasis.

The decarboxylation and aminpropyl transferase reactions are practically irreversible and hence, there is a distinct system that converts the higher polyamines back to putrescine. Spermidine/spermin N^1-acetyltransferase (SSAT) acetylates spermidine and spermine at primary amino groups and these intermediates are then converted to putrescine and spermidine respectively by polyamine oxidase (PAO)[105]. Relatively recently, an oxidase that could convert spermine to spermidine without the requirement for an acetylated intermediate was cloned and named spermine oxidase (SMO)[118]. SSAT is, similarly to ODC, a highly inducible enzyme[105] and it has been reported to respond to changes in polyamine levels through a polyamine response element (PRE) in the SSAT gene[119]. In addition, the oncogene K-RAS negatively regulates SSAT, contributing to the increased polyamine levels brought about by K-RAS activation[120].

Polyamine transport

The polyamine biosynthesis and uptake systems work in concert to ensure proper intracellular levels of polyamines, and inhibition of endogenous biosynthesis results in compensatory upregulation of uptake[121]. Exogenous polyamines are derived both from dietary intake[122] and from the intestinal flora[123].

Transport systems for polyamines have been cloned in E. Coli[124]. There is one putrescine specific uptake system and one spermidine-preferential transport system, both of which consist of channel forming and substrate binding proteins as well as ATPases[125]. In addition, there is a third transporter, specific for putrescine that is capable of both uptake and excretion[125].

Whereas the key enzymes responsible for polyamine biosynthesis and catabolism have been cloned and characterized, there is to date no mammalian polyamine transporter cloned and the mechanism by which polyamines enter

mammalian cells is still poorly understood. A large number of studies have been addressing this issue and collectively, they point towards an energy-dependent, carrier-mediated and saturable system[126]. Two types of carriers have been proposed, one with the preference for putrescine and one for spermidine and spermine[126] but it has also been suggested that all three polyamines enter cells through the same transporter. In either case, the transport system is non-specific, as demonstrated by the uptake of drugs with structures resembling the natural polyamines, *e.g* methylglyoxalbisguanylhydrazone (MGBG)[127] and the competitive inhibition by polybasic peptides (*e.g* the HIV-1 trans-activator of transcription (TAT) transduction peptide, Tat, paper **I**) and cationic lipids[128].

Earlier studies from our group have suggested an important role for cell surface heparan sulphate proteoglycans (HSPGs) (described more in detail below) in the uptake of polyamines. Spermine was shown to bind HS with an affinity 10 times that for DNA[129] and degradation of cell surface HSPGs by HS lyase treatment resulted in reduced uptake[130]. Polyamine uptake was not completely abrogated however, indicating additional HSPG-independent uptake pathways. The use of competitive inhibitors of HS synthesis inhibited polyamine-dependent cell growth *in vitro*. Interestingly, inhibition of polyamine synthesis resulted in an altered distribution of HSPGs between the extracellular and cell-associated pools with the latter containing increased levels of HSPGs with high affinity for spermine, as compared with control cells[130]. It was further shown that polyamine depleted cells synthesize glypican-1 HS chains with increased affinity for spermine[131]. Mutant CHO cells deficient in different aspects of HS synthesis[132] were more sensitive to growth inhibition by polyamine depletion *in vitro* and were unable to utilize exogenously added polyamine to restore growth[133]. The CHO mutant with the most severe phenotype, pgsA, formed almost no tumour foci in the presence of the polyamine biosynthesis inhibitor α-difluoromethylornithine (DFMO), as shown in a mouse model of haematogenous lung metastasis[133]. It has been suggested that cell surface associated, recycling glypican-1 might be a membrane carrier for polyamines and that the uptake is mediated through an endocytic pathway[134] involving caveolin-1 positive endosomes[135]. The notion of an endocytic uptake mechanism for polyamine uptake was disputed by Poulin and coworkers that argued for a two-step mechanism of polyamine uptake; the initial transport across the plasma membrane via a classical transporter, followed by sequestration in special vesicles in the late endocytic compartment[136]. These conclusions were based on the use of mutant cells deficient in clathrin-coated pit

dependent endocytosis and hence, do not rule out the involvement of clathrin-independent endocytosis. Recently, it was suggested that polyamines associate with lipid rafts and that they are internalized by a clathrin-independent, caveolin-1 regulated endocytic pathway[137]. Moreover, it was shown that oncogenes like K-RAS can regulate polyamine uptake by modulating caveolin-1 phosphorylation[137]. In summary, polyamine internalization in mammalian cells is a highly regulated process that works in concert with biosynthesis in order to maintain polyamine homeostasis. Growing evidence point towards the involvement of an endocytic uptake mechanism that, at least for the higher polyamines spermidine and spermine, is dependent on cell surface HSPGs. Accordingly, in paper **I** and **II** of this thesis**,** we show that the HIV-TAT transduction peptide, Tat, which is known to be internalized via PG-dependent pathways[138], and an epitope specific single chain variable fragment (ScFv) anti-HS antibody, respectively, interfere with polyamine uptake and polyamine-dependent cell growth.

The polyamine export system is ill-defined but is suggested to be a selective process, regulated by the growth status of the cell[88]. Recently, a diamine exporter was identified that mediates arginine uptake and putrescine efflux, suggesting an arginine/putrescine exchange reaction[139]. Moreover, the exporter was found to be associated with SSAT, indicating that the export and acetylation systems might be linked[139].

Polyamines and cancer

As discussed above, the link between polyamines and cancer development is now well established and the mechanistic basis for this association is gradually becoming clearer. ODC is a putative proto-oncogene and under cell culture conditions, overexpression of human ODC cDNA in mouse NIH 3T3 cells leads to transformation[140]. However, increased ODC activity alone does not appear to be sufficient for transformation as ODC-overexpressing transgenic mice, despite 20-50 times higher ODC activity than their syngenic littermates, did not display a differential incidence in spontaneous tumour formation compared to WT mice[141]. Instead, ODC deregulation seems to be an integral part of the transformation process rather than an initiating event and indeed, numerous means of deregulation have been reported to occur in response to oncogenic stimuli[142]. ODC is a transcriptional target of c-myc[107] and can mediate both myc-induced lymphomagenesis[143] and familial adenomatous polyposis coli (FAP)[144]. Evidence

for a causative rather than associative effect of a deregulated polyamine system in human colon cancer was provided by Martinez *et al.*[145]. They showed that a single nucleotide polymorphism (SNP) in the ODC gene reduced polyamine synthesis and was associated with reduced risk of colon-polyp recurrence in individuals taking aspirin[145]. Aspirin does not affect the transcription of ODC but instead induces the transcription of SSAT[120], resulting in increased catabolism of polyamines. Polyamine expansion, through severe deregulation of polyamine regulatory enzymes including ODC, has been suggested to be a hallmark of neuroblastomas with amplified MYCN[146]. Accordingly, targeting ODC with DFMO delayed tumour incidence and onset in a mouse model of neuroblastoma[147]. In addition, ODC expression was dramatically induced in response to RAS activation[148]. In a model of tumour progression and invasiveness, elevated levels of ODC were demonstrated to cooperate with the RAS/RAF/MEK/ERK pathway in the conversion of normal keratinocytes into invasive malignant cells[149], indicating a threshold of ODC activity required for tumour cell invasion.

The use of transgenic animal models provides a unique system to study the events and gene interactions taking place during tumour development. One of the most studied models for carcinogenesis is the two-stage skin carcinogenesis protocol. In this model, an initiation phase involving DNA damage that will lead to gene mutations is accomplished by topical application of a sub-carcinogenic dose of a carcinogen. Subsequently, a promotion phase involving proliferation of initiated cells is elicited by repeated treatment with a cancer promoting agent [142]. This and similar protocols have been applied in a large number of different polyamine-associated transgenic mice in order to study the role of polyamines in carcinogenesis. The different mouse lines and their cancer-related phenotypes are summarized in **Table 1**. In brief, in the majority of the animals, targeted expression of ODC, AZ and SSAT to skin has been achieved through expression from keratin promoters, and in some cases double transgenic mice have been created through breeding with animals with aberrant expression of certain oncogenes, *e.g.* RAS and MEK. Forced expression of ODC in this system increases the sensitivity for chemical- and UV-induced carcinogenesis, and overexpression of AZ inhibits tumour formation. Induced expression of SSAT has created ambiguous results with both increased and decreased susceptibility to carcinogenesis reported. These results collectively establish polyamines as crucial

players in carcinogenesis and neoplastic growth. What are the specific functions of the polyamines?

The polyamine status of a cell specifically influences the expression and stability of various growth related genes and the examples of *e.g.* c-myc[150, 151] and JunD[152] clearly illustrate how polyamines can influence cellular proliferation; increased polyamine levels induce c-myc which inhibits p21Cip1[150], an important regulator of cell cycle control and conversely, reduced polyamine levels stabilize JunD which inhibits cyclin-dependent kinase (CDK) 4[152], a protein essential for propagation through the cell cycle. Changes in polyamine levels have also been reported to specifically alter the expression of genes involved in cell-cell contact formation[153], with implications for tumour development. In addition, overexpression of ODC has been found to alter intrinsic histone acetyltransferase (HAT) and deacetylase (HDAC) activities both *in vitro* and *in* vivo[154, 155]. These effects were reversible upon DFMO treatment, implicating elevated polyamine levels in the regulation of chromatin structure. In addition to regulating tumour cell behaviour, polyamines are also recognised as being important modulators of stroma cells. As discussed above, angiogenesis is a pivotal process for tumour development and polyamines have proved necessary for blood vessel formation. ODC overexpressing cells form highly vascularised tumours in mice[156] and elevated ODC activity in the skin of ODC transgenic mice increased dermal vascularisation[157]. Accordingly, micro vessel sprouting and migration of primary human ECs were impaired upon polyamine depletion *in vitro*[158] and DFMO-mediated inhibition of tumour growth in a gastric cancer model was exerted partly through anti- angiogenic effects[159]. The role of polyamines in apoptosis appears to be bi-functional with reports of both protective and stimulatory effects of polyamine depletion on tumour and EC apoptosis[96, 160]. Similarly, tumour regression after DFMO treatment in skin tumour models has been reported to involve tumour cell apoptosis in some mouse lines[161] but not in others[162]. In paper **III** of this thesis, we provide a new piece to the puzzle by showing a novel role of the polyamine system in the adaptive response of tumour cells to hypoxic stress, implicating polyamines as important modulators of hypoxia-induced cell death.

The importance of polyamines in cancer development makes the polyamine pathway a suitable target for the development of anti-cancer drugs and has inspired the design and synthesis of a large number of polyamine biosynthesis inhibitors[163]. Due to their key, rate-limiting roles in polyamine biosynthesis, inhibitors of ODC and AdoMetDC have been of particular interest. DFMO is a highly specific

suicide inhibitor of ODC and was the first effective and rationally designed anti-proliferative drug targeting the polyamine system[164]. *In vitro* studies have demonstrated depletion of putrescine and spermidine whereas spermine levels are generally less affected[165]. Polyamine depletion results in growth arrest, and block in G_1 as well as prolongation of S phase have been reported[163]. Reduced proliferation however, will not automatically lead to cell loss and accordingly, the high expectations of DFMO from *in vitro* studies were not met *in vivo*. DFMO was actively evaluated, either alone or in combination with other agents and radiotherapy, in clinical trials during the 70's and 80's but due to the cytostatic rather than cytotoxic effects, DFMO was a rather unsuccessful anti-cancer agent[163]. The main reason for this failure has been attributed to a compensatory increase in uptake of polyamines from the circulation[121]. The greater therapeutic effect of DFMO in mice inoculated with mutant L1210 leukemia cells, deficient in polyamine uptake, than in mice inoculated with WT leukemia cells strongly supports this notion[166]. In addition, recycling of spermine[88] and amplification of the ODC gene[167] have been reported to contribute to the overcoming of the DFMO effects. Despite the overall disappointing results of DFMO on established tumours in clinical settings, it has shown some beneficial results in malignant gliomas[168] and has proven to be a potent inhibitor of carcinogenesis, making it attractive as a chemopreventive agent[169]. In addition, DFMO is an active anti-parasitic agent that is used in the treatment of African sleeping sickness[170]. It should also be mentioned that another type of drugs targeting the polyamine system is being evaluated in clinical studies, *i.e.* the polyamine analogues[171]. Polyamine analogues are similar enough to the parent polyamines to become internalized by the polyamine transporter but dissimilar enough to be unable to execute polyamine functions within cells[171].

The discussion above illustrates the necessity of combining polyamine biosynthesis inhibition with uptake inhibition in order to achieve effective anti-tumour effects. Indeed, in this thesis, evidence is provided that polyamine uptake inhibition enhances the effect of DFMO *in vitro* (paper **I** and **II**) and that combined targeting of biosynthesis and uptake efficiently reduces tumour growth *in vivo* (paper **I**).

Table 1. Phenotypes of polyamine-related transgenic mice

Transgene	Phenotype	Ref
K2 *General ODC overexpression*	Increased tumorigenesis in response to two-stage skin carcinogenesis protocol	172
K5/ODC / K6/ODC *Epithelial-targeted (predominantly skin) overexpression of ODC*	Spontaneous skin tumours; sensitive to topical application of numerous genotoxic carcinogens; polyamine-dependent increase in susceptibility to DMBA-induced carcinogenesis; increased susceptibility to photo carcinogenesis	162, 173-176
K6/ODCdn *Skin-targeted expression of dominant-negative form of ODC*	Equally susceptible to skin carcinogenesis as compared to WT littermates	177
$ODC^{+/-}$ *Heterozygous for ODC*	Decreased susceptibility to two-stage skin carcinogenesis protocol	178
RAS/ODC *Skin-targeted overexpression of ODC, general overexpression of RAS*	Polyamine-dependent formation of spontaneous skin tumours; increased apoptosis and decreased tumour vascularization in DFMO treated tumours	157, 179
$Ptch1^{+/-}$/ODC *Skin-targeted overexpression of ODC, heterozygous for PTCH1*	Increased susceptibility to UV carcinogenesis comp. to $Ptch1^{+/-}$ littermates	180
Eμ-MYC/$ODC^{+/-}$ *B-cell overexpression of MYC heterozygous for ODC*	Delayed MYC-induced lymphoma development	143
K5/AZ / K6/AZ *Epithelial-targeted (predominantly skin) overexpression of AZ*	Decreased susceptibility to two-stage skin carcinogenesis in different genetic backgrounds; reduced susceptibility to stomach carcinogenesis	181-183

MEK/K5-AZ / MEK/K6-AZ *Skin-targeted overexpression of MEK and AZ*	Decreased susceptibility to MEK-driven skin carcinogenesis	[184]
PTCH1$^{+/-}$/AZ *Skin-targeted overexpression of AZ, heterozygous for PTCH1*	Decreased susceptibility to UV carcinogenesis comp. to PTCH1$^{+/-}$ littermates	[180]
MT/SSAT *Targeted overexpression of SSAT from the metallothionein I promoter*	Decreased susceptibility to two-stage skin carcinogenesis protocol	[185]
K6/SSAT *Skin-targeted overexpression of SSAT*	ODC and putrescine-dependent increase in susceptibility to two-stage carcinogenesis protocol	[186, 187]
K6/SSAT / K6/AZ *Skin-targeted overexpression of SSAT and AZ*	Blocked SSAT-driven skin carcinogenesis after two-stage protocol	[187]
APC$^{+/-}$/SSAT *General overexpression of SSAT, heterozygous for APC*	Increased intestinal tumourigenesis comp. to APC$^{+/-}$ littermates	[188]
APC$^{+/-}$/SSATko *SSAT knock out, heterozygous or APC*	Decreased intestinal tumourigenesis comp. to APC $^{+/-}$ littermates	[188]
TRAMP/SSAT *SV40 antigens expressed from androgen-driven promoter, general overexpression of SSAT*	Decreased prostate tumour growth comp. to TRAMP littermates	[189]

ODC, Ornithine decarboxylase; K5, Keratin 5 promoter; K6, Keratin 6 promoter; DMBA, 7,12-dimethylbenz(a)anthracene; DFMO, α-difluoromethylornithine; PTCH1, Patched 1; AZ, Antizyme; MT, Metallothionein; SSAT, Spermidine/spermine N^1-acetyltransferase; APC, Adenomatous polyposis coli, TRAMP, Transgenic adenocarcinoma of mouse prostate

Proteoglycans

The proteoglycan (PG) superfamily comprises a large number of molecules with diverse functions spanning from regulation of cell growth and tissue organization to collagen fibrillogenesis. PGs consist of a core protein substituted with one or several glycosaminoglycans (GAGs), *i.e.* linear and highly diverse carbohydrate chains made up of 40-160 disaccharide units. PGs can be found both in the ECM, intracellularly and on the cell surface. The basement membrane PGs are primarily substituted with HS-GAGs and include perlecan, agrin and collagen XVIII[190]. Other ECM PGs are mostly substituted with the chondroitin sulphate (CS), dermatan sulphate (DS) or keratan sulphate (KS) GAGs and include hyalectans, molecules interacting with hyaluronan and lectins, and small leucine-rich PGs (SLRPs)[191]. The dominating cell surface PGs are the HS substituted families of the membrane spanning syndecans and the glycosylphosphatidylinositol (GPI)-anchored glypicans. There are four syndecans (syndecan 1-4) and six glypicans (glypican 1-6) identified in mammalians and they all modulate the actions of a large number of extracellular ligands. In this thesis, the function of cell surface HSPGs as internalizing receptors is addressed and the following presentation will focus on this class of PGs.

Heparan sulphate synthesis

HS synthesis is a complex process that takes place in a sequential manner by membrane spanning enzymes residing in the endoplasmic reticulum (ER) and the Golgi apparatus[192]. HS is synthesized by a) the formation of a linker region b) the generation of the polysaccharide chain and c) enzymatic modifications of the GAG chain.

Both HS and CS have an identical linkage tetrasaccharide as the initial sequence added to the core protein. This linkage region is initially formed through the addition of a xylose (Xyl) from UDP-Xyl to a serine residue in the core protein by xylosyltransferase. The recognition site in the protein for the xylosyltransferase

is a serine-glycine dipeptide and one or more flanking acidic residues[193]. The subsequent addition of two galactose (Gal) residues and one glucuronic acid (GlcA) completes the –GlcA-Gal-Gal-Xyl linker region[194]. After this step, the HS assembly diverges from that of CS where the addition of N-acetyl glucosamine (GlcNAc) to GlcA initiates HS synthesis (see **Figure 2**) whereas the addition of N-acetyl galactosamine (GalNAc) is the first step in CS synthesis. Elongation of the HS chain then proceeds through the sequential addition of GlcA and GlcNAc by the products of the exostosin (EXT) 1 and 2 tumour suppressor genes. Both proteins are thought to be important for HS polymerization and are probably a part of a larger heterodimeric complex[195], responsible for chain extension. As the chain extends, it can be enzymatically modified at various positions. This process seems to occur in a strictly orderly manner with the product of one modification serving as substrate for the next[196]. The initial modification enzyme, N-deacetylase/N-sulphotransferase (NDST), substitues the N-acetyl group with a sulphate group in clusters of GlcNAc (converting GlcNAc to GlcNS), leaving regions of the chain unmodified. Further modifications, which tend to occur after N-sulphation, are epimerization of GlcA into iduronic acid (IdoA) by C5-epimerase, 2-*O*-sulphation of IdoA and GlcA by 2-*O*-glucuronic acid/iduronic acid sulphotransferase and subsequently further sulphation at position C6 and, more seldom, C3 by 6-*O*-sulphotransferase and 3-*O*-sulphotransferase, respectively[197]. The modification reactions are clustered around the GlcNS-containing disaccharides and will thus result in a characteristic GAG domain structure of highly sulphated domains (NS domains) interspersed between largely unmodified regions (NA domains)[198]. This biosynthetic machinery results in tremendous diversity and it has been suggested that virtually all chains are structurally unique[199]. The structure of HS chains does not appear to be correlated to the core protein onto which they are assembled[200] and is though to be similar on different core proteins within the same cell[201]. The enormous diversity of HS is however, to some extent, regulated. Changes in HS composition have, for example, been recorded in association with developmentally related process in the embryonic brain[202], and DFMO induces structural changes of HSPG to accommodate increased polyamine uptake[131]. All HS biosynthetic enzymes except for C5-epimerase and 2-*O*-sulphotransferase exist in numerous isoforms and the amount or proportions of different isoforms in a given cell might also be critical for the structure of the HS chain[199]. Indeed, tissue regulated modification patterns have been observed by van Kuppevelt and co workers when studying HS epitope distribution using phage display-derived ScFv anti-HS

antibodies[203-205]. These antibodies recognize specific HS epitopes, 3-4 monosaccharides in length, several of which have been relatively well characterized. The development of antibodies for the study and mapping of HS epitopes instead of using traditional biochemical methods will most likely improve our understanding of the complex HS biology. In paper **II** of this thesis, some of these antibodies were employed in a first attempt to gain insight into the structural requirements for polyamine binding to HSPG. Moreover, they were used as a novel approach to inhibit polyamine uptake in cancer cells.

Functional interactions of heparan sulphate proteoglycans

The binding of proteins to cell surface HSPGs can serve numerous functions including immobilization of the ligand, protection from degradation, increase of the local concentration and change of conformation and presentation to cognate high affinity receptors[194]. The overall effect is usually to enhance receptor activation at low ligand concentration, and the diverse nature of ligands makes HSPGs important players in a large number of biological processes such as lipid metabolism, angiogenesis and organ development. An "on/off mechanism" for HSPG function has been proposed[206] in which the presentation of ligands in the right conformation to their receptors represents the "on" state of HSPG function. Notably, cell surface HSPGs are in a dynamic state and their internalization and lysosomal degradation[207] can also result in the clearance of bound ligands – the "off" mechanism[206].

In general, ligand binding depends on the arrangements of NS and NA domains and on the modified residues within the NS domains. Two types of sites for ligand interactions have been identified. Regions consisting of relatively common disaccharides arranged in a specific pattern, as in the case of FGF and FGFR[197, 208], can confer specific interactions. Alternatively, a specific, relatively uncommon modification can be recognized, which is exemplified by antithrombin that binds to a specific HS pentasaccharide containing an internal 3-O-sulphate[209]. The binding motifs for the negatively charged HS chains are usually protein domains containing a stretch of basic amino acids[210]. Numerous cytokines, growth factors and microbes contain these domains[194] facilitating interactions with cell surface HSPGs. In addition, cell surface HSPGs, particularly the transmembrane syndecans, can bind ECM components and the combined interaction with integrins

results in cytoskeletal reorganizations and the formation of *e.g.* focal adhesions, important for cell adhesion[211]. Moreover, secreted HSPGs also interact with growth factors, and perlecan that resides in the basement membrane, has a particularly important role in matrix sequestration of HS ligands[212].

Heparan sulphate proteoglycans and cancer

HSPGs have been shown to interact with a large number of molecules essential for tumour growth and angiogenesis and importantly, their own expression and overall metabolism have been demonstrated to be altered in malignant tissues[213].

HSPGs interact with cytokines such as IL-8[214] and SDF-1α[215] and control their activity by localizing them in the right extracellular compartment. Binding to HS also establishes cytokine gradients which are crucial when cells are required to migrate through tissues, as in the case of angiogenesis and metastasis. Growth factors such as FGF, VEGF, hepatocyte growth factor (HGF) and PDGF have all been shown to interact with HSPGs[197]. VEGF-A exists in several isoforms, two of which contain HS binding basic regions. These domains are required for proper retention of the growth factors in the matrix in order to direct EC migration during angiogenesis. Loss of these retention motifs results in misguided ECs and reduced capillary branch formation[216]. Furthermore, the release of heparanase from tumour cells[217] might promote cleavage of HS fragments and thereby release of bound growth factors together with soluble, growth promoting HS fragments[218], further supporting the growth and neovascularisation of the tumour. Malignant cells regulate the expression and structure of HSPGs to facilitate increased growth factor stimulation. Glypican-1 is overexpressed in pancreatic cancer cells[219] promoting the mitogenic response to growth factors such as bFGF and heparin binding EGF-like growth factor (HB-EGF). Moreover, syndecan-1 expression in tumour and stroma cells has been shown to be altered during tumour progression. Syndecan-1 is upregulated in stromal fibroblasts of infiltrating breast carcinomas[220] and its shedding contributes to a paracrine potentiation of bFGF stimulation of the breast carcinoma cells[221]. Simultaneous decrease of carcinoma cell syndecan-1 expression leads to reduced cell adhesion and increased invasive potential[220]. Interestingly, several of the above mentioned growth factors and cytokines are regulated by hypoxia, and HIF-1α mediated induction of these factors is important during tumourigenesis. As cell surface HSPGs act as intimate co-receptors and modulators of these factors, one might speculate that HSPGs

themselves are regulated by hypoxia. In support of this notion, it was shown that hypoxia regulates the expression of regulatory enzymes responsible for HS chain synthesis, resulting in increased responsiveness of hypoxic ECs to bFGF[222]. We hypothesised that tumour cell HSPG function could be similarly altered and in paper **III** of this thesis, it was indeed shown that hypoxia enhances polyamine uptake; a role for HSPGs in hypoxic induction of the uptake of polyamines and other HS binding ligands is likely but remains to be investigated in greater detail.

Internalization of macromolecules

Cell surface HSPGs not only bind extracellular ligands, but also facilitate the internalization of numerous macromolecules[206] (see **Figure 2**). Well known examples are the lipid-associated proteins apolipoprotein E and lipoprotein lipase[223], the growth factor bFGF[224, 225] and polyamines[130]. HSPGs have also been implicated in microbe invasion of host cells[206]. Yet another class of molecules that appear to use cell surface HSPGs for cellular entry are proteins containing so called protein transductions domains (PTDs) or cell penetrating peptides (CPPs) – short stretches (approximately 15 amino acids) rich in the basic amino acids arginine and lysine[226]. These peptides can be taken up on their own or be fused to other proteins and thereby mediate their internalization[138, 227]. The early indication that certain proteins, other than bacterial toxins, could efficiently enter cells came from the viral HIV-TAT protein. In 1987, Frankel and Pabo demonstrated that TAT could shuttle between cells and by entering the cytosol and the nucleus of cells adjacent to where it had originally been synthesized, TAT could activate dormant virus in recipient cells[228]. In the context of cancer therapy, the fusion of PTDs to different proteins with anti-cancer activity has been used as a delivery strategy[227] and examples of proteins or peptides delivered to cells in this manner include p53, p16, a pro-apoptotic Smac peptide and BH3 pro-apoptotic domain (for refs, see [227]). Of particular interest in the context of this thesis is the strategy developed by Harada *et al*.[229]. They created a TAT-ODD-caspase-3 fusion protein in order to induce cell death selectively in hypoxic regions of tumours. The ODD domain facilitates degradation of the protein in normally oxygenized cells whereas hypoxic cells will be subjected to caspase-mediated apoptosis. Accordingly, intraperitoneal injection of the fusion protein into tumour bearing mice resulted in stabilization of the protein in the solid tumours and reduction of tumour mass[229].

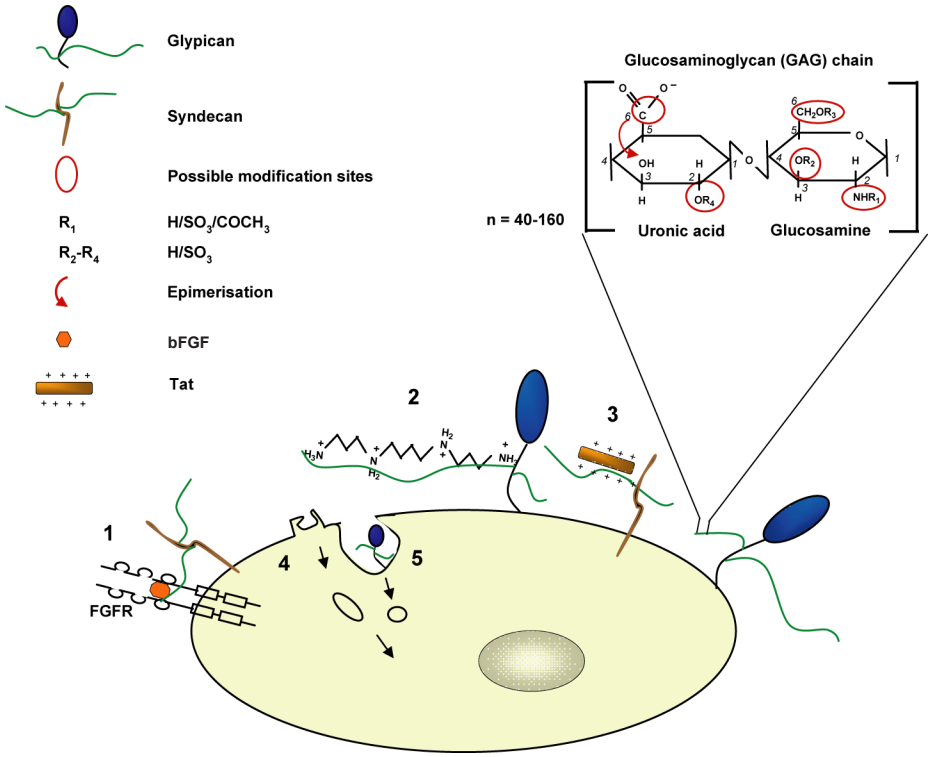

Figure 2. Cell surface HSPGs as internalizing receptors. The HS GAG chain is composed of uronic acid (glucuronic acid (GlcA) or iduronic acid (IdoA)/ glucosamine (GlcN) disaccharides. These sugar residues are modified by N-deacetylation and sulphation of GlcNAc (R_1), epimerization at the C5 position of GlcA into IdoA and by specific sulphotransferases (R_2-R_4). The modified chains are highly anionic and extremely diverse, giving rise to numerous interactions with cationic macromolecules. For example, HSPGs bind to both bFGF and FGFR, facilitating internalization of bFGF (1). The polybasic polyamines (2) are taken up by cells in an HSPG-dependent manner and internalization of the polybasic Tat peptide (3) also requires cell surface HSPGs. HSPG-dependent uptake involves endocytosis and both syndecans and glypicans can be endocytosed together with ligands. The exact endocytic pathway for HSPG-mediated cellular entry is still unknown and both macropinocytosis (4) and clathrin-independent pathways sensitive to membrane cholesterol depletion (5) have been suggested.
HS, heparan sulphate; HSPG, heparan sulphate proteoglycan; bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; Tat, TAT protein transduction domain.

Many PTDs can be used to deliver macromolecules both when fused to a cargo protein, as described above, and as an electrostatic complex with macromolecular cargo, *e.g.* DNA plasmid. Mislick and Baldeschwieler demonstrated that poly-lysine/DNA complex uptake was dependent on HSPGs as digestion of cell surface HSPGs drastically decreased complex uptake and expression of transfected DNA was approx. 50 times lower in PG deficient cells than WT cells[230]. Further studies by our group have shown a PG dependence for both cationic lipid- and Tat, mediated DNA uptake[231, 232].

Studies by our group and others have established that HSPG-dependent uptake of PTDs, either on their own or complexed to negatively charged macromolecules, involves endocytosis[226]. The exact pathway for HSPG-mediated cellular entry is however still debated. The ambiguity of the current characterization of the different endocytic pathways together with the use of unspecific drugs and varying experimental conditions probably contribute to the observed discrepancies between proposed pathways. Nevertheless, certain themes are starting to emerge; membrane cholesterol seems to be of importance, clathrin-mediated pathways are probably not a major route of entry and the actin cytoskeleton might be involved[225, 233, 234]. Until very recently, it has been an open question whether the cell surface HSPGs merely present ligands to their true internalizing receptors or if, in fact, the HSPGs themselves are the true internalizing entities. Endocytosis of glypican-1 has been implicated in the uptake of polyamines[134] and it has now convincingly been shown that both syndecans and glypicans are found in endocytosed vesicles, demonstrating HSPGs to be true internalizing receptors for various macromolecules[235].

In paper **II** of this thesis, we capitalized on the fact that Tat is taken up by cells in an HSPG-mediated manner[138] and explored the novel strategy of using this particular peptide as an inhibitor of polyamine uptake *in vitro* and *in vivo*.

Microvesicles

Biogenesis of microvesicles

The traditional view of cellular communication involves a) direct cell-cell contact where membrane proteins interact with membrane receptors on neighbouring cells in trans, b) cell-ECM contact whereby matrix components can regulate intracellular signalling events through interactions with cell surface receptors, c) signalling molecules like cytokines or growth factors secreted from one cell and deciphered by another upon cell surface receptor binding or d) as for steroid hormones, upon binding to intranuclear receptors after direct penetration of the plasma membrane.

This convention has been challenged by recent findings suggesting that secreted membrane vesicles, exosomes and shedding vesicles, constitute novel players in cell-cell communication[236-241]. These endogenous macromolecules emerge as pivotal mediators of information transfer between cells, conveying horizontal propagation of complex and multi-facetted messages. The existence of vesicle-like structures secreted by cells was first noticed in 1967 and referred to as "pro-coagulant particle matter" around platelets[242]. Reticulocyte-derived exosomes were later described as a means of removing transferring receptors during reticulocyte maturation[243]. Since these land marking reports, vesicles have been shown to be secreted by numerous cell types including dendritic cells, platelets, epithelial cells, B lymphocytes as well as numerous types of tumour cells[239, 241].

Vesicular secretion is a physiological phenomenon associated with cell growth and activation; besides constitutive secretion, vesicular release has been associated with cellular activation *e.g.* by thrombin for platelets[244], growth factors for prostate cancer cells[245] or upon transformation of glioma cells[246]. Intensive research over the last ten years has identified two vesicle-discharge processes, each leading to release of distinct types of vesicles; the release of exosomes from exocytosis of multivesicular bodies (MVBs) and the release of shedding vesicles by direct budding of the plasma membrane[237].

The exosomes are defined as small vesicles (30-100 nm in diameter) that have an endosomal origin. The MVB is an intermediate but well-defined cellular compartment that is formed from reversed budding of endosomes, giving rise to enclosed intraluminal vesicles (ILVs)[247]. Fusion of a MVB with the lysosome will result in degradation of ILV-associated proteins and lipids, whereas fusion of the MVB with the plasma membrane results in the release of the ILVs as exosomes[238]. Very little is known about the signals responsible for protein sorting to MVBs and currently, there is no common sorting signal identified. However, mono-ubiquitination has been shown to serve as a sorting signal for trafficking to MVBs[248]. Ubiquitinated cargos are recognised by the hetero-oligomeric protein complex endosomal sorting complex required for transport (ESCRT) I- II- and III that, together with additional proteins, promote the inclusion into MVBs[247].

The shedding vesicles are larger than exosomes, 100-1000 nm in diameter, and are formed through outward budding of the plasma membrane. As for exosomes, the sorting mechanisms for inclusion of cargos into shedding vesicles remain obscure. However, several pathways have partly been delineated in this process. Activation-induced increase of intracellular Ca^{2+} levels triggers vesicular shedding[249] and Ca^{2+} signalling has been postulated to have a central role in vesicle formation. The plasma membrane phospholipid asymmetry with *e.g.* phosphatidylserine (PS) and phosphatidylethanolamine (PE) located at the cytosolic side of the membrane is maintained by enzymes like translocases, scramblases and floppases[250]. Ca^{2+} released from the ER upon cell activation perturbs this asymmetry through regulation of several of these enzymes, which contributes to vesicle budding. As a result, shed vesicles display relatively large amounts of PS in their outer layer of the membrane[250]. In addition, Ca^{2+} activates cytosolic proteases, leading to the reorganization of the cytoskeleton, detachment of the plasma membrane from the cortical actin, and finally induction of vesicle shedding[239]. Moreover, studies using cholesterol depleting drugs have pointed to the importance of cholesterol-rich lipid raft domains in the biogenesis of shedding vesicles[251].

The composition of secreted vesicles seems to largely reflect their donor cells; vesicles secreted from antigen presenting cells, such as dendritic cells, contain MHC-I and –II molecules[252], tumour cells can secrete vesicles containing FasL[253] and reticulocyte-derived vesicles contain transferrin receptor[243]. Nevertheless, some proteins are located on the surface or in the lumen of almost all vesicles, independently of their cell of origin, and may thus be considered as vesicular

markers[241]. Although it is unlikely that exosomes and shedding vesicles that are produced by so fundamentally different processes would carry the same cargo, there are at present no distinct markers for exosomes and shedding vesicles, respectively. Some vesicle type-specific markers have been suggested, including β1 integrin and metalloproteinases for shedding vesicles and tumour necrosis factor receptor (TNFR)1 for exosomes[237]. Moreover, consistent with their endosomal origin, exosomes typically do not contain ER, mitochondrial or nuclear proteins. The most frequently used markers, however, are utilized to characterise both exosomes and shedding vesicles. These include, but are not limited to, cytoplasmatic proteins like annexins and heat-shock proteins like Hsp70 and Hsp90[241]. Members of the tetraspanin family, particularly CD63 and CD81 are widely used vesicular markers [241, 254] and in addition, the absence of *e.g.* mitochondria-residing proteins like cytochrome C or Golgi-associated proteins like GM130 has been used to illustrate the purity of isolated vesicle populations[255].

The research field of secreted vesicles is still in its infancy and the nomenclature of secreted, membrane-enclosed vesicles is still controversial. Proposed names for vesicles of different cellular origin include: ectosomes for neutrophils, microparticles for platelets and monocytes, exovesicles for dendritic cells and shedding vesicles for tumour cells[237]. Depending on cell type, experimental settings, markers and isolation protocol used, the isolated vesicle population may consist solely of exosomes, solely of shedding vesicles or, perhaps most likely, of a mix of both. In this thesis, the general term MVs will be used to define secreted, membrane-enclosed vesicles derived either from exocytosis of MVBs or from budding of the plasma membrane.

MVs are biologically active entities that may target cells in close proximity of their donor cells as well as cells of remote tissues. Potentially, they can interact with cell membrane receptors of targets cells, eliciting a signalling response, or fuse with the plasma membrane releasing their cargo into the cytoplasm or be taken up by the target cell through endocytosis. Alternatively, MVs can be disrupted, thereby releasing their intraluminal content in the extracellular space. The function of MVs depends on the cell type from which they derive and on their molecular composition. This thesis focuses on the function of cancer cell-derived MVs in angiogenesis and tumour progression and their potential role as biomarkers of tumour disease. A brief summary of some of the major functions of MVs will be given below.

Microvesicle functions

The majority of cells participating in the inflammatory process are known to secrete MVs and these MVs, in turn, also influence the inflammatory process. In 1996, it was shown that MVs from EBV-transformed B-cells could stimulate $CD4^+$ T-cells in an antigen-specific manner[256]. Two years later, it was demonstrated that established murine tumours could be eradicated in a T-cell dependent fashion by MVs derived from dendritic cells pulsed with tumour antigen[257]. On the other hand, tumour-derived MVs have been shown to induce apoptosis of T-lymphocytes[258], suggesting anti-inflammatory properties of MVs.

It has been estimated that platelet-derived MVs make up approx. 80 % of the MV population in plasma of healthy humans[259]. Upon activation, platelets secrete MVs containing tissue factor (TF), the main initiator of the coagulation cascade. TF forms a complex with factor VII and, when activated, this complex further generates factor Xa, which requires negatively charged phospholipids for continued activity[260]. Owing to the relatively large abundance of negatively charged PS in the outer layer of MV membranes[250], MVs are not only the major source of TF, but also provide the negatively charged surface required for sustained coagulation.

The highly interesting, and to some extent still controversial, concept of cell-to-cell transfer of nucleic acids has emerged through the study of nanotubes, apoptotic bodies and nucleic acid-binding proteins[236]. Several reports on MV-mediated intercellular RNA transfer provide further evidence in favour of this notion. In 2006, Ratajczak *et al.* demonstrated that embryonic stem cells secrete MVs enriched in specific mRNAs, which can be transferred to and induce phenotypic changes in hematopoietic progenitor cells[261]. A year later, transfer of functional mRNAs from EPCs to primary ECs was demonstrated[262]. The same phenomenon was described by Valadi *et al.* when they demonstrated the presence of translated mouse proteins in human mast cells after incubation of human cells with MVs derived from mouse mast cells[263]. In the same study, target cell specificity of mRNA transfer was suggested, as mast cell-derived MVs could transfer mRNAs to other mast cells but not to $CD4^+$ cells[263]. However, translation of a reporter mRNA in glioblastoma MVs was shown in ECs[264], arguing against cell type specific interactions. Interestingly, MVs have also been shown to contain miRNAs[263, 264] and considering the relative promiscuity of miRNAs for binding to mRNA sequences, the effect on the translational machinery in recipient cells could potentially be complex.

Microvesicles and cancer

A solid tumour can be viewed as a multicellular organism, in which cellular interactions between numerous different cell types are crucial for growth and progression. MVs and their cargo of biologically active proteins and RNA are emerging as pivotal entities in the tumour microenvironment, regulating as diverse processes as tumour cell proliferation, angiogenesis and metastasis. The different roles of MVs in the tumour microenvironment are illustrated in **Figure 3**.

The association of MVs with cancer was noticed already in the late 1970's[265, 266], and increased levels of MVs in plasma or serum in cancer patients have been well established[267-269]. The tumour suppressor p53 has been implicated in the regulation of MV secretion[270] and transformation has been suggested to increase MV release[246]. In addition, the secretion of lung cancer cell-derived MVs was shown to increase upon hypoxic stimulation[271]. MV-mediated transfer of a truncated, oncogenic form of the EGFR, EGFRvIII, between glioma cells resulted in *e.g.* anchorage-independent growth and upregulation of EGFR-target genes in recipient cells, illustrating how MV-mediated horizontal propagation of oncogene products can influence the tumour cell phenotype[246].

Tumour-derived MVs have been sugested to have a role in tumour cell escape from immune surveillance. MVs isolated from sera of patients with oral cancer were shown to contain FasL and to induce apoptosis in activated T lymphocytes[272]. In addition, the FasL content of tumour-derived MVs was suggested to be of prognostic significance, as the levels of vesicular FasL expression were associated with tumour stage[272].

Degradation of the ECM is essential for tumour growth, angiogenesis and metastasis and is mainly executed by the endopeptidases MMPs. MVs derived from tumour cells contain proteases, including MMPs and EMMPRIN (a membrane-associated glycoprotein know to induce the production of MMPs in fibroblast[273])[274-276] and rapid brake down of extracellular vesicles will release biologically active proteins to the tumour stroma. Acidic pH is a feature of many solid tumours due to their increased anaerobic metabolism and has been suggested to facilitate the lysis of MVs and thereby increased pro-invasive activity of tumour-derived MVs[277]. Tumour cell-derived MVs can also induce the secretion of MMPs from target cells, including ECs[278], and tumour cells can, in turn, be induced to secrete MMPs after stimulation with *e.g.* platelet derived-MVs[244]. The role of MVs in metastasis formation was analysed, comparing primary and metastatic human prostate tumours using microarray technology. A

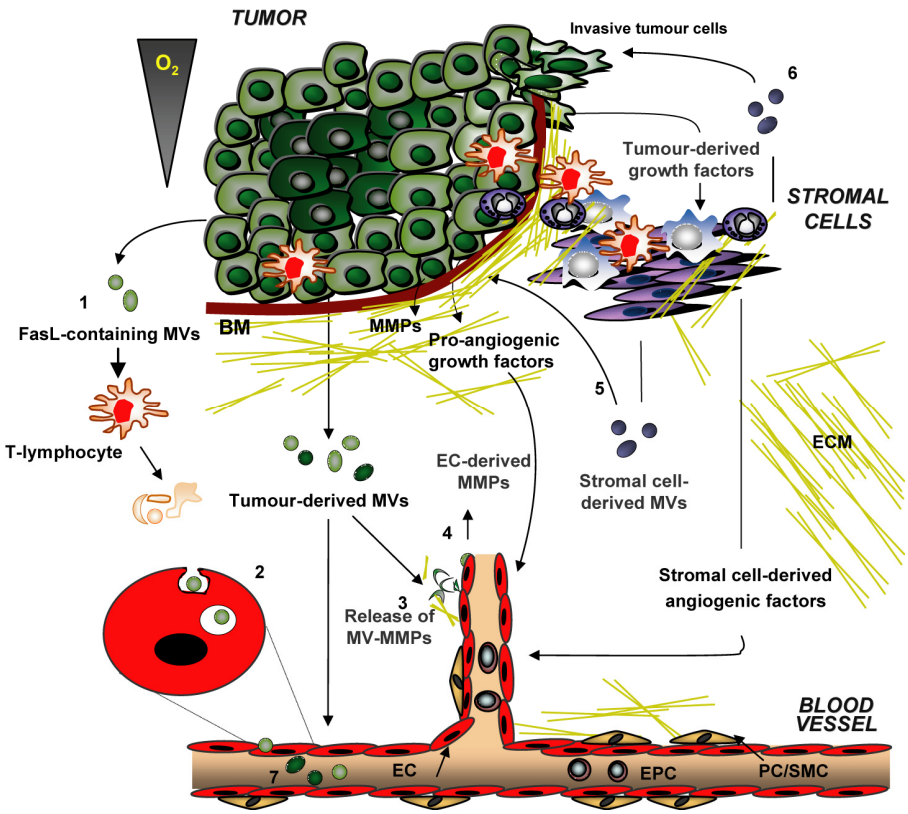

Figure 3. MVs in the tumour microenvironment. Tumour cell-derived MVs have been suggested to facilitate escape of tumour cells from immune surveillance by inducing apoptosis in activated T-lymphocytes (1). MV-mediated intercellular transfer (2) of functional RNAs and proteins have been suggested and in paper **IV** of this thesis, the existence of numerous angiogenic factors in glioma cell-derived MVs is demonstrated. Tumour cell-derived MVs can contain MMPs, which are released upon break down of the MVs (3). Moreover, ECs can be induced to secrete MMPs after stimulation with tumour cell-derived MVs (4). Stromal cell-derived MVs can stimulate tumour cells to release angiogenic factors and MMPs (5) and enhance their migration and invasion (6). Tumour cell-derived MVs have been suggested to enter the peripheral circulation (7) and might thereby affect recipient cells at distant sites. Please see text for further details and references.

MV, microvesicle; FasL, Fas ligand; MMP, matrix metalloproteinase; EC, endothelial cell; EPC, endothelial progenitor cell; PC, pericyte; SMC, smooth muscle cell; ECM, extracellular matrix. Adapted from [292].

higher deletion frequency of a locus encoding a protein implicated as a negative regulator of MV formation was revealed in metastatic tumours compared to primary, organ confined tumours[245], suggesting an association between MV formation and metastasis. The invasive potential of tumour cells can be influenced by the surrounding stroma and MV-mediated cross talk between cancer cells and fibroblasts can indeed promote the migration and invasion of prostate cancer cells.[274]. These reports reinforce the importance of intercellular communication for tumour development and the notion of MVs as crucial mediators of cellular communication within the tumour microenvironment.

In addition to stimulating tumour cell migration and invasion, matrix degradation by *e.g* MMPs also facilitates angiogenesis. Ovarian carcinoma cell-derived MVs can stimulate the expression and secretion of MMPs from ECs, enhancing their migratory and invasive potential[278] and MVs secreted from fibrosarcoma and prostate cancer cells induce *in vitro* capillary formation and *in vivo* angiogenesis in the chick CAM assay[279]. Increased expression of mRNAs for pro-angiogenic proteins (*e.g.* VEGF, HGF, IL-8) in lung cancer cells has been reported after stimulation with platelet-derived MVs[244] and MVs derived from glioblastoma cells contain numerous pro-angiogenic proteins, including IL-8, TIMP-1 and VEGF, which expression likely contribute to the pro-angiogenic capability of these MVs[264]. Interestingly, Al-Nedawi *et al.* showed the onset of VEGF expression, resulting in an autocrine VEGF stimulation, in ECs following the transfer of oncogenic EGFRvIII via MVs shed by glioblastoma cells[280]. As discussed above, hypoxia is a major trigger of tumour angiogenesis and at present, the prevailing view is that hypoxia-induced angiogenesis is mediated by soluble growth factors and cytokines secreted from malignant cells and tumour stromal cells. In paper **IV** of this thesis however, we suggest tumour-derived MVs as novel, hypoxia-induced mediators of pro-angiogenic signalling.

The emission of MVs to blood and urine may provide unique opportunities in the diagnosis, prognosis and treatment prediction of cancer disease. MVs are easily accessible through a blood or urine sample and could offer an alternative to invasive biopsies for *e.g.* molecular profiling of tumour cells. In addition, a population of tumour-derived MVs would most likely be more informative as to the overall tumour malignancy status of heterogeneous lesions than a biopsy taken only at one time-point from one region of the tumour. A pilot study by Smalley *et al.* in 2007 suggested that MVs can be used for the early detection of bladder cancer as nine proteins were differentially expressed in MVs isolated from cancer

patients compared with controls[281]. Similarly, cancer-specific MV mRNAs have been used as a marker for detection of cancer[264, 282]; EGFRvIII positive MVs have been isolated from serum of glioma patients[264] and the presence of two prostate specific mRNAs in MVs isolated form urine of prostate cancer patients[282] further supports the use of MVs as diagnostic tools in the management of cancer. Moreover, the miRNA profile of ovarian cancer cell-derived MVs isolated from peripheral blood exhibited a strong correlation with tumour-derived miRNA profiles, indicating that miRNAs contained within isolated MVs indeed reflect the miRNA profile of the tumour[269]. The same study showed that the miRNA profile of MVs from patients with malignant ovarian cancer was significantly different from the profile observed in patients with benign disease[269], suggesting that MVs could be used as diagnostic markers. In paper **IV**, we provide a novel aspect on the use of tumour-derived MVs as biomarkers of cancer by showing that hypoxia specifically regulates a subset of mRNAs/proteins within MVs derived from glioma cells. This implicates glioma cell-derived MVs as a potential source of a "hypoxia biomarker profile" that optimally could be used for prognostic and treatment response purposes in cancer patients.

The present investigation

Aims

The general objective of this thesis was to study the role of polyamines and MVs in tumour biology. As tumours are frequently presented with areas of hypoxia and as hypoxia is one of the major driving forces of tumour progression, a specific aim was to elucidate the potential roles of polyamines and MVs in the hypoxic stress response of cancer cells.

Specific aims:

I. To evaluate the possibility of using the polybasic HIV-TAT transduction peptide, Tat, as an inhibitor of polyamine uptake in human tumour cells and to investigate the effect of combined targeting of polyamine biosynthesis and uptake on tumour growth.

II. To study the HS epitope requirements for polyamine interaction with cell surface HSPGs and to explore the use of anti-HS antibodies as a means of interfering with polyamine uptake.

III. To elucidate the role of polyamines in hypoxia-induced adaptive responses in human tumour cells and to evaluate whether targeting the polyamine system is a feasible strategy to increase tumour cell sensitivity to hypoxic stress.

IV. To investigate the role of cancer cell-derived MVs in hypoxia-induced pro-angiogenic signalling and to address the possibility of using tumour cell-derived MVs as biomarkers of hypoxic signalling activation in cancer cells.

Methods

In this thesis, the following methods have been used (for a detailed description, see papers I-IV):

- Flow cytometry based assays for assessing cellular binding and uptake of macromolecules, MVs and antibodies.
- Radioactivity based assays for measurement of polyamine uptake.
- ODC activity assays.
- Measurement of polyamine levels.
- *In vitro* proliferation assays.
- *In vitro* cell survival assays.
- Affinity chromatography.
- Metabolic labelling and isolation of PGs.
- Fluorescence and confocal microscopy imaging.
- Electron microscopy.
- Immunblot assays.
- Quantitative real-time polymerase chain reaction (qPCR).
- RNA interference.
- Differential centrifugation for the isolation of MVs.
- Microarray analysis.
- *In vivo* xenograft models for tumour formation.

Results

Paper I – HIV-Tat protein transduction domain specifically attenuates growth of polyamine deprived tumour cells

Introduction - Polyamine biosynthesis inhibition by the specific inhibitor DFMO as a treatment of cancer patients has been largely disappointing due to compensatory cellular uptake of polyamines. Hence, efficient anti- tumour therapy directed against the polyamine pathway requires inhibition of both *de novo* synthesis and uptake. Polyamine internalization is dependent on cell surface HSPGs and earlier work showed dramatic effects on the formation of metastases by combined targeting of polyamine biosynthesis and HSPG-dependent uptake[133]. The polybasic HIV-TAT transduction peptide, Tat (GRKKRRQRRRPPQ), contains eight positive charges derived from arginine and lysine residues and enters cells via PG-dependent pathways. We hypothesized that the Tat peptide would be a potent, competitive inhibitor of polyamine-PG interactions.

Results - The affinity of Tat for heparin was greater than spermine affinity for heparin, as shown by affinity chromatography. DFMO treatment increased the uptake of both polyamines and Tat which, together with the fact that Tat efficiently inhibited polyamine uptake in a dose-dependent manner, suggest a common internalization pathway of these HS ligands. The biological consequences of Tat-mediated inhibition of polyamine uptake were investigated and we demonstrated inhibition of polyamine-dependent cell growth *in vitro*. Moreover, the combined treatment with DFMO and Tat peptide resulted in a 90% reduction of tumour growth in an animal tumour model.

We conclude that the Tat peptide efficiently competes with polyamines for HSPG-mediated uptake and that the combined inhibition of biosynthesis by DFMO, and uptake by Tat, inhibits carcinoma cell growth *in vitro* and *in vivo*.

Paper II – Single chain fragment anti-heparan sulphate antibody targets the polyamine transport system and attenuates polyamine-dependent cell proliferation

Introduction - Based on the conclusions from paper **I**, we next sought more specific methods to investigate the interaction between polyamines and HSPGs.

ScFv antibodies with preferences for various HS epitopes are potential tools to study specific HS structural requirements for polyamine binding.

Results - Both polyamine binding and uptake were inhibited by the anti-HS antibody RB4EA12 whereas two other antibodies studied had no effect, indicating epitope specific inhibitory activity of anti-HS antibodies. RB4EA12 recognizes relatively low sulphated HS domains, suggesting that such domains are important for polyamine binding and subsequent uptake. Under the conditions used, incubation of tumour cells with RB4EA12 resulted in compensatory upregulation of ODC, suggesting that uptake inhibition results in deficient levels of intracellular polyamines. Accordingly, inhibition of polyamine internalization by RB4EA12 significantly inhibited proliferation of tumour cells made dependent on exogenous polyamines, either through pharmacological (DFMO) or genetic (polyamine synthesis deficient $ODC^{-/-}$ cells) disruption of endogenous biosynthesis. Another finding was that combined treatment with DFMO and RB4EA12 resulted in cytostatic rather than cytotoxic effects on tumour cells.

In conclusion, epitope specific ScFv anti-HS antibodies can be used to reduce the bioavailability of extracellular polyamines.

Paper III – Hypoxia-mediated induction of the polyamine system provides opportunities for tumour growth inhibition by combined targeting of vascular endothelial growth factor and ornithine decarboxylase

Introduction - Hypoxia is a hallmark of solid tumours and hypoxic stress induces adaptive cellular responses through the regulation of genes involved in *e.g.* angiogenesis, metabolism and apoptosis. Hypoxic tumour cells are relatively resistant to chemo- and radiotherapy but the existence of hypoxic regions provides opportunities for tumour selective therapy targeting either hypoxia *per se* or cellular responses to hypoxia. Anti-angiogenic therapy of cancer provides proof of principle of such strategies. Increased biosynthesis and concentrations of polyamines have been described in several tumour types. We hypothesized that hypoxia may be partly responsible for these findings and that altered polyamine homeostasis may be an important part of hypoxia-mediated malignant progression.

Results - Hypoxia increased polyamine uptake in a transient and oxygen concentration dependent manner in several types of cancer cells. Interestingly, and

in contrast to what was first expected, increased polyamine uptake was accompanied with increased levels and activity of ODC, resulting in elevated levels of polyamines in hypoxic cells. Moreover, ODC levels were increased in hypoxic tumour regions *in vivo*. Hence, hypoxic induction of neither ODC nor polyamine uptake was secondary to downregulation of the other and our data point towards an important regulatory role of AZI.

The involvement of the polyamine system in the adaptive response to hypoxic stress was demonstrated by the sensitization of polyamine-deprived tumour cells to hypoxia-induced cell death *in vitro*. Specific caspase activation in hypoxic, DFMO-treated cells indicated an apoptotic mechanism of cell death. Importantly, the anti-tumour effect of anti-angiogenic therapy was drastically enhanced when combined with ODC inhibition in a human glioma xenograft model *in vivo*. Tumours from mice treated both with bevacizumab and DFMO displayed profound signs of apoptosis as compared to tumours from single treated mice, suggesting a protective role of increased polyamine levels in hypoxic tumour cells.

Paper IV – Microvesicles may constitute a novel marker of hypoxia driven pro-angiogenic signalling in cancer

Introduction - Hypoxia-induced angiogenesis is critical for primary tumour growth and metastasis and intensive research over the last decades has resulted in the development of angiogenesis inhibitors for the treatment of cancer. The expectations from pre-clinical data have, however, not been fulfilled in the clinical setting. This reflects the complexity of the hypoxia-driven angiogenic response but also the lack of valid, predictive biomarkers of anti-angiogenic treatment for the pre-selection of responsive patients or for the evaluation of treatment response. Recently, the involvement of secreted MVs in intercellular communication, especially within the tumour microenvironment, has been suggested. A large number of tumour cells have been shown to secrete MVs and they have been demonstrated to contain complex biological material such as mRNA, miRNA and proteins. Moreover, tumour-derived MVs have been suggested to elicit a biological response in adjacent cells and potentially also at the systemic level.

We hypothesized that MVs constitute a novel marker of hypoxia-induced signalling in cancer cells and that intercellular transfer of MVs may elicit a hypoxic, pro-angiogenic response in recipient ECs.

Results – MVs derived from hypoxic glioma cells stimulated the proliferation and the survival of primary ECs. Several angiogenesis-related proteins found in tumour-derived MVs, such as IL-8 and IGFBP-3, were upregulated by hypoxia whereas the levels of known anti-angiogenic proteins were downregulated. Tumour-derived MVs further contained numerous activated RTKs and the levels of EGFR, a receptor with large impact on glioma biology, were specifically upregulated in hypoxic MVs. Gene expression analysis of glioma tumour cells and corresponding MVs revealed an almost complete overlap between mRNAs expressed in MVs and donor cells, suggesting that MV-associated mRNAs closely reflect the transcriptional status of host cells. Importantly, this was true also at hypoxic conditions. Interestingly, hypoxia altered the levels of a specific subset of mRNAs in tumour-derived MVs, several of which have been associated with angiogenesis and tumour development.

Conclusions and discussion

In this thesis, the role of polyamines and MVs in tumour biology has been investigated, and below is a brief summary of the main conclusions. In addition, the major implications for therapeutic intervention of cancer are discussed.

Cell surface HSPG as a target for polyamine uptake inhibition (papers I, II and III)

The role of cell surface HSPGs as mediators of internalization of various ligands is well recognized[206, 226] and the role of HSPGs in the cellular uptake of growth promoting polyamines has been of specific interest in our lab. In papers **I** and **II** of this thesis, further evidence for this proposal is provided and two new strategies of interfering with HSPG-dependent polyamine uptake are presented. Tat inhibits polyamine uptake by competition for PG binding sites (paper **I**). Moreover, DFMO treatment increases cellular binding of both spermine and the anti-HS antibody RB4EA12 (paper **II**), suggesting that increased polyamine uptake upon DFMO treatment[121] involves increased levels of polyamine-binding cell surface HSPGs. The Tat peptide has been used to introduce therapeutic molecules into tumour cells[227]. As DFMO treatment increase Tat uptake (paper **I**) it is plausible that in addition to achieving efficient polyamine deprivation, increased concentrations, and potentially increased therapeutic effect, of delivered molecules would be obtained if Tat-mediated delivery of *e.g.* a pro-apoptotic protein was

combined with ODC inhibition. Another interesting possibility would be anti-HS antibody-mediated drug delivery in combination with DFMO treatment.

Inhibition of polyamine uptake by the anti-HS antibody RB4EA12 suggests the possibility of antibody-mediated targeting of HSPGs as a means of interfering with the polyamine system in tumour cells. As discussed above, HSPGs are important for a large number of essential biological processes and unspecific blocking of cell surface HSPGs is expected to result in serious adverse effects. However, the interaction between HSPGs and polyamines appears to be epitope specific and HSPGs containing these structures are upregulated upon DFMO treatment (paper **II**). Moreover, the hypoxia-mediated increase in polyamine uptake (paper **III**) implies that the level or structure of polyamine-binding, cell surface HSPGs may be altered in response to hypoxic stimuli. This is strictly speculative as no PG analysis was performed in paper **III** but, if this is the case, hypoxic cells, *i.e.* almost exclusively tumour cells, may alter their HSPG expression in order to facilitate increased polyamine uptake. This opens up the interesting possibility of specific targeting of hypoxic tumour cells using epitope specific anti-HS antibodies. Indeed, earlier studies have shown changed fine structure of HS chains on ECs in response to hypoxia[222]. Moreover, the importance of HSPGs for the activity of a large number of growth factors makes HSPGs an interesting target for antibody-based anti tumour-therapy. Further studies investigating the effect of hypoxia on cell surface HSPGs regarding polyamine binding capacity and affinity together with studies in animal models investigating the bioavailability of anti-HS antibodies *in vivo* as well as possible adverse effects will determine the feasibility of this approach. Nonetheless, antibody based therapy is a fast growing area in clinical medicine and the use of antibodies targeting *e.g.* HER-2, VEGF and EGFR are examples of successful targeted therapies of cancer.

Hypoxia-induced adaptive responses in tumour cells – novel roles for the polyamine system and tumour cell-derived MVs and implications for the use of anti-angiogenic therapies (paper III and IV)

In paper **III** of this thesis, a novel role for the polyamine system in the adaptive response to hypoxic stress is demonstrated (see **Figure 4**). Both uptake and biosynthesis of polyamines were upregulated in response to hypoxic stimuli. Our data suggested that these hypoxic effects were directly related to the supply of

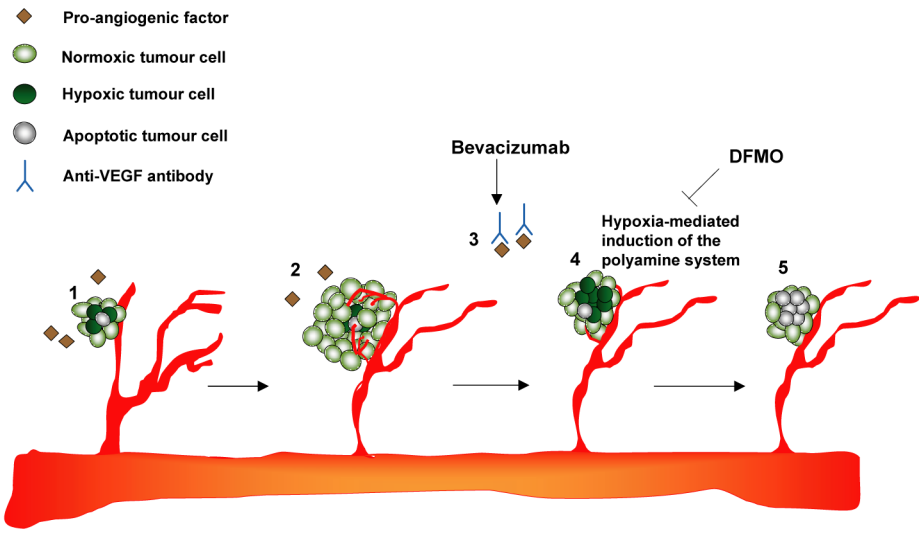

Figure 4. Interference with the polyamine system enhances the tumour inhibiting effect of anti-angiogenic therapy. Upon tumour expansion, hypoxic regions appear as a consequence of inadequate oxygen supply and hypoxic tumour cells secrete pro-angiogenic growth factors, *e.g.* VEGF (1), stimulating tumour angiogenesis (2). Treatment with the anti-VEGF antibody bevacizumab inhibits tumour angiogenesis and induces tumour hypoxia (3). The hypoxia-mediated induction of the polyamine system is blocked by DFMO (4) and as a result, tumour cells are sensitized to hypoxic stress and profound signs of apoptosis (5) can be seen in tumours from DFMO and bevacizumab treated mice, as compared to mice treated with either agent alone (paper **III** of this thesis).
VEGF, vascular endothelial growth factor; DFMO, α-difluoromethylornithine.

exogenous polyamines. A role for the polyamines as ancient stress molecules has been suggested[283]. According to this proposal, polyamines respond to various stress stimuli, such as reactive oxygen species and acidic environments, by playing a dual role turning on stress responsive genes and acting globally to shut down more redundant genes[283]. We showed that inhibition of polyamine synthesis sensitizes tumour cells to hypoxia-induced cell death, indicating a protective and adaptive role for polyamines at hypoxic conditions.

During hypoxia, general protein synthesis is suppressed[284] as a means of promoting energy homeostasis, and this is mediated through inhibition of cap-

dependent translation initiation. Although general translation is inhibited, the consequence at the individual gene level is highly variable. A few proteins important for proliferation and cell survival are in fact induced at hypoxic conditions. Under these circumstances, proteins can be translated by cap-independent mechanisms facilitated by internal ribosome entry sites (IRESs) within the 5' untranslated region (UTR) of the mRNA[285]. In 2000, Pyronnet *et al.* identified an IRES within the ODC mRNA and found it to be functional exclusively during G_2/M phase, when cap-dependent translation was impaired[286]. This suggests that ODC translation can occur even at hypoxic conditions when general protein synthesis is suppressed which, in turn, would indicate an important role for ODC and polyamines in hypoxia-induced responses. In analogy to this, VEGF, which is a survival factor of ECs, has ben suggested to be translated by IRES-dependent mechanisms under hypoxic conditions[287].

The mechanisms behind the anti-tumour effects of anti-angiogenic therapy are debated and have been suggested to derive either from induction of severe tumour hypoxia upon blocked neoangiogenesis or from a normalization of the vasculature, resulting in increased delivery of chemotherapeutic drugs. In paper **III**, we show substantial hypoxic induction in glioma tumours in mice treated with the anti-VEGF antibody bevacizumab (Avastin), as compared to similar sized tumours from control mice. Hypoxia induces apoptosis in normal cells but as tumour cells frequently evolve ways of evading apoptosis, the effect of hypoxic stress on tumour cells probably depends on their genetic make-up. Indeed, hypoxia has been shown to select for cells with diminished apoptotic potential[46] which has raised the concern of using anti-angiogenic therapy, especially in the neoadjuvant or adjuvant setting. Hence, a treatment strategy that selectively targets the adaptive response of tumour cells to hypoxia would be an important complement to anti-angiogenic therapy. Given the effects of hypoxia on the polyamine system, interfering with this system could represent one such strategy and accordingly, inhibition of ODC by DFMO enhances the anti-tumour effect of bevacizumab in a glioma xenograft model (paper **III**). Importantly, tumours from mice treated with DFMO and bevacizumab showed a substantial increase in the number of apoptotic cells as compared to tumours from mice treated with either drug alone, indicating that the combined targeting of VEGF and ODC is a feasible strategy to kill hypoxic tumour cells (see **Figure 4**). Bevacizumab was recently approved for clinical use in recurrent glioblastomas and some encouraging results have been achieved by combining DFMO and chemotherapy in patients with anaplastic

gliomas[168]. The possibility of testing the combined effect of DFMO and bevacizumab in the clinical setting is currently under investigation.

The polyamine transport system has been a target for anticancer drug delivery for more than twenty years. The rationale for this approach is to improve targeting of tumour cells by "harnessing the established activity of known anticancer drugs by attaching them to molecules that are transported into cancer cells via a selective transport system"[288]. Increased effectiveness of *e.g.* etoposide has been achieved by the conjugation to spermine[289]. The induction of the polyamine system at hypoxia implies that increased delivery of conjugated drugs might be achieved under hypoxic conditions. One interesting possibility would be to combine bevacizumab (to induce tumour hypoxia) and DFMO (to block polyamine synthesis and to further increase HSPG-dependent uptake in hypoxic cells) with the TAT-ODD-caspase 3 fusion protein[229] in order to achieve efficient and highly selective targeting of hypoxic tumour cells. This could be of special interest in the context of glioma, the tumour type studied in paper **III**, since systemic administration of HIV-TAT has shown a wide tissue distribution, including the central nervous system[290].

In paper **IV**, a novel role for tumour cell-derived MVs as mediators of the hypoxia-induced adaptive response is suggested. MVs derived from hypoxic cancer cells stimulate the proliferation and survival of ECs and the enhanced effect of hypoxic MVs, compared to normoxic MVs, probably depends on the altered molecular composition of hypoxic MVs. Tumour angiogenesis is pivotal for primary tumour development and metastasis and is in large driven by hypoxic signalling. In addition to the traditional view of soluble growth factors and cytokines being the mediators of hypoxia-induced angiogenesis, paper **IV** provides evidence for an important role of secreted MVs in the communication between malignant and stromal cells in the tumour microenvironment. This finding imposes an even higher degree of complexity to the angiogenic process but also opens up new possibilities of therapeutic targeting of tumour angiogenesis. One of the major rationales for targeting ECs has been the genetic stability of these cells and thereby the lack of acquired resistance to treatment. However, the reported transfer of functional mRNAs and miRNAs between cells through secreted MVs complicates the picture. In our ongoing study (paper **IV**), we have yet to show the transfer of MV content to ECs but one might speculate that the pro-angiogenic effects of hypoxic MVs involve the transfer and biological action of pro-angiogenic mRNAs, miRNAs and proteins. Nevertheless, glioma cell-derived MVs indeed

exhibit pro-angiogenic effects and targeting MV uptake might be a feasible strategy to improve the efficacy of traditional anti-angiogenic therapy. In support of this notion, the use of Diannexin, which is thought to impair docking and uptake of MVs, delayed EGFR-driven glioma tumour growth and reduced tumour microvascular density in immunodeficient mice[280].

Tumour-derived MVs as potential biomarkers for tumour disease (paper IV)

In paper **IV**, we identified a subset of transcripts that were specifically upregulated in hypoxic glioma cell-derived MVs. As the transcriptional profile of glioma-derived MVs largely reflects the transcriptional status of their donor cells, it is proposed that MVs may serve as a potential source of a "biomarker profile" for hypoxic signalling in cancer cells. Tumour hypoxia has been associated with the aggressiveness of solid tumours and to date, tumour hypoxia is measured either with Eppendorf needle electrodes or by immunohistochemical analysis of hypoxia markers on tissue sections[12]. Both methods are invasive in nature and thus limited in the clinical situation. Numerous studies have reported on the secretion of tumour-derived MVs to the peripheral circulation and hence, MVs secreted from tumours could potentially constitute a readily accessible biomarker for the management of cancer (see **Figure 5**). Contamination of bladder cancer-derived MVs isolated from urine with MVs secreted from activated blood cells has been reported[281] and to avoid contamination and dilution problems, specific isolation of tumour-derived MVs is required. As of yet, there are no specific tumour MV markers identified but the use of a hypoxic MV profile, as suggested in paper **IV** of this thesis, to monitor tumour development or treatment response might confer an additional level of specificity. Ongoing glioma xenograft studies in the lab investigate the possibility of using glioma cell-derived MVs as markers for tumour hypoxia.

An important issue that limits the rational use of anti-angiogenic therapy is the lack of predictive markers for selection of responsive patients prior to start of treatment as well as lack of early markers for treatment response. Numerous transcripts associated with angiogenic signalling were upregulated in hypoxic glioma cell-derived MVs (paper **IV**) and might therefore reflect the angiogenic activity within the tumour. Their use as biomarkers for angiogenesis and treatment

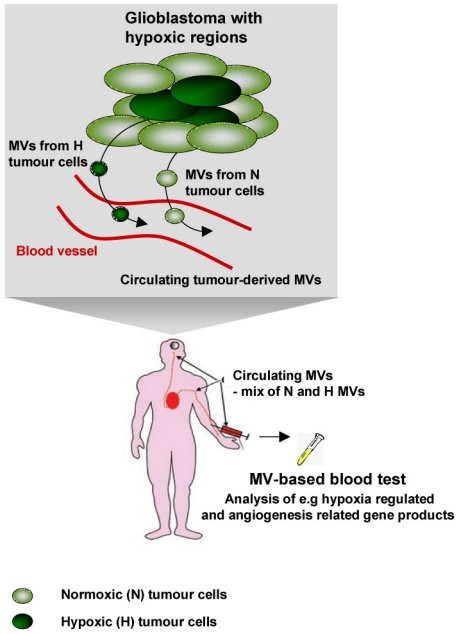

Figure 5. Tumour-derived MVs as potential biomarkers in cancer. MV-associated mRNAs largely reflect the transcriptional status of their donor cells both at normoxic and hypoxic conditions (paper **IV** of this thesis) and it is thus suggested that MVs might serve as biomarkers of hypoxic signalling activation in cancer cells. Tumour-derived MVs can be isolated from peripheral blood and may represent a readily accessible source of information regarding *e.g.* hypoxic signalling and angiogenic activity of the growing tumour. Adapted from [293].

response to anti-angiogenic therapy is a theoretical, yet highly interesting possibility.

Metastasis is the major cause of cancer-related deaths. Although genetic alterations of malignant cells are determinant factors for metastatic potential, a receptive microenvironment is a prerequisite for establishing tumour growth at distant sites. Recruitment of bone marrow-derived hematopoietic progenitor cells (HPCs) and subsequent EPCs have been suggested to be necessary for the establishment of a "pre-metastatic niche"[291], priming target tissues to receive incoming tumour cells. Due to their proven pro-invasive properties and presence in the peripheral circulation, tumour-derived MVs have been suggested to play an important role in the formation of such niches. Interestingly, platelet-derived MVs increased the metastatic potential of lung cancer cells[244], indicating the importance of MV-mediated interplay between different cell types within the tumour microenvironment. Moreover, MVs secreted from EPCs have been demonstrated to induce proliferation and survival of ECs and to stimulate both *in vitro* and *in vivo* angiogenesis[262]. These effects of MVs could potentially have important implications for the formation of a pre-metastatic niche as stimulation of

angiogenesis facilitates the establishment of tumours at distant sites. Hence, strategies to interfere with MV transfer might also be beneficial in the context of anti-angiogenic manipulation of the pre-metastatic niche.

Populärvetenskaplig sammanfattning

De vanligaste sätten att behandla cancer idag är med en särskild typ av läkemedel, så kallade cytostatika, som stoppar cancercellernas förmåga att dela sig eller med strålning av cancertumören med syfte att döda cancercellerna. Båda dessa behandlingsformer medför många och svåra biverkningar. En förklaring till varför vissa av biverkningarna uppstår är att det inte enbart är cancerceller som påverkas av läkemedlen eller strålningen utan även andra, helt friska och normala celler. För att allvarliga biverkningar inte ska uppstå behövs läkemedel som endast skadar eller dödar cancerceller och som lämnar friska och normala celler opåverkade. Utvecklingen av sådana läkemedel kräver ökad kunskap om hur cancerceller fungerar och på vilka sätt de skiljer sig från normala celler.

Cancertumörer växer snabbt och ofta hinner kroppen inte bilda nya blodkärl i samma takt som tumören växer. Blodkärl behövs för att försörja tumören med syre och näring, och när det inte finns tillräckligt många kärl drabbas vissa cancerceller i tumören av syrebrist. Ett annat ord för syrebrist är hypoxi. Hypoxi är något som i princip bara drabbar celler i tumörer eftersom de är de enda cellerna som växer så snabbt att nybildningen av blodkärl inte hinner med. Hypoxi är dödligt för alla typer av celler och för att kunna fortsätta växa och föröka sig har cancercellerna kommit på många smarta sätt att anpassa sig till en tillvaro präglad av lite syre. Cancercellerna kan till exempel anpassa sig till hypoxi genom att öka tillverkningen av, eller ändra utseende på, vissa livsnödvändiga molekyler. Eftersom hypoxi i princip bara drabbar celler i tumörer skulle ett angrepp mot cancercellers sätt att anpassa sig till hypoxi kunna vara ett sätt att specifikt döda cancerceller samtidigt som normala celler lämnas opåverkade.

I den här avhandlingen undersöks hur hypoxi ändrar cancercellers sätt att använda polyaminer och mikrovesikler (MV:er), två typer av molekyler som är kopplade till uppkomst och tillväxt av tumörer. Polyaminer är små, positivt laddade molekyler som är absolut livsnödvändiga för alla typer av celler men som används i större mängder av cancerceller än av normala celler. Anledningen till

detta är troligtvis att polyaminer är viktiga för att celler ska kunna dela sig och blir fler, och cancerceller delar sig fortare än vanliga celler. Utan att vi tänker på det äter vi polyaminer nästan dagligen. De finns till exempel i mjölkprodukter. Polyaminerna finns runt om i vår kropp och tar sig in i kroppens alla celler på ett sätt som hittills inte är helt klarlagt, och de tillverkas dessutom inne i cellerna. Det är dock känt att en viss typ av molekyler på cellytan, så kallade heparan sulfat proteoglykaner (HSPG:er), är viktiga för att polyaminerna effektivt ska kunna ta sig in i celler. HSPG:er består av både proteiner och kolhydrater.

Det har gjorts försök att behandla cancer med ett läkemedel som heter α-diflormetylornitin (DFMO) vilket stänger ner cellernas egen tillverkning av polyaminer. Tyvärr har det inte gett så bra resultat som man hoppats på. Det beror på att cancercellerna som kompensation tar upp mer polyaminer från sin omgivning om deras eget tillverkningsmaskineri stängs av med hjälp av läkemedel. För att effektivt hindra all tillförsel av polyaminer till cancercellerna krävs därför en kombinerad blockering av både tillverkningen och upptaget.

I den här avhandlingen visas att vissa specifika strukturer på kolhydratdelen av HSPG:n är viktiga för att polyaminer ska kunna ta sig in i cancerceller, och också att mängden polyaminer som en cancercell kan ta upp minskar om dessa strukturer blockeras. Dessutom har en bit av ett protein, en peptid som kallas Tat och som också tas upp av celler med hjälp av HSPG:er, använts för att konkurrera ut upptaget av polyaminer in i cancercellerna. Kombinationsbehandling av möss med DFMO, som stänger av cellernas egen tillverkning av polyaminer, och Tat, som blockerar upptag av polyaminer från omgivningen, hade en kraftigt dämpande effekt på tumörtillväxten. En annan mycket intressant upptäckt var att hypoxi instruerade cancercellerna att både tillverka mer polyaminer och att ta upp mer polyaminer från omgivningen. När cancercellernas polyamintillverkning stängdes av dog de av hypoxi i högre utsträckning än de celler som tilläts tillverka stora mängder polyaminer. Att öka mängden polyaminer som finns inne i cellen verkar därmed vara en anpassningsmekanism som cancerceller använder sig av för att överleva i hypoxi. Denna hypotes testades genom att möss med tumörer behandlades både med ett läkemedel som förhindrar att blodkärl nybildas, vilket gör att många celler i tumören drabbas av syrebrist (blir hypoxiska), och med DFMO som ju förhindrar cancercellernas försök till ökad polyamintillverkning. Denna kombinationsbehandling resulterade i en kraftigt minskad tumörtillväxt och ett ökat antal döda cancerceller i jämförelse med tumörerna i de möss som bara fick ett av läkemedlen.

En tumör kan liknas vid en hel organism bestående av många olika typer av celler som alla måste samarbeta och kommunicera för att nå det gemensamma målet, tillväxt av tumören. Kommunikation mellan cellerna sker genom att de släpper ut proteiner och andra molekyler som påverkar celler i närheten. På senare tid har vår kunskap om en relativt ny typ av molekyler, MV:er, ökat och insikten att de kan vara viktiga för hur celler kommunicerar med varandra i en tumör har börjat växa fram. MV:er kan beskrivas som små blåsor som knipsas av från celler och som i denna process får med sig en del av cellens innehåll i form av genetiskt material och proteiner. Denna avhandling visar att hypoxi ändrar innehållet i MV:er som knipsas av från cancerceller, och också att MV:er som kommer från cancerceller som drabbats av hypoxi därmed påverkar de celler som bygger upp blodkärl, så kallade endotelceller, på ett sätt som gör att fler blodkärl skulle kunna växa in i tumören. Att stimulera inväxt av blodkärl in i tumören är en välkänd anpassningsmekanism som cancerceller använder sig av när de utsätts för hypoxi, och upptäckterna som presenteras här skulle kunna betyda att MV:er representerar en tidigare okänd del av denna anpassningsmekanism. Eftersom innehållet i MV:er speglar innehållet i cancercellen den kommer ifrån presenteras även förslaget att MV:er skulle kunna användas som källa till information om de anpassningsmekanismer som sker inne i en hypoxidrabbad cancercell.

Sammanfattningsvis pekar den här avhandlingen på möjligheten att använda en strategi där cancercellers försök att anpassa sig till en miljö med lite syre förhindras. Detta skulle kunna vara ett möjligt sätt att åstadkomma cancercell-specifika läkemedel. Polyaminer och MV:er från cancerceller föreslås som tänkbara angreppspunkter för att göra cancerceller mer känsliga för hypoxi, eller som informationskälla till vad som händer inuti cancerceller.

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