Mechanistic Studies On the Role of Polyamines and Microvesicles in Tumor Growth and Hypoxia-mediated Angiogenesis

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Mechanistic Studies On the Role of Polyamines and Microvesicles in Tumor Growth and Hypoxia-mediated Angiogenesis

Katrin Svensson

Division of Oncology
Department of Clinical Sciences
Lund University
2012
To my Family
Solid tumors are composed of cancer cells, as well as resident or infiltrating non-malignant cells that contribute to the malignant state in an ecosystem generally accepted as the tumor microenvironment. Microenvironmental tumor hypoxia is the foremost leading cause of angiogenesis, *i.e.* the formation of new blood vessels from pre-existing vasculature, and its correlation to tumor growth and aggressiveness has included hypoxia-induced angiogenesis into the hallmarks of cancer. Accordingly, anti-angiogenic therapy was developed for the treatment of cancer patients; however, clinical studies have shown benefit only for a small group of patients, thus challenging the preceding expectations on anti-angiogenic targeting of tumors and underscoring the complex biology of human cancer disease.

This thesis aims at investigating mechanistic roles of polyamines and microvesicles in tumor biology and hypoxia-mediated angiogenesis. We considered the option of inhibiting cancer cell proliferation by the combination of polyamine synthesis inhibition with phage display-derived antibodies targeting the polyamine uptake system. Further, we found a novel role of polyamines in the hypoxic stress response of cancer cells. The findings resulted in a strategy for enhancing the sensitivity of polyamine biosynthesis inhibition in experimental glioblastoma, *i.e.* highly aggressive brain tumors, through simultaneous anti-angiogenic induction of tumor hypoxia. We have elucidated the role of protease-activated receptors in the hypoxic responses of endothelial cells, and found a specific role of protease activated receptor-2. We show that hypoxic coagulation system activation in glioblastoma cell derived microvesicles elicits pro-angiogenic signaling in hypoxic endothelial cells through protease activated receptor-2. Further studies investigated the uptake mechanism of glioblastoma cell-derived microvesicles, and present the still hypothetical possibility of multicellular transfer of complex, molecular information through microvesicles.

In conclusion, further studies aim at understanding the limitations of current anti-angiogenic treatments as well as identifying new targets or combinations of therapies. Data presented in this thesis identify new pathways of hypoxia-mediated tumor development with possible implications for therapeutic intervention.
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>5</td>
</tr>
<tr>
<td>LIST OF PUBLICATIONS</td>
<td>8</td>
</tr>
<tr>
<td>Publications not included in the thesis</td>
<td>9</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>10</td>
</tr>
<tr>
<td>TUMOR DEVELOPMENT</td>
<td>11</td>
</tr>
<tr>
<td>The cancer cell phenotype</td>
<td>11</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>12</td>
</tr>
<tr>
<td>Role of polyamines in transformation and tumor development</td>
<td>14</td>
</tr>
<tr>
<td>Definition and functional role of polyamines</td>
<td>14</td>
</tr>
<tr>
<td>Metabolic pathways and transport of polyamines</td>
<td>15</td>
</tr>
<tr>
<td>Proteoglycan-mediated polyamine uptake</td>
<td>18</td>
</tr>
<tr>
<td>Polyamines and cancer</td>
<td>19</td>
</tr>
<tr>
<td>Targeting the polyamine system in cancer</td>
<td>20</td>
</tr>
<tr>
<td>TUMOR MICROENVIRONMENT</td>
<td>21</td>
</tr>
<tr>
<td>Angiogenesis, hypoxia and coagulation in the tumor microenvironment</td>
<td>21</td>
</tr>
<tr>
<td>Tumor angiogenesis</td>
<td>22</td>
</tr>
<tr>
<td>Mechanisms underlying angiogenesis</td>
<td>22</td>
</tr>
<tr>
<td>The biology behind vascular endothelial growth factor</td>
<td>25</td>
</tr>
<tr>
<td>Anti-angiogenic therapy</td>
<td>26</td>
</tr>
<tr>
<td>Resistance mechanisms to anti-angiogenic therapy</td>
<td>27</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>28</td>
</tr>
<tr>
<td>Hypoxia-inducible factors (HIFs)</td>
<td>28</td>
</tr>
<tr>
<td>Coagulation</td>
<td>29</td>
</tr>
<tr>
<td>The nature of cancer-related coagulopathy</td>
<td>30</td>
</tr>
<tr>
<td>Regulation of tissue factor (TF) expression</td>
<td>31</td>
</tr>
<tr>
<td>Hypoxic induction of TF</td>
<td>32</td>
</tr>
<tr>
<td>Mechanisms of TF-induced tumor angiogenesis</td>
<td>33</td>
</tr>
<tr>
<td>Clotting-independent PAR activation by coagulation proteases</td>
<td>33</td>
</tr>
<tr>
<td>Targeting the coagulation system in cancer therapy</td>
<td>37</td>
</tr>
</tbody>
</table>
EXTRACELLULAR VESICLES SHAPE THE MICROENVIRONMENT ....... 38
Extracellular membrane vesicles as mediators of cellular communication .......... 38
Biogenesis of extracellular membrane vesicles .................................................. 39
Composition of extracellular membrane vesicles .............................................. 41
Extracellular membrane vesicles in health and disease ........................................ 42
Extracellular membrane vesicles in physiology .................................................. 43
Extracellular membrane vesicles in cancer ....................................................... 43
TF-bearing microparticles as systemic activators of coagulation ....... 43
Extracellular vesicles as modulators of tumor microenvironment ... 44
Mechanisms of extracellular membrane vesicle uptake .................................... 45
Vesicular uptake pathways ............................................................................. 46
Intracellular sorting and multivesicular body formation .................................... 49
THE PRESENT INVESTIGATION ....................................................................... 51
Aims of study ............................................................................................... 51
Methodology .................................................................................................. 52
Results .......................................................................................................... 53
Paper I
Single chain fragment anti-heparan sulfate antibody targets the polyamine system and attenuates polyamine-dependent cell proliferation .......... 53
Paper II
Hypoxia-mediated induction of the polyamine system provides opportunities for tumor growth inhibition by combined targeting of vascular endothelial growth factor and ornithine decarboxylase ....... 53
Paper III
Hypoxia triggers a pro-angiogenic pathway involving cancer cell microvesicles and PAR-2 mediated HB-EGF signaling in endothelial cells ............................................................................. 54
Paper IV
Microvesicle transfer involves PI3K/MAPK-dependent nonclassical endocytosis and microtubule-mediated sorting to recirculating multivesicular body compartments ............................................ 55
Concluding remarks and future perspective .................................................. 56
POPULARIZED SUMMARY IN SWEDISH ....................................................... 64
ACKNOWLEDGEMENT .................................................................................... 66
REFERENCES ....................................................................................................... 68
List of publications

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


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Publications not included in the thesis


## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ</td>
<td>Antizyme</td>
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<tr>
<td>AZI</td>
<td>Antizyme inhibitor</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CLIC</td>
<td>Clathrin and dynamin independent carriers</td>
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<tr>
<td>CtxB</td>
<td>Cholera toxin subunit B</td>
</tr>
<tr>
<td>dcSAM</td>
<td>Decarboxylated S-adenosylmethionine</td>
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<tr>
<td>DFMO</td>
<td>α-Difluoromethylornithine</td>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGF/EGFR</td>
<td>Epidermal growth factor/Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinases</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF</td>
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<tr>
<td>GEEC</td>
<td>GPI-AP enriched early endosomal compartments</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin binding epidermal growth factor-like protein</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HS</td>
<td>Heparan sulfate</td>
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<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
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<td>LMWH</td>
<td>Low molecular weight heparin</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>MV</td>
<td>Microvesicle</td>
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<td>MVB</td>
<td>Multivesicular body</td>
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<td>NF-1</td>
<td>Neurofibromatosis type-1</td>
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<td>ODC</td>
<td>Ornithine decarboxylase</td>
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<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain</td>
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<tr>
<td>PAR</td>
<td>Protease activated receptor</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>PyMT-MMTV</td>
<td>Polyoma Middle T antigen Mammary Tumor Virus</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SAMDC/AdoMetDC</td>
<td>S-adenosylmethionine decarboxylase</td>
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<tr>
<td>ScFv</td>
<td>Single chain variable fragment</td>
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<tr>
<td>SSAT</td>
<td>Spermidine/Spermine N1-acetyltransferase</td>
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<tr>
<td>Tat</td>
<td>TAT protein transduction domain</td>
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<tr>
<td>TEM</td>
<td>Tetraspanin enriched domains</td>
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<tr>
<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Cancer is a term for a large assembly of different diseases, defined by an uncontrollable growth of cells that invades the environment of the affected organ. But a tumor is more than just a simple mass of proliferating cells – instead, a tumor is encompassed of a multifaceted, heterogeneous tissue composed of distinct cell types. What phenotypic features distinguish cancer from non malignant tumors? The following section will introduce the hallmarks of cancer, as defined by Hanahan and Weinberg\textsuperscript{1,2}, to provide a general background to the biology of tumor development.

The cancer cell phenotype

The most fundamental feature of cancer cells involves their ability to sustain proliferative signaling. Normal tissues control growth rate to remain the proliferating and apoptotic cells in an adequate and balanced homeostasis. Cancer cells, for a number of reasons, can shift this balance towards augmented proliferation. For example, they are able to produce their own growth factors, resulting in an autocrine loop of proliferative stimuli, creating independence on external growth stimuli. Excessive proliferative signaling is often a result of overexpression of Myc, Ras or Raf proteins that subsequently counteract senescence or apoptosis signals\textsuperscript{3}. Somatic mutations such as of B-raf will lead to constitutive activation of downstream mitogen-activated kinase (MAPK) signaling irrespective of a previous ligand-receptor interaction\textsuperscript{4}. Other mechanisms of sustaining proliferation are achieved by the disruption of negative-feedback mechanisms that attenuate proliferative signaling. For example, the commonly mutated RAS oncprotein leads to impaired Ras GTPase activity, whose normal activity is to make certain that activation of Ras is transient. Loss-of-function of PTEN phosphatase, often as a result of promoter methylation, has the consequence of amplifying phosphoinositide 3-kinase (PI3K) signaling, thus promoting cell survival and tumor growth\textsuperscript{5}.

The mechanisms of growth suppressor evasion and resistance to cell death are used by cancer cells to circumvent negative regulation of cell proliferation.
The classical tumor suppressors retinoblastoma-associated (RB) and TP53 regulate cellular fate in terms of proliferation, senescence and apoptosis. Upon loss-of function of RB, cells lack their control of cell-cycle progression, leading to persistent cell proliferation, and in the case of TP53 loss, cells overlook irreparable damage and subsequent apoptosis signals. Loss of TP53 is frequently seen in many tumor types, including glioma tumors of the brain. Other important hallmarks include enabling of replicative immortality, genome instability and mutation, tumor-promoting inflammation, reprogramming energy metabolism as well as evading immune destruction. In addition, the hallmarks of invasion and metastasis are multistep processes including local invasion, intravasation into blood and lymphatic vessels in close proximity, release of cancer cells systemically, followed by extravasation of cancer cells to their final destination of colonization.

One of the most determining and fundamental hallmarks of tumor development involves the induction of angiogenesis, i.e. the formation of blood vessels from pre-existing vasculature, primarily as a result of tissue hypoxia, or low oxygen levels. Importantly, tumors usually become clinically manifest after they have become angiogenic and thus expanded in size. A major focus in this thesis work is to investigate the mechanistic aspects of the role of polyamines and microvesicles in tumor development and hypoxia-induced angiogenesis in the most aggressive and common primary brain tumor type, glioblastoma (GBM).

**Glioblastoma**

Gliomas constitute a group of brain tumors with specific biologic features, and are originally derived from glial cells that may have stem cell features. Classification of gliomas is based on cell of origin, location, histopathological features and lineage markers. High grade gliomas include anaplastic astrocytoma/oligodendroglioma (WHO grade III), and GBM (WHO grade IV), where the latter is the most frequent primary neoplasm in the central nervous system. Characteristic features of GBM include heterogeneous histopathological appearance, endothelial cell (EC)/microvascular proliferation leading to dilated, leaky vessels, necrosis and pseudopallisading areas surrounding necrotic foci, as well as a hypercoagulative state. Patients diagnosed with GBM most commonly receive three different treatment modalities; surgery, radiotherapy and chemotherapy, however using all of these modalities, median overall survival of GBM patients is only about 15 months. It is an immense challenge to treat GBM due to several complicating factors; many drugs cannot cross the blood-brain barrier, and even if they do,
these tumor cells are exceptionally resistant to conventional therapies, while the normal brain is susceptible to damage and has limited capacity to repair itself\textsuperscript{23}.

In spite of the striking heterogeneity of these tumors, the underlying pathogenesis of GBM includes abnormalities in a set of common cellular pathways. Two distinct molecular pathways of GBM progression have been suggested; primary \textit{de novo} formation of GBM, or less commonly, secondary GBM developing from low grade astrocytoma\textsuperscript{24-26}. Unlike many systemic tumors, high grade gliomas do not express mutant RAS\textsuperscript{27}. Genetic analyses of primary GBM have made known that the majority displays overexpression, amplification or mutation of epidermal growth factor receptor (EGFR)\textsuperscript{28-31}. Also, deletion of PTEN as well as aberrations in the p53 pathway, such as p14\textsuperscript{ARF}, has been described\textsuperscript{32-34}. Secondary GBMs involve transformation from astrocytoma and anaplastic astrocytoma, and are very often followed by platelet derived growth factor receptor (PDGFR) overexpression and p53 deletion. Genetically modified mice have been developed to study tumor progression on the basis of common alterations in human GBM. Mutations in neurofibromatosis type-1 (NF-1) and p53 accelerate astrocytoma formation\textsuperscript{35}, and glial fibrillary acidic protein (GFAP)-v-src transgenic mice develop spontaneous gliomas\textsuperscript{36}. A recent publication reports on high grade glioma mouse models with mutations in p16/p19 with or without loss of p53 and PTEN\textsuperscript{37}. However, no single genetic change defines GBM, which points towards a strategy where multitargeting of signaling pathways in gliomas or individualized therapy will be required. High grade gliomas have recently been the subject for anti-angiogenic treatment, which will be further discussed under the angiogenesis section.
Role of polyamines in transformation and tumor development

As mentioned in the introductory section, transformation due to overexpression of oncogenes frequently results in the onset of proliferative signaling. The amino acid derived polyamines have long been associated with cell proliferation and cancer by the regulation of specific oncogenes and tumor-suppressor genes, and their function in cancer have made them attractive targets for anti-cancer treatment.

**Definition and functional role of polyamines**

The polyamines were observed already in 1678; however, it was not until 1968 the finding of elevated levels of polyamines in tumors was made\(^3\)\(^8\). Polyamines are naturally occurring, organic, cationic compounds having two or more amino groups, and have been identified in bacteria, fungus, plants and all types of eukaryotic cells\(^3\)\(^9\). Their conservation across evolution argues for their significance in cellular functions. The major polyamines putrescine (a diamine), spermidine and spermine are critical for cell proliferation and continuation of life in virtually all known cell types. Extensive research in the polyamine field has lead to substantial knowledge of the direct connections of polyamines with cellular proliferation, differentiation, cell death, and normal tissue functions\(^3\)\(^8\). However, a more detailed understanding of their mechanisms of action is still required.

Owing to their small size and water soluble characteristics with multiple positive charges at physiological pH, polyamines can bind numerous, polyanionic macromolecules, such as DNA, RNA, enzymes, soluble and membrane proteins, and polyphosphorylated molecules in the cytoplasm\(^3\)\(^9\). Hypotheses regarding their regulation of intracellular events in mammalian cells include DNA/genome and protein/enzyme binding, and regulation of translation and signal transduction. Being cations, polyamines bind to and change conformational properties of DNA\(^4\)\(^0\). Polyamines are also able to augment the binding of DNA to transcription factors such as nuclear hormone receptors\(^4\)\(^1\), and to indirectly influence the transcription of other genes by mediating the expression of transcription factors. This can be exemplified by decreased expression of the proto-oncogenes c-myc, c-fos and c-jun upon polyamine depletion in rat small intestinal crypt cells\(^4\)\(^2\). A significant finding is the formation of the amino acid hypusine specifically by spermidine in the eukaryotic initiation factor 5A (eIF-5A), leading to eIF-5A-dependent protein synthesis, mRNA stability and RNA transport\(^4\)\(^3\). Polyamines are also
modulators of a variety of ion channels, e.g. N-methyl-D-aspartate (NMDA), ionotropic glutamate transmembrane receptors, and potassium channels. Other proposed functions of polyamines involve endocytosis and membrane trafficking regulation through the binding of the substrate for PI3K, and by cytoskeleton rearrangement mediated by the small GTPases Rac and RhoA.

Cell death in response to elevated polyamine levels has been demonstrated by overexpression studies of the polyamine biosynthesis enzyme ornithine decarboxylase (ODC), which resulted in apoptosis mediated by cytochrome C release. Furthermore, polyamines are key regulators of the cell cycle. Variations in enzyme activities of ODC and subsequent changes in polyamine levels have been detected during the different phases of the cell cycle. Consequently, depletion of intracellular polyamines by the irreversible, and highly specific inhibitor of ODC, α-difluoromethylornithine (DFMO), results in growth arrest. The necessity of polyamines for regulation of apoptosis and cell proliferation is clearly illustrated by the phenotypes of mice deficient in enzymes related to polyamine synthesis. Knockout of key genes involved in the anabolism of polyamines results in increased apoptosis and/or decreased proliferation, and early embryonic lethality.

**Metabolic pathways and transport of polyamines**

Intracellular levels of polyamines are highly regulated by biosynthesis, catabolism, export and uptake of extracellular polyamines. The key enzymes in the biosynthesis and degradation of polyamines in mammalian cells are well characterized. The primary precursors of polyamine biosynthesis are arginine and methionine. Arginine is processed by arginases to form ornithine, the primary substrate of putrescine biosynthesis. Methionine is converted to S-adenosylmethionine (AdoMet) by methionine adenosyltransferase (MAT) and is then included in the biosynthesis of the higher polyamines. The critical and rate-limiting enzyme in the biosynthetic pathway is ODC, a cytosolic enzyme that catalyses the decarboxylation of ornithine into the diamine putrescine. Spermidine and spermine are formed from putrescine by the attachment of aminopropyl groups, a process catalyzed by the aminopropyl transferases spermidine and spermine synthase.

The catabolic conversion of the higher polyamines spermidine and spermine into putrescine involves several enzymatic steps. Spermidine/spermine N\(^1\)-acetyltransferase (SSAT) acetylates spermidine and spermine at primary amino groups, followed by the action of polyamine oxidases (PAO) generating spermidine and putrescine, respectively.

Regulatory control of polyamine biosynthesis includes modulation of (i) transcription, (ii) mRNA stability, (iii) translation and (iv) protein degradation.
Transcription of ODC can be induced by e.g. growth factors, hormones and tumor promoters. ODC mRNA has a long 5’ untranslated region (UTR), and ODC is therefore enhanced by elevated levels of active eIF4-E, which also may contribute to its transforming properties. More importantly, translation of ODC is facilitated by a eukaryotic mRNA 5’ cap-independent mechanism, as demonstrated in HeLa cells by Pyronnet et al. Cap-independent internal ribosomal entry site (IRES) exists in the mRNA of ODC, which ensures elevated polyamine levels even under conditions with low cap-dependent translation efficiency, such as during G2/M.

Protein degradation represents one of the major control systems for the regulation of polyamine levels. The half-life of the enzymes ODC and AdoMetDC are among the shortest known, ranging from 10 min up to 1 h, arguing for a great degree of post-translational regulation of enzyme activity. Antizyme (AZ) is a major regulator of intracellular polyamine levels through down-regulation of ODC levels. Elevated polyamine levels results in the complete AZ protein translation via a unique +1 frameshifting mechanism on AZ mRNA. The AZ protein binds to monomeric ODC, which results in its inactivation through ubiquitin-independent degradation by the 26S proteasome. The first discovery of the inhibitory effect of AZ on ODC was found upon the presence of excess levels of putrescine in rat hepatoma cell cultures.

AZ is in turn regulated by the ODC homologue antizyme inhibitor (AZI). Although it is homologous to ODC, AZI has no enzymatic activity, but instead binds AZ with greater affinity than ODC, saving ODC from degradation. High AZI levels leads to a positive regulation of polyamine levels, and is indispensable for survival. In conclusion, ODC is subjected to positive and negative feedback mechanisms regulated mainly by intracellular polyamine levels where high and low polyamine levels leads to decreased and increased ODC activity, respectively. Figure 1 summarizes the different processes of biosynthesis, catabolism and transport of polyamines as well as some of the regulatory control mechanisms.
Figure 1. Key features of polyamine metabolism. (1) Polyamine levels are regulated by biosynthesis (white area) and degradation (light blue area). Arginine is converted by arginases into ornithine, the primary substrate for putrescine biosynthesis. (2) ODC catalyses the decarboxylation of ornithine into the diamine putrescine. ODC activity is mostly regulated by protein degradation. AZ is induced by high intracellular polyamine levels, and binds to monomeric ODC, which results in inactivation and ubiquitin-independent degradation of ODC by the proteasome. AZ is in turn negatively regulated by the ODC homologue AZI, which binds AZ with greater affinity than ODC. The inhibitor DFMO binds irreversibly to ODC, thus inhibiting its activity. (3) L-methionine is converted into AdoMet by MAT, and is then included in the biosynthesis of the higher polyamines spermidine and spermine. (3) Spermidine and spermine are formed from putrescine by the aminopropyl transferases SDS and SMS. (4) Catabolism (dotted lines) involves SMO, SSAT and PAO. SMO converts spermine directly to spermidine. SSAT is the first, regulatory step in the retroconversion process, and acetylates spermidine and spermine at primary amino acid groups. The intermediate acetylderivatives, $N^1$-acetyl spermine and $N^1$-acetyl spermidine, are then substrates for PAOs generating spermidine and putrescine. (5) The export mechanism of polyamines is largely unknown, while the mechanism of import (6) is facilitated by HSPG, and can in addition be negatively regulated by AZ. For references, see text. Abbreviations: AdoMet, Adenosyl methionine; AZ, antizyme; AZI, antizyme inhibitor; HSPG, heparan sulfate proteoglycan; MAT, methionine adenosyl transferase; MTA, 5’ methylthioadenosine, ODC, ornithine decarboxylase; PAO, polyamine oxidase; SDS, spermidine synthase; SMO, spermine oxidase, SMS, spermine synthase; SSAT, spermidine/spermine $N^1$-acetyltransferase.
Proteoglycan-mediated polyamine uptake

Polyamines can in addition to be synthesized, also be imported from exogenous sources, e.g. dietary intake\textsuperscript{60} and the intestinal flora\textsuperscript{61}. The transport systems for polyamine uptake are characterized in yeast, bacteria and plant cells\textsuperscript{62}; however, the mechanisms for polyamine uptake in mammalian cells are not fully understood, and the receptor for the polyamines has so far not been characterized. Mounting evidence speaks in favor of a non-specific uptake system as natural polyamine-resembling structures e.g. polybasic peptides\textsuperscript{63} and methylglyoxal bis-guanylhydrazone (MGBG)\textsuperscript{64} are internalized through the same pathway. Previous work has demonstrated that heparan sulfate proteoglycans (HSPGs) are involved in the uptake of polyamines\textsuperscript{65}. HSPGs are a superfamily comprised of a core protein attached with one or several glycosaminoglycans (GAGs). Cell surface bound HSPGs are involved in the uptake of numerous macromolecules besides polyamines\textsuperscript{65}, such as bFGF\textsuperscript{66}, apolipoprotein E\textsuperscript{67} and the HIV tat transduction peptide Tat\textsuperscript{68,69}. However, additional, HSPG-independent pathways are likely, based on the fact that polyamine uptake was not completely abrogated in HSPG-deficient mutant cells\textsuperscript{70} or upon enzymatic degradation of cell surface HSPGs\textsuperscript{65}. Interestingly, blocking polyamine biosynthesis using DFMO induces structural changes of HS GAGs to accommodate increased uptake of polyamines\textsuperscript{68,71}, thus adding mechanistic support of the compensatory uptake in DFMO treated cells.

Polyamines may be internalized in a two-step process involving initial transport across the plasma membrane via a classical transporter, followed by sorting into late endocytic compartments\textsuperscript{72}. The cell surface associated HSPG glypican-1 has indeed been shown to trigger endocytic uptake of polyamines\textsuperscript{73}, and the endocytosis of HSPGs have been suggested to involve lipid-raft dependent caveolin-regulated uptake pathways\textsuperscript{74}.

To conclude, the identification of molecules involved in the uptake of polyamines may have considerable impact on the design of new anti-cancer therapeutics. Studies of HS modification patterns by phage display derived single chain variable fragment (ScFv) anti-HS antibodies developed by van Kuppevelt and co-workers have improved our understanding of HS biology\textsuperscript{75}. In paper I of this thesis, we demonstrate that concomitant inhibition of ODC by DFMO, and HSPG-dependent polyamine uptake using an HS specific antibody results in reduced cancer cell proliferation even in the context of high extracellular polyamine levels.
Polyamines and cancer

In 1968, Russel and Snyder reported on the connection between increased ODC activity and cancer. Today, the relationship between cell growth and polyamines in cancer is widely accepted and ODC is a putative proto-oncogene. Elevated ODC activity is found in most human cancers, and is frequently associated with neoplastic transformation caused by oncogenes. Indeed, overexpression of ODC in the immortalized mouse fibroblasts NIH-3T3, was found to be sufficient to cause transformation, whereas an already transformed oncogene driven phenotype can be reversed by the inhibition of ODC activity of the same cell line. It is now well established that the ODC gene is a target of the oncogene c-myc, and that elevated levels of ODC is a result of increased activity of the Myc/Max transcription complex. Besides from Myc, ODC is also under control by the Ras oncogene, as activation of Ras increases both mRNA and protein levels of ODC. In addition, germline loss of function mutation in the tumor suppressor gene adenomatous polyposis coli (APC) results in an increase in ODC activity and polyamine biosynthesis. In support of these data, inhibition of polyamine biosynthesis in Apc Min/+ mice was shown to suppress intestinal tumor formation. Polyamines have also been shown to promote angiogenesis in a variety of settings, e.g. ODC overexpressing cells in mice formed tumors that were highly vascularized and dermal vascularization was increased in transgenic mice overexpressing ODC.

As an inhibitor of both polyamine biosynthesis and uptake, AZ acts as a negative regulator of cell proliferation and has an apparent role in tumor development. In a study using keratin promoter elements for targeted expression of AZ in the skin (K6), the authors demonstrated that AZ suppresses tumor growth. The antitumor effects seen in transgenic mice due to AZ expression can be explained by the reversion of the increase in ODC and polyamine levels that are needed for neoplastic formation, although other mechanisms such as AZ effects on protein turnover cannot be ruled out. Conversely, skin targeted overexpression of ODC in RAS/ODC mice, results in polyamine-dependent formation of spontaneous skin tumors. Given the fact that the genomic region including AZI often is mutated in ovarian and prostate cancer, the role of AZI in cancer has been under investigation. Indeed, AZI knockdown has been shown to decrease in vivo prostate tumor growth, and future studies on AZI knockout mice will hopefully contribute to our understanding of the mechanistic role of AZI in tumor development.

In conclusion, current understanding predicts polyamines to act rather causative than associative in their role in cancer, thus making ODC, AZ and AZI valid therapeutic targets for cancer treatment.
Targeting the polyamine system in cancer
The irreversible ODC inhibitor DFMO was at date of discovery presented as an efficient chemotherapy for hyperproliferative disorders, including cancer\textsuperscript{38}. DFMO was found to exert remarkable inhibitory effects \textit{in vitro} as well as on carcinogen-induced cancers in a number of mouse and rat models. However, despite these effects, early monotherapy using DFMO resulted in rather discouraging results in some cancers\textsuperscript{90}. The major reasons for the poor effect of DFMO are ODC amplification creating resistance to DFMO, or more importantly, that inhibition of synthesis results in a compensatory uptake of polyamines. DFMO was in a study used in combination with HSPG-binding Tat, previously shown to efficiently inhibit polyamine uptake, which resulted in a 90\% reduction in tumor burden in a xenograft model\textsuperscript{63}. This study, together with numerous other investigations\textsuperscript{70,91}, demonstrate as a proof of principle that efficient tumor inhibition using DFMO relies on the fact that extracellular uptake of polyamines is concomitantly hindered. To this date, there are no such strategies available in the clinic.

Several monotherapy studies or studies using combination of polyamine inhibitors with other drugs are currently in clinical trials\textsuperscript{92}. A recent phase 3 study of skin cancer prevention by DFMO in patients with a previous history of skin cancer found a reduction in the recurrence of basal cell carcinoma using DFMO as compared to placebo\textsuperscript{93}. Another clinical study found reduced recurrent adenomatous polyps using DFMO in combination with the non-steroidal anti-inflammatory drug (NSAID) sulindac for the prevention of sporadic colorectal adenomas\textsuperscript{94}. DFMO has recently made substantial advancement in neuroblastoma treatment, and future studies will evaluate the benefit of DFMO as mono or combination therapy\textsuperscript{90,95}. In paper II, we found a novel role of polyamines in the hypoxic response, which formed a rationale for a treatment combination of DFMO and the monoclonal anti-VEGF antibody bevacizumab.
Cancer cells are not isolated, but rather co-exist and co-evolve with resident cells in the tumor microenvironment. These cells are conclusively demonstrated to have importance for tumor initiation and progression. Highlighted cell types in this context include cancer-associated fibroblasts building up the extracellular matrix, endothelial cells and pericytes responsible for vessel formation and function, and immune cells that can either obliterate or support the tumor. In addition, stem cell like cells capable of differentiation exist in or are recruited to the tumor area. The microenvironment is a central regulator in the fate of a tumor, and recent research in this field has truly facilitated the general understanding of the role of cancer cell-stromal interactions in tumor biology.

Angiogenesis, hypoxia and coagulation in the tumor microenvironment

Tumor angiogenesis is the most evident support that the stromal compartment contributes to tumor development. The onset of the angiogenic switch is regulated by hypoxia - an inevitable and early event upon tumor growth. Interestingly, the activation of coagulation and angiogenesis is coregulated as a consequence of microenvironmental hypoxia, by upregulation of foremost the pro-angiogenic protein vascular endothelial growth factor (VEGF) and the major coagulation initiator tissue factor (TF). VEGFs are capable of rapidly inducing TF expression, and conversely, oncogenic and modulatory factors of TF expression contribute to angiogenesis by upregulation of VEGF and direct signaling activation. Conclusively, the complex interrelationships between the constituent processes of hypoxia, angiogenesis, hemostasis and cellular signaling in the tumor microenvironment result in the establishment of multiple feedback loops connecting angiogenesis and coagulation activation in malignancies.
Tumor angiogenesis

Pioneered by studies of the laboratory of Judah Folkman about four decades ago, we now acknowledge tumor vasculature as indispensable for neoplastic expansion. Folkman’s statement that “solid tumors are far more dependent upon new capillary sprouts than we had previously believed” widely expanded our conception of tumor development. The process in which new capillaries sprout from existing vessels is called angiogenesis. Remarkably, the vasculature is quiescent in adults, with exceptions for in the female reproductive system and in pathophysiological situations such as fracture and wound healing, arthritis and ischemia of the heart and brain. Nevertheless, in response to a stimulus, endothelial cells (ECs) can be activated to multiply and develop new vessels. This intricate process is regularly induced by hypoxia, and mechanisms underlying this process will be described in detail in this chapter.

Mechanisms underlying angiogenesis

Even with the genetic abnormalities of cancer cells as discussed above, the limit for tumor formation is determined by their capability of initial recruitment of blood vessels. Failure of cancer cells to recruit vessels will lead to a nonexpanding, relatively harmless “dormant” tumor. The biology of tumor dormancy is poorly understood, but animal models have revealed that microscopic neoplastic cells can remain in an occult, asymptomatic and non-detectable state. These tumors may remain dormant indefinitely unless circumstances in the tumor microenvironment activate the angiogenic switch. Angiogenesis is required not only for continued tumor growth, but also for the ability of a tumor to metastasize, thus making angiogenesis indispensable in malignancy.

So how do tumors develop a blood supply? To investigate the underlying mechanisms behind this phenomenon, Folkman and co-workers already in their early work suggested that tumors secrete “diffuse” tumor activating factors, TAFs. This finding was followed by studies on neovascularization of implanted tumors into cornea, suggesting that cancer cells secrete soluble factors that facilitate the ingrowth of new vessels. Angiogenic factors have ever since been extensively studied using angiogenesis models in order to identify central tumor angiogenesis factors. The molecular basis of the angiogenic switch involves increased production of multiple pro-angiogenic factors and loss of angiogenic inhibitors. These factors can be produced by cancer cells and by inflammatory or stromal cells, e.g. macrophages and fibroblasts; in some tumors, dominant oncogenes, such as Ras and Myc, can upregulate angiogenesis related proteins in cancer cells. Examples of highly upregulated factors are basic and acidic fibroblast growth factor (aFGF, bFGF) and VEGF.
Hypoxia is a major inducer of VEGF in nearly all cell types tested, and induction of VEGF stimulates mitogenic effects on ECs and increases vascular permeability\(^\text{100}\).

The initial step in vessel sprouting involves the activation of ECs in response to gradients of VEGF mainly secreted by cancer cells, followed by the conversion of a previously quiescent cell into a tip cell\(^\text{107}\). The tip cell is a migrating cell that develops a new capillary sprout, as well as synergizing the secretion of proteolytic enzymes \textit{e.g.} matrix metalloproteinases (MMPs) and urokinase plasminogen (uPAR), together with tumor cells, in order to degrade the basement membrane\(^\text{108,109}\). Angiopoietin-1 (ANGPT1) binds to the Tie2 receptor on endothelial stalk cells, contributing to blood vessel maturation and stability\(^\text{108}\). Tumor cells secrete angiopoietin-2 (ANGPT2), which by competition of ANGPT1 for binding to Tie2, facilitates sprout formation\(^\text{110}\). Integrins on tip cells facilitate EC binding to extracellular molecules and are thus necessary for tube formation, elongation and remodeling of the vessel. Maturation of the vessel involves the recruitment of mesenchymal cells and the differentiation into pericytes by TGF-\(\beta\) and ANGPT1 stimulation. However, in tumor vasculature, the ECs are poorly covered with pericytes as well as displaying a reduced basement membrane. ECs are known to be interdependent with pericytes for proper vessel development\(^\text{111}\), as pericytes function as mechanical and physiological support building up normal vasculature\(^\text{112}\). Given that pericytes have maintained plasticity, they are allowed to differentiate into various cell types, \textit{e.g.} smooth muscle cells, fibroblasts or other mesenchymal cells, and studies have revealed a function of pericyte coverage in the maintenance of an efficient tumor vascular system\(^\text{113,114}\). In addition, sprouts can develop from bone marrow-derived cells with stem cell like properties, such as endothelial progenitor cells (EPCs), through postnatal vasculogenesis\(^\text{110,115}\).

The existence of endogenous angiogenesis inhibitors could present a natural barrier to angiogenesis as shown by a number of groups, \textit{e.g.} \(\alpha\)-interferon\(^\text{116}\) inhibited EC motility and platelet factor-4\(^\text{117,118}\) could interfere with EC proliferation. The secreted glycoprotein trombospondin-1 (TSP-1) has also been shown to inhibit angiogenesis in EC chemotaxis assays and \textit{in vivo} using the corneal pocket assay\(^\text{119}\). Due to the very fast induction of angiogenesis in tissues upon damage, stored angiogenic regulators are believed to be released by enzymatic degradation of components that sequester VEGFs and other growth factors in the matrix\(^\text{120}\). Sequestered angiogenesis inhibitors are, however, stored as cryptic parts of larger proteins that are not inhibitors themselves. This is exemplified with the 28 kDa fibronectin fragment or the 16 kDa prolactin fragment that significantly inhibited EC proliferation, while the full length proteins had no such effect\(^\text{121,122}\), or the very potent angiogenesis inhibitor...
angiostatin, which is a fragment of plasminogen\textsuperscript{123,124}. These naturally occurring peptides may have potential for future therapeutic applications. However, key questions of their regulation and under which circumstances these cryptic inhibitors are released remain to be elucidated.

The skewed balance between pro and anti-angiogenic factors presented in the tumor microenvironment is one reason for the structural abnormalities frequently seen in tumor vessels. Tumor vasculature is dilated and often displays a disorganized pattern of growth. Pathological tumor vessels are enlarged compared to the fine capillary network present in normal tissue, and exhibit leakiness as well as torturous “dead” ends, which may lead to fluctuating oxygen levels even in those areas that are restrained with vessels\textsuperscript{125}. Figure 2 summarizes the angiogenic onset by hypoxia in the tumor microenvironment.

\textbf{Figure 2. Induction of the pro-angiogenic response in the tumor microenvironment.} (1) Oncogenic changes in cancer cells induce transformation leading to cancer cell proliferation and tissue expansion. (2) Hypoxia is induced in areas far away from existing or functional vessels. (3) Hypoxic cells acquire a more migratory phenotype by the production of matrix degrading enzymes \textit{e.g.} MMPs that remodulate the tumor stroma. (4) Hypoxic cancer cells and host cells upregulate hypoxia inducible factors (HIFs) in
response to low oxygen levels and start the transcription of HIF target genes. The hypoxic response leads to release of pro-angiogenic factors, e.g. VEGF, FGF, PI GF and EPO. (5) ECs respond to ligand binding through the activation of RTKs, e.g. VEGFR-2 and FGFR, resulting in the induction of EC proliferation, migration and survival. (6) Angiogenic sprouting initiates with tip cell migration towards a chemotactic gradient of growth factors. Newly formed tumor vessels are dilated, leaky and display tortuous ends, vascular leakage and fluctuating hypoxia. (7) In addition, inhibition of anti-angiogenic responses such as TSP-1 and Ang-1 further augments the angiogenic drive. (8) Anti-angiogenic treatment aims at targeting soluble ligands of RTKs or RTKs directly on ECs. For references, see text. Ang-1; angiostatin-1, ECM; extracellular matrix, EPO; erythropoietin, FGF; fibroblast growth factor; HIF; hypoxia inducible factor, MMP; matrix metalloproteinase, PI GF; placental growth factor, RTK; receptor tyrosine kinase, TSP-1; thrombospondin-1, VEGF; vascular endothelial growth factor.

The biology behind vascular endothelial growth factor

VEGFs are important stimulators in de novo formation of the embryonic circulatory system, vasculogenesis; and the growth of blood vessels from pre-existing vasculature, angiogenesis. VEGF is a subfamily of the platelet derived growth factor (PDGF) family of cysteine-knot factors. Among the most important members of the VEGF family is VEGF-A. Other members are placental growth factor (PI GF), important for vasculogenesis, as well as angiogenesis upon inflammation, ischemia, wound healing and cancer. VEGF-B plays a less pronounced role in the vascular system, VEGF-C is important in lymphangiogenesis, and VEGF-D in the development of the lymphatic vasculature in lung bronchioles. It should be emphasized that the exact roles of the various VEGF isoforms remain to be fully elucidated. The biological effects of VEGF-A are mediated by two receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2. Both receptors have an extracellular domain, a single transmembrane region and a consensus TK sequence domain. VEGFR-3 (fms-like-tyrosine kinase-4, or flt-4) is a member of the same family of RTKs, however receptor for VEGF-C and VEGF-D.

The function of VEGF-A in angiogenesis is performed through increased migration and mitosis of ECs, creation of blood vessel lumen and fenestrations, as well as increased αVβ3 integrin activity. VEGF-A is also chemotactic for granulocytes and macrophages and induces vasodilatation indirectly through nitric oxide (NO) release. In addition, VEGF is known as a vascular permeability factor, based on its capability to induce vascular leakage. For ECs, VEGF is a survival factor that acts through activation of the PI3K-pathway and by the induction of the anti-apoptotic proteins Bcl-2 and A1. In physiology, VEGF has a key role in embryonic and early postnatal development, as shown by defective vascularization and a reduced number of nucleated red blood cells in the yolk sac in Vegf−/− embryos.
VEGF also has a pronounced role in a number of pathologic conditions besides its effect in neoplastic disease. VEGF upregulation has been implicated in the development of brain edema as a response to focal cerebral ischemia\textsuperscript{137}. Myocardial ischemia leads to hypoxia-inducible factor-1α (HIF1-α) accumulation and stimulates angiogenic sprouting through VEGF\textsuperscript{138}. In addition, VEGF is central for the development of diabetic retinopathy associated with retinal ischemia, which may result in hemorrhages, retinal detachment and blindness. Angiogenesis during wound healing is triggered by HIF-dependent VEGF signaling, and other cytokines and growth factors secreted by inflammatory cells, such as TNF-α, which may further accelerate HIF-α accumulation\textsuperscript{139}. The current progress in the molecular mechanisms behind angiogenesis and the broader biological understanding of blood vessel formation and development raise expectations for the development of more efficient therapeutics of angiogenesis-related non-cancerous pathological disorders as well as for malignancies.

**Anti-angiogenic therapy**

The fundamental objective of anti-angiogenic therapy is to inhibit the progression of pathologic angiogenesis. Since the discovery of the underlying mechanisms of tumor angiogenesis, a series of drugs targeting this system have been developed. Drugs approved for cancer treatment by the U. S. Food and Drug Administration (FDA) are the monoclonal antibody bevacizumab (Avastin\textsuperscript{®}), which inhibits VEGF-A by direct binding, and the approval is extended for use as monotherapy in recurrent GBM\textsuperscript{140} and for use in combination therapy for metastatic colorectal cancer, non-small cell lung cancers, and metastatic renal cell cancer\textsuperscript{141}. Sorafenib (Nexavar\textsuperscript{®}) is a small molecule inhibitor of several RTKs, such as VEGFR and PDGFR as well as intracellular Raf kinases, and is approved for hepatocellular carcinoma and metastatic renal cell cancer\textsuperscript{142}. Sunitinib (Sutent\textsuperscript{®}) is used in the treatment of metastatic renal cell cancer and neuroendocrine tumors, and targets multiple RTKs, including VEGFR, PDGFR, RET proto-oncogene, colony stimulating factor-1 receptor (CSF-1R) and fms-like tyrosine kinase (flt3)\textsuperscript{142}. Pazopanib (Votrient\textsuperscript{®}) is approved for metastatic renal cell cancer and also targets multiple RTKs\textsuperscript{140,142,143}. In addition, Thalidomide inhibits bFGF activity and is used in combination with dexamethasone in patients with newly diagnosed multiple myeloma\textsuperscript{144}. Inhibition of tumor growth with the mTOR inhibitors everolimus and temsirolimus may partly be explained by indirect effects on angiogenesis through mTOR-mediated HIF activation. These drugs are now approved by FDA for use in advanced renal cell carcinoma\textsuperscript{145}.
The effects of anti-angiogenic therapy in GBM patients have so far been elusive. The rationale for treatment of GBMs with anti-angiogenic strategies is based on the fact that they are highly vascularized tumors, and microvascular density has previously been demonstrated to be a marker of low and high grade glioma. Encouraging responses in patients with recurrent GBM using bevacizumab was demonstrated in 2007. Later the same year, Vredenburgh et al. presented a phase II trial of bevacizumab concomitantly with the topoisomerase inhibitor irinotecan in recurrent GBM, which led to almost instant clinical implementation. A current randomized, double blinded, multicenter phase III trial (AVAGLIO) will elucidate if there is a benefit from adding bevacizumab to standard therapy in first-line treatment of GBM patients.

Resistance mechanisms to anti-angiogenic therapy
Preclinical studies have demonstrated that lowering the amount of VEGF in the tumor results in decreased permeability in the vessels, which results in normalization of the chaotic tumor vasculature. Potential advantages of normalization and increased oxygen supply would evidently be the possibility of increased bioavailability of chemotherapy into the tumor and increased potency of ionizing radiation therapy. However, treatment modalities that target angiogenic pathways are currently associated with difficulties, e.g. the phenotype of the tumor may shift towards a more infiltrative behavior and increased metastasis as a result of vessel retraction and aggravated hypoxia. It is still a controversy whether these adaptive-evasive responses truly occur, but emerging data from GBM models point towards recurrent tumors with significantly increased infiltrative and migratory behavior following bevacizumab treatment. A very recent publication demonstrated that although anti-tumor effects are seen by a blocking VEGFR2 antibody and sunitinib, increased invasiveness and sometimes even augmented distant metastases were observed in the RIP-1Tag2 model of pancreatic neuroendocrine cancer. Tumors, in particular brain tumors, may use alternative ways to retain blood supply. Studies from 2000 showed that anti-VEGF treatment in a GBM model indeed prolonged survival, but resulted in increased vascular cooption. Additional resistance mechanisms include redundancy of angiogenic pathways and decreased sensitivity to the administered drug by the production of pro-survival or anti-apoptotic factors, such as surviving in ECs in response to anti-VEGF treatment. Hypoxia can select for tumor cells with mutations in p53, thus changing the vascular dependence of the tumor and further therapeutic responses. In addition, significant challenges in other aspects remain; limitations in tumor vasculature drug delivery are still an
important issue, as well as finding a way to predict tumor response to anti-
angiogenic therapies.

In conclusion, anti-angiogenic strategies have allowed for significant
advancement in patient survival in sub-groups of patients in certain cancer
types, as well as for a deeper, yet far from complete, understanding of tumor
biology.

Hypoxia

Solid tumors are prone to develop hypoxic regions as a consequence of a fast
outgrowth of the existing vascular network\textsuperscript{165,166}. It is important to keep in mind
that the threshold of hypoxia is relative and varies between tissues and does not
represent a specific value although initial studies have approximated tumor
hypoxia to occur at approximately 1 \% O\textsubscript{2}\textsuperscript{167}. The adaptation to hypoxia
includes a phenotypic shift; cells modify their metabolism towards increased
glycolysis and anaerobic metabolism, while universal mechanisms such as
protein translation, DNA repair and cell growth in many cases are reduced.
Hypoxic tumors are more resistant to radio and chemotherapy\textsuperscript{168}, and as a result
of selection pressure, hypoxia may increase the aggressiveness and the genomic
instability of many tumors\textsuperscript{169}.

\textit{Hypoxia-inducible factors}

Transcriptional upregulation of pro-angiogenic factors are mediated by HIF\alpha
and HIF\beta subunits\textsuperscript{170}. There are currently three known oxygen-sensitive HIF\alpha
subunits; HIF1\alpha, HIF2\alpha and HIF3\alpha. HIF1\beta is a member of the Aryl
Hydrocarbon Receptor Nuclear Translocation (ARNT) family. Both subunits
are constitutively expressed at the mRNA levels independently of oxygen levels.
HIF\alpha is however targeted by the oxygen-dependent prolyl hydroxylase enzymes
PHD1, PHD2 and PHD3. Hydroxylation of HIF\alpha subunits allows for
recognition by the von Hippel Lindau (VHL) E3 ligase complex\textsuperscript{171}. Thus,
ubiquitination results in proteosomal degradation of HIF\alpha subunits under
normoxic conditions. In addition, hydroxylation at an asparagine residue in the
C-terminal region of HIF1\alpha subunit by factor inhibiting HIF (FIH) leads to
disruption of HIF association with transcriptional co-activators\textsuperscript{172}. Hence,
HIF1\beta levels are readily detected at normoxic conditions, while almost no
expression of HIF\alpha protein is seen. Due to strict oxygen dependence of PHDs
and FIH, HIF\alpha hydroxylation is greatly reduced at hypoxia. HIF1\alpha and
HIF2\alpha proteins rapidly accumulate in the cytoplasm and are transported to the
nucleus where they form complexes with the ARNT/HIF1\beta subunit. This
complex further recruits a number of co-regulators, e.g. CREB-binding protein (CBP/p300), and activates transcription through association with hypoxia responsive elements (HREs) in promoter regions of HIF target genes.\textsuperscript{173-175}

The notion that hypoxia affects biological parameters in tumor biology and may modify the malignant potential of tumor cells\textsuperscript{169,176} gains support from the fact that HIF1α is associated with increased aggressiveness of gastrointestinal\textsuperscript{177,178}, colorectal\textsuperscript{179} and GBM\textsuperscript{180} tumors. Conflicting reports indicate that the overall relationship between HIF1α expression and prognostic impact in breast and pancreatic cancer seems difficult to evaluate.\textsuperscript{181-184} HIF1α and HIF2α are both expressed in human GBM\textsuperscript{185}, but their respective roles in tumor progression remain unclear.

Deletion of HIF1α and HIF2α has been demonstrated to result in aberrant organ vascularization during development.\textsuperscript{186} However, the expression of HIF1α and HIF2α is not redundant, and deletion of HIF1α in vascular ECs resulted in disturbed hypoxia-induced transcription, as well as delayed wound healing and reduced proliferation. Interestingly, loss of HIF1α in ECs resulted in a significant reduction in tumor growth.\textsuperscript{187} Similarly, upon targeting of HIF1α or HIF2α in experimental tumor models, the effect seems to be a reduction in overall tumor burden and more severe necrosis\textsuperscript{188}. Somewhat contradictory to these findings, excessive HIF levels do not necessarily result in increased vessel formation, as supported by defective placental vasculogenesis in VHL\textsuperscript{−/−} mice.\textsuperscript{189} The same phenomenon however is seen in VEGF-A overexpressing mice, suggesting that expression of angiogenic factors requires a narrow window for normal vascular development.\textsuperscript{190}

In summary, HIFs are crucial regulators of hypoxia-dependent tumor development, and further investigations will evaluate the possibilities of using HIFs for diagnostic/prognostic and therapeutic interventions.

### Coagulation

Coagulation is by definition the process during which blood forms solid clots. Malignant tumors are thrombogenic, \textit{i.e.} they are associated with activation of the coagulation system and, importantly, this activation coincides with angiogenesis. The nature of cancer associated thrombosis and activation of the pathways involved in coagulation related angiogenesis and tumor development, as well as treatment strategies, will be highlighted below.
The nature of cancer-related coagulopathy

Already in 1861, Armand Trousseau reported about the association between malignant disorders and thromboembolism. While the report concerned visceral cancer, today we have expanded the term “Trousseau’s syndrome” to be applicable to the occurrence of venous thromboembolism (VTE) in numerous solid tumors and hematological cancers\(^{191,192}\). Of all cancer patients, 10-15 % develops symptomatic thrombosis, and this number is substantially higher in certain tumors, e.g. lung, pancreas, stomach, colon, ovarian, and CNS cancers\(^{193-195}\). Up to 90 % of all metastatic cancers\(^{196}\) display abnormalities in any of the clinical measurements or in laboratory coagulation variables e.g. the circulating fibrin degradation product d-dimer, circulating thrombin-antithrombin complexes or TF\(^{197-199}\). Also, treatment itself may influence on thromboembolic events, e.g. post-operative thromboembolism is a major risk upon tumor excision. Some therapeutic agents such as VEGF inhibitors, thalidomide and tamoxifen may also provoke thrombogenic effects, which motivates strategies for prevention, detection and management of patients receiving these treatments\(^{200}\).

Thrombosis involves three major components: the vascular wall (particularly the endothelium), platelets and the coagulation cascade. The major initiator of the coagulation cascade is TF (also known as factor III and tromboplastin), a 47 kDa glycoprotein located at the plasma membrane. TF belongs to the cytokine receptor II family and consists of 263 amino acids, including 1-219 amino acid residues in the extracellular domain responsible for ligand binding, a 22 amino acid long transmembrane domain, and a 20-21 amino acid long cytoplasmic domain\(^{201}\) that can be phosphorylated and plays a key role in intracellular signaling\(^{202,203}\). Soluble factor VII forms a complex with TF, forming an activated complex, TF-VIIa. Unbound factor VIIa has limited activity, i.e. the protease function of VIIa in vivo is completely TF dependent\(^{204}\). TF-VIIa activates factor X into Xa, which together with factor Va assembles into the prothrombinase complex that converts factor II (pro-thrombin) into IIa (thrombin), completing the hemostatic response by clot formation\(^{205}\).

Apart from hemostasis, TF participates in numerous biological processes, such as embryogenesis, inflammation, cell migration, angiogenesis and metastasis\(^{206-208}\). In normal physiology, TF is essential for the induction of angiogenesis, as the absence of TF in mice leads to embryonal lethality due to impaired vascular integrity and abnormal development of the yolk sac\(^{209-212}\). This phenotype is comparable with that of VEGF-deficient embryos, indicating that TF and VEGF regulate analogous functions\(^{135,136}\).
In pathologic conditions, TF also has a significant role, as it has been found to be overexpressed in numerous cancers, e.g. in colorectal cancer\textsuperscript{213,214}, malignant gliomas\textsuperscript{215}, breast cancer\textsuperscript{216,217}, pancreatic cancer\textsuperscript{218,219} and acute promyelocytic leukemia\textsuperscript{220}. These tumors also have an elevated capacity to induce coagulation and angiogenesis\textsuperscript{207,221}. Interestingly, TF expression appears to be fundamentally critical for metastasis, as metastatic melanoma and colorectal cancer cells may express up to 1000-fold more TF than their nonmalignant counterparts\textsuperscript{222,223}. Thus, TF has a vital position both in physiological and pathological conditions.

As mentioned above, malignant disease frequently associates with a high incidence of macro or microvascular thrombotic manifestations. On the other hand, it has been speculated why the aberrant TF expression in tumor cells not always seems to cause intratumoral thrombosis and occlusion of the tumor vasculature. Instead, thrombosis is seen in distant organs despite the fact that the tumor interstitium contains high levels of local activation of e.g. TF, thrombin activity, and deposition of cross-linked fibrin\textsuperscript{224}. The mechanisms behind this paradox are not clear, however, an explanation for this phenomenon may be intratumoral elevated activity of the fibrinolytic system, such as plasmin and plasminogen activators. The involvement of the fibrinolytic system in malignancy has been demonstrated in tumor growth, angiogenesis and metastasis\textsuperscript{105,225-227}. Cancer patients with elevated levels of proteins involved in plasminogen activated pathways e.g. urokinase plasminogen activator (u-PA), urokinase plasminogen activator inhibitor-1 (PAI-1) and urokinase plasminogen receptor (u-PAR), seem to have worse prognosis\textsuperscript{228}. However, the localization of the fibrinolytic proteins in the tumor remains to be elucidated, although u-PAR and u-PA have been detected in ECs in GBM\textsuperscript{229} and astrocytoma\textsuperscript{230}, respectively.

Regulation of Tissue factor (TF) expression

In general, TF is induced at the level of gene transcription and probably by posttranslational mechanisms e.g. by regulation of mRNA stability\textsuperscript{231}. TF is classified as an immediate early gene, i.e. transcription activation is rapid and transient in response to various stimuli. VEGF can induce TF expression in ECs through early growth response gene product-1 (EGR-1)\textsuperscript{232}, and this is also true for cancer cells\textsuperscript{233}. Conversely, aberrant TF expression in tumor cells can contribute to the angiogenic phenotype by upregulating VEGF and downregulating TSP\textsuperscript{234,235}. Some transcription factors, in particular NF-κB and specificity protein-1 (SP-1) are involved in the regulation of both VEGF and TF, which may explain their coregulation in many tumors\textsuperscript{231,236-238}. Hence, the correlation between VEGF and TF has been confirmed in a variety of settings; higher levels of TF in tumors resulted in stronger VEGF expression and
microvessel density, and VEGF and TF were found to colocalize in both breast and lung cancer.

Quiescent ECs have generally no expression of TF, due to the fact that promoter elements in the TF gene repress the transcription under basal conditions. Other findings point toward an “encrypted” TF on the surface of ECs, meaning that although TF is detected on the cell surface, it displays no pro-coagulant activity. Blum et al. proposed that a continuously activated PI3K-Akt signaling pathway results in the suppression of TF in ECs under physiological conditions. The opposite is seen in cancer cells, where the frequent loss of the tumor suppressor gene PTEN leads to a constitutively active PI3K-Akt signaling pathway, and in consistency with numerous other oncogenic events in tumors, inactivation of PTEN is associated with elevated risk of thrombosis by inducing the expression of TF. As mentioned above, EC generally do not express TF, but under stress conditions such as stimulation with the bacteria toxin lipopolysaccharide (LPS) and cytokines, a prothrombotic microenvironment is triggered by the synthesis of TF and PAI-1. TNF-α may induce TF expression in ECs, and in response to inflammatory stimuli enhanced TF transcription involves nuclear factor kappa B (NF-κB) activation. However, to this date, the role of ECs in coagulation activation in malignancy is ill-defined.

Conclusively, VEGF can induce TF expression and vice versa, which contributes to the creation of a vicious cycle connecting angiogenesis and coagulation activation in tumors.

Hypoxic induction of TF
Cancer cells and tumor-associated stroma are frequently exposed to hypoxia as well as displaying an activation of the coagulation system by the overexpression of TF. The mechanism of TF induction however, seems to be HIF-independent. As mentioned above, VEGF can induce TF expression through EGR-1, and it is established that EGR-1 is rapidly upregulated at hypoxia in vitro as well as in GBM tumors. EGR-1 is central for the transcription of TF, as shown by the absence of both TF and fibrin deposition in EGR-1 null mice. In addition, PTEN inactivation and hypoxia, two characteristics of GBM, have been shown to upregulate the expression of TF and subsequent plasma coagulation in GBM. Cultured or native endothelium exposed to hypoxic conditions has been reported to generate a procoagulant factor Xa generating phenotype. Importantly, it is unclear if hypoxia induces the expression of TF in ECs, but one study reports on the an additive activation of TF by hypoxia upon endotoxin stimulation, while others demonstrate an increased procoagulative activity dependent on factor X.
The oncogenic events that are capable of deregulating the hemostatic system thus proceed in parallel with various tumor microenvironmental factors such as hypoxia and inflammation, which together with responses to different therapies may hamper disease treatment.

Mechanisms of TF-induced tumor angiogenesis

It is well established that TF contributes to tumor angiogenesis and progression through clotting-dependent mechanisms, which mainly involve thrombin-mediated fibrin generation and platelet activation\(^{207,225,257-259}\). However, recent work has extended the understanding of the versatile effects of TF and its mechanistic roles in the modulation of cancer cells and non-malignant host cells through clotting-independent events.

Clotting-independent PAR activation by coagulation proteases

Clotting-independent mechanisms have emerged as important in the regulation of cancer progression, and are now believed to include three TF-dependent signaling pathways via (i) thrombin generation (ii) intracellular signaling upon TF-VIIa interaction and (iii) phosphorylation of the cytoplasmic domain of the TF receptor\(^{260,261}\).

These pathways involve the activation of a family of G-protein coupled receptors (GPCRs), \textit{i.e.} protease activated receptors (PARs)\(^{262,263}\). To date, the expression of four PARs, PAR-1-4, has been identified in most tissues and in numerous cells\(^{264}\). All PARs except for PAR-4 are expressed on the endothelium\(^{265,266}\). PARs are involved in numerous pathophysiological events, \textit{e.g.} thrombosis, atherosclerosis, inflammation and tumor metastasis. The activation of PARs occurs through a unique proteolytic cleavage within the first extracellular loop, in which the N-terminus of the receptor interacts with the ligand at a specific amino acid sequence\(^{267,268}\). This irreversible cleavage generates a conformation change and a new N-terminus, which in turn leads to self-activation through a tethered ligand mechanism\(^{269}\). The widely expressed PAR-1 activates by cleavage at Arg\(^{31}/\text{Ser}^{42}\) by thrombin, TF-VIIa-Xa complex, granzyme A, trypsin and factor Xa, while the more cell specific expressed PAR-3 and PAR-4 mainly are cleaved by thrombin\(^{270-273}\). Both thrombin and trypsin are secreted frequently by tumor cells, and are believed to contribute to the metastatic potential of tumor cells. PAR-2 is expressed in a variety of tissues, \textit{e.g.} liver, kidney, intestine, colon, pancreas, lung and skin, and cleavage occurs at Arg\(^{42}/\text{Asp}^{43}\) residues, not by thrombin, but instead by TF-VIIa, TF-VIIa-Xa complex, factor Xa, trypsin and mast cell tryptase\(^{274-278}\). Synthetic peptides containing the first five or six amino acids, for PAR-1, SFLLR, and for PAR-2,
SLIGKV\textsuperscript{279} serve as a tethered ligand independently of receptor cleavage\textsuperscript{280}. The profound effects of PAR agonist stimulation on diverse cells lie in the differential activation of intracellular signaling pathways\textsuperscript{263}. Upon PAR activation, signaling pathways such as phosphoinositide hydrolysis, protein phosphorylation, cytosolic Ca\textsuperscript{2+} release are induced, and cAMP synthesis is suppressed. PARs are associated with heterotrimeric $\alpha\beta\gamma$ G proteins, in which the $\alpha$-subunit in a quiescent state is GDP-bound\textsuperscript{281}. Stimulation of the receptor activates the G-protein by hydrolysisation of GTP by GTPase activating proteins (GAPs). Interestingly, phenotypic effects related to these signaling pathways involve actin reorganization\textsuperscript{282}, integrin activation\textsuperscript{283}, cell proliferation\textsuperscript{284}, cell motility\textsuperscript{275}, immune responses and inflammation\textsuperscript{285}.

PAR-1 has been found to be involved in the development of embryonic vasculature, since lack of PAR-1 expression in ECs resulted in maturation defects in blood vessels\textsuperscript{286}. PAR-1 is capable of inducing direct modulation of vascular integrity by thrombin, or by the indirect induction of angiotensin and VEGF PAR-1 was upregulated in prostate cancer\textsuperscript{287} and appears to be primarily involved in thrombin-mediated effects in tumors. The expression of PAR-1 correlates to invasiveness in breast carcinoma cell lines and human breast metastatic tissues\textsuperscript{288}. Moreover, thrombin induced platelet-tumor adhesion and pulmonary metastasis through PAR-1 activation in an experimental model\textsuperscript{283}. High efficiency of metastatic lesions was seen in PAR-1 deficient mice, suggesting that the primary target for PAR-1 signaling involves clotting-independent pathways and effects on the cancer cell\textsuperscript{289}. In a more recent study of primary prostate cancer and bone metastases, PAR-1 was found to be upregulated in reactive stroma\textsuperscript{290,291}.

Importantly, TF can also mediate pro-angiogenic signaling independently of thrombin by direct TF-VIIa, TF-VIIa-Xa or Xa-mediated cleavage of PARs, particularly PAR-1 and PAR-2. TF-VIIa is believed to cleave PAR-2 exclusively, whereas TF-VIIa-Xa and factor Xa alone can activate both PAR-1 and PAR-2, although with different kinetics\textsuperscript{262,292,293}. The TF-VIIa-PAR-2 signaling axis promotes angiogenesis by the secretion of several factors such as interleukin-8 (IL-8) and VEGF\textsuperscript{294}. Signaling by Xa may be of relevance for the migration of smooth muscle cells\textsuperscript{295}, and the induction and release of IL-6, IL-8, monocyte chemotactic protein (MCP-1), and nitric oxide in EC\textsubscript{s}\textsuperscript{296}. These data have been corroborated by numerous studies verifying that these cellular effects are mediated by PARs\textsuperscript{262,297,298}. Tumor cell TF-VIIa-PAR-2 signaling appears to be more important for tumor growth than coagulation effects of TF, as shown in xenograft breast tumor growth in mice targeting either the signaling or coagulative functions of TF, using the monoclonal antibodies 10H10 or 5G9, respectively\textsuperscript{299}. 

34
Numerous studies demonstrate that PAR-2 signaling is greatly involved in tumor development and angiogenesis. Studies by Milia et al. demonstrated enhanced angiogenesis by PAR-2 mediated vasodilation, EC proliferation, and plasma protein extravasation in a model of hindlimb ischemia. More direct evidence of a specific role of the TF cytoplasmic domain (TFCD) in developmental and tumor angiogenesis was shown by enhanced TF-VIIa-PAR-2 dependent angiogenesis upon genetic deletion of the TFCD. Genetic evidence for the importance of TF-PAR-2 signaling in tumor angiogenesis and development was confirmed in a model of mammary tumor virus (MMTV) promoter-driven expression of the Polyoma Middle T antigen (PyMT) that results in spontaneous development of breast cancer in mice. Deficiency of PAR-1 in this model did not delay development of breast cancer relative to wild type; however PAR-2 null mice exhibited delayed tumor vascularization. Intriguingly, in a positive feed-back loop, activation of PAR-2 phosphorylates the TFCD, which leads to attenuation of TFCD-dependent suppression of PAR-2. These data were supported by the correlation between high TFCD phosphorylation and worse prognosis in human breast cancer. However, it is still controversial whether this pathway plays a role in primary tumor growth. Furthermore, in an in vivo model of metastasis, it was shown that the TFCD of cancer cells significantly contributed to metastasis. Later studies confirmed this finding by showing that the TF-VIIa complex cooperates with specific functions of the TFCD in cancer cells to support metastasis.

Taken together, TF participates in cancer-related thrombosis and tumor-associated angiogenesis and metastasis (for an overview of selected pathways, see figure 3). Although TF does not seem to possess any transforming properties, it clearly has the ability to modulate tumor cell behavior, most likely through the interference with the host microenvironment. The above findings reinforce the significance of coagulation-independent TF signaling in tumor development. In paper III, we investigated the role of PAR-2 in hypoxia-induced angiogenesis in ECs, and found that hypoxia upregulated PAR-2 that subsequently triggered HB-EGF expression important for the angiogenic process. Thus, TF-VIIa or factor Xa signaling via PAR-2 may contribute to pro-angiogenic responses in the appropriate cellular microenvironment.
Figure 3. The many faces of TF-dependent signaling in cancer. The coagulation cascade is associated with tumor growth and metastasis by the induction of angiogenesis. There are two pathways for the induction of TF-dependent angiogenesis, clotting-dependent (light blue area) and clotting-independent (white area). (1) Coagulation involves thrombin and platelet activation, fibrin deposition and subsequent clot formation that induce angiogenesis, either by direct effects of clotting, or by platelet-derived growth factors. (2) In addition, thrombin can induce angiogenesis via clotting-independent pathways, e.g. through the activation of PAR-1 mainly on tumor cells leading to increased migration and metastasis, but also to vascular remodeling of ECs. (3) Other clotting-independent pathways involve activation of PAR-2, mainly by the TF-VIIa complex. Activation of PAR-2 by cleavage leads to self-activation through a tethered ligand mechanism, and downstream signaling pathways involving MAPK and PI3K among others. Intracellular signaling cascades contribute to the transcription of angiogenesis-related genes, which induce immune response, proliferation, migration, and prevent apoptosis. (4) TF-bearing microvesicles released from tumor cells and platelets can escape into the bloodstream and induce coagulation. (5) TFCD in cancer cells and tumor-associated stromal cells also regulate...
metastasis and angiogenesis, in the latter case in a PAR-2 dependent manner. Additionally, anticoagulative factors in this system are TFPI and ATIII. ECs are the major source of TFPI that inhibits TF-VIIa and TF-VIIa-Xa complex as well as factor Xa. ATIII mainly inhibits factor Xa and thrombin under physiological conditions. Abbreviations: ATIII; anti-thrombin III, HS; heparan sulfate, MVs; microvesicles, PAR; protease-activated receptor, TFCD; TF cytoplasmic domain, TFPI; TF pathway inhibitor; VEGF; vascular endothelial growth factor.

Targeting the coagulation system in cancer therapy

Several groups have suggested that targeting TF in vivo using antibody-mediated therapy or targeted delivery of TF to the tumor may be efficient tools for therapeutic intervention\textsuperscript{305,306}. However, no clinical trials using this approach have been evaluated to this date. Napc2, nematode anticoagulant protein c2, binds to the active site of VIIa, and thus stabilizes the two complexes TF-VIIa-X and TF-VIIa-Xa\textsuperscript{307,308}. Napc2 has been shown to reduce tumor growth and angiogenesis in an in vivo model of melanoma\textsuperscript{309}, while no effect was seen using the Xa inhibitor Nap5. Ongoing clinical studies are evaluating the effect of recombinant Napc2 on coronary diseases, but no clinical trials in cancer patients have so far been completed\textsuperscript{310}. Thrombin-targeted, anticoagulant therapy may affect prothrombotic properties as well as growth and metastatic potentials of a tumor. Direct targeting of thrombosis may be achieved by the anticoagulants TFPI-1, coumarin, or low molecular weight heparin (LMWH). Some evidence from metaanalyses encompassing retrospective studies of VTE prophylaxis and treatment trials did indeed indicate that in particular LMWH may increase survival in cancer patients independently of reduced thrombosis-associated mortality\textsuperscript{311}. Ongoing studies will provide additional insights into the potential tumor-inhibiting effects of using antithrombotic agents in patients with cancer. Inhibitors of PAR-1 and PAR-4 have been suggested to block thrombin-induced cellular responses in vitro\textsuperscript{312}, but targeting the PARs is associated with some difficulties due to the differential expression of PARs, and owing to the co-expression in platelets, PAR inhibition creates a rather unspecific receptor blockade. Future studies on this subject will elucidate whether targeting of the coagulation pathway might prevent tumor progression and metastases in cancer patients.
Extracellular vesicles shape the microenvironment

All cells must communicate and quickly respond to changes in their environment. Hence, the communication between cells in the tumor microenvironment is essential for the development of a tumor. Extracellular membrane vesicles (EMVs) as a means of exchange of information have attracted attention in the context of tumor formation, due to recent discoveries of new mechanisms biologically and evolutionarily relevant for intercellular communication. The formation, role and future implications of EMVs in tumor development and progression will be discussed in this chapter.

Extracellular membrane vesicles as mediators of cellular communication

The preceding view on how multicellular organisms communicate with each other on a cellular level have mainly been characterized by the secretion of soluble factors activating cell surface receptors on neighboring cells, cell-cell contact dependent on adhesion proteins, or cell-cell contact and transfer of membrane fragments via nibbling, trogocytosis or tunneling nanotubes. Notably, a vast amount of data now supports the concept that cell derived secreted membrane vesicles are important players in biology, and we now appreciate the importance of EMVs in the induction of cellular responses and in the transfer of material between cells\textsuperscript{313-315}. The release of vesicles seems to be an evolutionary inherent mechanism, due to the fact that vesicle release is not only essential for eukaryotic cells, as also bacteria have the competence to shed vesicles, which signifies the extent by which organisms communicate, within or with each other\textsuperscript{316}.

The modes of vesicular communication have been suggested to involve direct activation of cell surface receptors by vesicles\textsuperscript{317,318}, transfer of functional proteins to recipient cells\textsuperscript{319,320}, transfer of functional mRNA that can be
translated into protein\textsuperscript{321}, effects of transferred miRNA on expression levels of mRNA targets\textsuperscript{322}, transformation of cells\textsuperscript{319}, or the induction of cell signaling pathways upon internalization, or alternatively, upon activation by released material from disrupted vesicles\textsuperscript{323}. Accordingly, many cells produce EMVs, either upon activation, as for ECs and platelets, or constitutively, as for many cancer cells\textsuperscript{313}. Thus, EMVs can be viewed as conveyors of cell responses, and are implicated in processes of local communication in humans\textsuperscript{324}, as well as at a systemic level owing to the fact that they have been found in blood plasma\textsuperscript{325}, urine\textsuperscript{326,327}, saliva\textsuperscript{328,329}, breast milk\textsuperscript{328} and ascites fluid\textsuperscript{330}. The functional consequences of such transfer may cover an immense width involving immune system response, inflammation, hemostasis and a range of additional processes involved in tumor development. Their presence in various body fluids opens up the potential relevance of EMVs as biomarkers, and as new targets in anti-cancer therapy.

Biogenesis of extracellular membrane vesicles

Membrane vesicles are spherical structures enclosing soluble cargo by a lipid bilayer. EMVs contain cytosol and display the same topology of membrane proteins as a regular plasma membrane. Upon release from living cells, irrespective of their origin, vesicles are collectively termed extracellular membrane vesicles (EMVs)\textsuperscript{313}. The discovery of various types of EMVs has created heterogeneity in the terminology of secreted vesicles isolated in different studies\textsuperscript{313}. The formation of EMVs occurs through two distinct processes; exosome formation or microvesicle (MV) shedding. In this thesis, EMV will be used as a term of both types of vesicles, while the terms exosomes or MVs will be used according to the isolation procedure presented in the corresponding report. Microparticles (MPs) are generally defined as MVs released from platelets and monocytes by shedding and are the most abundant vesicles in blood. Due to their highly coagulative capacity, they are involved in activation of the coagulation system and although some of the descriptions for MV shedding also relates to MPs, the biogenesis of the latter will not be discussed here.

Exosomes are 30-150 nm sized particles released from multivesicular bodies (MVBs) upon their fusion with the plasma membrane. The characteristic composition of exosomes is discussed below\textsuperscript{315,331}. The earliest evidence of vesicular release of transferrin receptor (TfnR) was presented by Johnstone et
al., where they described the release of exosomes from multivesicular endosomes (MVE) during reticulocyte maturation\textsuperscript{332}. MVBs are formed upon the fusion of intraluminal, late endosomal vesicles as a result of endocytosis. In fact, they are the transport intermediate between early and late endosomes\textsuperscript{331,332}. The MVB pathway represents one of the most central turnover systems for cell surface transmembrane proteins and lipids, in which proteins and nucleic acids are selectively incorporated to form new vesicles. A part of cell surface proteins that enter the endosomal system are intended for inclusion into MVBs. However, the mechanisms that control the fate of the proteins going into a secreting MVB are largely unknown, but processes involving sorting signals such as monoubiquitination have been suggested to promote the inclusion of proteins into MVB. Fusion of the MVB with the lysosome leads to degradation, whereas the fusion of the MVB with the plasma membrane will release the vesicles as exosomes\textsuperscript{333} (for a more detailed discussion, see the chapter of endosomal sorting and MVB formation).

Extracellular vesicles with features of exosomes are not only formed in MVBs, but also have their origin from the plasma membrane. These shedded plasma membrane-derived vesicles, 50 nm to 1000 nm in size are termed MVs or sometimes oncosomes or shedded vesicles if derived from tumor cells\textsuperscript{334,335}. The EMVs used in the studies of the present thesis (papers III and IV) are classified as “MVs with exosome-like characteristics” according to the presence of several of the features observed for exosomes; however, we most definitely have a mixed composition of exosomes and MVs according to our analyses and previous published composition data by others\textsuperscript{336}.

As for both exosome and MV biogenesis, the mechanisms underlying EMV release or shedding is incomprehensible. Activation of exosomes and MV release has been shown to increase upon rise in intracellular Ca\textsuperscript{2+}, which activates cytosolic proteinases responsible for degradation of cortical actin and thus allowing for membrane budding\textsuperscript{337}. In addition, cholesterol depletion has been shown to reduce MV release, indicating that lipid raft domains are central in MV biogenesis\textsuperscript{313}. The different pathways of EMV formation are briefly described in figure 4.
Figure 4. Biogenesis and release of extracellular membrane vesicles. The formation of EMVs occurs via two distinct pathways. By coordination of the protein trafficking between the organelles, the endosomal system directs vesicles from (1) endocytosis, (2) early endosomal and recycling compartments, as well as vesicles from TGN and (3) late endosomes/MVBs. (4) A subset of late endosomes are directed to the plasma membrane for exocytosis of exosomes, a process shown to be dependent on monoubiquitination and to some extent ESCRT. (5) Exosomes are formed upon the fusion of a MVB with the plasma membrane, releasing the vesicles into the extracellular space, whereby they are named exosomes. (6) Vesicles from late endosomes may also fuse with lysosomes for degradation. (7) MVs are formed by budding off from the plasma membrane in a process that involves calcium release, intact cytoskeleton and lipid rafts. Exosomes and MVs display the same topology but may differ in certain cargo. Abbreviations: CGN; cis-golgi network, ESCRT; endosomal sorting complex required for transport, MVB; multivesicular body, TGN; trans-golgi network, UB; ubiquitin.

Composition of extracellular membrane vesicles
The composition of MVs and exosomes is rather overlapping and they share many similarities. Summaries of comprehensive proteomic analyses, although with deficiencies in stringency of some protocol parameters, have revealed clusters of proteins residing in these vesicles. The traditional exosomal markers are proteins originally derived from the MVB compartment, e.g.
tetrapanins and the ESCRT proteins tsg101 and alix. The tetraspanins CD63, CD81, CD9 are highly enriched in most exosomes, and subsequently used for flow cytometry-based analysis and isolation of exosomes. In addition, annexins, rab proteins and chaperones are common constituents of exosomes. The endosomal origin of exosomes makes them devoid of double stranded DNA, nuclear proteins and other organelle markers, such as the ER, Golgi and mitochondrial markers. The main marker proteins of MVs are suggested to be integrins, selectins and CD40. In addition, MVs have been demonstrated to arise from specific domains previously described as endosome-like domains that presented an enrichment of the exosomal and endosomal proteins CD81 and CD63. In addition, MVs have been suggested to arise from lipid rafts, which is similar to the lipid raft associated protein sorting occurring in exosome biogenesis. Furthermore, exosomes and MVs both contain high amounts of cholesterol, sphingolipids, phospholipids, as well as presenting phosphatidyl serine on their surface. At present, there are no markers to distinguish between exosomes and MVs; however, cell-specific markers may prove useful in some settings. Apart from the highly enriched markers discussed above, the composition of EMV principally reflects the donor cell. For example, reticulocyte-derived vesicles contain transferring-receptor, vesicles derived from dendritic cells display MHC-I and II molecules, tumor cell derived vesicles may carry EGFR, native or mutated. In addition to proteins and lipids, coding and non-coding RNA and single stranded DNA have been found in the lumen of EMVs.

**Extracellular membrane vesicles in health and disease**

The function of EMVs largely depends on their cargo and which cells that will internalize them. EMVs are secreted by numerous cells in the body; however, the underlying mechanisms for the release of vesicles are so far not fully characterized. Normal cells secrete vesicles in small amounts, while cancer cells constitutively secrete a large amount of EMVs. Elevated levels of vesicles are also found to be of importance in other situations, e.g. pregnancy, hemostasis, immune system regulation, diabetes, inflammation and as very recently described, in stem cell differentiation. Below follows a brief overview of some of the major functions of EMVs in physiology and in disease, as well as putative mechanisms of EMV uptake.
Extracellular membrane vesicles in physiology

Many of the studies conducted with tumor cell derived MVs are based on results originating from research elucidating the role of MPs in hemostasis and exosome modulation of the immune system. MPs were first found in plasma and were described as procoagulant particles derived from platelets. Platelet derived MPs constitute up to 80% of total vesicle count in blood plasma in healthy humans, and are involved in normal hemostasis as well as in hypercoagulability during disease. The role of exosomes in immune surveillance was initiated already in 1988, when Sims et al. showed that exosome release could be triggered by complement activation. Ten years later, the first description of exosomes in immune regulation showed that exosomes carry antigen presenting peptide-MHC complexes. Furthermore, a study investigating the role of exosomes in physiology was conducted using pregnancy-associated exosomes in the modulation of immune response. This study provides support for a physiological role of exosomes in vivo, as the authors noted that full term delivering women had higher levels of exosomes with T cell inhibiting activity as compared to pre-term delivering women. Finally, the use of dendritic exosomes as a tumor vaccine in a mouse model prompted studies investigating the implications of exosomes for therapeutic intervention. Besides their role in immune regulation, EMVs also have attracted increasing interest among stem cell researchers. In 2006, Ratatjczak et al. demonstrated that embryonic-stem cell derived MVs were capable of reprogramming of hematopoetic stem cells upon the transfer of mRNA and proteins residing in MVs. In support of these data, another group demonstrated the activation of an angiogenic program involving the PI3K/AKT pathway by the transfer of MVs derived from EPCs.

Extracellular membrane vesicles in cancer

TF-bearing microparticles as systemic activators of coagulation

As discussed in the coagulation chapter, TF expression has been correlated to cancer patient outcome, and these studies were supported by several others, showing that TF present in plasma from cancer patients correlates to poor prognosis. In recent years, accumulated evidence that TF may be circulating as an alternatively spliced variant, or as part of MPs has arised. Langer et al. showed that circulating, platelet-derived, TF-positive MPs from patients with an underlying malignancy had procoagulative capacity. In fact, plateleterived MPs display 50 to 100-fold higher specific procoagulant activity than activated platelets. This result can be put into a highly relevant context by the
finding that emission of TF-bearing MPs into the circulation probably serves as a vehicle for systemic coagulopathy in colorectal cancer, prostate cancer, pancreatic cancer and GBM\textsuperscript{214,356,359-362}. In a prospective study with GBM patients, procoagulant activity of circulating MPs was measured pre and post-operatively. It was concluded that GBM patients displayed higher procoagulative MP activity compared with healthy controls\textsuperscript{362}. Higher levels of TF-bearing MPs were also found in patients with onset of VTE compared to those without VTE\textsuperscript{363}, suggesting that procoagulant MP activity may represent one of several factors predisposing for thrombosis. The discovery that circulating MPs correlate with VTE supports the possibility of using TF-bearing MPs as diagnostic/predictive biomarkers of VTE\textsuperscript{364}.

Extracellular vesicles as modulators of the tumor microenvironment
The link between EMVs and cancer was first suggested from studies on a cell line derived from a patient with Hodgkin’s disease in 1978\textsuperscript{365}. Today, the potential effects of cancer cell derived EMVs have been suggested in numerous settings, e.g. escape from immune attack and apoptosis, or enhanced angiogenesis, altered microenvironmental remodeling and promotion of metastasis. These effects are on the cancer cell itself, or on stromal cells of the surrounding microenvironment as well as systemically. This is exemplified by the transfer of mutated epidermal growth factor receptor (EGFR), EGFRvIII, to indolent cells, whereby cells acquire a transformed phenotype\textsuperscript{320}. Transformation of fibroblasts and epithelial cells was also seen upon uptake of GBM and breast cancer cell-derived MVs, which could be connected to the transfer of fibronectin and transglutaminase\textsuperscript{319}. In a study of nasopharyngeal carcinoma it was demonstrated that tumor virus utilizes exosomes as vehicles for intercellular communication to activate ERK and AKT signaling pathways\textsuperscript{323}.

Furthermore, cancer cells may release MVs in order to escape from complement induced lysis\textsuperscript{366} and, depending on their cell-of origin, may expose Fas ligand to counteract the effects of the adaptive immune system\textsuperscript{317,318}. MVs may also play roles in the survival against cellular stress as MVs have been found to contain high levels of the potent pro-apoptotic protein caspase-3 from conditioned medium, without being detectable in donor cells. Thus, it was speculated that cells may release caspase 3-loaded MVs as a cellular escape from apoptosis\textsuperscript{367}. The involvement of MVs in multidrug survival has been indicated when membrane shedding-related genes were found to be upregulated in doxorubicin-insensitive cancer cell lines, and their corresponding MVs contained enriched levels of doxorubicin\textsuperscript{368}. In consistency with these data, cisplatin-insensitive cancer cells demonstrated abnormal lysosomal trafficking
with subsequent release of MVs with 2.6-fold higher levels of cisplatin compared to cisplatin-sensitive cells\textsuperscript{369}.

Cancer cell derived MVs may promote angiogenesis due to the fact that they contain mRNA for VEGF and hepatocyte growth factor (HGF)\textsuperscript{370}, and studies by Skog \textit{et al.} demonstrated that GBM-derived exosomes containing mRNA, miRNA and angiogenic growth factors that may promote angiogenesis\textsuperscript{371}. Further studies have corroborated these data. For example, melanoma derived exosomes were found to elicit pro-angiogenic responses in endothelium in a paracrine manner by the secretion of inflammatory cytokines\textsuperscript{372}, and the notch ligand delta-like 4 (Dll4), normally upregulated during angiogenesis, has been shown to be transferred via exosomes to ECs, which upon transfer acquire pro-angiogenic properties\textsuperscript{373}. In addition, procoagulative MPs or MVs have been demonstrated to have supportive roles in angiogenesis by the formation of fibrin and indirectly by the release of matrix-bound growth factors, but no studies mention the potential role of TF-bearing EMVs as inducers of TF signaling in the context of tumor development and angiogenesis. Notably, further studies should evaluate the effect of these vesicles, not only in thrombosis, but also in other TF-dependent signaling processes. In paper III of this thesis, we capitalized on the fact that GBM-derived MVs contain procoagulant TF, and investigated the possibility of MV-induced, TF-PAR signaling dependent angiogenesis.

Invasive growth and metastasis have been speculated to be linked to effects driven by EMVs. For example, EMVs may promote metastasis by modulating the stroma owing to the fact that they contain proteases, including uPA, MMP-2 and MMP-9\textsuperscript{374}. The procoagulant properties of EMVs may contribute to hematological spread through P-selectin-dependent activation of platelets, which support fibrin formation further facilitating tumor adhesion on the vessel wall\textsuperscript{356,374}. An intriguing phenomenon, but yet to be confirmed by others, is the fact that EMVs may contribute to metastasis by “priming” the pre-metastatic niche, as shown in lymph nodes and other distant organs upon pre-treatment with EMVs\textsuperscript{375,376}.

As EMVs are secreted into various body fluids and often represent their tissue of origin, their clinical value in terms of biomarkers or predictors of disease stage or treatment response has emerged as a promising alternative. Successful isolation of cancer specific RNA or protein in EMVs from cancer patients has allowed for the evaluation of cancer-associated EMVs as biomarkers that may provide guidance for individualized cancer therapy\textsuperscript{326,365,371}. 
Mechanisms of extracellular membrane vesicle uptake

Given the functional effects of EMV transfer and the fact that the delivery of material via membrane vesicles has been proposed to serve as a possible tool for the delivery of targeted therapeutics or drugs, detailed studies revealing the mechanisms involved in MV release, delivery and transfer remain an imperative challenge. Studies investigating uptake mechanisms aim to precede the development of MV-based delivery of therapeutic molecules. Below follows a brief overview of mechanisms of cellular entry and a discussion of the current standing of EMV uptake.

Vesicular uptake pathways

The plasma membrane continuously adjusts the composition as a response to needs. Endocytosis is by definition a mechanism where a cell ingests materials by the conversed process of invagination of plasma membrane components into intracellular endosomes. It is a well organized process that regulates numerous biological functions, such as the composition of proteins of the plasma membrane by negative regulation of receptors, but also signalling from the plasma membrane and during endocytosis. The high redundancy of involved proteins in this process indicates the significance of this mechanism. Pathways of entry consist of clathrin-dependent pathways, caveolin-dependent, clathrin-independent “non-classical” endocytosis, macropinocytosis and cell specific uptake via phagocytosis. Mammalian cells are able to make use of several pathways and although some proteins enter cells via a specific pathway, most cargoes can enter cells by several of the described pathways under certain conditions.

Clathrin-mediated endocytosis (CME) is the most well-characterized endocytosis pathway, and involves internalization of receptor-ligand complexes and membrane carrier proteins. CME can be constitutive, as for transferrin receptor (TfnR), or ligand induced, as for G-protein coupled receptors (GPCRs). The uptake of Tfn and low-density lipoprotein (LDL) in complexes with their respective receptors represent the most well-established markers of CME. The discovery of LDL uptake outlined the process of CME to include recognition of a transmembrane receptor by a ligand, whereby the cytoplasmic domain of the receptor crosslinks the clathrin coat and triggers the internalization to generate vesicular structures. Pathogens that utilize CME as entry into the host cells include semliki forest virus and adenovirus. Besides the formation of a clathrin coat surrounding the pit, CME is also dependent on adaptor proteins e.g. AP2 and the small GTPase dynamin.
Some of the non-CME endocytosis pathways are associated with lipid rafts and are classified based on the involved proteins; caveolin, the small GTPases RhoA, Cdc42, Arf6 and potentially other, not yet identified pathways. Caveolin-dependent endocytosis is the most defined non-CME entry pathway and engages one or several of the three caveolin members, in order to produce flask-shaped invaginations coated with caveolin. Caveolae share many characteristic with membrane rafts, i.e. high cholesterol and glycosphingolipid content, which suggests a common endocytic entry. Cholera toxin subunit B (CtxB), simian virus 40 (SV40) and glycosylphosphatidylinositol (GPI)-linked proteins are implicated as cargoes of caveolin-mediated endocytosis.

The non-clathrin, non-caveolin-dependent (“non-classical”) entry routes involving the small GTPases are not yet fully established, however recent findings point towards a regulatory role in endocytosis. Some proteins enter the cell by a mechanism involving the IL2Rβ receptor and RhoA and Rac1. This pathway appears to be dynamin-dependent but the transport regulation of cargo implicated in this pathway remains to be elucidated. Clathrin-independent, carrier/GPI-enriched early endosomal compartment (CLIC/GEEC) has a tubular or ring-like vesicular morphology, and implicated proteins in this pathway are Cdc42, actin, and ARHGAP26. The entry pathway producing vesicular and tubulovesicular structures mediated by Arf6 is rather unclear, but Arf6 has been implicated in recycling of vesicles to the plasma membrane, and seems to be reliant of intact dynamin. Major histocompatibility complex (MHC) class I proteins, CD59, carboxypeptidase are examples of cargoes that are suggested to enter through Arf6-mediated endocytosis. All of the above mentioned pathways are dependent on cholesterol, and consequently, depletion of cholesterol leads to a disruption and inhibition of cargo uptake of the entry pathways.

Macropinocytosis is a receptor-independent uptake of large volumes of bulk fluid, often in response to activation by growth factors e.g. EGF. This process is associated with membrane ruffling and protrusion due to actin remodelling, followed by a protruding membrane that encloses large volumes of extracellular fluid forming an macropinosome. Studies inhibiting CME revealed that the cells upregulated non-CME fluid phase uptake, suggesting that the regulation of endocytosis may be compensatory. Phagocytosis is a process related to pinocytosis, but whereas macropinocytosis is unspecific in the cargo it internalizes, phagocytosis is specific. For example, apoptotic cell remnants or pathogens are engulfed by specialized phagocytic cells, such as macrophages. Many of the above mentioned endocytosis pathways involve actin or tubulin cytoskeleton. In addition, endocytosis induces a variety of signaling pathways, such as mitogen-activated protein kinase (MAPK) and PI3K-Akt, both of which
have been demonstrated to be initiated at the cell surface and continue during endosomal sorting, and more recent data suggest that signalling is a required element of endocytosis\textsuperscript{382}. The discussed pathways of entry and examples of their respective cargo are summarized in Table 1.

<table>
<thead>
<tr>
<th>Endocytosis mechanisms</th>
<th>Primary carriers</th>
<th>Dyn dep.</th>
<th>Small G-protein involved</th>
<th>Earliest endosomal compartment</th>
<th>Implicated cargos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clathrin-dependent pathways</td>
<td>Clathrin-coated pits</td>
<td>Yes</td>
<td>Rab5, Arf6</td>
<td>Early endosome</td>
<td>GPCRs*, RTKs, TfnR, LDLR</td>
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<tr>
<td>Clathrin-independent pathways</td>
<td>50-80 nm caveolar structures</td>
<td>Maybe</td>
<td>Unclear</td>
<td>Caveosome or early endosome</td>
<td>SV40, EV1, CtxB, GPI-linked proteins</td>
</tr>
<tr>
<td>Caveolar</td>
<td>Clathrin-and dynamin independent carriers (CLIC)</td>
<td>No</td>
<td>Cdc42, Arf1</td>
<td>GPI-AP enriched early endosomal compartments (GEEC)</td>
<td>Fluid phase markers, CtxB, GPI-linked proteins</td>
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<tr>
<td>Cdc42-regulated</td>
<td>Uncoated vesicles</td>
<td>Yes</td>
<td>RhoA, Rac1</td>
<td>Early endosome</td>
<td>IgE receptor, IL-2R-αβ, IL-2R-β</td>
</tr>
<tr>
<td>RhoA-regulated</td>
<td>Unknown</td>
<td>No</td>
<td>Arf6</td>
<td>Early tubular recycling compartment</td>
<td>MHC I, CD59, β1 integrin, E-cadherin, GPI-AP</td>
</tr>
<tr>
<td>ARF6-regulated</td>
<td>Ruffled regions form 0.5-5 µm vesicles</td>
<td>No</td>
<td>Rac1</td>
<td>Late endosome</td>
<td>Fluid phase markers, RTKs</td>
</tr>
<tr>
<td>Macropinocytosis</td>
<td>0.75 µm vesicles</td>
<td>Yes</td>
<td>Arf6, cdc42, Rac1, RhoA</td>
<td>Early phagosome/endosome</td>
<td>Pathogens, apoptotic remnants</td>
</tr>
</tbody>
</table>

Table 1. Pathways of entry into cells. Mechanisms of entry in mammalian cells include CME endocytosis that can be ligand induced as for GPCRs, or constitutive as for RTKs, TfnR and LDLR. Clathrin-independent pathways involve the most common, caveolin-mediated endocytosis, or the recently characterized “non-classical” non-caveolin mediated pathways involving small GTPases. Macropinocytosis involves larger uptake of liquids, while phagocytosis specifically recognizes pathogens for destruction. See text for references. Abbreviations: CME, clathrin-mediated endocytosis; GPCR, G-protein coupled receptor; GPI, glycosylphosphatidylinositol; LDLR, low density lipoprotein receptor; MHC, major histocompatibility complex; RTK, receptor tyrosine kinase; TfnR, transferrin receptor.
Putative mechanisms of EMV uptake have been discussed in the literature; however, data supporting the hypotheses are vague. To this date, it is not established whether EMV uptake involves membrane fusion or endocytosis, although it has been speculated that uptake of vesicles occurs though membrane fusion, due to the fact that RNA is directly delivered into the cytoplasm, and by studies on platelets indicating that MPs may fuse with activated cells, but no mechanistic data support these hypotheses. Very recent data point toward a mechanism of membrane fusion, based on lipid-fusion studies on mouse dendritic cells allowed to ingest miRNA containing exosomes. However, due to limitations in the lipid based assay, one cannot exclude the possibility of a dual mechanism involving hemifusion or complete membrane fusion followed by endocytosis. In fact, electron microscopy studies of the uptake of TF-bearing MVs into platelets showed that the internalization does not occur via membrane fusion, but rather into the open canalicular system of resting platelets. Some groups have studied EMV uptake in more detail, however, due to rather contradictory data, there is no conclusive evidence of the entry mechanism. Studies investigating the uptake of EMVs have proposed that ovarian cancer cells internalize EMVs through CME and lipid raft-mediated endocytosis, e.g. nonspecifically through all known pathways. In addition, it is still controversial whether EMV uptake may be cell type specific. Studies on oligodendrocyte-derived exosomes have indicated that exosomes are internalized via macropinocytosis instead of phagocytosis in the phagocytic-competent cell microglia. However, according to a study comparing phagocytic cells with a number of normal and cancer cell lines, the uptake of exosomes occurs through phagocytosis rather than endocytosis. Furthermore, conclusions from studies on exosomal uptake in mast cells have led to the assumption that exosome uptake may be cell type specific. The two latter studies are, in contrast to previously published data on EMV uptake, performed in various cell lines, arguing against cell-type specific uptake of EMVs. Indeed, in paper III of this thesis, we confirm that GBM cell-derived MVs are internalized into ECs. In paper IV, we found that GBM-derived MV entry into ECs and GBM cells occurs through a lipid raft-mediated endocytosis mechanism, and discuss the potential implications for EMV transfer.

Intracellular sorting and multivesicular body formation
Recent advances have uncovered mechanisms for targeting of proteins into the MVB compartment, and as all endosomal trafficking, sorting of vesicles into MVB is a highly regulated event. Downregulation of EGFR and GPCRs from the cell surface is a result of the addition of a single ubiquitin to a substrate, a process called monoubiquitination. Ubiquitin functions as a
positive sorting signal for the selection of cargo into the MVB, but seems to be removed before proteins enter the MVB by a de-ubiquitinating enzyme called Doa4. ESCRT-I, composed of Vsp23, Vsp28, and Vsp37 selects ubiquitinylated cargo, and ESCRT-II directs the ESCRT-III complex to the appropriate membrane, while ESCRT-III seems to have a role in concentration and sequestration of the cargo\textsuperscript{389}. For example, ESCRT sorting has lately been shown to be partially regulated by the tetraspanin CD63\textsuperscript{390}. CD63 is a main component of MVBs, and is known to cycle between endosomal and secretory MVB compartments, and therefore being one of the major markers of exosomes. In paper IV of this thesis, we investigate the fascinating idea that sorting of MV content involves MVB inclusion to generate a second generation of MVs, by following the transfer of CD63.
Present investigation

Aims of study

The overall aims of this study were to investigate the mechanistic roles of polyamines in tumor growth, and to unravel MV-mediated interplay between cancer cells and ECs in the context of hypoxia-driven tumor angiogenesis.

Specific aims of the included papers were:

I. To investigate the possibility of inhibiting cancer cell proliferation by the combination of polyamine biosynthesis and HSPG uptake inhibition

II. To study the role of polyamines in hypoxia-induced stress in cancer cells and to evaluate the effect of combined targeting of the polyamine system and anti-angiogenesis therapy in a GBM xenograft model

III. To elucidate the role of PARs in the hypoxic responses in ECs and if coagulation activation in GBM cells may elicit paracrine, pro-angiogenic signaling in ECs through GBM derived MVs

IV. To unravel the mechanism of uptake of GBM cell-derived MVs and to address the possibility of multicellular, horizontal transfer of MV-derived information
Methodology

Below follows a description of each method with reference to the papers in which they were used. For details, see in the respective “Materials and methods” sections.

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenesis array (III)</td>
<td>Protein based array for angiogenesis-related proteins</td>
</tr>
<tr>
<td>Actichrome assay (III)</td>
<td>Measurement of active TF by cleavage of factor X into Xa in a chromogenic assay</td>
</tr>
<tr>
<td>Confocal laser scanning microscopy (I, III, IV)</td>
<td>Microscopic technique equipped with CO₂ and 37°C incubator enabling scanning through samples in sections of the z-plane on live cells</td>
</tr>
<tr>
<td>Cell survival/apoptosis assays (I, II, III)</td>
<td>Cell survival assays in vitro by lactate dehydrogenase (LDH) release measurement or by TUNEL labeling or Annexin V staining</td>
</tr>
<tr>
<td>Epifluorescence microscopy (II, III)</td>
<td>Immunofluorescence-based microscopic technique with above observation of the specimen</td>
</tr>
<tr>
<td>Flow cytometry (I, II, III, IV)</td>
<td>Laser based single-cell analysis using fluorochrome conjugated antibodies or MVs</td>
</tr>
<tr>
<td>Immunohistochemistry (II, III)</td>
<td>Microscopic localization of specific antigens in tissues by staining with antibodies labeled with fluorescent or pigmented conjugates.</td>
</tr>
<tr>
<td>In vitro migration assays (III)</td>
<td>Migration assays based on “scratch” wound assay or transwell pore migration system</td>
</tr>
<tr>
<td>In vivo xenograft models (II)</td>
<td>Tumor model with glioblastoma cell inoculation subcutaneously into SCID mice</td>
</tr>
<tr>
<td>Microvesicle labeling (III, IV)</td>
<td>Fluorescent labeling of microvesicles with lipid-based fluorescent dyes</td>
</tr>
<tr>
<td>Microvesicle purification (III, IV)</td>
<td>Isolation of microvesicles from medium by consecutive ultracentrifugations</td>
</tr>
<tr>
<td>ODC activity assay (II)</td>
<td>Radioactivity based assay for measurement of conversion of ornithine into putrescine</td>
</tr>
<tr>
<td>Phosphokinase array (III, IV)</td>
<td>Protein based array for phosphorylated proteins</td>
</tr>
<tr>
<td>Plasmid transfection (III, IV)</td>
<td>Non-viral method for the introduction of nucleic acids into mammalian cells</td>
</tr>
<tr>
<td>Polyamine levels (II)</td>
<td>Measurement of polyamine levels using an amino acid analyzer</td>
</tr>
<tr>
<td>[14]C-polyamine uptake (I, II)</td>
<td>Radioactivity based assay for measurement of polyamine binding or uptake</td>
</tr>
<tr>
<td>q-RT-PCR (II, III)</td>
<td>mRNA expression analysis by quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RNA interference (II, III, IV)</td>
<td>Silencing of expression by lipofectamine mediated RNA transfection</td>
</tr>
<tr>
<td>³H-thymidine incorporation (I, III)</td>
<td>Proliferation assay in vitro by radioactive isotope incorporation</td>
</tr>
<tr>
<td>Tissue factor ELISA (III)</td>
<td>Measurement of nM concentrations of TF in conditioned medium or plasma</td>
</tr>
<tr>
<td>Transmission electron microscopy (TEM) (III, IV)</td>
<td>Microscopic technique for morphological analyses of microvesicles and subcellular localization of microvesicles and proteins</td>
</tr>
<tr>
<td>Tube formation assay (III)</td>
<td>Endothelial tube formation assay using matrigel to evaluate the tube forming capacity</td>
</tr>
<tr>
<td>Western blot (I, II, III, IV)</td>
<td>Gel electrophoretic separation of proteins and transfer to protein binding membranes for enzyme-conjugated antibody detection</td>
</tr>
</tbody>
</table>
Results

Paper I - Single chain fragment anti-heparan sulfate antibody targets the polyamine system and attenuates polyamine-dependent cell proliferation

Introduction

Inhibition of polyamines with the use of DFMO as cancer treatment has been largely unsatisfactory mainly due to enhanced compensatory import of polyamines. Efficient anti-tumor therapy thus requires inhibition of both pathways, and strategies to inhibit the internalization of polyamines may have implications for treatment of cancer. Based on previous studies demonstrating the involvement of cell-surface HSPGs in the uptake of polyamines, and that combined targeting of biosynthesis and uptake attenuated tumor growth in vitro and in vivo, we sought to investigate the interaction between polyamine and HS in more detail.

Results

In order to study the interaction of polyamines and cell surface HSPG, we made use of single chain variable fragment antibodies with preferences for various HS epitopes. The anti-low-sulfated HS antibody RB4EA12 inhibited polyamine binding and uptake, while two other antibodies had virtually no effect. Inhibition of polyamine uptake with RB4EA12 resulted in an upregulation of ODC, which suggests that the intracellular levels of polyamines were reduced. Inhibition of internalization of polyamines by RB4EA12 significantly reduced tumor cell proliferation in DFMO-treated cells or cells deficient in ODC in the presence of excess extracellular polyamine. We could conclude that epitope specific anti-HS antibodies may be used to reduce the bioavailability of polyamines and that cell-surface HSPG is an applicable target for antibody-mediated inhibition of polyamine uptake.

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

Introduction

The hypoxic signaling response mainly serves to restore oxygen and nutrient supply in order to rescue cells from cell death. Hypoxic tumors are relatively resistant to radio and chemotherapy and are associated with a more aggressive phenotype, but the
hypoxic environment also provides opportunities for specific targeting of tumor cells. Polyamines are frequently found to be increased in tumor tissue, and although ODC is transcriptionally regulated by oncogenes, there is no unifying explanation to the universal induction of polyamines in tumor tissue. Given the fact that hypoxia and the induction of polyamine levels are common features of various malignancies, we hypothesized that hypoxia may influence the polyamine regulation as a part of hypoxia-mediated malignant progression.

Results
We found that hypoxic induction simultaneously upregulated the biosynthesis and uptake of polyamines in several cancer cell lines, and ODC expression correlated with hypoxic areas in experimental and clinical tumors. This resulted in an overall increase in polyamine levels, most notably putrescine. The upregulation of polyamine biosynthesis and uptake was found to be regulated by AZI. Importantly, combined targeting of ODC and anti-angiogenic therapy resulted in a drastic attenuation of tumor growth. In addition, tumors treated with bevacizumab and DFMO displayed profound effects on vascularization and survival, as shown by reduced vessel and increased apoptosis staining, respectively. These data suggest that polyamines protect cells under hypoxic stress, and that upregulation of the polyamine system is part of the cellular adaptive response to hypoxia.

Paper III - Hypoxia triggers a pro angiogenic pathway involving cancer cell microvesicles and PAR-2 mediated HB-EGF signaling in endothelial cells

Introduction
High grade gliomas present a histological appearance of pathophysiological vessels, hypercoagulation and necrosis. TF-dependent PAR-2 signaling has been associated with angiogenesis in physiological models as well as in tumors203,300. Although these studies suggested a specific involvement of PARs in angiogenesis, the relationship between hypoxia, abnormal vasculature and onset of intratumoral coagulation remains to be elucidated. We set out to investigate how these phenomena of the tumor microenvironment may be linked at the molecular level during tumor development.

Results
We present data showing that hypoxia upregulates PAR-2 in ECs, which elicits an angiogenic EC phenotype by the upregulation of the potent pro-angiogenic factor HB-EGF. This PAR-2 dependent HB-EGF induction was associated with ERK1/2 phosphorylation, and inhibition of ERK1/2 phosphorylation attenuated PAR-2-dependent HB-EGF induction as well as EC activation. TF was upregulated in hypoxic
cancer cells, while below detection limit in ECs. Thus, ECs are not self-sufficient in TF-dependent PAR-2 activation even at prolonged hypoxia. Interestingly, hypoxic GBM cells were shown to release substantial amounts of TF that was mainly associated with secreted MVs. GBM cell-derived MVs were further shown to trigger TF/VIIa-dependent activation of hypoxic ECs in a paracrine manner. This is, to our knowledge, the first study linking hypoxia-induced coagulation activation in cancer cells to PAR-2-mediated activation of ECs. The identified pathway may constitute a motivating target for the development of additional strategies to treat aggressive brain tumors.

Paper IV - Microvesicle transfer involves PI3K/MAPK-dependent nonclassical endocytosis and microtubule-mediated sorting to recirculating multivesicular body compartments

Introduction
EMVs have increasingly been recognized as important mediators of intercellular communication in tumor development through transfer of cancer cell derived genetic material and signaling proteins, and may at the present be regarded as delivery vehicles carrying tumor-derived molecular information. The functional effects upon treatment with MVs most likely rely on the fact that they are internalized in order to release their content. However, the mechanisms of their uptake by the receiving cells, and how this pathway may be targeted remain unclear. Thus, revealing the mechanisms involved in MV information transfer remain an important challenge. In this study, we aimed to elucidate the uptake mechanism of GBM cell-derived MVs and to investigate intracellular trafficking and sorting of MVs upon internalization.

Results
We found that MV uptake follows a nonclassical endocytotic pathway and that MVs undergo intracellular trafficking along microtubules to late endosomal and lysosomal compartments. We further show that PI3K and downstream activation of ERK1/2 were indispensable for cellular uptake of MVs. Importantly, a significant fraction of MVs was sorted to CD63-positive MVBs, and we present data that introduce the concept of exchange of MV cargo and integration of information from multiple cells in a next generation of secreted MVs. These results broaden our general understanding of MV-dependent horizontal transfer of biologically relevant information, with implications for anti-tumor effects upon local or systemic blocking of MV transfer.
Concluding remarks and future perspective

In this thesis, the role of polyamines and MVs in tumor biology has been investigated. Below follows a brief summary of the main conclusions and the major implications for therapeutic intervention of cancer.

Enhanced sensitivity of polyamine biosynthesis inhibition by concomitant targeting of polyamine uptake or tumor angiogenesis

Antibody-based therapy is a rapidly growing area in clinical oncology. Examples of some successful targeted therapies of cancer, besides from bevacizumab, are HER-2 and EGFR antibodies. Specific targeting of tumor cells enables interference with a greater efficiency, and perhaps more importantly less side-effects, compared to conventional chemotherapy. As mentioned in the introduction, the expectations of DFMO since its discovery have not been fulfilled in the clinic. Upregulation of polyamine uptake was an unexpected outcome of biosynthesis inhibition using DFMO. Thus, in paper I, we hypothesized that cell-surface HSPG may be a feasible target for antibody-mediated polyamine uptake inhibition. We conclude that treating cells with the anti-HS antibody RB4EA12 reduced proliferation dependent on extracellular polyamines. This effect was cytostatic rather than cytotoxic at the concentrations used, suggesting that it would possibly be more preventative. HSPGs are involved in numerous biological processes such as binding growth factors and cytokines, and further studies need to evaluate the anti-tumor effects as well as the potential adverse affects using these antibodies in vivo. However, a part of the enhanced polyamine uptake in response to DFMO was reversible with RB4EA12, which suggests that the interaction of HSPGs and polyamines is epitope specific. Based on previous publications demonstrating significant inhibiting effects in vivo by combined biosynthesis and uptake inhibition in human bladder carcinoma and in the K6/ODC mouse model of squamous cell carcinoma, antibody-mediated targeting of the uptake pathways of polyamines may be an interesting approach to increase sensitivity of DFMO in cancer treatment.

Another mode of enhancing sensitivity of DFMO treatment is by the induction of hypoxia. In paper II, we addressed the fact that elevated levels of polyamines are found in tumors, and that hypoxic areas are regularly found in solid tumors. We demonstrated that inhibition of polyamine synthesis sensitizes cells to hypoxia-induced cell death in vitro, indicating that polyamines have an adaptive and protective role during hypoxia. Furthermore, our results suggest that the hypoxic effects were directly related to the supply of exogenous
polyamines, as cell death induced by hypoxia in polyamine-depleted cells could be reversed by the addition of exogenous polyamines.

Tumor hypoxia regularly leads to inefficient treatment response due to enhanced drug resistance and low drug bioavailability in the tumor. It is still a debate whether anti-angiogenic treatment increases hypoxia and/or promotes normalization of the vasculature. Normalization would be of benefit for the access of drugs and radiation efficacy, and in many cases clinical symptoms are tempered by anti-angiogenic therapy. However, in accordance with a study using an orthotopic GBM model, we could in paper II conclude that anti-angiogenic treatment provokes hypoxia, as tumors treated with bevacizumab displayed substantial hypoxic induction as compared to corresponding control tumors. A treatment strategy that specifically targets the adaptive mechanisms of tumor cells to hypoxia is becoming more and more vital. Accordingly, combined treatment with DFMO and bevacizumab in vivo strongly augmented the effect as compared to each treatment alone. Further studies are warranted to investigate the different modes of adaptive responses in clinical settings of GBM, and the possibility of using the combination of DFMO and bevacizumab is an interesting alternative. On the basis of the above mentioned results, together with the inhibition of the transport system, one could speculate that combined targeting of polyamine biosynthesis using DFMO together with uptake inhibition using HS-specific antibodies (paper I) in cells sensitized by anti-angiogenic treatment (paper II) would prove more efficient.

Conclusively, these data emphasize the need for continued development of specific inhibitors and antibodies and reinforce the notion that targeting of the polyamine system is a strategy of multiple levels.

Hypoxia-induced responses in glioblastoma – role of the polyamine system and protease activated receptor-2

The highly necrotic and hypoxic manifestations of GBM tumors substantiate the fact that hypoxia shapes the environmental situation in the tumor. Current data support the concept that hypoxia can drive genetic instability and the acquisition of mutations. A drawback with present models as a basis for the design of new therapies is the failure to distinguish between the different biological effects of acute versus chronic hypoxia, and the complex fluctuating "cyclic" hypoxic microenvironment resulting in reoxygenation. The different systems are likely to induce diverse direct or indirect effects on biological processes such as transcription and translation of proteins in vitro compared to patient tumors. However, difficulties in estimating the hypoxic conditions in the clinical situation obviously hamper the setup of hypoxic studies in vitro. In
our studies, we used constant hypoxic treatment without reoxygenation, and define acute hypoxia up to approx. 8 h, and prolonged, chronic hypoxia between 24-48 h. We found that ODC in cancer cells and PAR-2 in ECs was upregulated by acute hypoxia in paper II and paper III, respectively. The transient upregulation of ODC in cancer cells (paper II) and PAR-2 in HUVEC, HUAEC and HBMEC ECs (paper III) point to a potential role of HIFs, in particular HIF1α. ODC mRNA levels were however not found to be upregulated by hypoxia. In the case of PAR-2, upregulation was found on mRNA and protein level, and HIF-stabilizing agents mimicking the hypoxic effects did in fact suggest an effect of HIFs both in paper II and paper III. However, a direct transcriptional effect of HIF1α on the expression of ODC, AZ, AZI or PAR-2 was not the case, which does not exclude that hypoxia indirectly mediates HIF-dependent changes leading to upregulation of these proteins. Further studies clearly will have to determine the mechanisms underlying hypoxic upregulation of ODC and PAR-2.

During hypoxia, general cap-dependent protein translation initiation is suppressed in favor for processes regulating survival and stress responses, meaning that regulation of individual genes may be highly variable. As mentioned in the introduction, cap-independent translation is facilitated by internal ribosome entry sites (IRES) in the mRNA of ODC during G2/M cell cycle phase, but also during hypoxia. This mechanism of action indicates that ODC possesses important functions during cellular stress. Moreover, we found that the mRNA levels of AZ and AZI were unaffected by hypoxia, which does not preclude posttranslational upregulation of AZI or downregulation of AZ. By RNA interference experiments in paper II, we found AZI to be involved in regulating polyamine homeostasis in hypoxic cells. With the data presented, it may be speculated that levels of AZI and AZ would be increased or decreased, respectively in response to hypoxic stress. We were unfortunately not able to investigate protein levels of AZ and AZI due to the lack of well-functioning antibodies.

We have studied the effect of polyamine depletion in cancer cells, but are cancer cells the primary targets of DFMO treatment in vivo? One could argue that hypoxia-mediated responses most likely also would affect the tumor vasculature, based on the fact that ODC-overexpressing cells form highly vascularized tumors in mice85. Indeed, treatment with DFMO inhibited tumor growth and metastasis by inducing apoptosis and reducing the angiogenic potential in a human gastric cancer model392. Moreover, reduced vessel density was seen in ODC/Ras mice upon DFMO treatment86. In contrary to these results, recent studies point toward that polyamines function as modulators of EC survival during hypoxic stress393, as polyamine depletion resulted in
increased survival during hypoxic stress, which may suggest that hypoxic tumors containing large amounts of polyamines would cause apoptosis in the surrounding ECs. However, other tumor microenvironmental factors may influence the effect of hypoxia on tumor ECs upon polyamine depletion, as polyamines can either promote or prevent apoptosis depending on the conditions. In the light of these findings, in paper II, we found that DFMO treatment made tumors more susceptible to anti-angiogenic therapy due to aggravated hypoxia. The in vitro data directly suggest that these hypoxic effects were related to the levels of polyamines. Increased levels of polyamines are found in serum, urine and cerebrospinal fluid (CSF) in cancer patients. Already thirty years ago, polyamines were found in CSF of patients with astrocytoma and GBM; however, CSF polyamine levels were found not to be an indicator of tumor progression in GBM, as reported for medulloblastoma where polyamines were found to have the greatest potential utility for diagnosis. In addition, more recent reports show that although ODC activity and putrescine levels correlate to malignancy in brain tumors, low activity does not certify that the tumor is benign. In a study from 2001, ODC activity was suggested to represent a biochemical marker of malignancy in brain tumors, and future studies will evaluate the polyamine system as prognostic factors. Trials using DFMO as mono or combination therapy is challenged by the difficulties to predict treatment response. However, more recently, Levin et al. proposed a method for the quantification of ODC protein levels in formalin-fixed tissues to identify patients likely to respond to DFMO treatment.

The hypoxia-induced responses in GBM demonstrated in paper III, argues for significant functions in hypoxia-driven EC activation through PAR-2 signaling. The functional effects are specific for PAR-2 as PAR-1 expression was unaffected by hypoxia in ECs and the specific agonist peptide for PAR-1 had no additional effect on migration and proliferation of ECs as compared to control. In addition, there was a correlation between high expression of PAR-2 in CD31 and αVβ3-positive activated pathological vessels in GBM, whereas there was no such correlation in low grade astrocytoma, suggesting that PAR-2 expression is indeed induced in activated tumor endothelium. These data are in accordance with previous findings, demonstrating that PAR-2 expression co-associates with neovasculature in a mouse model of developmental angiogenesis. Furthermore, we show that PAR-2 in this environment acts through a pathway involving p-ERK1/2 mediated induction of the EGFR ligand HB-EGF. In vivo functions of HB-EGF are largely undefined, but recent findings suggest that HB-EGF is involved in tumorigenic signaling. The mechanism of HB-EGF upregulation through PAR-2 was not fully elucidated; however we found the transcription factor CREB to be upregulated as a response to PAR-2
stimulation. In analogy to our data, Versteeg et al. found that TF-VIIa signaling results in CREB upregulation via PAR-2\textsuperscript{401}. It is conceivable that CREB induction could lead to the transcription of HB-EGF based on the fact that CREB has a putative binding site in the promoter of HB-EGF; however, no such analyses were performed based on the preliminary finding that knockdown of CREB had no effect on HB-EGF upregulation upon PAR-2 stimulation (unpublished data). In accordance to the above data, Camerer et al. showed that TF-dependent signaling in keratinocytes induced the expression of HB-EGF mRNA\textsuperscript{402}. The receptor of HB-EGF, EGFR, is as earlier discussed, frequently mutated in GBM, resulting in an autocrine loop contributing to cell autonomy. In a gene expression analysis on GBM expressing the mutated form of the receptor, EGFRvIII, it was revealed that this receptor induces the expression of HB-EGF that can signal through the functional, non-mutated receptor\textsuperscript{403}. In addition, the histological presence of EGFRvIII correlates to HB-EGF expression, providing insights into the development of autocrine loops and sustainable signalling in GBM. These findings, together with the fact that antibody-mediated neutralization of HB-EGF has shown inhibitory effects in ECs (paper III) and GBM cells\textsuperscript{403} should stimulate studies on targeting of HB-EGF in GBM.

GBM cell-derived MVs mediate TF dependent pro-angiogenic signaling through PAR-2

A communication route based on MVs may constitute a multipurpose system to influence malignant and stromal cells in the surrounding microenvironment during tumor development. In paper III, we show that hypoxic cancer cells release TF/VIIa-bearing MVs that trigger upregulated PAR-2 on hypoxic vascular ECs, resulting in increased levels of the pro-angiogenic growth factor HB-EGF, thus providing new support for the link between coagulation, hypoxia and angiogenesis in GBM during tumor development. We thus present for the first time that MVs are implicated in non-coagulative TF-dependent signaling processes in ECs, and that GBM cell-derived MVs may constitute an important signaling mechanism in hypoxia-driven modulation of non-malignant cells residing in the tumor. In addition to the functional TF and VIIa detected in MVs, soluble VIIa is expected to exist at nM concentrations in the tumor microenvironment. The fact that exogenous VIIa potentiated the effect of MVs on p-ERK induction (paper III), speaks in favor of that the induction of p-ERK is TF-dependent and that the effects of MVs in vivo may be even greater than those shown in vitro.

The mechanisms of MVs release are currently not known, but GBM cells are as discussed earlier, releasing substantial amounts of MVs. Interestingly, loss of
p53 is frequent in GBM patients\textsuperscript{25,32}, and loss of p53 in mouse models results in increased release of TF-bearing exosomes, suggesting that p53 may be involved in this process\textsuperscript{404}. These results are supported by data demonstrating that senescence as a consequence of DNA damage may induce a p53-dependent increase in the biogenesis of exosome-like vesicles\textsuperscript{405}. Thus, regulation of vesicle release by p53 may be one of the factors contributing to the high levels of shed, TF-bearing MVs from GBM tumors.

Except from HB-EGF, other proteins have been shown to respond to TF-VIIa signaling, \textit{e.g.} IL-1\textbeta, IL-8 and FGF-5\textsuperscript{402}. Although not proven at this point, cytokines such as IL-1, IL-23, GM-CSF, IL-8, are examples of proteins induced by MVs\textsuperscript{372}, and some of these effects may be induced through the TF pathway. Blocking of this pathway may influence not only the pro-angiogenic signaling, but also affect the pro-inflammatory response known to affect tumor development. Additionally, it would be highly interesting to explore the effects of TF-VIIa bearing MVs on GBM development in an orthotopic model to test the hypothesis in a more relevant microenvironmental context, or even more exiting, in the context of PAR-2 deficiency of the host.

Emerging data point towards the idea that TF-enriched MVs secreted from platelets\textsuperscript{357,360,361} and activated ECs\textsuperscript{406} are the underlying mechanism of the high incidence of thromboembolism in cancer patients. TF-bearing MPs have thus been suggested to be implicated as a predictive biomarker of VTE. Indeed, in paper III, we found that plasma levels of TF correlated with tumor mass in a GBM xenograft mouse model, indicating that TF released by GBM cells escapes into the circulation. Another possible, although speculative, event might be the contribution of TF-enriched MVs secreted from hypoxic cancer cells, which conceivably may provide a non-invasive marker that reflects a more hypoxic and aggressive tumor as well as treatment prediction by the measurement of circulating, MV-associated TF in plasma in cancer patients. Treatment responses following anti-angiogenic treatment is hampered by the lack of markers for the prediction of tumor response. The presence of cancer cell derived, TF-bearing EMVs in blood lend support to the idea of using TF as a predictive marker of tumor progression or treatment response.
Transfer and signaling of GBM cell-derived MVs

Genetic and epigenetic changes in the multistep process of tumor progression have in recent times become more and more multifaceted. The transfer of MVs between cells adds further complexity to this process. Transfer of EGFRvIII has been shown to be incorporated into cells lacking the mutated form of the receptor, thus contributing to the horizontal transfer of oncogene products[^320]. An interesting idea for future exploration is the transfer of functional TF from cancer cell derived MVs into ECs that normally lack TF. In that case, PAR-2 would be independent of external, TF-inducing stimuli for the support of tumor angiogenesis.

In paper III, we found MVs to activate PAR-2 and further to be co-internalized into the same compartment. Based on the above findings, we next set out to clarify the mechanism of uptake of MVs in paper IV, and found that MV uptake occurs through endocytosis, and identified explicit signaling pathways implicated in and essential for the uptake process. As previously discussed, the mechanism of EMV uptake is controversial, and data provided so far are contradictory. On the basis of electron microscopy data, silencing experiments and drug inhibition of endocytosis pathways, we hereby conclude that MV uptake involves lipid-raft mediated endocytosis and not membrane fusion as discussed earlier. The fact that MVs were further sorted to CD63-positive compartments lends further support to this mechanism. The described pathway has similarities with the recently described CLIC-GEEC endocytic pathway characterized by clathrin, caveolin and dynamin-independence and Cx3B co-localization. Ongoing studies will evaluate the impact of the small GTPase Cdc42 in the endocytosis of MVs. In analogy with the identified uptake pathway, MVs and exosomes are shown to be arising from lipid raft domains[^341] and being dependent on cholesterol[^313]. Despite the fact that CD63 is very abundant in late endosomes/lysosomes, the knockout mouse of CD63 does not display lysosomal abnormalities, probably due to high redundancy between family proteins of tetraspanins[^407]. Although CD63 is enriched in TEMs in the plasma membrane, we did not find CD63 to be involved in the uptake of MVs (paper IV). The complex localization pattern and the high abundance of CD63 suggest an important function in intracellular trafficking; however, the functions of tetraspanins in general are not fully understood and future investigations will hopefully elucidate the role of CD63 in exosome biogenesis.

The reduction in HB-EGF mRNA and protein levels, as well as the reversed cell proliferation of PAR-2 stimulation by the MEK inhibitor U0126 in paper III, suggest that p-ERK1/2 is involved in this signalling cascade leading to the
phenotypic effects. In this paper we did not study if the uptake of MVs were unchanged in the presence of U0126, but as discovered in paper IV, PI3K and MEK inhibitors most probably also affect the uptake of EMVs. On the basis of the data presented in paper IV, one could speculate that the endocytosis of MVs is required for signalling through p-ERK1/2, and subsequent blocking of uptake will abolish the functional effects. For example, mesenchymal derived stem cell exosomes were found to support tumor growth in vivo, mainly promoted by p-ERK1/2 dependent induction of VEGF and CXCR4. In blocking experiments using U0126 in the same study, the reversed functional effects may not only be dependent on downstream effects of ERK phosphorylation, but also on p-ERK1/2 for efficient internalization. Thus, therapeutic strategies targeting key signalling systems in cancer cells, e.g. MEK or PI3K inhibitors would presumably also affect the capability of cells to secrete and internalize EMVs.

Additionally, we present preliminary data indicating that MVs are sorted to MVB compartments of recipient cells as shown by the co-localization of MVs with the MVB markers CD63 and tsg101 on confocal and electron microscopy, respectively. Although full mechanistic data was not provided in this paper, it clearly displays the possibility that MV cargo may be transferred between multiple cells.

Conclusively, these data emphasize new intriguing roles of MVs in tumor angiogenesis, and reveal a possible mechanism of uptake. Further analyses will hopefully disclose additional components involved and advance our understanding of this potentially key pathway involved in tumor development and progression.
Populärvetenskaplig sammanfattning


Hindra tumörcellernas räddningsmekanism

Vanliga cancerläkemedel orsakar flera och svåra biverkningar på grund av deras påverkan på alla snabbt delande celler utan att särskilja mellan normala och canceromvandlade celler. Vid snabb tillväxt i en fast vävnad uppstår syrebrist, en mycket ogästvänlig miljö för de flesta celler. Men cancerceller har olika räddningsmekanismer för att undkomma undergång när de utsätts för denna typ av stress. Räddningsmekanismer som bland annat genom att utsöndra faktorer som främjar blodkärlsinväxt, minskar celldöden, ökar överlevnaden och hindrar immunförsvaret från att ingripa. Därtill har vi funnit att tumörceller genom att öka tillverkningen av polyaminer; små, livsnödvändiga molekyler - sedan tidigare kända för att vara förhöjda i olika tumörformer - kan överleva när de utsätts för stress i form av syrebrist. Att öka mängden polyaminer genom en ökning av både tillverkning innuti och upptag utifrån visade sig vara ett sätt för cellerna att anpassa sig till den ogästvänliga tumörmiljön. Genom att tumörbärande möss behandlades med en kombination av två redan etablerade cancerläkemedel; ett som motverkar polyamintillverkning och ett som hindrar blodkärlstillväxt som en strategi att göra cancercellerna mer känsliga för syrebrist, har vi kunnat visa på väsentligt minskad tumörtlöften, och i synnerhet att tumörerna innehöll synnerligen fler döda celler på grund av minskad kärlinväxt. Att kunna stänga av tumörcellernas räddningsmekanism belyser möjligheten för förbättringar av den effekt vi ser idag av nuvarande läkemedel för blodkärlsstrypning.
Kommunikation främjar tumörtillväxt


Sammanfattningsvis belyser min avhandling potentialen med att använda en av cancercellers viktiga räddningsmekanismer och deras förmåga att kommunicera med omgivningen som tänkbara angreppspunkter för utvecklingen och användningen av nya eller redan etablerade cancerläkemedel i behandlingen av cancer.
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