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Mechanistic studies on the role of extracellular vesicles in the tumor microenvironment

Christianson, Helena

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Abstract

The transition from normal into malignant cells with acquired uncontrolled growth is a process where cancer cells viciously develop adaptive strategies to overcome microenvironmental and metabolic stress. Hypoxia, or deficient oxygen supply, is a common feature of the expanding tumor which alters cell metabolism and consecutively causes lowered intracellular pH. To alleviate hypoxic and acidotic stress, cancer cells respond by promoting blood vessel formation, angiogenesis, and by activating mechanisms for increased acid-base control. These adaptive processes involve complex modes of cell communication in the tumor microenvironment.

The aim of this thesis was to investigate the role of extracellular vesicles (EVs) in the hypoxic tumor microenvironment and the mechanisms by which EVs mediate cell-cell communication as well as regulatory aspects of hypoxia-induced acidosis. In the first part (papers I and II), we provide new insights into the mechanisms of EV transfer between cells. It is demonstrated that a significant proportion of cancer cell-derived EVs is transferred into recipient cells by binding to the glycan chains of the cell surface receptors, heparan sulfate proteoglycans (PGs). We further show that EVs are internalized through a lipid raft-mediated endocytosis pathway negatively regulated by caveolin-1. In the second part (papers III and IV), we show that EVs originating from hypoxic cancer cells exhibit a specific molecular profile and induce a more pronounced pro-angiogenic response in endothelial recipient cells and in an *in vivo* tumor model as compared to normoxia-derived EVs. EVs may thus serve as biomarkers wherein tumor specific conditions, such as hypoxia, are reflected. Finally, we found that EVs may constitute a functionally active route for the release of the major pH regulatory enzyme, carbonic anhydrase IX (CAIX). Molecular studies revealed that CAIX in cells and in EVs can be substituted with specific glycosaminoglycan chains, defining CAIX as a part-time PG.

These findings advance our understanding of the role of EV-dependent signaling in the tumor microenvironment. Data presented in this thesis identify molecular mechanisms by which EVs mediate cell-cell communication that constitute potential targets for therapeutic interventions.

Key words Extracellular vesicle, exosome, microvesicle, glioblastoma, hypoxia, angiogenesis, acidosis, proteoglycan, endocytosis, carbonic anhydrase IX, biomarker.

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Cover

Image illustrating the cycle of compartmentalized cell-cell communication. Intracellular compartments (yellow) serve as receivers to which internalized extracellular vesicles (blue) home after their journey through the extracellular space.

Confocal microscopy picture taken by Dr. Katrin Svensson

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Till Erik

"The future is already here, it's just not evenly distributed"
William Gibson

Abstract

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Table of contents

List of papers

This thesis is based on the following original papers and manuscript, referred to in the text by their roman numerals indicated below:

I. Svensson KJ, **Christianson HC**, Wittrup A, Bourseau-Guilmain E, Lindqvist E, Svensson LM, Mörgelin M, Belting M. Exosome uptake depends on ERK1/2-heat shock protein 27 signalling and lipid raft-mediated endocytosis negatively regulated by caveolin-1. *J Biol Chem.* 2013 288:17713-17724.

II. **Christianson HC**, Svensson KJ, van Kuppevelt TH, Li JP, Belting M. Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc Natl Acad Sci U S A.* 2013 110:17380-17385.

III. Kucharzewska P, **Christianson HC**, Welch JE, Svensson KJ, Fredlund E, Ringnér M, Mörgelin M, Bourseau-Guilmain E, Bengzon J, Belting M. Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. *Proc Natl Acad Sci U S A.* 2013 110:7312-7317.

IV. **Christianson HC**, Lidfeldt J, Shevela D, Lindqvist E, Messinger J, Belting M. Evidence of Carbonic Anhydrase IX as a hypoxia regulated part-time proteoglycan sorted to exosomes: Novel modes of structural and spatial regulation. *Manuscript.*

List of papers not included in this thesis

- Kucharzewska P, **Christianson HC**, Belting M. Global profiling of metabolic adaptation to hypoxic stress in human glioblastoma cells. *Under revision.*

- **Christianson HC**, Belting M. Heparan sulfate proteoglycan as a cell-surface endocytosis receptor. *Matrix Biol.* 2014 35:51-55. *Review.*

- **Christianson HC**, Svensson KJ, Belting M. Exosome and microvesicle mediated phene transfer in mammalian cells. *Semin Cancer Biol.* 2014 28:31-38. *Review.*

- **Christianson HC**, van Kuppevelt TH, Belting M. ScFv anti-heparan sulfate antibodies unexpectedly activate endothelial and cancer cells through p38 MAPK: implications for antibody-based targeting of heparan sulfate proteoglycans in cancer. *PLoS One.* 2012:e49092.

- Svensson KJ, **Christianson HC**, Kucharzewska P, Fagerström V, Lundstedt L, Borgquist S, Jirström K, Belting M. Chondroitin sulfate expression predicts poor outcome in breast cancer. *Int J Oncol.* 2011 39:1421-1428.

- Svensson KJ, Kucharzewska P, **Christianson HC**, Sköld S, Löfstedt T, Johansson MC, Mörgelin M, Bengzon J, Ruf W, Belting M. Hypoxia triggers a proangiogenic pathway involving cancer cell microvesicles and PAR-2-mediated heparin-binding EGF signaling in endothelial cells. *Proc Natl Acad Sci U S A.* 2011 108:13147-13152.

Abbreviations

CAIX	Carbonic anhydrase IX
CAV-1	Caveolin-1
CME	Clathrin-mediated endocytosis
CS	Chondroitin sulfate
DS	Dermatan sulfate
DC	Dendritic cell
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinase
EVs	Extracellular vesicles
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GalNac	N-acetylgalactosamine
GBM	Glioblastoma multiforme
GlcA	Glucuronic acid
GlcNac	N-acetylglucosamine
HGF	Hepatocyte growth factor
HIFs	Hypoxia-inducible factors
IdoA	Iduronic acid
LMWHs	Low molecular weight heparins
MMP	Matrix metalloproteinase
MVBs	Multivesicular bodies
PAR-2	Protease-activated receptor-2
PG	Proteoglycan
PS	Phosphatidylserine
SULF	Sulfatase
TCA	Tricarboxylic acid
TF	Tissue factor
TGF-β	Transforming growth factor
VEGF	Vascular endothelial growth factor
VTE	Venous thromboembolism

Introduction to the tumor microenvironment

Cancer development is the consequence of damages to the DNA, the blueprint that defines and controls life. Sequential accumulation of unrepaired changes to the nucleotide sequence caused by defects in cell cycle checkpoint controls essentially lead to tumor transformation and genomic instability [1]. These alterations enable cancer cells to acquire capabilities different from normal cells, most importantly, uncontrolled cell division and malfunctioning DNA repair. The model of clonal evolution proposed by Nowell in 1976 suggests that cancer development occurs through accumulation of mutations that confer cellular growth and/or survival advantages [2]. Hence, favorable genetic aberrations that promote *e.g.* resistance to growth inhibition and cell death will drive the selection of cancer cells with aggressive phenotypes and give rise to tumor heterogeneity.

Cancer cells evolve several ways to deregulate the normally tightly controlled system of growth signals transmitted through transmembrane receptors. To this end, cancer cells circumvent external control mechanisms and achieve sustained proliferation by overexpressing growth signals and cognate receptors or by inducing constitutive signaling downstream of receptor activation. Amplification or overexpression of the human epidermal growth factor receptor 2 (HER2) oncogene is for example found in 20-30% of breast tumors, and is associated with a more aggressive disease and increased mortality if not properly targeted by trastuzumab or equivalent treatments [3].

Uncontrolled cell division may also be fueled by inactivation of proteins that negatively regulate cell proliferation. Retinoblastoma protein [4] and TP53 [5], *i.e.* tumor suppressor proteins that normally act as gatekeepers to prevent damaged or stressed cells to progress through the cell-cycle, are frequently mutated in cancer. Abnormal cell growth is normally limited by the action of programmed cell death, apoptosis, by which defective cells are removed. Cell stress, such as excessive genomic damage, inadequate oxygen or energy levels and hyperactive oncoprotein signaling triggers the apoptotic program through the activation of a cascade of proteolytic caspases. This process of controlled self-digestion is regulated by pro- and anti-apoptotic members of the Bcl-2 protein family. However, cancer cells may avoid apoptosis by increasing the expression of anti-apoptotic regulators and survival signals or by downregulating pro-apoptotic regulators.

Despite these intrinsic cellular capabilities that promote tumor growth, the transition from hyper-proliferating cancer *in situ* to a lethal, metastatic disease will not occur without the assistance of a supportive micro and macroenvironment of the host. Tumor progression from invasion of the surrounding tissue followed by intravasation into a blood or lymphatic vessel and finally extravasation at a distant site indeed require functional interactions between cancer cells and their surrounding microenvironment. In addition to transformed cancer cells, the tumor is composed of a variety of non-malignant cells as well as extracellular matrix (ECM) components. Tumor resident cells include *e.g.* fibroblasts, endothelial cells, pericytes, mesenchymal stem cells and immune cells that together influence various processes of tumorigenesis [6]. Cancer cells utilize persuasive cell communication to co-ordinate these cells to promote their own growth and survival, including direct cell-cell contact and secretion of soluble factors such as growth factors, cytokines and extracellular vesicles (EVs).

Oncogenic stimuli have been shown to activate and convert tumor infiltrating fibroblasts into so called cancer-associated fibroblasts. This kind of priming of cancer-associated fibroblasts exemplifies the vicious modes of action by which cancer cells use accessory cells to set the stage for a beneficial microenvironment [7]. The normal functions of fibroblasts, *e.g.* stroma remodulation under wound healing and tissue regeneration, are reprogrammed to favor the generation of tumor stroma that facilitates invasion [8], and blood vessel formation [9], and to induce immunosuppression [10].

Another important example of a network of cells that becomes hijacked in the tumor microenvironment is the immune system. Cancer cells undertake various mechanisms to evade immune recognition and suppress immune reactivity [11]. For example, human tumor T regulatory cells have been shown to suppress tumor-specific T cell immunity which contributes to enhanced tumor growth and reduced survival [12]. Further, several tumor-derived factors may mediate functional impairments of tumor resident dendritic cells (DC) that fail to prime T cells to the same degree as under physiological conditions [13, 14]. Paradoxically, it seems that immunosuppressive cues can occur in parallel with inflammation-induced carcinogenesis. Oncogenic signaling such as aberrant activation of the protein tyrosine kinase, RET [15], and the RAS-RAF-MAPK pathway [16] has been shown to foster a pro-inflammatory environment in which elevated levels of inflammatory cytokines promote angiogenesis, speed up the cell cycle and prevent cell death, all of which augment tumor growth [15-17]. Drugs that target cancer-related inflammation [18, 19] and immunotherapies aiming to prime the adaptive immune system are currently under intense investigation for anticancer therapies [20].

These examples illustrate the critical cellular interplay that takes place within the tumor microenvironment. It is thus increasingly recognized that tumor associated non-malignant cells and conditions arising as consequences of rapid cancer cell proliferation shape the characteristics of the tumor milieu. These features play critical

roles in malignant progression and influence the response of conventional anti-tumor therapies. Characteristics of the tumor microenvironment that are of particular importance to this thesis are next discussed in further detail.

Tumor hypoxia

Sufficient oxygen levels are fundamental for normal cellular metabolism and energy homeostasis in mammalian organisms. However, the chaotic environment of solid tumors is associated with areas of hypoxia, *i.e.* insufficient oxygen supply. Hypoxia first arises as a result of the rapidly expanding tumor tissue wherein the existing vasculature cannot meet the increasing need for oxygen. Consequently, cells located further from the maximum oxygen diffusion distance of the nearest capillary can encounter significantly lower oxygen tension with levels reaching below 2.5 mmHg [21]. In addition, tumor-associated thrombotic events frequently cause temporary vascular occlusion and subsequent acute hypoxia, characterized by transient episodes of hypoxia followed by reoxygenation. Cancer cells located in areas of low oxygen tension have to co-opt adaptive mechanisms to survive the hypoxic stress. Hypoxia-induced adaptions include a switch to oxygen-independent metabolism, increased angiogenesis, up-regulation of pH regulatory mechanisms to manage intracellular acidification, as well as increased migration and invasion to escape the hypoxic areas. Adaptive mechanisms in the tumor microenvironment and their pathological consequences are outlined in **Figure 1**.

Hypoxia inducible factors (HIFs) form a family of transcription factors that play essential roles in the maintenance of oxygen homeostasis [22, 23]. The HIFs have been shown to drive a wide variety of adaptive responses at reduced oxygen levels in tumorigenesis, but also in tissue ischaemia in general [24, 25]. HIFs are composed of two subunits, HIF-α and HIF-β. The HIF-β subunit is regarded as insensitive to changes in oxygen levels and is constitutively expressed, whereas HIF-α is acutely up-regulated in hypoxia by the inactivation of two types of oxygen sensors; prolyl hydroxylase (PHD) enzymes and factor inhibiting HIF-1 (FIH). In the presence of oxygen, PDH catalyzes hydroxylation of HIF-α that enables polyubiquitylation mediated by the von Hippel–Lindau and E3 ubiquitin complex [26] and, in turn, targets HIF-α for proteasome degradation. FIH-mediated hydroxylation, on the other hand, blocks the interaction between HIF-α and the transcriptional activator p300/CBP [27]. However, at hypoxic conditions, PHDs and FIH are inactive because of lack of available oxygen. Consequently, HIF-α is stabilized and translocated to the nucleus where it heterodimerizes with HIF-β. In the nucleus, HIF heterodimers form complexes with additional cofactors and bind to the hypoxia-response elements in the promoter and/or enhancer regions of hypoxia-responsive

genes enhancing the transcription of proteins that are crucial in mediating the hypoxic stress response [28].

There is ample evidence that hypoxia and subsequent pathophysiological consequences thereof are closely linked to poor cancer prognosis; hypoxic adaption may serve as one of the most important microenvironmental determinants for tumor cell dissemination and metastasis [29, 30]. Invasion and metastasis are the most common causes of cancer-related morbidity and mortality. Although the underlying mechanisms of hypoxia-induced metastatic potential are not entirely understood, it may be linked to HIF associated induction of epithelial to mesenchymal transition (EMT) [31]. The process of EMT is associated with loss of cadherins and adhesive junctions and cytoskeletal reorganization resulting in the transition from adhesive cells to non-polarized mesenchymal-like cells with a mobile and invasive phenotype [32, 33]. Hypoxia-induced secretion of the collagen crosslinking enzyme lysyl oxidase is suggested to regulate the invasive properties of hypoxic cancer cells through focal adhesion kinase activity and cell-matrix adhesion, thereby providing another mechanism that facilitates metastasis [34]. Further, hypoxia influences the capacity of cancer cells to promote extracellular matrix remodeling and basement membrane digestion by increasing the expression of for example matrix metalloproteinases (MMPs) and heparanase [35-37].

The clinical relevance of tumor hypoxia is also linked to therapy resistance. The most obvious reason for resistance in the hypoxic tumor microenvironment is inadequate drug distribution as a consequence of distant or perturbed vasculature. Also, the less proliferative nature of hypoxic cells protects them from the mode of action of most anti-proliferative chemotherapies. Further, the multi-drug resistance protein, p-glycoprotein, is a HIF target [38] that has been shown to reduce the brain tumor penetration of for example erlotinib [39]. Tumor hypoxia also represents a major obstacle for radiotherapy mainly due to lack of oxygen-generated free radical species, which are responsible for radiation induced DNA damage.

Importantly, the recognition of hypoxia as a negative prognostic factor of survival and response to treatment has urged initiatives that exploit tumor cell adaptive responses to hypoxia in the development of new cancer therapies [40]. Also, hypoxia-induced molecular signatures may provide information that could be valuable in directing appropriate therapies; this concept is explored in paper **III**.

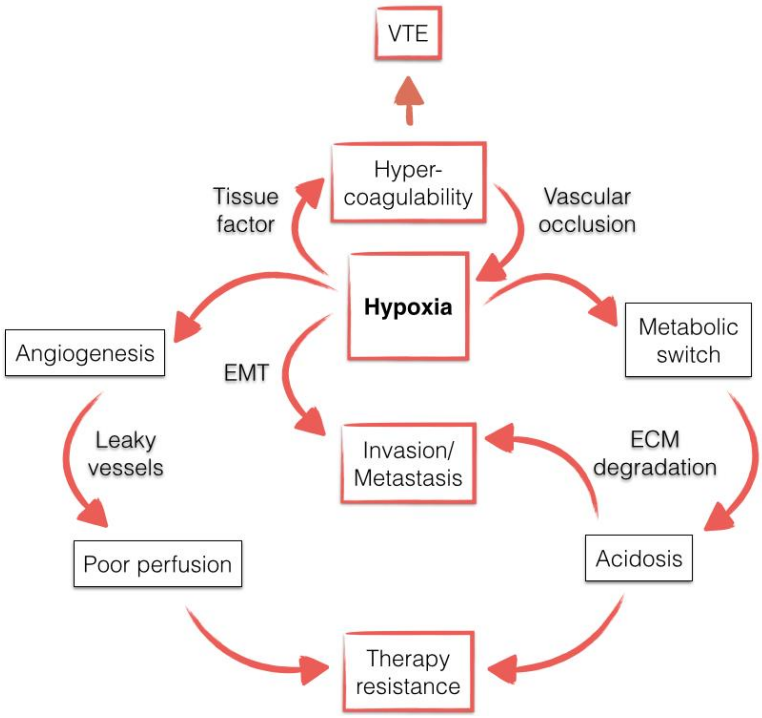

Figure 1. Vicious cycles of hypoxia-induced tumor progression. Hypoxia is a key driver in tumor development which is associated with several pathophysiological features. Cancer cells secrete pro-angiogenic growth factors to create a malfunctioning vasculature characterized by dilated and leaky vessels that give rise to increased tumor interstitial fluid pressure and edema. These abnormalities lead to poor tumor drug delivery and impaired efficacy of chemotherapy and radiotherapy. Hypoxic cancer cells undergo a metabolic switch towards increased glycolysis, which results in acidification of the tumor microenvironment due to extensive production of lactic acid and carbon dioxide in combination with inadequate metabolite clearance. Tumor acidosis aggravates therapy resistance due to reduced drug efficacy at low pH. Hypoxia-induced cellular adaption drives the selection of aggressive and invasive cancer cells, in part by induction of EMT. In addition, tumor acidosis facilitates tumor invasion by ECM remodulation, which allows motile cancer cells to escape the primary site, disseminate and ultimately metastasize. Damaged endothelium, as a consequence of aberrant angiogenesis, and hypercoagulation significantly increase the risk of venous thromboembolism (VTE) in cancer patients. Hypoxia-induced expression of the principal initiator of coagulation, tissue factor, is central to cancer associated thrombotic events, and increased clotting in tumors leads to vascular occlusion and sustained hypoxia. Taken together, tumor hypoxia contributes to cycles of events that viciously fuel tumor development and progression. Abbreviations; EMT, epithelial to mesenchymal transition; ECM, extracellular matrix.

Angiogenesis

Angiogenesis, *i.e.* the formation of new blood vessels from pre-existing vessels, is a hypoxia-induced process which is essential for further tumor progression. The moment at which a tumor begins to overexpress pro-angiogenic factors is generally referred to as the "angiogenic switch" [41]. Following its onset, a complex, multistep program for endothelial cell recruitment and blood vessel formation is triggered to restore tumor perfusion. The initial events of angiogenesis involve stimulation of the endothelial cell proliferation by the action of soluble, pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF). The second step of the angiogenic process, known as sprouting, involves the release of proteases resulting in ECM degradation. The so called endothelial tip cells then escape into the underlying tissue and migrate towards the chemotactic gradient of angiogenic stimuli. These sprouts are accompanied by stalk cells that proliferate to form a vessel lumen [42]. The Notch signaling pathway is essential for vascular development and has been implicated in various aspects of vessel maturation [43, 44]. Transcription of the Notch ligand JAG2 is induced by hypoxia in a HIF-1α dependent manner, and tumor-derived JAG2-mediated Notch activation contributes to the formation of endothelial capillary-like structures [45]. Thus, vessel formation normally involves tightly coordinated migration, proliferation and tube formation, all of which can be studied *in vitro* to assess angiogenic responses. Following the establishment of vascular sprouts, pericytes are recruited to stabilize the maturing vessel [46]. The balance between anti- and pro-angiogenic factors is tipped over towards an excessive pro-angiogenic drive within the hypoxic tumor microenvironment which ultimately leads to the formation of disorganized, highly permeable blood vessels with frequent blind ends, all contributing to insufficient perfusion and sustained hypoxia. In addition to being a supply system for nutrient and oxygen delivery, tumor-associated vasculature also serves as a route for cancer cells to exit into the systemic circulation. Accordingly, angiogenesis represents one of the key events during the transition from localized into disseminated disease and the expression of several pro-angiogenic factors and their cognate receptors has been found to correlate with a poor prognosis in many cancer types [47, 48].

The concept of interfering with blood vessel formation in order to prevent tumor growth was described in the early 1970s [49]. Since then, numerous reports on angiogenesis inhibition have demonstrated compromised tumor growth and many anti-angiogenic agents are currently undergoing clinical development. The first anti-angiogenic molecule that was approved for clinical use was bevacizumab (Avastin), a humanized neutralizing antibody targeting VEGF. Targeting of single pathways has unfortunately been associated with acquired drug resistance [50]. Several studies

reveal changes to the cancer cells or the tumor microenvironment, *e.g.* activation and/or up-regulation of alternative pro-angiogenic signaling pathways, whereby cancer cells acquire mechanisms for evasive resistance [51, 52]. The development of small molecule receptor tyrosine kinase inhibitors targeting several interconnected pro-angiogenic signaling pathways might circumvent drug resistance and result in increased therapeutic benefit. However, the clinical benefits associated with the use of these drugs have, so far, been very limited [53, 54]. In fact, some studies indicate that anti-angiogenic therapy might promote selection of a more aggressive tumor phenotype as a consequence of increased hypoxia [55-57]. Instead, utilizing more modest anti-angiogenic approaches to balance the abundant angiogenic stimuli and thereby induce vessel normalization could potentially improve drug distribution and the effects of radiotherapy [58]. Surely, the outcome of clinical studies and the experience from the use of angiogenesis inhibitors in clinical practice have so far been a disappointment, partly due to lack of predictive and early response biomarkers.

Tumor Acidosis

Well-oxygenated tissues typically use highly efficient aerobic respiration to generate ATP. This includes a set of metabolic reactions and processes where the glycolysis end-product, pyruvate, is oxidized into carbon dioxide and water within the tricarboxylic acid (TCA) cycle while at the same time reducing NAD into NADH. NADH is further utilized to drive the electron transport chain across the mitochondrial membrane wherein most ATP is produced as part of oxidative phosphorylation. The lack of oxygen prevents hypoxic cells from utilizing oxidative phosphorylation; instead, they rely on oxygen-independent glycolysis as a primary mechanism for ATP production. Hence, an important hypoxia-induced adaptive mechanism is the shift from aerobic to anaerobic metabolism. HIF-mediated transcriptional regulation drives the metabolic adaption by inhibiting TCA cycle activity and promoting glycolysis during hypoxia [59, 60]. For example, HIF induces the expression of pyruvate dehydrogenase kinase 1, which inhibits pyruvate dehydrogenase, *i.e.* the enzyme that catalyzes the generation of the TCA cycle substrate, acetyl-CoA. Also, hexokinase 2 which commits glucose to the glycolytic pathway is upregulated. In the absence of oxygen, pyruvate is used as a hydrogen acceptor to regenerate NAD^+ from NADH which is required during glycolysis. This redox reaction converts pyruvate to lactic acid that results in potentially toxic, intracellular acidification to which hypoxic cells must adapt in order to survive.

Reprogramming of cancer cell energy metabolism and subsequent tumor acidosis are acknowledged as cancer hallmarks that support tumor progression [61, 62]. A key player in the regulation of the intracellular acid-base control is the hypoxia-induced cell-surface enzyme, CAIX. CAIX protects tumor cells from acidic stress and high CAIX expression correlates with poor survival in several tumor types [63-65]. CAIX

assists in maintaining the intracellular pH level by catalyzing the reversible hydration of carbon dioxide into bicarbonate and protons at the extracellular side. Bicarbonate is imported across the plasma membrane by bicarbonate transporters where it is converted back to water and CO_2, thus buffering the elevated intracellular levels of protons. Efficient shunting of lactic acid mediated by monocarboxylate transporter and diffusion of CO_2 into the extracellular microenvironment lead to intracellular alkalization at the expense of extracellular acidification. Hence, the combined effect of increased lactic acid production from glucose breakdown and defective vasculature, lead to inadequate metabolite clearance and acidification of the tumor microenvironment [66]. Selected parts of the hypoxia-induced pH regulatory system and the functions of CAIX are presented in **Figure 2.**

Acidosis correlates with induced chromosomal instability [67] that may drive adaptive advantages, and several mechanisms by which acidosis promotes phenotypic changes towards clinically more aggressive tumor characteristics have been proposed. Low extracellular pH may increase the enzymatic activity of proteases that degrade ECM constituents and thus ease cell migration [68]. Although many cancer cells acquire acid-resistant phenotypes that allow them to survive and proliferate, this toxic condition is detrimental to adjacent normal stroma causing concomitant cell death that may provide space for tumor cells to invade [69]. The clinical significance of tumor acidosis is evident by resistance to cancer therapies due to reduced cellular drug uptake, *i.e.* acidosis will result in protonation of weak base drugs that will accumulate in the extracellular space as the plasma membrane is less permeable to charged species [70][71].

Therapeutic approaches involving pH regulated pro-drugs and tumor targeting strategies that interfere with components of the pH regulatory machinery are emerging as promising anti-cancer strategies. CAIX has earned great interest as a biomarker due to its hypoxia-regulated expression and association with tumor progression. Its remarkable catalytic activity with an optimal activity at pH 6.5 [72], a pH value commonly found within the hypoxic tumor tissue, and its cell surface location implicate CAIX as one of the most important proteins involved in tumoral extracellular acidification and makes it suitable for therapeutic targeting. However, development of highly selective inhibitors for CAIX is still a challenge due to the structural similarities between the thirteen known mammalian CA isoforms [73-75]. Further elucidation of the structure and function of CAIX is clearly needed, which is the aim of paper **IV**.

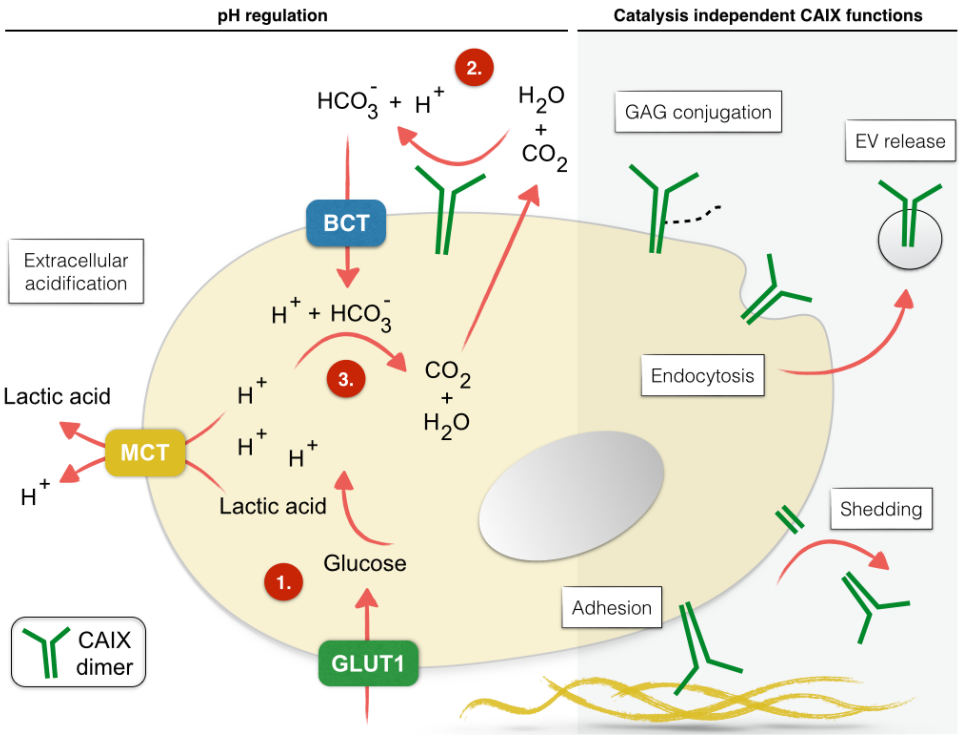

Figure 2. Functions and cellular regulation of Carbonic Anhydrase IX.
(1) Hypoxia-induced GLUT1 expression facilitates glucose uptake which contributes to increased anaerobic glycolysis at insufficient levels of oxygen. This results in elevated lactic acid production. MCT expidites the excretion of lactic acid and protons which leads to extracellular acidification. (2) CAIX catalyzes the hydration of CO_2 into HCO_3^- and a proton after which BCT transports HCO_3^- across the plasma membrane. (3) HCO_3^- import counterbalances the high proton levels at the expence of extracellular acidification as a result of CO_2 diffusion. In addition to its function in the pH regulatory machinery, CAIX is involved in a number of catalysis independent functions and processes (light grey). Cell surface CAIX availability is regulated by endocytosis and proteolytic shedding of the extracellular region. In paper **IV**, CAIX was found to be secreted on extracellular vesicles (EV). CAIX was also shown to be conjugated with glycosaminoglycan (GAG) polysaccharide chains (dotted line), thus defining CAIX as a new part-time proteoglycan. CAIX has been proposed to play a role in cell adhesion, however, the mechanism of this activity is not clear. Abbreviations: BCT, bicarbonate transporter; GLUT1, glucose transporter 1; MCT, monocarboxylate transporter.

Glioblastoma Multiforme

Gliomas are primary brain tumors arising from glial cells and are clinically divided into four grades, according to the WHO, based on histological features. Glioblastoma multiforme (GBM) are tumors classified as grade IV and are the most common and most aggressive type of primary brain tumor in adults. Of note, the grade IV classification is largely determined by factors directly related to hypoxia and acidosis, *i.e.* necrotic areas and endothelial hyperproliferation, which are not found in low grade tumors. Treatment of GBM usually consists of surgical resection, radiotherapy with concomitant chemotherapy (temozolomide) and adjuvant chemotherapy [76]. In spite of intense treatment regimens, the prognosis is poor with a median survival time of approximately 14 months [77]. While most cancer related deaths are caused by metastasis, GBM tumors seldom metastasize but are associated with a highly infiltrative growth pattern and are therefore difficult to eradicate surgically, leading to virtually inevitable recurrence.

As indicated, GBM tumors display a heterogeneous histopathological appearance characterized by the presence of necrotic areas surrounded by hypoxic, pseudopalisading cells and hyperplastic blood vessels [78-80]. Abnormal regulation of the vasculature and hemostasis is central in GBM pathology. Uncontrolled angiogenesis gives rise to microvascular hyperplasia and leaky vessels that result in entry of fluid into the parenchyma and subsequent edema. Hypercoagulability and increased levels of coagulation factors such as tissue factor (TF) and tissue type plasminogen activator are common in GBM tumors [81, 82]. Microvascular thrombotic occlusion occurs in the majority of GBM [83] and this highly procoagulant feature is associated with systemic thromboembolic disease [84]. The risk of venous thromboembolism (VTE) is greatly elevated in GBM patients with a postoperative risk of 7-28% over a 1-year period [84] and results in significant morbidity and mortality. Hemostasis has been suggested to be affected by the secretion of tumor-derived procoagulant components into the circulation, and TF-bearing microparticles, *i.e.* large EVs, may play a significant role in these events [85, 86].

The characteristics of GBM constitute several complicating factors for efficient treatment; many cancer drugs do not pass the blood-brain barrier and the increased interstitial pressure due to vasogenic edema worsens the already poor drug distribution. Due to its extremely vascular nature and elevated levels of VEGF, GBM was an appealing candidate for anti-angiogenic treatment. Bevacizumab is currently approved by the food and drug administration for use as mono-therapy in recurrent GBM but the potential benefit has been a subject of debate. Also, phase III clinical trials have failed to show any significant benefit of adding bevacizumab to standard therapy in the treatment of primary GBM patients.

In addition to the distinct morphological features of GBM, considerable cellular and molecular heterogeneity exists between GBM tumors and even within lesions with cancer cell subpopulations of diverse genetic variants. Several genetic aberrations have been found in GBM tumors, including missense mutations of the PTEN gene, overexpression, amplification or activating mutations of the EGFR proto-oncogene [87, 88], deletions in the NFκB pathway [89] and genetic alterations of receptor tyrosine kinase pathways, *e.g.* PDGFR-beta [77, 90]. Research leading to an understanding of the molecular mechanisms and gene mutations involved in GBM pathogenesis has led to more promising and tailored therapeutic approaches. For example, temozolomide recently proved to significantly prolong survival in a subgroup of GBM patients with tumors displaying epigenetic silencing of the MGMT (O6-methylguanine-DNA methyltransferase) gene encoding the enzyme O6-alkylguanine DNA alkyltransferase (AGT) [91]. Reduced MGMT expression compromises DNA repair and thereby improves the therapeutic efficacy of temozolomide. Further understanding of the genetic drivers and the molecular heterogeneity of GBM will be of great importance for improved prediction of prognosis and response to therapy as well as for the development of targeted therapies.

Next, the specific topics covered in the present thesis will be discussed in greater detail.

Proteoglycans

The proteoglycans (PGs) form a diverse family of proteins with the common characteristic of one or more covalently attached glycosaminoglycan (GAG) polysaccharide chains which governs a great part of their functional features. The GAG chains consist of repeating disaccharides that can be structurally modified by the addition of sulfate groups. The lengths, sulfation patterns and numbers of GAG chains as well as the distinctive core proteins together contribute to the functional diversity of the PG family of proteins. The diversity is also reflected in the many biological processes involving PGs. Almost all mammalian cells produce PGs that can be secreted to the ECM, including small leucine-rich PGs and aggrecan, and to the basement membrane where perlecan, agrin and collagen type XVIII are found. These PGs determine many of the biological properties of the embedded cells as well as physical characteristics of tissues. Serglycine is the only known obligatory intracellular PG contained within secretory vesicles as scaffold for retention of key inflammatory mediators [92]. Syndecans and glypicans are the two main families of cell surface PGs. However, it is becoming increasingly recognized that additional cell surface proteins can exist with or without substitution with one or more GAG chains, being so called part-time PGs [93-95]. This thesis work describes a new role of cell-surface PGs in the cellular internalization of EVs (paper **II**) and identifies CAIX as a new part-time, cell surface PG (paper **IV**).

Glycosaminglycan biosynthesis

GAG chains are composed of repeating hexosamine-uronic acid disaccharide units. Depending on the type of disaccharide building blocks, the PG superfamily is subdivided into three major groups; chondroitin/dermatan sulfate (CS/DS) PGs, heparin/heparan sulfate proteoglycans (HS) PGs and keratan sulfate (KS) PGs. The following discussion will mainly focus on the role of HSPGs and CSPGs in the tumor microenvironment. CS and HS GAGs are synthesized through the combined action of a number of endoplasmic reticulum and Golgi-resident enzymes. GAG chain polymerization is initiated by the transfer of a xylose to the hydroxyl group of specific serine residues in the core protein. Xylose transfer is followed by the addition of two galactose saccharides and one glucuronic acid (GlcA), together forming a tetrasaccharide linker region that serves as attachment site for further GAG

polymerization. The linker region is recognized by two separate transferase enzymes, responsible for the addition of N-acetylglucosamine (GlcNac) or N-acetylgalactosamine (GalNac), respectively, as the fifth saccharide. This will commit the synthesis to either HS or CS synthesis. Alternating transfer of GlcA and hexosamines generates a growing GAG chain, reaching a length of about 40-300 disaccharides, which is subsequently subjected to sequential structural modifications involving epimerization and sulfation. The HS and CS GAG disaccharide units consist of GlcA linked to GlcNac and GalNac, respectively. GlcA in CS can be modified by epimerization into iduronic acid (IdoA), thereby converting CS into the GAG DS. CS/DS GAGs will hereafter be collectively referred to as CS.

Glycosaminoglycan structural diversity

The variable sulfation of GAG chains results in complex and highly negatively charged structures along the polysaccharide chain. HS and CS do not share the same modifying enzymes; consequently some sulfation patterns are CS and HS specific. The addition of sulfate groups to HS are mediated by a set of sulfotransferases that act in a somewhat hierarchical order, meaning that the sulfated saccharides of one reaction are substrates for the next sulfotransferase. The first modification of HS is the replacement of the N-acetyl group of GlcNAc for a sulfate groups (GlcNS). Some deacetylated GlcNAc residues are left unsulfated yielding aminosugars (GlcN). N-sulfation of the GlcN allows for the binding of HS epimerase that converts GlcA residues adjacent to a GlcNS residue into IdoA. Next, the hydroxyl group at the C2-position of IdoA or, more rarely, residual GlcA may be sulfated (2-*O*-sulfation), followed by 6-*O*-sulfation and occasionally 3-*O*-sulfation of GlcNS. Completion of these ordered sulfation steps generates clusters of sulfate groups in domain-like structures around the initial sites of N-deacetylation, interspersed with larger domains that are relatively unmodified. However, the extent to which the modifications are completed varies, resulting in a tremendous sequence diversity of the final HS chain.

The CS modifications do not include N-sulfation and 3-*O*-sulfation whereas 4-*O*-sulfation of GalNac is specific to CS [96, 97]. The main players of the machinery responsible for CS and HS biosynthesis and modifications are summarized in **Table 1**. Most of these enzymes exist in several isoforms with divergent substrate specificities and tissue distribution, further adding to the complexity of GAG biosynthesis. Moreover, the non-templated process of GAG synthesis and subsequent modifications thereof increase the structural and functional diversity of PGs at several levels; by regulating the chain composition (*e.g.* CS vs. HS), the GAG fine structure and the number of attached chains as well as the chain length.

Table 1. Essential GAG synthesis and modulating enzymes and their catalytic activities

Linker region	**Catalytic activity**
Xylosyl transferase	Transfers xylose to the OH-group of serine within the protein core
Gal transferase I	Transfers Gal to the xylose residue
Gal transferase II	Transfers Gal to the -GalGal-Xyl residue
GlcA transferase I	Transfers GlcA to the –Gal-Gal-Xyl residue
HS synthesis	
GlcNac transferase I	Transfers the initial GlcNac to the linker region
HS polymerase (EXT 1/2)	Stepwise addition of GlcA and GlcNAc residues
NDST	Converts GlcNAc into GlcNS
HS epimerase	Epimerizes GIcA residues into IdoA
2-OST	Transfers SO_3^- to C2 of GlcA or IdoA
6-OST	Transfers SO_3^- to C6 of GlcNAc
3-OST	Transfers SO_3^- to C3 of GlcNAc
CS synthesis	
CS GalNac transferase I	Transfers the initial GalNac to the linker region
CS synthase	Stepwise addition of GlcA and GalNAc residues
DS epimerase	Epimerizes GIcA residues into IdoA which converts CS into DS
GlcA/IdoA-2-OST	Transfers SO_3^- to C2 of GlcA or IdoA
C4-ST	Transfers SO_3^- to C4 of GalNAc
C6-ST	Transfers SO_3^- to C6 of GalNAc
Extracellular modifications	
Sulfatase	Removes the GlcNAc 6-O SO_3^- from HS chains
Heparanase	Degrades HS chains

Abbreviations: N-deacetylase/N-sulfotransferase, NDST; O-sulfotransferase, OST; C4-ST, Chondroitin-4-sulfotransferase; C6-ST, chondroitin 6-sulfotransferase

The arrangement of the negatively charged sulfate groups and orientation of the carboxyl groups along the polysaccharide chain give rise to enormous structural

diversity which allow for interactions with basic amino acid residues within numerous proteins. The subsequent conformational modulation of the GAG-interacting protein is usually the trigger for a specific biological function. Hence, the generation of distinct GAG structures for selective ligand interactions represents extensively regulated processes.

Due to technical difficulties in structural analyses of GAGs and the lack of sensitive high throughput sequencing methods, less is known about protein-specific GAG interactions. GAG sequences identified as required for specific binding of a variety of proteins are reviewed in [98]. A well-studied example of specific HS-ligand interactions is that of antithrombin, *i.e.* a key protease of the coagulation pathway. Antithrombin is pharmacologically regulated by heparin, *i.e.* oversulfated HS, which induces a conformational change of its active site. As a result, antithrombin's ability to cleave and inactivate thrombin and thereby inhibit blood coagulation is substantially increased. The specific orientation of a few essential sulfate groups within a HS pentasaccharide sequence has been identified as responsible for this activity [99, 100], demonstrating the general principle of the 'specificity' within protein-GAG interactions. Collectively, GAG structural diversity as regulated by the expression of a repertoire of modifying enzymes together with the specific features of the core protein govern multiple functions of fundamental biological importance.

Functions of cell surface proteoglycans

The major representatives of cell surface PGs are divided into the two families of transmembrane syndecans and glycosylphosphatidylinositol (GPI)-anchored glypicans. Glypicans are devoid of intracellular domains while the cytoplasmic domain of syndecans contains two highly conserved regions, C1 and C2, and a variable region (V) situated between C1 and C2 that is unique to each syndecan. In mammals, the syndecans [101] and glypicans [102] comprise of four and six members, respectively. Typically, cell surface PGs are preferentially substituted with HS chains, and single CS chains sometimes decorating the surface-near part of syndecan-1 and-3. Specific GAG structures interact with diverse groups of proteins including chemokines, growth factors, ECM proteins, enzymes, lipoproteins, and microbial proteins and thereby mediate activation, intracellular degradation or immobilization of the ligands. Given their localization, cell surface PGs modulate the responses of various extracellular cues. PGs are thus instrumental in directing processes such as cell migration, macromolecular internalization, proliferation and invasion. These activities are largely involved in tumor progression and aberrant PG expression and GAG regulation have been demonstrated in malignant tissues [103]. Specific functions and modes of cell surface PG regulations are highlighted in **Figure 3.**

Proteoglycans as co-receptors of cell signaling

PGs regulate multiple signaling pathways by serving as co-receptors for GAG-binding growth factors including acidic and basic fibroblast growth factors (FGF) [104, 105] transforming growth factor β (TGF-β) [106] and VEGF [107]. FGF has been thoroughly investigated in this context and several syndecan isoforms and glypican have been shown to aid the formation of ligand-receptor complexes and enhance subsequent receptor signaling. HS binding increases the affinity of basic FGF for FGF receptor (FGFR); it has been established that the formation of FGF-HS-FGFR complexes is a prerequisite for efficient activation of the signaling receptor [108]. A similar relationship has been identified for hepatocyte growth factor (HGF), in that the interaction of HGF with HS on syndecan-1 strongly promotes activation of the HGF receptor, Met, and enhances downstream signaling [109]. The relevance of PG-mediated functions in tumorigenesis is further exemplified by the fact that HS was essential for the induction of growth promoting activities by epidermal growth factor (EGF)-family ligands in a multiple myeloma model [110]. The concept that GAGs act as modulators of the ligand binding affinity is also demonstrated for chemokine receptor as shown by a 40-70% decrease in the binding of RANTES, MCP-1, IL-8 and MIP-1alpha when removing GAGs from cells expressing the respective chemokine receptors [111]. In line with this, induction of IL-8/syndecan-1 complex shedding was accompanied by inhibition of neutrophil transendothelial migration [112].

In addition to direct presentation of ligands to their cognate receptors, HSPG-binding and retention of ligands serve to make up reservoirs from which HS-bound ligands can be released by proteolysis of the PG core protein or by heparanase-mediated cleavage of the HS chains [113]. HS sequestration has been shown to protect growth factors, chemokines and cytokines from proteolysis [114]. Also, cell surface PG-mediated ligand sequestration on the lumenal surface of endothelial cells constitutes concentration gradients of different chemo-attractants, which are required for successful inflammatory cell migration across the endothelium [115, 116]. Interestingly, studies in Drosophila and mice reveal opposite outcomes of glypican-mediated regulation of morphogen signaling [117]. While the Drosophila glypican analogue Dally-like protein is required for efficient Hedgehog binding and signaling via its receptor Patched [118], glypcican-3-mediated endocytosis of Sonic Hedgehog in mice negatively regulates signaling activation by continuous removal of Sonic Hedgehog from the cell surface [119]. Accordingly, HSPG-mediated ligand binding govern diverse biological processes such as angiogenesis, immune modulation, developmental processes and tumorigenesis by controlling signaling complex assembly, ligand sequestration in the ECM, and presentation of ligands to their cognate cell surface receptors.

Role of proteoglycans in cell adhesion

The role of cell surface PGs in cell adhesion is most thoroughly investigated with syndecans, and several studies implicate syndecans in focal adhesion formation in cooperation with integrins [120, 121]. Focal adhesion assembly requires fibronectin binding to the HS chains of syndecans [122], which induces the formation of syndecan oligomers. Oligomerization enables binding and activation of protein kinase Cα [123] followed by modulation of Rho GTPases [124] which, in turn, regulate cytoskeleton rearrangements. Furthermore, the V region of syndecan-4 provides a direct link to the cytoskeleton through the binding of the actin bundle protein α-actinin [125]. Accordingly, cell surface PGs possess both structural and signaling properties that link the cell exterior to the internal cytoskeletal network and intracellular signaling activities which together regulate adhesion and cell migration.

CD44 is a cell surface part-time PG that can be modified by HS or CS substitution [126]. CD44 constitutes a multistructural receptor that can interact with both the HS-binding domain of fibronectin [127] and hyaluronic acid GAGs within the ECM; interactions that have been implicated in tumor invasion and progression [128, 129]. In paper **IV**, we identified CAIX as a new part-time CS/HSPG with one GAG attachment site situated in its so called PG domain. This particular domain has been shown to impact cell adhesion and migration [130, 131]. Further, we show that CAIX strongly interacts with immobilized heparin, thus revealing HS-binding properties. In analogy with the dual GAG interaction capacity of CD44, these newly identified features of CAIX may enable ECM interactions which would provide explanations to the observed role of CAIX in cell adhesion.

Proteoglycan mediated ligand internalization

There are multiple cellular entry pathways for uptake of extracellular components that can vary in type of internalized cargo as well as required adaptor proteins and signaling molecules. Endocytosis is an umbrella term for a range of different vesicular internalization pathways. The different routes of endocytosis can broadly be divided into clathrin dependent (classical) or clathrin independent (non-classical). Clathrin independent mechanisms include macropinocytosis which primarily serves in the uptake of extracellular fluid, caveolin-mediated endocytosis and processes involving plasma membrane lipid rafts. Lipid rafts are microdomains rich in cell surface signaling receptors, sphingolipids and cholesterol that act as organizing centers for the assembly of intracellular signaling and scaffolding molecules.

Different kinds of internalizing receptors and a substantial number of accessory proteins are involved in these ingestion processes and in the intracellular transportation of internalized cargo. Some receptors display high ligand selectivity whereas other uptake receptors show more promiscuous cargo selection. HSPGs have

been shown to mediate binding and internalization of a large number of macromolecular cargo including lipoproteins [132], viruses [133-137], DNA [138, 139] and polyamines [140]. Although the mode of HSPG-mediated internalization may differ between ligands, a variety of HSPG-binding cargos, *e.g.* FGF2 [141], the cationic antimicrobial peptide LL-37 [142], DNA polyplexes [143] and apoE-VLDL [132][144] have all been reported to commence through caveolin-independent lipid raft mediated-uptake. Similarly, in papers **I** and **II** of this thesis, it is shown that a substantial proportion of EVs is internalized through a HSPG-dependent, lipid raft-mediated endocytosis pathway.

Several small GTPases including Rac1, RhoA, RhoG and ARF6, as well as actin microfilament interactions play critical roles in the regulation of HSPG internalization [145-147]. Also, various adaptor proteins of the syndecan cytoplasmic domain, including the PDZ-domain proteins syntenin and synbidin, have been shown to participate in HSPG-mediated intracellular transportation such as recycling through the endosomal compartment to the cell surface [146], and the recruitment of intracellular vesicles to postsynaptic sites [148]. The internalization process following ligand interaction has been described in detail for syndecan-1 wherein ligand binding results in extracellular signal-regulated kinase (ERK) activation and redistribution to lipid rafts. Lipid raft-resident Src kinases then phosphorylate tyrosyl residues within the syndecan-1 transmembrane and cytoplasmic regions which trigger the recruitment of cortactin and initiate actin-dependent endocytosis [149]. Interactions and signaling events that orchestrate internalization of GPI-anchored glypicans, devoid of transmembrane and cytoplasmic domains, remain less studied. Uptake of glypican-bound ligands is reported to proceed primarily through caveolin-dependent endocytosis [150], and the GPI-anchor is proposed to act as an endocytosis signal. This was suggested by the inability of mutant glypican-3 proteins, where the GPI-anchoring domain was lacking or replaced by the syndecan-1 transmembrane domain, to regulate hedgehog signaling [119].

The mode of internalization and the biological response of internalized cargo are, in addition to possible core protein specific regulation, also influenced by the HS-ligand interactions. This notion is supported by a study demonstrating that recombinant non-HS binding adeno-associated virus vectors enter recipient cells more efficiently through clathrin-dependent endocytosis, resulting in higher transgene expression, as compared to clathrin-independent uptake of HS-binding vectors [151]. Internalized HS-bound cargo may be subject to several possible fates, *e.g.* transportation to the lysosome for proteolytic degradation, recycle back to the cell surface [146], targeting to the nucleus, or recycling for extracellular secretion [152], together implicating cell-surface PGs in directing intracellular cargo sorting and membrane trafficking.

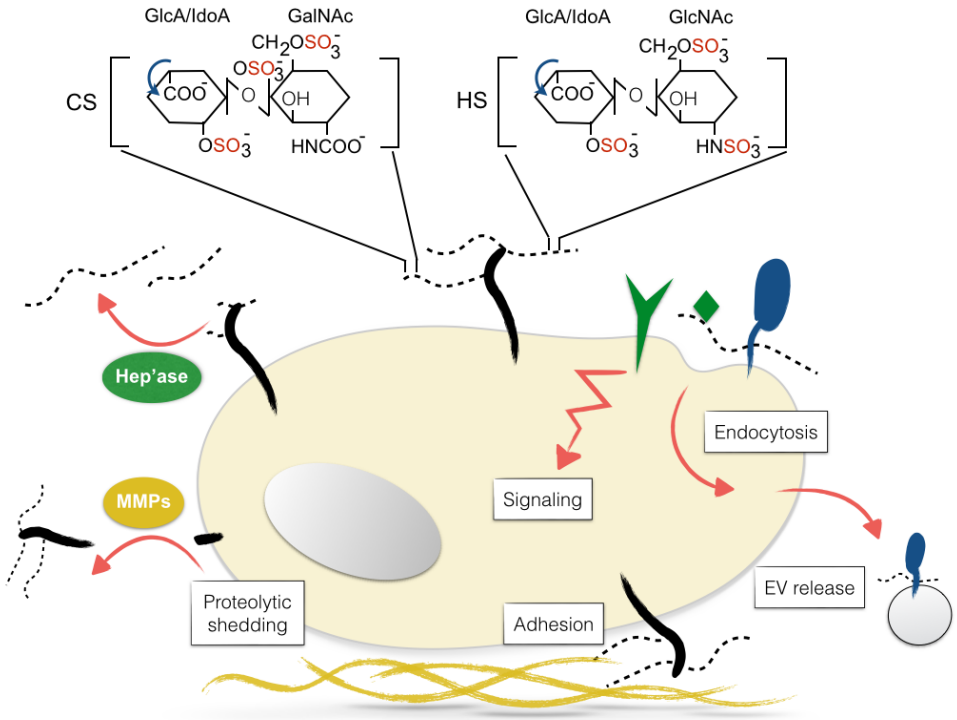

Figure 3. Cellular processing and functional activities of cell surface PGs.
Cell surface PGs are substituted with HS and/or CS GAG chains. Shown is the position for C5 epimerization of GlcA into IdoA (blue arrows), and possible sulfation positions (red) of the sugar residues constituting the CS and HS polysaccharides. HS chains can be digested by heparanase (Hep'ase) and cell surface PGs can be released through protease-mediated cleavage (MMPs). In papers **II** and **IV** of this thesis, PGs are demonstrated to be secreted on extracellular vesicles (EV). The anionic GAG chains give rise to numerous interactions with cationic molecules, and both syndecans (black) and glypicans (blue) are involved in diverse processes; *e.g.* growth factor binding and co-receptor signaling, cell adhesion and cargo internalization through endocytosis.

Multiple levels of proteoglycan regulation

PG expression, cellular localization and GAG chain structure may be regulated by several processes all contributing to the fine tuning of PG-dependent activities. In higher organisms, many of the GAG building and modification enzymes occur in multiple isoforms that differ in substrate specificity and spatial and temporal expression. Tissue specific enzyme expression may contribute to tissue diversity in GAG fine structure that could serve specific physiological functions. The significance of proper HS regulation is reflected by abnormal development or even early

embryonic lethality in mice lacking enzymes responsible for HS biosynthesis and particular sulfation modifications [153].

PG core protein expression is also tissue-specific [154] and developmentally regulated, with different isoforms involved in distinct biological activities; syndecan-1 participates in early development [155-157] and wound healing [158], syndecan-2 induces maturation of neuronal dendritic spines [159] and angiogenic sprouting [160], syndecan-3 is involved in skeletal development [161] and syndecan-4 participates in focal adhesion assembly [121]. Although specific functions are reported for individual PGs, functional redundancy between PG core proteins is suggested as phenotypes of knock out mice are generally mild [153]. Overlapping functions between syndecans and glypicans may in fact reflect the importance of a robust system for PG-mediated functions.

Cell surface proteoglycan availability

The availability of cell surface PGs is regulated by endocytosis as well as ectodomain shedding. The extracellular domains of glypicans can be shed from the cell surface by GPI-specific lipases [162] and syndecans are cleaved by a variety of MMPs [163-165]. Release of soluble PG ectodomains can influence PG-dependent functions by sequestration of GAG binding ligands, making them unavailable for receptor signaling and cellular uptake. Conversely, PG shedding may also translocate bound ligands to neighboring cells for paracrine signaling activation. Several microbial pathogens release proteases to stimulate shedding of host cell PG ectodomains. This virulence mechanism facilitates sequestration of cationic, HS-binding antimicrobial peptides, thus preventing pathogen lysis [166, 167]. Surface availability of cell adhesion and signaling molecules can also be regulated by cell surface recycling; syntenin-mediated syndecan recycling has been suggested to control intracellular integrin turnover and cell spreading [146]. In papers **II** and **IV**, we describe yet another mode of cell surface PG regulation, mediated by EV secretion. PGs of both syndecan and glypican types are transferred to EVs, and the newly identified part-time PG, CAIX, was found to be correctly oriented in the EV membrane with its ectodomain facing outwards.

Extracellular glycosaminoglycan modifications

Once translocated to the cell surface, GAGs can be further modified by extracellular endo-6-O-sulfatases (SULF1 and SULF2) or by the endo-β glucuronidase, heparanase. The sulfatases remove 6-*O*-sulfate groups from GlcNAc residues and heparanase cleaves the HS GAGs into smaller fragments for release. Extracellular modification of GAGs in the tumor microenvironment has structural and functional consequences, which significantly impact cancer progression. SULF1 expression was

shown to be transcriptionally deregulated under hypoxic conditions in breast cancer cell lines resulting in increased FGF signaling, cell migration and invasion [168]. SULF2 has shown similar promotion of FGF signaling as well as of additional HS-binding signaling proteins, including VEGF [169], Wnt [170, 171] and SDF1 [169]. However, contrasting effects have been reported for sulfatase activity; SULF1 was shown to be downregulated in many cancer cell lines and to inhibit tumor cell growth [172], presumably owing to reduced growth factor affinity for the modified GAG.

The release of HS-bound growth factors by heparanase cleavage facilitates their access to cell surface receptors and increased signal transduction [173, 174]. Heparanase-mediated HS fragmentation may even improve the steric conditions of solubilized growth factor-HS complexes and promote the formation of the active FGF-HS-FGFR ternary complex. In addition to regulation of cell-surface PGs, heparanase is instrumental in remodeling the surrounding ECM, linking heparanase to increased angiogenesis, cell invasion and metastasis. Accordingly, heparanase overexpression has been associated with worse patient outcome in several cancer types [175-178].
In summary, the complex nature of PG regulation including distinct cell and tissue expression patterns, structural GAG alterations as well as cellular modes of PG internalization and release clearly contributes to the functional outcome of PG-dependent activities and emphasizes the biological importance of correct maintenance of PGs.

Therapeutic implications of cell surface proteoglycans

PG-dependent functions have been explored as strategies for therapies in various diseases and approaches targeting the role of PGs as cell surface endocytosis receptors are of particular interest in the context of this thesis work. Following the discovery of HSPGs as uptake receptors for DNA/cationic complexes [179, 180], HS-binding transporters, *e.g.* cationic polymers, polybasic peptides and lipids, have been the subject of intense research as a means for therapeutic gene delivery. Another group of cationic ligands that exploits PGs for cellular entry is the polyamines [181]. Polyamines are involved in the regulation of cell growth, survival and migration primarily through ionic interactions with numerous polyanions including DNA, RNA, proteins and phospholipids [182]. Intracellular levels of polyamines are tightly regulated by *de novo* synthesis and uptake from the extracellular environment [183]. However, elevated polyamine levels have been associated with various cancers and other hyperproliferative conditions [184]. Indeed, inhibition of PG glycosylation was shown to reduce polyamine uptake and to enhance the anti-tumor effects of polyamine synthesis inhibitors *in vivo* [185].

There are several ways of interfering with PG functions, either by competitive GAG interactions using exogenous GAGs and other polyanionic compounds; by inhibition of GAG modifying enzymes such as heparanase; or using false xylose derivatives that prevent GAG priming onto core proteins. However, the promiscuous binding capacity of GAGs and severe phenotypes of GAG knockout animals point to the need for highly specific strategies to interfere with PG functions. Hence, efforts are made to synthesize oligosaccharides that exclusively impact GAG interactions associated with pathological processes [186]. Difficulties with low immunogenicity of non-protein structures have for long hampered the development of antibodies for analysis and targeting of the GAG compartment. Utilizing phage display technology, van Kuppevelt and colleagues have generated a series of single chain variable fragment anti-GAG antibodies. These tools may enable detailed analyses of GAG epitope distribution in malignancies [187-189], and could prove useful in the search for putative GAG targets in the tumor microenvironment.

HSPGs expressed at the cell surface of endothelial cells lining the blood vessel lumen contribute to the control of normal hemostasis through binding and activation of antithrombin. The integrity of the tumor vasculature is often impaired resulting in loss of the anticoagulant surface which together with additional procoagulant changes results in an increased risk of thrombotic events in cancer patients. Currently, the only clinically established use of GAGs in the management of cancer patients is low molecular weight heparins (LMWHs) for prophylaxis or treatment of VTE [190]. Taken together, GAG and PG components constitute candidates for targeted drug delivery, inhibition of virus and macromolecular entry or interference with oncogenic signaling implicating these complex components of the glycocalyx as interesting targets of future cancer therapies.

Extracellular vesicles

Membrane derived vesicles including exosomes and microvesicles (MVs, here collectively referred to as extracellular vesicles; EVs) are released by most cell types. Several lines of evidence suggest EVs as important players in cell-cell communication. EVs are lipid membrane enclosed spheres harboring diverse molecular components including RNA [191], functionally active proteins [192], DNA sequences [193] as well as lipids [194]. Secreted EVs may target cells in close proximity of their donor cell as well as recipient cells remote from their site of release. EVs thus represent mediators of information exchange conveying horizontal transfer of complex molecular messages.

EV influences recipient cells by *e.g.* interaction with cell membrane receptors, eliciting signaling activation, and by transfer of their content of bioactive molecules upon cell binding and entry. The functions of EVs depend on their molecular composition, directly influenced by the cell type from which they are derived, as well as the nature of the recipient cell. Different biological functions have been attributed to EVs including in development [195], and immune response [196] as well as in diseases, such as cancer [197, 198], and spreading of viruses [201] and prions [202, 203]. EV release appears to be a general biological process and EVs have been detected in biological fluids like blood, breast milk, saliva [204, 205] and urine [206]. Although standardized procedures are still lacking, EVs can be recovered from biological fluids and cell cultures for monitoring of specific pathophysiological processes, suggesting a potential of EVs as biomarkers [200, 207]. This thesis work aims to investigate different aspects of GBM cell-derived EVs in tumorigenesis including i) the mechanisms of EV internalization, ii) EV mediated functions in angiogenesis and tumor development and their potential role as biomarkers, and iii) EVs as emitters of pH-regulatory, enzymatic activity. The population of vesicles isolated in these studies constitutes a mix of exosomes and MVs as determined by vesicle size analysis and the presence of established exosome markers, thus, referred to as EVs with exosome-like characteristics. An overview of the current understanding of EV formation, mechanisms of EV transfer as well as their implication in tumor development will be given below.

Extracellular vesicle biogenesis

The formation of exosomes, *i.e.* small vesicles typically in the size range of 30-100nm, is initiated by plasma membrane invagination followed by the generation of endosomes. Internalized endosomal cargo is transported through the endosomal compartments and sequestered together with cytoplasmic constituents in multivesicular bodies (MVB). Fusion of exocytic MVBs with the plasma membrane results in the release of intraluminal vesicles (ILVs) as extracellular exosomes. Shedding MVs are larger vesicles (100-1000nm in diameter) that are generated through outward budding and fission of the plasma membrane. The mechanisms for MV budding are generally less well studied than exosome formation although some players have been identified recently, including Rac1/Cdc42/PAK-regulated actin cytoskeleton contraction [208]. Hyaluronan synthase 3, the enzyme responsible for the production of the GAG hyaluronic acid at the cell membrane has been suggested to contribute to MV release [209]. Notably, HAS isozymes are enriched and active in different types of actin-rich cellular protrusions including microvillus-like regions [210], filopodia [211] and lamellipodia [212], and invadopodia have been described as MVB docking sites for exosome secretion [213]. This may suggest that exosome and MV formation are partly controlled by common mechanisms. Further evidence of GAG interactions in EV formation was reported by Baietti *et al.* who demonstrated a role of syndecans in supporting intraluminal budding of endosomal membranes and found that exosome release was reduced when disrupting HS biosynthesis or sulfation [214]. In this context, the well-established role of syndecans as an endocytosis receptor indicates a link between initial PG-mediated endosome formation and subsequent exosome release.

Additional players known to participate in intracellular vesicle trafficking and membrane fusion belong to the family of Rab GTPases. Rab27a and Rab27b promote exosome secretion by controlling MVB docking at the plasma membrane [215]. The suggested role of EVs in tumor formation and progression was reinforced in both a carcinoma and a melanoma mouse model as demonstrated by reduced tumor growth and lung metastasis when disrupting Rab27a expression and subsequent exosome secretion. [198, 216]. The closely related protein, Rab27b appears to act in a compensatory manner when Rab27a is absent [217]. Also, Rab22a was recently identified to drive hypoxia-induced MV synthesis [218], again suggesting possible overlapping mechanisms between exosome and MV biogenesis.

Endosomal Sorting Complexes Required for Transport (ESCRT) controls the sorting of ubiquitin tagged proteins and assembly of ILVs into MVBs. Silencing of different ESCRT machinery components results in decreased exosome secretion suggesting an ESCRT-dependent pathway for exosome formation [219]. ESCRT-independent

exosome secretion has also been described to involve lipid interactions and tetraspanins. For example, reduced exosome release after treatment with neutral sphingomyelinase inhibitors suggests the sphingolipid ceramide as an important mediator of exosome biogenesis, possibly by aiding escape from lysosomal digestion for a favored release of MVB contents as exosomes [220]. A contrasting report proposes a role of glycosphingolipids and flotillins, rather than ceramide, in the release of exosomes from prostate cancer cells [221], indicating that the role of sphingolipids in exosome formation may be cell-type specific. Additionally, lipid domains within the plasma membrane, *i.e.* lipid rafts, have been reported as regions for MV budding [222, 223]. These cholesterol and sphingolipid-rich microdomains concentrate numerous cell surface receptors and serve as hubs for endocytosis invagination, thus, possibly contributing to subsequent generation of exosomes. Oncogenic activation seems to be a general mechanism for vesiculation as suggested by increased exosomal-like EV production by H-Ras overexpression [224] and oncosome release by EGFR activation [225] as well as increased exosome production as a result of aberrant p53 stimulation [226].

Although these and other studies have provided some insight, it is clear that the mechanisms of EV formation and release, and how this may be regulated in the context of cancer remains to be fully elucidated.

Context dependent extracellular vesicle characteristics

In spite of distinct sites of origin for MV and exosome biogenesis, some features in their formation seem connected. Further, the separation of exosomes and MVs by size is hampered by overlapping sizes of small MVs and large exosomes. The molecular composition of exosomes most likely depends on the initial event triggering endocytosis, *e.g.* receptor activation or macromolecular cargo ingestion. Likewise, distinct membrane sites for MV formation, including lipid rafts and invadopodia, and different physiological cues inducing MV shedding conceivably contribute to diverse MV characteristics. Importantly, once MVs are taken up by endocytosis in recipient cells, this class of vesicles will inevitably be physically connected with the exosome biogenesis pathway, potentially resulting in the formation of hybrid vesicles. Collectively, similarities between the two EV populations and putative differences within the respective type of vesicle as well as crude isolation techniques, so far, significantly obstruct efforts to study specific types of vesicles. The complex and ever-changing "vesiculome" may thus be more instructive to study in specific biological contexts rather than as subpopulations of distinct size or biogenesis origin. Supporting this view, several studies demonstrate altered molecular content and function of EVs when isolated from different conditions. Two reports utilizing blockade of E-cadherin and EGFR stimulation to induce EMT found an increased emission of TF and EGFR-containing exosome like vesicles [227] and significant

alterations of the EV proteome [228]. Similarly, exosomes isolated from cisplatin treated cells contained more cisplatin when culturing cells at low pH as compared to normal pH levels, which was reversed when pre-treating cells with a proton pump inhibitor [229]. Further, in paper **III** we describe a role of tumor hypoxia in dictating the molecular set-up of EVs and in driving EV-mediated pro-angiogenic effects. Thus, the ultimate composition and functional importance of EVs are seemingly attributed to the phenotypic status of the donor cancer cell, which is the net result of its mutational landscape and its microenvironmental context. The molecular information reflected within the total EV population may constitute a more comprehensive source of biomarkers as opposed to distinct subgroups. This is illustrated by the fact that only 3% of the extracellular miRNA content of plasma is associated with exosomes [230], the fraction that is harbored by MVs and other EVs has yet to be evaluated.

Exosome biogenesis appears to be a more regulated process than MV shedding. However, in analogy with a proposed cooperation between different endocytosis pathways for ligand internalization, there may also be overlapping and compensatory mechanisms for EV secretion. This could in fact provide a more robust control of vesicle emission. A better understanding of EV biogenesis could advance our general knowledge of EV biology. However, identification of pathological alterations of these mechanisms, *e.g.* induction of Rab22-dependent MV formation at hypoxic conditions [218], seems of more importance when addressing the functional relevance of EVs from a clinical perspective.

Role of extracellular vesicles in tumorigenesis

Cell-cell communication mediated by intercellular vesicle transfer is a complex story with multiple layers of functional outcomes. Mounting evidence suggests EVs as implicated in a large array of processes aiding tumorigenesis, including escape from immune attack [231] and apoptosis [232], EV-mediated receptor signaling activation [199], decoying and re-routing of drugs for extracellular disposal [233, 234], induction of angiogenesis [235] and increased metastatic potency [198]. An early study that fueled the interest of EVs in cancer cell-cell communication demonstrated that MVs expressing mutant EGFR (EGFRvIII) triggered oncogenic signaling and the onset of EGFRvIII-regulated gene expression in recipient cells [192]. EGFvIIIR was detected on the surface of recipient cells, which introduced the concept of MVs as mediators of horizontal propagation of oncogenic constituents.

Many EV surface proteins may serve as adaptors for cell surface docking and receptor interactions and would supposedly elicit cell responses without the need for cellular internalization. The glycoprotein TF is a key coagulation activator that has been found to be secreted on the surface of EVs [236] and on circulating microparticles

[237]. TF is also a major activator of protease-activated receptor 2 (PAR-2) signaling and TF expression is induced at hypoxic conditions. Work by our group revealed a mechanism by which TF-bearing MVs link pro-angiogenic signaling and hypercoagulation in the context of tumor hypoxia [236]. GBM cell-derived, TF-bearing MVs were shown to trigger PAR-2 activation resulting in rapid induction of ERK signaling followed by up-regulation of heparin binding-EGF-like growth factor and proliferation in hypoxic endothelial cells. Moreover, electron microscopy studies revealed PAR-2 and MV-TF to co-localize within endosomes of endothelial cells. Hence, specific EV-cell surface interactions seem of importance for EV-mediated intracellular translocation and turn-over of hypoxia-induced cell surface receptors. The study presented in paper **III** further reinforces the role of EVs in the hypoxic tumor microenvironment by demonstrating potent stimulation of angiogenesis and tumor development by hypoxic GBM derived EVs.

Intriguingly, EVs may impact tumor development by protecting cancer cells from the cytotoxic effects of chemotherapy and have been proposed to constitute a route for the transfer of drug resistance properties to nearby cells. This is exemplified by acquired docetaxel insensitivity in docetaxel-sensitive cell lines treated with exosomes from docetaxel-resistant cells, which may be explained by the transfer of P-glycoprotein, mediating efflux of many anti-cancer agents [234]. Other studies propose a mode by which cancer cells package drugs into exosomes to evade cytotoxicity [229, 238]. Moreover, HER2-expressing exosomes counteracted trastuzumab induced proliferation inhibition of HER2-overexpressing cells [233]. Thus, secreted HER2-exosomes may serve as decoy targets that prevent cellular trastuzumab-HER2 interaction.

EV-mediated biological functions that have received less attention involve the ability to execute intercellular enzymatic activities. In fact, several metalloproteinases including MMPs, a disintegrin and metalloproteinases (ADAMs), and ADAM with thrombospondin motif (ADAMTSs) have been detected in exosomes from malignant ascites and tumor cell lines [239], and recent studies have revealed that proteolytic processing can occur on the surface of exosomes. Exosomes derived from cultured fibrosarcoma and melanoma cells were reported to contain a catalytically active form of MMP-14 that activated pro-MMP-2 and degraded type 1 collagen and gelatin [240]. Further, Lee and co-workers showed that MMP-15 is released on exosomes and mediates tumor suppressive activities by binding to integrin αvβ3 thereby impairing cell adhesion and migration as well as *in vivo* tumor growth [241]. In paper **IV**, we found that EVs harbor CA activity, largely contributed by the hypoxia-induced, tumor specific CA isoform, CAIX involved in pH regulation.

Initial studies demonstrating exosomes as carriers of mRNAs and microRNAs (miRNAs) [191] have received great attention. Exosomes are thought to protect enclosed RNAs from degradation during transit through the extracellular environment, thus enabling transmission of RNAs between cells. Indeed, radiolabelled exosomal mRNA was shown to be transferred to recipient cells and

exosome transfer resulted in detection of new proteins, which may indicate translation in the recipient cells. However, evidence of functional effects of EV-mediated genetic exchange is based on overexpressed reporter gene systems [242, 243], and the biological relevance of transfer of endogenous RNAs has yet to be proven. Further, many studies ascribe observed EV functions to single miRNAs although thousands of miRNAs with numerous targets are detected in EVs [244]. This seems unlikely as EVs constitute complex entities composed of multifaceted biological information and macromolecular building blocks. In fact, a recent study that quantified the number of miRNAs per exosome from different biological sources found that the average number of the most abundant miRNAs is far less than one copy per exosome [230]. However, a recent report by Kalluri and co-workers seemingly is at odds with these findings; it was suggested that exosomes from breast cancer cells and breast cancer patient sera exhibit a profound, autonomous ability to process pre-miRNAs into mature miRNAs, and that the transfer of this activity transformed non-malignant epithelial cells to form tumors [245]. In any case, the total EV-associated RNA signature and protection of EV enclosed RNAs from degradation may still render EVs as a suitable biomarker source of circulating, tumor-derived RNAs.

Mechanisms of extracellular vesicle internalization

While there has been an initial focus on the functions and biogenesis of EVs, the mechanisms of cellular EV capture and internalization still remain elusive. For long, transfer of vesicular cargo was claimed to occur through fusion with the plasma membrane of the recipient cell although little evidence for this hypothesis has been presented. Also, many studies ascribe observed EV effects to intracellular release of EV cargo without actually confirming EV internalization. Understanding the mechanisms of EV transfer and target cell selection could improve the prospect for therapeutic targeting of EVs and for development of EVs as therapeutic delivery vehicles. A summary of reported EV uptake mechanisms is outlined below.

Extracellular vesicle uptake receptors

The initial event preceding EV internalization is the docking of ligands present on the EV surface onto the recipient cell surface. A number of different cell surface receptors have been proposed for the internalization or binding of EVs. We found that cell surface HSPGs function as receptors of EV uptake (paper **II**). Perturbed HSPG function in genetic mutants and competitive HSPG inhibition did not completely block EV uptake, which suggests the existence of additional, non-HSPG-dependent uptake pathways.

Exosomes have been implicated as vehicles for tumor antigen display, and exosome capture by DCs has been reported to require the cell surface receptor LFA-1 and exosome-bound ICAM-1 for antigen transfer between DCs *in vivo* [246]. It should be noted that in some cases the functional effects mediated by EVs do not require internalization of the vesicle. In favor of this idea, exosomes shed from antigen presenting cells were shown to mediate $CD8^+$ T cell stimulation through ICAM and B7-1-dependent engagement of MHC class I/peptide complexes [247]. Integrins and immunoglobulins abundantly expressed on immune cells have been suggested as EV receptors, including CD11a and integrins αv and β3 [248]. A role of C-type lectin with ligand specificity for mannose was suggested by reduced exosome uptake by competitive inhibition with D-mannose and D-glucosamine [249].

Directed EV uptake is most likely dependent on the combination of specific receptor expression on the recipient cell and complementing EV surface ligands. An early study on the functional effects of EVs in tumor angiogenesis suggested a role of phosphatidylserine (PS) on the surface of MVs for cellular binding and internalization [250]. Pre-treatment of MVs with PS-blocking Annexin V or Diannexin decreased MV-mediated ERK and Akt signaling, VEGF receptor phosphorylation and tumor growth. Later, the PS-binding receptor TIM4, which is responsible for macrophage engulfment of apoptotic bodies, was suggested by Miyanishi *et al.* to associate with surface PS on exosomes [251]. These results were supported by an approximate 30% reduction in exosome uptake with a TIM4 blocking antibody in mouse macrophages [252]. In addition to phospholipid-protein interactions, inhibitory effects of protein K treatment of EVs support the role of protein-protein interactions in EV attachment and uptake [253]. For example, the expression of distinct tetraspanin profiles on EVs contributed to target cell selection as evident by differential tissue homing of exosomes with different tetraspanin set-ups [254]. Taken together, EV uptake is most probably not restricted to one particular class of uptake receptor, and differs depending on the EV surface characteristics.

Uptake pathways involved in extracellular vesicle transfer

A number of reports demonstrate a dramatic reduction of EV internalization when cells are incubated at 4°C, suggesting that EV uptake is not a passive process (paper **I**, paper **II** and [253, 255, 256]). An active EV uptake pathway is also supported by the requirement of an intact cytoskeleton for efficient EV internalization, as shown by decreased uptake by inhibition of actin filament depolymerization (paper **I** and [242, 252, 253, 257]). However, membrane fusion with cholesterol-rich plasma membrane microdomains has also been observed when using a fluorescent lipid dequenching technique. Fusion of cell membranes and fluorescently labelled exosomes was measured as dilution of a self-quenching probe leading to increased detection of the fluorescent signal [242, 258]. Few studies corroborate quantitative internalization

measurements of fluorescently labelled EVs with qualitative imaging approaches. In paper **I** and in a previous report by our group [199], electron microscopy data reveal double membrane structures of internalized EVs residing in intraluminal vesicles. These organelles were positive for the MVB marker Tsg-101 after short-term incubations, which is indicative of an endocytic uptake process. In my view, some EVs may utilize an alternative fusion-based entry route, although the majority of research reports support a dominating endocytic mechanism for EV uptake. Methods used to discriminate between different cellular entry mechanisms and suggested uptake pathways are listed in **Table 2**.

Several endocytosis pathways have been proposed for EV uptake and a range of chemical inhibitors have been used to block specific uptake pathways to tease out the molecular mechanisms involved in EV entry. Clathrin-mediated endocytosis (CME) expedites the internalization of transmembrane receptors and their ligands. CME commences through the assembly of clathrin-coated vesicles that invaginate and pinch off from the plasma membrane. Inhibition of clathrin-coated pit formation using the chemical inhibitor chlorpromazine has in some cases been shown to decrease the uptake of EVs [252, 253].

Caveolae are plasma membrane domains rich in cholesterol, sphingolipids and the scaffolding type proteins, caveolins and cavins that mediate endocytosis through the formation of small cave-like invaginations [259]. Contradictory results have been reported for caveolin-mediated endocytosis; while caveolin-1 (CAV-1) gene knock down significantly impaired the uptake of exosomes derived from Epstein-Barr virus-infected cells [260], EV uptake was increased in CAV-1 deficient mouse embryonic fibroblast cells and glioma cells exhibiting lentiviral-mediated CAV-1 knock down (paper **I**). It can be concluded that CAV-1 is implicated in the regulation of EV uptake although the precise role may vary between target cells and EV types.

Macropinocytosis is involved in the ingestion of extracellular fluid and components of the extracellular space without the need for direct membrane contact with the internalized material. Macropinocytosis requires Na^+/H^+ exchanger activity [261] and inhibition of this process may prevent EV uptake [257]. However, other studies did not find a role of macropinocytosis in the uptake of EVs [260, 262]. Lipid raft-mediated endocytosis requires cholesterol and is therefore sensitive to cholesterol depletion agents such as filipin, methyl-β-cyclodextrin and simvastatin all of which have been reported to diminish EV uptake ([242, 252, 253, 258] and paper **I**). Notably, treatment with single inhibitors of one particular endocytosis pathway frequently fails to completely abrogate internalization; thus, EV uptake most probably occurs in parallel through more than one endocytic mechanism.

Table 2. Summary of reported EV entry mechanisms

Mechanism/Pathway	Inhibitor	Target /note	Reference
Lipid raft	MβCD Filipin Simvastatin	Cholesterol removal Cholesterol removal Cholesterol synthesis inhibitor	[252, 253, 263], paper I [242], paper I Paper I
Caveolin	siRNA	CAV-1 knock down CAV-1 overexpression	[260], Paper I Paper I
Macropinocytosis	Amiloride Annexin-V	Na^+/H^+ exchange inhibitor PS binding	[252, 253, 257], paper I [257, 264]
Clathrin mediated endocytosis	Chlorpromazine	Dopamin antagonist	[252, 253]
Actin	Cytochalasin Latrunculin	Actin polymerization inhibitors	[242, 248, 249, 252, 253, 257, 265, 266], paper I [252], paper I
Microtubuli	Nocodazole	Microtubli polymerization inhibitior	Paper I
Dynamin	NSC 23766 Dynasore siRNA	Rac1 inhibitor GTPase inhibitor	[257] [257, 266] [252]
Membrane fusion		Fluorescence self-quenched probe	[242, 258]
ERK1/2	U0126	MEK inhibitor	Paper I
PI3K	Wortmannin LY294002	PI3K inhibitor PI3K inhibitor	[252], paper I [252], paper I
Calcium	EDTA	Ca^{2+} chelation Ca^{2+} free condition	[242, 248, 249, 265, 266] Paper II

Abbreviations; Methyl-β-cyclodextrin, MβCD; Phosphatidylserine, PS.

Cell signaling is elicited shortly after start of EV incubation, which partly represents receptor activation. We showed that several kinases are activated by EVs followed by a transient increase of *e.g.* ERK phosphorylation (paper **I**). Notably, intact ERK activity was required for EV internalization as pre-treatment with the MEK inhibitor UO126 substantially decreased EV uptake. Similarly, phagocytic internalization of exosomes by macrophages was reported to depend on phosphatidylinositide 3-kinase (PI3K) signaling [252]. Induction of cell signaling upon EV binding followed by sustained signaling during cellular entry in combination with EV-resident signaling components paints a picture of internalized EVs as multilevel "signalosomes". It

remains to be elucidated how these complex networks of signaling activities contribute to the functional effects mediated by EV transfer. The level of complexity of intercellular vesicle communication is most likely not limited to the mechanisms of EV formation and EV cargo selection but may extend to the multitude of processes of how endocytc invagination, intracellular trafficking and compartmentalization together impact the signaling and biological outcome of EV transfer.

Intracellular fate of internalized extracellular vesicles

Downstream of cellular engulfment, the route for EV sorting and processing is versatile. EVs may be further metabolized by lysosomal degradation, by endosomal escape for cargo release into the cytosol, or even maturation within MVBs of recipient cells and recycling to new exosomes for extracellular emission. Some studies suggest the majority of internalized exosomes to localize to the lysosomal compartment [252, 257]. However, functional signaling proteins and RNA contained within EVs most likely must convey their functional effects in the cytosol. Intriguingly, data presented in paper **I** indicate that internalized EVs and endogenous exosomal components may mix in the recipient cell when recycled through the MVB system to generate multipart vesicles consisting of new and recycled EV constituents. A schematic overview of possible routes for intercellular EV translocation and intracellular processing is illustrated in **Figure 4**.

Actually, very little is known regarding the intracellular fate(s) and putative mechanisms for endosomal EV cargo escape. This is problematic given the number of reports claiming specific and profound effects of EV lumenal contents, most importantly miRNAs. Trafficking of endosomal cargo into the cytoplasm represents a major rate-limiting step for many drug delivery approaches and this is most likely also the case for transfer of EV cargo. Acidification plays a regulatory role of endosome maturation and trafficking in which V-ATPase proton pumps control the pH gradient across the endosomal membrane [267]. In paper **IV**, we demonstrate that the pH regulatory enzyme CAIX can be sorted to EVs and is enriched in EVs secreted from hypoxic cells. In addition to the possible functional effects of CAIX-bearing EVs in the extracellular space, it is tempting to speculate that CAIX may impact intracellular processing of internalized EVs by influencing the endosomal pH control.

Viruses utilize sophisticated mechanisms to achieve intracellular delivery of their genome including fusogenic peptides [268] and pore formation [269]. Interestingly, many EV features may converge with those of viruses [270] including i) the viral exploitation of EV biogenesis pathways for virus particle production [271, 272], ii) the role of viruses and potentially EVs in genetic material exchange, and iii) common mechanisms for cellular entry involving *e.g.* HSPG-binding and a regulatory role of CAV-1 in uptake restriction [273, 274]. A fascinating hypothesis proposes that intracellular compartmentalization in eukaryotes may have ascended as a remnant of

viral infection. Virus-mediated transmission of genes that introduced the capacity of vesicle formation and release is suggested to explain similarities between different aspects of virus and EV formation [270]. Building on this concept, mechanisms of virus endosomal escape may be interesting to explore as strategies for EV cargo release. Collectively, the spatial sorting of internalized EVs and their specific signaling competent cargo may together determine further intracellular processing and their functional effects. A better insight into these levels of regulation should benefit the design of EVs for targeted drug delivery.

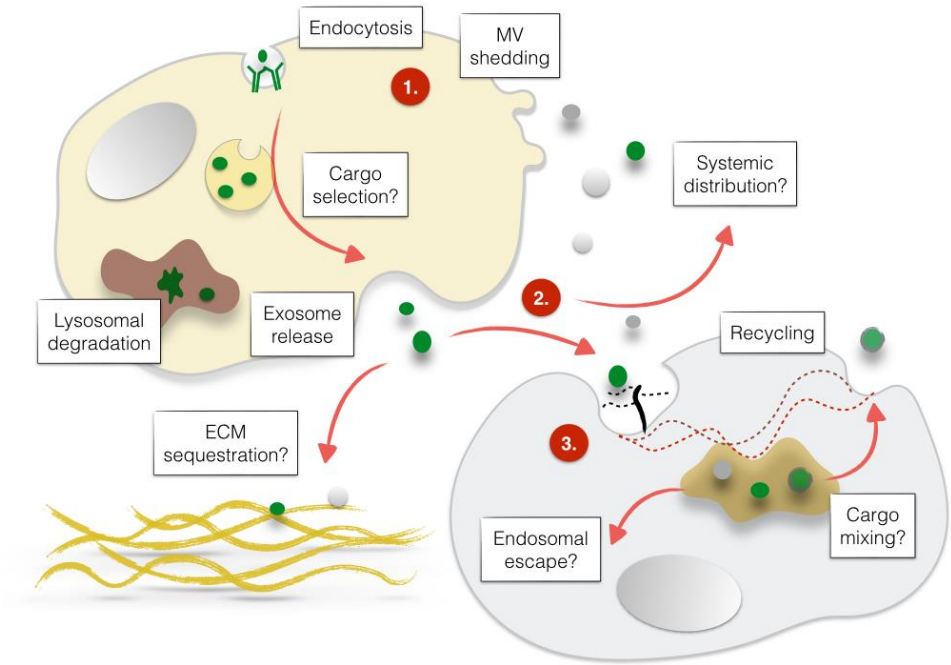

Figure 4. Biogenesis, intercellular transfer and intracellular processing of extracellular vesicles. (1) EV formation commences through either outward plasma membrane budding (MV shedding) or following endocytosis. Endocytic vesicles are processed through early and late endosomes followed by formation of intraluminal vesicles (ILVs; green) within multvesicular bodies (MVBs). MVBs that escape maturation to lysosomes may be destined for fusion with the plasma mebrane and thereby release of ILVs as exosomes. The process whereby exosome cargo is selected still remains to be determined. (2) Released EVs can be internalized by neighboring cells or cells at more distant sites. EVs may also become sequestered in the ECM or escape into the circulation for systemic distribution. The half life of EVs and the fraction of secreted EVs that makes it into the circulation remain to be elucidated. (3) EVs are internalized primarily through endocytosis; decreased EV uptake by blocking of different cell surface proteins indicates a receptor-mediated entry pathway (an HSPG is depicted here). In paper **I**, intracellular EV transport is demonstrated to proceed on microtubuli, and a process of EV cargo mixing and recycling is proposed. Abbreviations: MV, microvesicle; ECM, extracellular matrix.

Extracellular vesicles in cancer diagnosis and therapy

The notion that tumor-derived EVs contain RNA transcripts and proteins of tumor origin has spurred attempts to establish EVs as markers of cancer diagnosis and treatment prediction. Development of methods for non-invasive monitoring ("liquid biopsy") of tumor characteristics, progression or genetic evolution by evaluation of a circulating molecular signature is an appealing alternative to tissue biopsies. An early study demonstrated that tumor-specific EGFRvIII was detected in serum MV from GBM patients [275]. However, the mutant transcript was not detected in serum MVs of all patients that expressed the mutation in the tumor. Paper **III** revealed that EVs derived from hypoxic cells mirror the molecular profile of hypoxic donor cells. This supports the concept of using tumor-derived EVs as a source of liquid biopsies to assess tumor aggressiveness and progression. Improved isolation and analysis techniques are clearly required before the exciting concept of EVs as diagnostic biomarkers may become clinical reality.

In addition to a possible use of EVs as biomarkers of cancer and other pathological conditions, attempts have been made to engineer exosomes as therapeutic delivery vehicles. Manipulation of DC exosomes to express the membrane protein, Lamp2b, fused to the neuron-specific RVG peptide may allow siRNA delivery and target mRNA knock down specifically in neurons, microglia and oligodendrocytes after intravenous injection [243]. More recently, a similar approach demonstrated inhibition of tumor growth in an orthotopic mouse model using doxorubicin-loaded exosomes expressing a targeting peptide for αv-integrin [276].

EVs also represent an important communication mechanism in the interplay between immune cells. Exosomes from antigen-presenting cells have been shown to activate T cells [277], B cells [278] as well as natural killer cells [279] due to their expression of MHC molecules, stimulatory ligand or antigens. These features can be utilized to trigger anti-tumor immune responses by engineering the presentation of specific antigens. Both addition of proteins or peptides to DC cultures, resulting in indirect exosome-loading of antigens [280, 281], or direct replacement of MHC-bound antigens have been shown to stimulate T-cell responses as well as B-cell activation [281, 282]. In a mouse model of melanoma, it was recently reported that treatment with DC-derived exosomes loaded with the immune cell activating ligand, α-galactosylceramide, decreased tumor growth and increased median survival, presumably due to increased antigen-specific $CD8^+$ T-cell tumor infiltration [20]. These promising results of exosomes as tools in directing the immune response supports a future application in cancer immunotherapy. Accordingly, a clinical trial on patients with non-small cell lung cancer showed disease stabilization in some patients and activation of immune effectors when treated with tumor antigen loaded DC-derived exosomes [283]. However, converse functions of EVs in immune regulation are also reported. A model in which ligands of the immune stimulatory

receptor, NKG2D, residing on the exosome surface trigger down-regulation of the cognate receptor on natural killer and $CD8^+$ T cells was proposed to induce immune evasion of both cancer cells and human placenta explant cultures [231, 284].

The bio distribution and the ability of EVs to reach the tumor site or immune organs most probably impact their therapeutic potential as drug delivery vehicles or immune activators. The *in vivo* trafficking of exogenous EVs is affected by the route and site of administration. Injected exosomes were shown to disappear quickly from the blood circulation with a half-life of no more than two minutes [285]. Further, intravenous and intraperitoneal injection of exosomes resulted in distribution primarily to the liver followed by the lung, kidneys and spleen whereas exosomes delivered by intranasal administration were detected in the brain and intestines [285, 286]. In addition to the mentioned sites of tissue distribution, Peinado *et al.* found that exosomes administrated by tail vein injection home to the bone marrow wherein recipient cells are reprogrammed, or educated, to accelerate tumor growth [198]. Also, exosomes enhanced vascular leakiness in the lung, one of the primary sites of homing. This exemplifies tumor exosome-mediated priming effects at the systemic level that may impact the metastatic potential.

A better understanding of the processes of EV uptake, the contribution of EV surface components for binding of cell surface receptors in a cell and tissue-specific manner as well as EV *in vivo* distribution would detail the picture of intercellular EV-mediated communication and even aid the development of EVs as therapeutic vehicles.

Present Investigation

Aims of the thesis

The overall aims of this thesis were to elucidate the underlying mechanisms of EV cell internalization, the biological function of cancer cell-derived EV transfer in angiogenesis and tumorigenesis, and to determine the potential role of EVs as biomarkers. Specifically, the roles of PGs and hypoxia in the regulation of EV functions were investigated.

Specific aims of the included papers were:

I. To elucidate the endocytosis mechanism of EV internalization and study intracellular EV sorting and processing.

II. To determine the role of HSPGs in EV uptake and the functional relevance of this uptake receptor.

III. To study the functional role of EVs in hypoxia-mediated angiogenesis and to investigate a possible utility of a hypoxia-specific EV signature as biomarkers in GBM.

IV. To gain an understanding of the structural modifications and cellular regulation of CAIX and to address the functions of EV-resident CAIX.

Methods

The following list describes the methods used in the present thesis with reference to the paper/s in which they were used. For a detailed description, see the "Materials and Methods"section of the respective paper.

Method (paper)	**Description**
Western blotting (I-IV)	Gel electrophoresis separation and membrane transfer of protein for antibody-specific detection
Confocal laser scanning microscopy (I-IV)	Microscopy technique for z-scanning, 3D image reconstruction and live cell imaging
Immunohistochemistry/ Immunofluorescence (I-IV)	Antibody based technique for detection of specific antigen within cells and tissues
Extracellular vesicle isolation (I-IV)	Consecutive high speed centrifugation of conditioned cell culture medium or patient plasma
Fluorescent EV labelling (I-IV)	Labeling technique using membrane intercalating fluorophore-conjugated aliphatic lipid dyes
Electron microscopy (I-III)	High voltage electron beam image detection for ultrahigh resolution and magnifications
Multiplex antibody array (I, III)	Membrane based protein detection technique
Transfection and transduction (I, II, IV)	Lipofectamine/nucleic acid complex and virus based cell delivery for DNA plasmid or RNA introduction
Matrigel tube formation assay (III)	*In vitro* assessment of endothelial cell tube formation
Xenograft tumor model (III)	Subcutaneous tumor cell inoculation in SCID mice
In vitro survival assay (III)	7-amino-actinomycin D apoptosis marker detection
Ex vivo mouse aortic ring assay (III)	Microvessel sprouting measurement
In vitro proliferation assay (III)	^{3}H-thymidine DNA incorporation measurement
Laser capture microdissection (III)	Microscopy directed tissue isolation by laser cutting and capture
Quantitative real-time PCR (III)	Primer directed mRNA amplification and quantification
Site directed mutagenesis (IV)	Primer-directed introduction of site-specific DNA changes
Gene expression microarray (III)	Quantitative global mRNA transcript analysis
Proteoglycan extraction (II, IV)	Anion exchange and gel filtration chromatography
Immunoprecipitation (IV)	Immobilized antibody-directed protein capture
Biotinylation (IV)	Covalent attachment of biotin to protein for streptavidin-mediate capture or detection
Membrane inlet mass spectrometry analysis (IV)	Online measurement of conversion of isotope labelled chemical species for CA activity assessment

Results and discussion

Paper I

Cells communicate through several modes of action, *e.g.* cell-cell contact, ECM interactions and secretion of soluble factors. Secretion of EVs is now generally accepted as an important contribution to information exchange between cells. However, the mechanisms by which the receiving cell internalizes EVs still remain elusive. In this study, we aimed to investigate the cellular processes involved in EV uptake and intracellular transportation.

Results in short

- EVs are internalized into recipient cells in an energy dependent manner.
- Cholesterol depletion attenuates EV uptake while inhibitors of CME and macropinocytosis do not affect EV uptake.
- EV uptake depends on an unperturbed actin cytoskeleton as well as intact PI3K and ERK signaling.
- CAV-1 silencing and knock down augment EV uptake whereas CAV-1 overexpression decreases EV uptake.
- Internalized EVs reside in endosomes positive for the raft marker flotillin-1and are transported intracellularly along microtubules.
- EVs are sorted to CD63 and Tsg-101-positive compartments wherein cargo exchange may occur.

Role of lipid rafts and microtubule transportation in endocytosis and intracellular EV processing

Compartmentalization of cellular processes that allow for organized communication and exchange of molecular cargo between cells are of outmost importance in multicellular organisms. Intracellular processes utilized for this purpose involve endocytosis and vesicle trafficking that proceeds through early endosomes and maturation into late endosomes wherein endosome cargo is sequestered in ILVs and form MVBs. This compartment either evolves to lysosomes for protein degradation or recycles to the plasma membrane and thereby releases ILV as exosomes. Membrane fusion is central in these intracellular processes and key mediators of vesicle docking and fusion, including SNARES, SNAP adaptor proteins and NSF fusion ATPase have been extensively studied [287]. However, the intercellular counterpart of

compartmentalized communication, *i.e.* transfer of EVs still remains poorly described and the mechanism by which EVs are internalized into recipient cells has been a matter of debate. In analogy with a well-documented role of membrane fusion in intracellular vesicle trafficking, EV transfer has been suggested to proceed through membrane fusion [223, 242, 258]. This may occur in certain cell types or contexts; however, in paper **I** we found that a substantial proportion of GBM-derived EVs was internalized by endothelial cells through a lipid raft-mediated endocytosis pathway. Minor residual uptake of EVs at conditions of lipid raft disruption indicates alternative mechanisms of entry that may dynamically compete for EV internalization. However, in our cell system, membrane fusion seems unlikely as suggested by the observation of internalized EVs travelling on microtubule and the lack of fluorescently labelled plasma membrane after short time EV incubation. Internalization through endocytosis is also supported by detection of EVs in double membrane compartments and by the subsequent sorting of EVs to Tsg-101 and CD63 positive late endosome/MVB compartments.

This study further proposes an intracellular route for EV cargo exchange followed by recycling of mixed, multipart EVs destined for extracellular emission. This is indicated by the detection of fluorescently double-labelled EVs in recipient cells after uptake of re-isolated second generation EVs following incubation of PKH-labelled EVs with CD63-YFP expressing cells. A putative process in which internalized EVs mix with new cell constituents may extend the mode of action by which EVs contribute to intercellular propagation of oncogenic information. This still hypothetical concept warrants future studies that verify acquired protein markers directly on the population of re-secreted vesicles.

Caveolin-1 is the doorman of EV entry

We found that lipid raft-mediated EV internalization is negatively regulated by CAV-1. This agrees well with a previously reported role of CAV-1 as a negative regulator of endocytosis by stabilizing caveolar vesicles at the plasma membrane [274, 288], and thereby slowing down internalization. Notably, CAV-1 was recently shown to negatively regulate polyamine uptake in vascular smooth muscle cells [289]. Moreover, efficient polyamine internalization depends on HSPG-mediated uptake [140]. As described in paper **II**, EV uptake is also largely HSPG-dependent. Negative regulation of HSPG-mediated internalization by CAV-1 may thus be a general mechanism controlling macromolecular uptake.

No co-localization was detected between CAV-1 and internalized EVs as analyzed by confocal and TIRF microscopy, indicating that direct interaction is not required for CAV-1-mediated negative regulation. CAV-1 is, in addition to a major structural component of caveolae, a scaffolding protein involved in the organization and concentration of certain signaling molecules [290, 291] and receptors [292-294] within lipid rafts. CAV-1 negatively regulates several signaling molecules, including the small GTPase H-Ras and the c-Src kinase [291] as well as receptor tyrosine

kinases [293, 294]. Cell signaling activities upstream of EV-mediated ERK activation still remain to be revealed. Hence, CAV-1 may associate with proteins that interact directly with internalizing EVs or with signaling molecules required for uptake. Although ERK1/2 was not detected in a CAV-1 immunoprecipitate after short time EV incubation (paper **I**), suggesting that CAV-1 does not directly regulate this particular kinase, an indirect regulation of upstream signaling is still an interesting possibility.

Even though no direct connection between the lipid raft marker CAV-1 and EVs was demonstrated during the uptake process, both cholesterol depleting agents and cholesterol biosynthesis inhibitors were shown to reduce EV uptake, signifying lipid rafts to assist in EV internalization. EVs were indeed found in endosomes positive for flotillin-1, a marker of lipid rafts and caveolae [295]. Flotillin-1 may also reside in lipid rafts distinct from CAV-1 positive ones and has been shown to mediate endocytosis independently of CAV-1 [296, 297]. This notion supports a model wherein lipid rafts serve as the primary sites for EV internalization and specific constituents fine tune their entry; flotillin-1 may allow for rapid EV uptake while CAV-1 restricts the uptake. Collectively, our data together with the reported role of lipid rafts in MV shedding [222, 223] signify these membrane structures as hubs for both the reception and emission of vesicle-enclosed information.

Paper II

We and others have identified cell surface HSPGs as endocytosis receptors of a number of macromolecular cargos through mainly raft-mediated pathways. This encouraged us to explore a possible role of HSPGs as EV uptake receptors.

Results in short

- Internalized EVs reside in HSPG-expressing endosomes.
- EV internalization consumes HS-epitopes from the cell surface, and enhanced HSPG expression results in increased EV uptake.
- Soluble HS chains block EV uptake in a size and charge dependent manner and attenuated cell surface HS expression decreases EV uptake.
- Syndecan and glypican HSPGs are sorted to EVs but do not contribute to their internalization.
- HSPG-mediated EV internalization induces cell migration and ERK activation.
- Pharmacological inhibition of HS synthesis prevents EV-induced migration and ERK activation.

Cell surface HSPGs are major EV uptake receptors

Different EV uptake pathways and receptors are described in the literature, presumably depending on the EV donor cells, recipient cells, experimental set-up and contexts investigated. In paper **II**, we identified HSPGs as major receptors involved in the capture and internalization of EVs. We found that EV uptake was decreased by approximately 50% in the presence of low concentrations of exogenous HS, in HS-deficient cells, and by enzymatic degradation of cell surface HS. HSPG expression, surface availability, and characteristics of the GAG structure are known to influence the uptake of HS-binding macromolecules [298]. The same regulation seems to apply to EV entry. Firstly, highly sulfated and long HS chains exhibit an increased blocking capacity on EV uptake as compared to low sulfated and short HS chains. Secondly, pre-treating cells with the polyamine synthesis inhibitor, α-difluoromethylornithine, which causes concomitant alterations of PG synthesis [299] and increases HSPG-mediated polyamine uptake [140] was also shown to increase EV uptake. However, the entire EV population does not seem to require HSPGs for cellular entry. This may suggest specific uptake receptors for certain EV populations or reflect redundancy between uptake receptors that compete for uptake of available EVs. Alternatively, EV capture and entry may occur through cooperation between binding and internalizing receptors. The concept of cooperating receptors is well established for clearance of lipoprotein particles; HSPG can serve as lipoprotein uptake receptor alone or participate in the initial sequestration of the particle followed by internalization via the LDL receptor-related protein as a tertiary complex [300, 301]. A similar mechanism in which cooperating receptors would synergistically regulate uptake could be a possible model for EV internalization. Also, the size and complexity of EVs indicate that several potential receptor interactions could target various EV surface components for binding and internalization. As opposed to specific protein-protein interaction, the structural diversity of HS chains allows for binding to a large number of polybasic protein patches, providing almost unlimited binding opportunities. The components that were identified in papers **I** and **II** to play critical roles in the regulation of EV internalization are outlined in **Figure 5**.

The exact mechanism that triggers cargo internalization following PG-mediated binding of *e.g.* growth factors is not fully known. In a similar manner, there are probably a proportion of EVs that remains bound to the cell surface without undergoing internalization. In paper **II** we found that soluble CS, *i.e.* a GAG closely related to HS, failed to block EV uptake while cell surface CS digestion resulted in increased uptake. CS-mediated interactions play multiple roles in for example cell adhesion but are less well described in endocytosis. It may be speculated that CS would capture EVs and retain them at the cell surface. CS depletion would thereby increase the ratio of possible internalizing HS interactions to non-internalizing CS interactions.

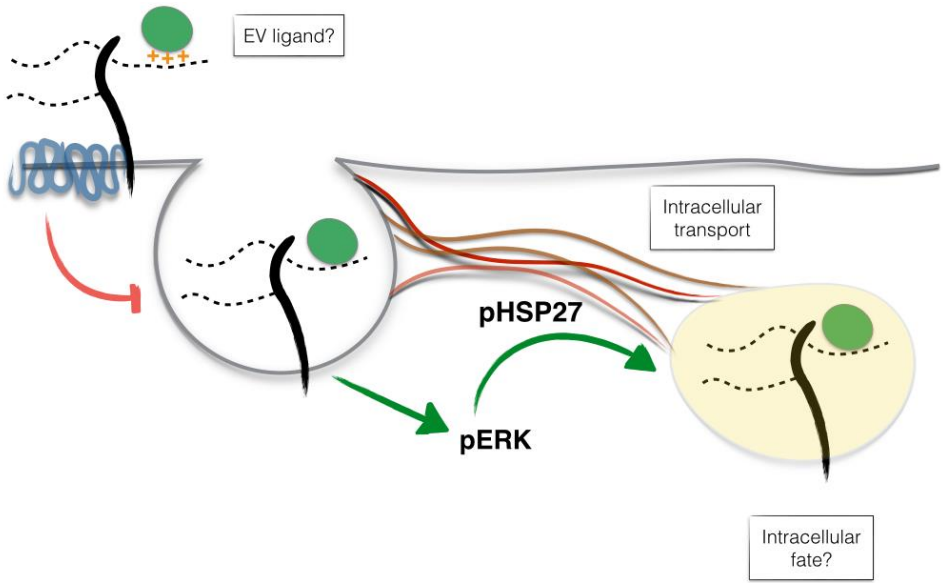

Figure 5. Proposed mechanism for EV internalization based on papers I and II.
A substantial proportion of EV uptake commences through binding of EVs to the HS chains of cell surface PGs. The EV surface ligands (yellow) responsible for this interaction remains to be identified. EV binding triggers the initiation of lipid raft-associated endocytosis which is under negative control by CAV-1 (blue). CAV-1 overexpression suppresses EV internalization as well as EV-mediated ERK activation and downstream HSP27 signaling. Intact actin cytoskeleton (red/brown) and HSP27 signaling are required for efficient EV uptake, and internalized EVs are transported along microtubuli. EV components and cellular processes that dictate the intracellular fate of internalized EVs are yet to be determined.

A recent publication by Thompson *et al.* [302] proposes a role of heparanase in EV formation; however, the data presented should be considered in light of HSPGs being major EV binding and uptake receptors. The study reports increased exosome release from heparanase overexpressing cells as compared to heparanase low cells. It may be argued that these data actually represent an increased release of HSPG-bound EVs from the cell surface upon HS degradation. Also, digested, soluble HS chains may influence the uptake by sequestering EVs in the extracellular space, as suggested by reduced EV uptake in the presence of exogenous HS chains (paper **II**).

Unknown role of EV resident proteoglycans

Data presented in paper **II** demonstrate that syndecan and glypican PGs are sorted to EVs but the extent to which PGs decorate the surface of EVs are not known and most likely differ between EV populations. EV-PGs were not found to impact EV uptake in our cell system. This can however not be excluded in other contexts or for certain EV populations. Knowing that EVs can serve as a route for the delivery of receptors to the surface of recipient cells [197], it would be highly interesting to investigate

possible EV-dependent transfer of PGs to their natural site of action as a positive feed-forward mechanism of increased EV uptake.

EV-mediated presentation of surface bound antigens has been shown to trigger immune responses; similarly, GAG-binding growth factors and cytokines may be located on the EV surface as bound to PGs. Indeed, protein analysis of isolated EVs performed in paper **III** detected soluble, HS-binding factors such as IL-8 and PDGF. PG-mediated presentation of these factors on EVs could allow for direct receptor interactions, providing a mechanism that would broaden the concept of EV-PGs as carriers of bioactive components.

Functional EV uptake receptor is looking for binding partner(s)

As demonstrated in papers **I** and **II**, EV internalization induces ERK activation. HSPG-mediated EV uptake was shown to contribute to EV-induced ERK signaling as suggested by reduced ERK phosphorylation by perturbed HSPG function. Further, disrupted HSPG functions diminished EV-mediated cell migration which implicates HSPGs as relevant mediators of EV transfer. However, the functional importance of this pathway remains to be shown *in vivo*. This source of EV capture may even constitute an obstacle for *e.g.* systemic delivery of therapeutic EVs. High HSPG expression on for example endothelial cells or hepatocytes could sequester and scavenge substantial amounts of EVs.

Despite numerous proteomic studies mapping EV constituents, few, if any, studies have specifically addressed the EV surface proteome for the identification of binding ligands in the context of EV uptake. One study suggests tissue transglutaminase and fibronectin interactions to be required for cancer cell-MV transfer and induction of mitogenic signaling activities [303]. Notably, both tissue transglutaminase and fibronectin bind to HSPGs [304, 305].

Paper III

GBM tumors are characterized by the presence of areas of severe hypoxia and abnormal vessel formation. Given the well-established role of angiogenesis in tumor progression and mounting evidence of EVs as implicated in cell-cell communication, we hypothesized that GBM-derived EVs would contribute to hypoxia-mediated pro-angiogenic responses. The hypoxia-dependent molecular EV characteristics were also investigated as a means to identify a possible biomarker profile of tumor oxygenation.

Results in short

- GBM patient plasma EVs display a distinct molecular profile as compared with controls.

- The molecular characteristics of EVs isolated from hypoxic conditions closely reflect the state of hypoxic donor cells.
- Hypoxic EVs contain elevated levels of hypoxia-induced mRNAs and proteins that correlate with poor prognosis.
- EV-mediated angiogenic activities are more pronounced for hypoxic EVs as compared to normoxic EVs.
- EVs reprogram the endothelial cell-derived secretome which induces pericyte activation.
- Hypoxic EVs enhance *in vivo* tumor growth and angiogenesis to a larger extent than normoxic EVs.

EVs serve as means for hypoxia-dependent intercellular communication

As of yet, no selection machinery for specific loading of EV cargos has been established. Instead, paper **III** and other studies suggest distinct molecular EV profiles to depend on intrinsic processes (*i.e.* EMT; [228]) or extrinsic conditions (*i.e.* low pH levels; [229]) that affect the phenotypic state of the donor cell. In paper **III**, we show that hypoxia results in the enrichment of hypoxia-induced proteins as well as RNA transcripts in EVs which promotes more potent stimulation of angiogenesis and tumor growth *in vitro* and *in vivo*. However, this study did not identify any single effector molecule as responsible for the enhanced functional effects of hypoxic EVs. Moreover, we did not discriminate between possible differences between surface-bound and intralumenal EV constituents in triggering pro-angiogenic effects. Several proteins of the hypoxic molecular signature are membrane bound (CAV-1 and CD26) or soluble factors (IL-8, PDGF and MMP-9) that may convey functional effects as bound to the EV surface. In addition, several thousands of enclosed RNA transcripts may contribute to EV-mediated cell responses. It is generally believed that a major mechanism by which EVs mediate cell communication is through protection of encapsulated constituents from degradation allowing for transfer of their cargo to distant recipient cells. This argues that the complete biological effects of EVs are only fully exerted following cellular entry and cargo release. However, it is not known whether initial signaling activation upon EV binding or cargo transfer mediates the main effects.

We detected rapid EV-mediated activation of EGFR, VEGFR2 and EphA2 receptor in endothelial cells, all of which are known to support angiogenesis. A recent publication links a pro-angiogenic role of CAV-1 with EphA2 receptor activation, AKT signaling and basic FGF expression [306]. Co-immunoprecipitation studies revealed that CAV-1 and EphA2 interact and CAV-1 silencing resulted in displacement of the receptor from the membrane to the cytosol. The authors propose that interaction between EphA2 and CAV-1 is necessary for receptor activation and downstream AKT-mediated FGF production and endothelial cell migration. CAV-1

is a well-established scaffolding protein involved in the assembly and signaling regulation of numerous partner proteins. Our findings of hypoxia-induced up-regulation of CAV-1 in EVs and the enrichment of CAV-1 in EVs isolated from GBM patient plasma, as compared to healthy controls, implicate CAV-1 in EV-mediated cancer cell communication. Although highly speculative, an intriguing idea would be a role of EV-resident CAV-1 in recruiting binding partners that elicit receptor activation on the surface of recipient cells. In this model receptor complex interactions would act in *trans* to activate RTK signaling in recipient cells.

Mixed messages in the cellular cross-talk of tumor angiogenesis provide potential biomarkers

The established fact that tumor progression is reliant on hypoxia-driven angiogenesis has prompted extensive studies of the processes regulating angiogenesis aiming to identify potential targets for cancer therapies. It is now clear that predictive biomarkers are needed to select groups of patients that are most likely to benefit from a specific anti-angiogenic treatment. In paper **III** we found several hypoxia-induced, pro-angiogenic proteins and RNA transcripts to be elevated in EVs from hypoxic GBM cells as well as from GBM patient plasma (proteins). The molecular reflection of the donor cell lends support to the idea of using circulating EVs as biomarkers to determine the oxygenation state, angiogenic profile and potential aggressiveness of the tumor.

In addition, we found that hypoxic EVs induced strong pro-angiogenic responses in endothelial cells. This suggests EVs as possible targets for the inhibition of hypoxia-dependent tumor angiogenesis. Interestingly, while robust effects were observed for EV in endothelial cells, the vessel mural cells, pericytes, were irresponsive. This discrepancy is quite intriguing and supports cell specific functional effects of EVs. Instead, GBM derived EVs were shown to reprogram endothelial cells to exert enhanced paracrine stimulation of pericytes; EV pre-conditioning of endothelial cells and subsequent treatment of pericytes with their conditioned media resulted in enhanced pericyte proliferation and migration. Perhaps EV-mediated endothelial cell activation more closely reflects the cellular cross-talk occurring *in vivo*? Indeed, hypoxia-derived EV stimulation increased pericyte-mediated tumor vessel coverage in a xenograft tumor model. Given the selection for more aggressive cancer cells by tumor hypoxia, the aim of anti-angiogenic therapies may instead be to achieve vessel normalization. Counteracting the excessive angiogenic drive within the tumor to promote the formation of an intact vasculature has been proposed to offer a window of opportunity that could improve drug delivery and radiotherapy efficacy. In this context, hypoxic EVs may actually reinforce the effects of conventional oncological treatments. Data presented in paper **III** support a role of EVs in vessel stabilization and illustrate the complex cross-talk that take place in the tumor microenvironment in which EVs constitute an important communicator.

Paper IV

An increased utility of glycolysis for anaerobic energy production at hypoxic conditions results in intracellular acidification of the tumor microenvironment. Hence, sufficient acid-base control is essential in order for cancer cells to adapt to this potentially toxic condition. The hypoxia-induced pH regulatory enzyme, CAIX, has been detected at elevated levels in the circulation of cancer patients and may associate with cancer progression. In this study, we investigated different means of cellular processing of CAIX that may be of importance for its function and for therapeutic CAIX targeting in cancer.

Results in short

- CAIX constitutes a part-time PG, conjugated with a single CS or HS chain.
- Glycosylated CAIX appears to be processed in a similar fashion as compared to unglycosylated CAIX.
- CAIX displays HS-binding properties.
- CAIX is sorted to EVs in a hypoxia-dependent manner.
- EVs show CA activity.
- EVs mediate intercellular transfer of CAIX.

CAIX links the sweet and sour of tumor hypoxia

CAIX is one of the most catalytically efficient enzymes described, and is rapidly up-regulated at hypoxia with a half-life extending 36 hours [307]. These robust features imply that its function is of great importance in relieving hypoxia-induced cellular acidosis. How different modes of post-translational regulation may contribute to the fine-tuning of its activity remains ill-defined. We discovered that CAIX is partly modified by the conjugation with one single GAG chain of either the CS or HS type. Approximately half of the CAIX pool constituted so called part-time PGs (PG-CAIX), and the GAG attachment site was found to be located within the extracellular distal part of the protein. In fact, not much is known about the regulation of part-time PGs, and it remains to be investigated what controls CAIX GAG glycosylation. Could hypoxia influence the CAIX glycosylation? No apparent differences in the proportions of PG-CAIX and unglycosylated CAIX were detected in hypoxic as compared to normoxic cells. This does, however, not exclude the possibility that structural modifications such as epimerization and sulfation are altered at hypoxia. Ongoing studies in our lab are focused on understanding what regulates the ratio of CAIX to PG-CAIX, the proportion of HS to CS carried by PG-CAIX as well as possible hypoxic regulation of the GAG chain structure.

GAG glycosylation is a common post-translational modification that renders distinct functional features of proteins. Most functions involve intermolecular interactions,

for example, focal adhesion assembly, binding and internalization of extracellular ligands and regulation of intracellular vesicle trafficking [214]. In line with this, CAIX is involved in cell adhesion and its membrane distribution has been shown to be regulated by endocytosis. It is thus tempting to speculate that the GAG substitution would contribute to these activities. Another sweet idea is a possible intramolecular GAG interaction that could contribute to functional regulation of the enzyme. The role of this GAG chain is yet to be determined, and investigations are currently ongoing to elucidate a possible function of glycosylation in CAIX's catalytic activity, *i.e.* the potential role of sugars in the regulation of the sour tumor.

EVs as intercellular transmitters of enzymatic activity and possible mediators of paracrine pH regulation

CAIX seems to be processed much like classical cell surface PGs, *i.e.* by endocytosis and ectodomain shedding. We have previously demonstrated that cell surface PG turn-over may also be regulated by EV secretion (paper **II**). In paper **IV**, CAIX was found to be sorted to EVs wherein CAIX was enzymatically active. EV-bound CAIX was transferred to recipient cells where it primarily localized to the cell-surface. It would be of great interest to further investigate if EV-mediated CAIX transfer could contribute to paracrine pH regulation as a part of the adaptive responses to tumor hypoxia and acidosis. Internalization of EV-CAIX and translocation to its natural site of action may suggest such a mechanism. However, CAIX uptake and surface membrane orientation may not be required for putative EV-mediated pH regulation. The presence of EVs in the extracellular space and their production of bicarbonate ions may have the potential of conferring increased resistance to acidotic stress in adjacent cells. The biological relevance of EV-mediated CAIX catalysis clearly warrants further study.

Concluding remarks and future perspectives

Unresolved issues in the regulation of extracellular vesicle communication

A remaining question is whether or not EV uptake is a cell type–specific process. It seems as though the expression of specific internalizing receptors are likely to favor uptake of EVs and supposedly a preferred uptake of certain EVs depending on distinct EV constituents. The role of EV ligands in cell specific uptake is indicated by the fact that EVs expressing different molecular tetraspanin complexes are selectively enriched in distinct cells and organs *in vivo* [254]. Another clue in the context of possible cell type specificity is the notion that overall HS composition varies greatly between cell types and that GAG chain substitution can vary in response to growth conditions [308]. This may influence the efficacy of HSPG-mediated internalization. Moreover, little information exists on the ratio of EV binding and/or internalization

between different cell populations. One study proposes that phagocytic cells internalize EVs more efficiently as compared to non-phagocytic cells [252]. The half-life of circulating EVs and the distance from the donor cell may be limited by phagocyte-mediated EV-clearance and HS sequestration. Unpublished data from our group indicate that the total cell surface proteome turn-over is decreased under hypoxic conditions, and that hypoxic cells utilize differential endocytosis pathways as compared to normoxic cells, presumably due to altered expression of key endocytosis proteins such as CAV-1. Accordingly, EV internalization may commence through diverse pathways depending on microenvironmental cues like hypoxia. Given that the mode of internalization and subsequent compartmentalization may influence the cellular response upon EV uptake, it is tempting to speculate that EVs would induce diverse functional outcomes when internalized by hypoxic cells as compared to normoxic cells, independent of the EV cargo or the molecular demand of the recipient cell. On this note, the requirement of intact ERK signaling for efficient EV internalization may suggest a role of the cellular signaling status as implicated in regulating EV transfer. Consequently, overactive oncogenic signaling cold potentially permit increased EV uptake. Taken together, in addition to the expression of cell surface receptors and EV ligands, environmental factors and the signaling state of the receiving cell conceivably impact the cellular capacity to internalize EVs.

The mechanism by which EVs convey downstream functional effects is another issue that presently remains unclear. What is the contribution of initial signaling activation upon encountering between EVs and the recipient cells? To what extent is subsequent cargo transfer involved? Or is it a combination that may be proportionally different depending on EV characteristics and recipient cell status? We found a rapid, EV-mediated onset of receptor activation and downstream ERK and AKT phosphorylation followed by delayed cell responses such as proliferation and migration. A delayed response was also observed in the lag phase preceding accelerated tumor growth mediated by hypoxic EVs. EV constituents transferred to recipient cells may be processed through autophagosomes or lysosomes for energy and macromolecule recovery. These pathways may be of particular importance for EV-induced proliferation and survival during nutrient shortage. Then again, excessive oncogenic signaling including ERK and AKT activation are common anomalies known to drive tumorigenesis and link extracellular signaling induction to a plethora of downstream cell responses. The question is to what extent EVs contribute to signal induction in relation to the vast pool of hormones, growth factors and cytokines within the tumor microenvironment. In addition to activities mediated by EV-cell contact and transfer, data presented by us (paper **IV**) and others [240, 241] suggest that secreted EVs carry catalytically active enzymes. The functions of circulating EVs as transmitters of enzymatic activity remains an intriguing and yet unexplored area of investigation.

Considerations for clinical implications of extracellular vesicles

The role of EVs in tumor development has been demonstrated repeatedly by *e.g.* inoculation of exogenous EVs ([198] and paper **III**) resulting in enhanced tumor growth, and by decreased tumor growth when disrupting exosome formation by Rab27 silencing [198, 216]. In a clinical setting, however, genetic modification of tumor cells to prevent EV formation is currently not an option. To my knowledge, no clinical trials have been purposely conducted aiming to block EV uptake. We found that EV uptake is significantly suppressed by low molecular weight heparin (LMWH). Notably, LMWH is frequently used in cancer patients to prevent and treat VTE which is a common cause of morbidity and mortality in many cancer types. Interestingly, in addition to anti-coagulant effects, LMWH treatment has also been proposed to have direct anti-tumor effects [309, 310]. Could these effects involve altered EV communication? Cancer patients who present with VTE sometimes have increased levels of TF-bearing microparticles [311]. It would be most interesting to measure the amount and composition of circulating EVs in cancer patients, before and after LMWH treatment, to assess a possible systemic inhibition of EV uptake.

There is an unmet need of predictive biomarkers for the selection of patients who are most likely to benefit from a given therapy prior to or early after start of treatment. As the tumor characteristics most likely change over the course of a treatment, sequential biomarker analysis could provide information on early markers for treatment response. However, the feasibility of repeated tumor tissue sampling is limited as tumors often are physically inaccessible, particularly in the case of tumors of the CNS. This has prompted efforts aiming to investigate cancer cell-derived EVs in biofluids, such as plasma, cerebrospinal or lymph fluid and urine, as biomarkers. The total pool of EVs will inevitably contain EVs from non-malignant cells, which may constitute a disturbing source of unspecific information. On the other hand, the molecular profile of such a mix may provide a more comprehensive picture of the oncogenic activities taking place in the tumor microenvironment as well as the potential toxic side-effects of any given treatment. The potential recycling of cancer cell-derived EVs into multipart EVs, as suggested in paper **I**, would indicate that EVs secreted from non-malignant cells also harbor tumor specific constituents. Moreover, hypoxic GBM cell-derived EVs were shown to prime endothelial cells and impact their secretome to convey phenotypic alterations of pericytes in a paracrine manner (paper **III**). This supports the idea that stromal cells, directed by cancer cell-derived EVs, could generate secreted information that would reflect stress-induced adaptations within the tumor microenvironment. A so called vascular niche, established by tumor resident endothelial cells, has been proposed to promote tumor growth through an "angiocrine" mechanism mediated by secreted growth factors and adhesive molecules [312]. It is conceivable that hypoxic cancer cell-derived EVs would instruct the outcome of this angiocrine mechanism. Targeting this endothelial-derived mechanism might offer a means to reduce angiogenesis-mediated tumor acceleration while circumventing unwanted side effects associated with hypoxia.

In addition to angiogenesis, hypoxia-associated dysregulation of pH within the tumor microenvironment may represent an Achilles' heel of cancer cells. Key regulators of acid-base homeostasis are highly interesting as cancer biomarkers, and the cell surface localization of CAIX and additional membrane metabolite transporters makes them accessible treatment targets. However, the secretion of CAIX-bearing EVs may obstruct specific cellular delivery of CAIX targeting drugs. Elevated plasma CAIX levels have been proposed as potential prognostic markers [313-316], and EV-bound CAIX most likely contributes to the pool of CAIX detected in the circulation of cancer patients. In the context of EVs as a possible platform for monitoring tumor presence and characteristics, identification of CA activity in EVs together with previous reports on EV-mediated proteolysis [240, 241] and coagulation activation [199] may expand the prevailing view of EVs as mere carriers of molecular markers. Hence, the collective release of membrane bound enzymes could potentially constitute an opportunity to explore EV-mediated catalytic activities for the development of future diagnostic and predictive biomarker strategies in cancer. To what extent such EV-associated activities have a role in the malignant process at the systemic level remains an important question.

Populärvetenskaplig sammanfattning

Cancerceller är celler som på grund av mutationer, felaktigheter i cellernas DNA, har erhållit förmågan att dela sig ohämmat. Celler behöver tillgång till syre och näring för att överleva, vilket levereras via kroppens blodkärl. Den snabba tillväxten av tumörvävnad leder dock till att befintlig blodtillförsel inte räcker till och delar av tumörerna drabbas därför av syrebrist, så kallad hypoxi. Hypoxiska cancerceller signalerar då till omgivande blodkärlsceller att börja bilda nya kärl för att förbättra blodtillförseln. Cancercellerna har även utvecklat andra mekanismer för att hantera syrebrist. Genom att t.ex. ställa om sin ämnesomsättning till en ökad nedbrytning av socker kan de generera nödvändig energi på ett sätt som inte kräver tillgång till syre. Dessvärre leder nedbrytning av socker till en överproduktion av restprodukter som gör cellerna sura. Karbanhydras IX (CAIX) är ett enzym som hjälper celler att reglera mängden sura ämnen. CAIX finns i förhöjda nivåer i tumörvävnad vilket är kopplat till sämre prognos i många cancertyper. CAIX är således ett tänkbart mål för cancerterapi och det är därför viktigt att förstå hur CAIX regleras i tumören.

Cancercellerna anpassar sig alltså efter den ogästvänliga miljön som råder i tumören och kommunicerar med de omgivande cellerna, t.ex. blodkärlsceller, för att på bästa sätt justera sina förutsättningar för tillväxt till det bättre. Celler kommunicerar genom att skicka olika signalsubstanser till varandra, dessa kan liknas vid små brev med instruktioner till mottagarcellen om hur de ska bete sig. En typ av cellkommunikation som nyligen upptäckts sker via frisättning av små membranomslutna blåsor, så kallade extracellulära vesikler (EVs). Till skillnad från en enskild signalsubstans innehåller EVs en stor mängd molekylär information och kan liknas vid stora paket med instruktioner. EVs som skickas från tumörceller har visat sig bidra till en ökad blodkärlsbildning och tumörtillväxt. Vad som påverkar vesiklernas innehåll och hur det överförs till mottagarceller är inte helt känt. Detta avhandlingsarbete har undersökt hur EVs från cancerceller tas emot av mottagarceller. EVs påverkan på blodkärlsbildning och reglering av sura ämnen har även studerats samt hur detta påverkas av hypoxi.

Överföring av extracellulära vesikler mellan celler

Vi har funnit en process där kolesterolrika regioner i cellens membran är viktiga för att EVs ska tas in i mottagarcellen över cellens membran. Proteinet Caveolin-1, som finns i dessa membranregioner, visade sig kontrollera upptaget så att mindre EVs tas upp. Det är tidigare visat att denna typ av region i cellens membran kan fungera som

centrum för bildning av EVs. De kolesterolrika membranregionerna fungerar på så sätt som en slags postcentral för mottagning och utskick av paket med information.

Vidare har vi identifierat ett sorts cellyteprotein, heparansulfate-proteoglykaner (HSPGs), som en viktig mottagare för upptag av EVs. EVs fastnar på långa, negativt laddade sockerkedjor som sitter på HSPGs, vilket gör att HSPGs tar med sig den bundna vesikeln in i cellen. Det visade sig också att det väletablerade läkemedlet heparin, som liknar sockerkedjorna på HSPGs, kan hämma en stor del av EV-upptaget i celler och sannolikt även deras tumörstimulerande effekter, genom att konkurreara med bindningen mellan EVs och HSPG.

Syrebrist påverkar extracellulära vesiklers innehåll och funktion

I den andra delen av detta avhandlingsarbete studerade vi hur hypoxi påverkar innehållet i EVs och hur EVs från hypoxiska cancerceller påverkar mottagarceller jämfört med EVs från normalt syresatta celler. Experiment med blodkärlsceller visade att hypoxiska EVs får cellerna att efterlikna processer som förekommer vid blodkärlsbildning i större utsträckning än normala EVs. Hypoxiska EVs kan alltså öka tumörens förmåga att nybilda blodkärl och på så sätt även dess tillväxt. Vissa molekyler som enbart fanns i hypoxiska EVs återfanns även i EVs som isolerades från cancerpatienters blod, vilket kan innebära en möjlig användning av EVs som markörer för cancersjukdom. Vi upptäckte även att CAIX transporteras på EVs från cancerceller. Frisättning av CAIX via EVs kan på så sätt reglera nivån av sura ämnen, på avstånd från celler som uttrycker CAIX. EVs skulle därmed kunna bidra till en bättre tumör miljö och på så sätt gynna tumörens förutsättningar för fortsatt tillväxt.

Sammanfattningsvis har vi kartlagt en rad processer som styr hur cancercellerna påverkar sin omgivning med hjälp av EVs samt identifierat viktiga komponenter som kontrollerar cellens upptag av EVs. Detta utgör en viktig funktion i överföring av information mellan celler i tumörvävnaden, vilken skulle kunna utnyttjas för att förhindra en central del av cancercellernas kommunikation. Resultaten som presenteras i den här avhandlingen belyser även möjligheter att använda EVs för att få information om tumörens tillstånd, något som är viktigt för att kunna avgöra patienternas prognos och hur de svarar på olika behandlingar.

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"Many times I've wondered how much there is to know"
Led Zeppelin