Adaptive mechanisms in the hypoxic tumor microenvironment. Functional role of proteoglycans and identification of potential treatment targets

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Adaptive mechanisms in the hypoxic tumor microenvironment

Functional role of proteoglycans and identification of potential treatment targets

Julien Menard

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Title and subtitle: Adaptive mechanisms in the hypoxic tumor microenvironment. Functional role of proteoglycans and identification of potential treatment targets

Abstract

Cancer cells reside in a complex microenvironment comprising stromal cells and immune cells embedded in an extracellular matrix (ECM). Early on in tumor progression, cells reach a critical volume beyond which blood supply is impaired, leading to a decrease in oxygen levels (hypoxia) and nutrient supply. This situation triggers a stress response characterized by a metabolic switch to glycolysis, which will in turn induce acidification of the extracellular environment. Under hypoxia and acidosis, cancer cells adapt their integration of nutrients, signaling molecules, and more generally their exchanges with the extracellular environment not only to survive but also enhance tumor aggressiveness, metastasis and treatment resistance.

The overall aim of this thesis was to gain a better understanding of cancer cell adaptive mechanisms in the tumor microenvironment under hypoxic and acidic stress, that could be exploited therapeutically. We focused here on proteoglycan (PG)-dependent uptake mechanisms and cell surface proteins as targets of drug delivery.

In paper I, we provided evidence for a role of heparan sulfate PGs in increased uptake of lipoproteins linked to enhanced pro-tumorigenic signaling and acquisition of a lipid droplet loaded phenotype under hypoxia and acidosis, associated with increased tumor-forming capacity. In the follow-up paper II we investigated the functional effects of this phenotype during post-hypoxic stress and found increased tumor aggressiveness, macrophage recruitment and angiogenesis in glioma mouse models, correlating with enhanced expression of pro-angiogenic and pro-tumorigenic factors (e.g. VEGF, HGF, CAIX, VIM) *in vitro*. In paper III, the global effects of hypoxia on tumor cell surface proteome internalization were studied. We showed downregulation of the surface and internalized proteome at hypoxia, involving caveolin-1 negative regulation of endocytosis. Importantly, we identified several surface proteins that were actively internalized at hypoxia, including CAIX. We then provided proof of concept that these proteins could be hypoxia-specific target candidates of antibody drug conjugate (ADC) treatment. In paper IV, the CAIX target protein was identified as a part-time PG. Mechanistically, glycosaminoglycan (GAG) conjugation of CAIX negatively regulated its internalization through increased association with caveolin-1 membrane domains, which was partially alleviated in acidic conditions. We then showed that CAIX GAG depletion enhanced its internalization and that this could be used to potentiate the cell killing effect of anti-CAIX ADC treatment.

Altogether, these studies further demonstrate the important role of PGs in adaptive mechanisms to tumor microenvironmental stresses at different levels, and pave the way for the evaluation of novel, potential therapeutic strategies.

Key words: Hypoxia, Acidosis, Proteoglycan, Endocytosis, Glioblastoma, Lipid, Caveolin-1, Antibody drug conjugate

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"*Pour tirer le meilleur parti des connaissances acquises, pour en extraire toute la richesse, il importe de ne pas s'y habituer trop vite, de se laisser le temps de la surprise et de l'étonnement*." Hubert Reeves

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I. Metastasis Stimulation by Hypoxia and Acidosis-Induced Extracellular Lipid Uptake Is Mediated by Proteoglycan-Dependent Endocytosis.
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II. Effects of extracellular lipids on immune cell recruitment and pro-angiogenic signaling under tumor microenvironmental stress in glioma.
Menard, J. A., Enriquez, J., Indira Chandran, V., Johansson, M.C., Bång-Rudenstam, A., Lidfeldt, J., Siesjö, P., Darabi, A. and Belting, M. *Manuscript.*

III. Hypoxia regulates global membrane protein endocytosis through caveolin-1 in cancer cells.
Bourseau-Guilmain, E., **Menard, J. A**., Lindqvist, E., Indira Chandran, V., Christianson, H. C., Cerezo Magaña, M., Lidfeldt, J., Marko-Varga, G., Welinder, C. and Belting, M. *Nature Communications*. 7, 11371. (DOI:10.1038/ncomms11371). **2016.**

IV. Tumor antigen glycosaminoglycan modification regulates antibody-drug conjugate delivery and cytotoxicity.
Christianson, H. C.*, **Menard, J. A.*,** Chandran, V. I., Bourseau-Guilmain, E., Shevela, D., Lidfeldt, J., Mansson, A. S., Pastorekova, S., Messinger, J. and Belting, M. *Oncotarget*. 8, 66960-66974 (DOI:10.18632/oncotarget.16921). **2017.**
***Equal contribution.**

List of papers not included in the thesis

I. Targeting of non-cycling cells of hypoxic tumors.
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II. Functional role of extracellular vesicles and lipoproteins in the tumor microenvironment. **Menard, J. A.,** Cerezo Magaña, M. and Belting, M. *Phil.Trans,B.* (DOI:10.1098/rstb.2016.0480). **2017.** (Review, in press).

III. Deubiquitination of gamma-tubulin by BAP1 prevents chromosome instability in breast cancer cells.
Zarrizi, R, **Menard, J. A.**, Belting, M, Massoumi, R. *Cancer Research*. 74, 6499-508. (DOI:10.1158/0008-5472.CAN-14-0221). **2014.**

Abbreviations

ADC: Antibody-Drug Conjugate

Apo: Apolipoprotein

aSMA: alpha Smooth Muscle Actin

BBB: Blood Brain Barrier

CA: Carbonic Anhydrase

CE: Cholesteryl Esters

CHO: Chinese Hamster Ovary

CNS: Central Nervous System

CS: Chondroitin Sulfate

CSC: Cancer Stem Cell

CSPG: Chondroitin Sulfate Proteoglycan

CME: Clathrin-mediated endocytosis

EC: Endothelial cell

ECM: Extracellular Matrix

EGFR: Epidermal Growth Factor Receptor

EVs: Extracellular vesicles

GAG: Glycosaminoglycan

GalNAc: N-acetylgalactosamine

GBM: Glioblastoma

GlcA: Glucuronic acid

GlcNAc: N-acetylglucosamine

GlcNS: N-sulfoglucosamine

GM-CSF: Granulocyte Macrophage Colony Stimulating Factor

GPC: Glypican

H: Hypoxia

HA: Hyaluronan

HIF: Hypoxia-Inducible Factor

HS: Heparan Sulfate

HSPG: Heparan Sulfate Proteoglycan

LD: Lipid Droplet

LDL: Low-Density Lipoprotein

LMWH: Low Molecular Weight Heparin

LPL: Lipoprotein Lipase

MAPK: Mitogen-Activated Protein Kinases

MDSC: Myeloid Derived Suppressor Cells

MMP: Matrix Metalloproteinase

N: Normoxia

PCR: Polymerase Chain Reaction

PFA: Paraformaldehyde

pO_2: Oxygen partial pressure

ROS: Reactive Oxygen Species

RTK: Receptor Tyrosine Kinase

SDC: Syndecan

SR-B1: Scavenger Receptor Class B Type 1

TAG: Triacylglycerol

TAM: Tumor-Associated Macrophage

TME: Tumor Microenvironment

VEGF: Vascular Endothelial Growth Factor

WB: Western Blot

Abstract

Cancer cells reside in a complex microenvironment comprising stromal cells and immune cells embedded in an extracellular matrix (ECM). Early on in tumor progression, cells reach a critical volume beyond which blood supply is impaired, leading to a decrease in oxygen levels (hypoxia) and nutrient supply. This situation triggers a stress response characterized by a metabolic switch to glycolysis, which will in turn induce acidification of the extracellular environment. Under hypoxia and acidosis, cancer cells adapt their integration of nutrients, signaling molecules, and more generally their exchanges with the extracellular environment not only to survive but also enhance tumor aggressiveness, metastasis and treatment resistance.

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Altogether, these studies further demonstrate the important role of PGs in adaptive mechanisms to tumor microenvironmental stresses at different levels, and pave the way for the evaluation of novel, potential therapeutic strategies.

Glioblastoma

This section will give a brief introduction to glioblastoma (GBM), the main tumor type studied in the present thesis work.

Epidemiology

Gliomas are CNS tumors that arise from the glial tissue and are the most frequent primary brain tumors, *i.e.* not resulting from the spread of tumors from distant sites, and represent about 80% of all malignant brain tumors[1].

GBM, grade IV according to the official WHO (World Health Organization) classification, is the most common and aggressive form of primary malignant glioma (about 45% of gliomas diagnosed)[2]. As of 2015, the average age-adjusted incidence of GBM is 3.2 cases for 100.000 people[3]. Though relatively low, this number is to relate to the associated poor prognosis, with an average median survival of 15 months after diagnosis, which has only improved by a few months within the last decade with the current standard of care[4]. Primary GBM affects patients aged 62 on average, while secondary GBM, evolving from lower grade astrocytomas (grade II-III) or oligodendrogliomas (grade II-III) usually affects younger patients (45 years median age)[5,6]. Most patients are diagnosed with GBM after experiencing headaches, confusion, blurred vision or seizures. The diagnosis is established by magnetic resonance imaging (MRI) showing gadolinium enhancement in T1-weighted images *(Figure 1)*, often with oedema, necrosis and/or haemorrhage[7,8].

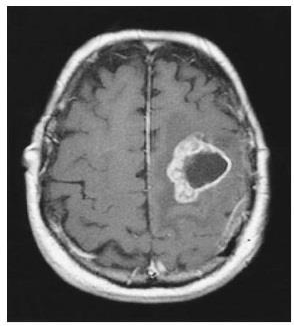

Figure 1: GBM MRI with Gadolinium contrast enhancement
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Histological analysis of biopsies or resected material and molecular subtyping (discussed further below) confirm the diagnosis and grading, *i.e.* the presence of pseudopalisading patterns around necrotic regions as well as vascular hyperproliferation is a feature of GBM *vs.* lower grades *(Figure 2)*.

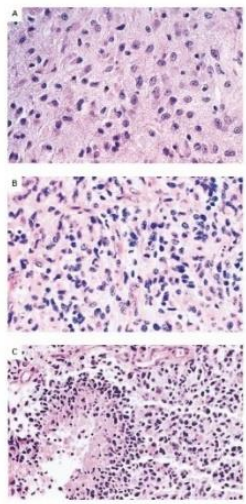

Figure 2: Histology of gliomas
Fibrillary astrocytoma (A), Anaplastic astrocytoma (B) and GBM (C), displaying pseudopalisading necrosis. Reproduced with permission from DeAngelis LM, N Engl J Med. 2001 Jan 11;344(2):114-23. Copyright Massachusetts Medical Society.

The etiology of GBM is still largely unknown though several possible causes have been discussed. Hereditary syndromes such as Li-Fraumeni, neurofibromatosis 1 and 2 and Von-Hippel Lindau disease are associated with an increased risk of

GBM[9], for which susceptibility loci have been identified in genome-wide association studies[10]. Nevertheless, heredity explains only about 5% of all gliomas[11]. Ionizing radiation, electromagnetic fields or pesticides have also been pointed out as factors increasing GBM onset in occupational studies[12,13] but others did not show any effects[14,15]. In another study, height and high BMI (Body Mass Index) were shown to increase the risk of GBM[16].

Histological and molecular aspects

GBM is histologically characterized by large areas of necrosis, surrounded by extensive, hypoxic pseudopalisading cell areas, which is a hallmark of GBM, grade IV tumors[17]. Grade IV classification depends also on the presence of sustained mitotic activity, microvascular proliferation (neovascularization) and nuclear atypia[18]. The tumor edges are poorly delineated and the degree of tumor cell infiltration very high, with invasion into the brain parenchyma by so-called "guerilla" cells[19]. Because of their glial origin, gliomas will frequently, but not always, stain positive for the GFAP (Glial Fibrillary Acidic Protein) marker, and the expression tends to be inversely correlated to tumor grade, even though this is not associated with increased tumor development *per se*[20].

Of particular relevance for the present studies, the brain extracellular matrix (ECM) has a specific composition compared to other organs, with very limited stiff structures of collagen and laminins, but enriched in PGs, that will be further discussed in the PG section, and hyaluronan[21]. This mesh of carbohydrate structures is particularly important for the trapping of soluble factors involved in proliferation and angiogenesis[22]. During the course of GBM progression, extensive ECM remodeling occurs, through matrix metalloproteinase (MMP) enzymatic activity[23], the secretion of fibrous proteins enhancing GBM cell motility[24,25] and angiogenesis[26].

At the genetic level, GBM has been profiled by the The Cancer Genome Atlas (TCGA) Research Network in a publicly available database[27]. This project as well as previous studies[28] further evidenced the heterogeneity and complexity of GBM, but established key genetic alterations. For instance, epidermal growth factor (EGFR) overexpression and specific EGFRvIII mutation[29], PTEN loss, TERT mutation, CDKN2A-p16^{INK4a} loss and 10q chromosome loss in primary GBM, IDH1/2 mutation, TP53 mutation and 19q, 10q and 22q chromosome loss in secondary GBM[30,31].

These mutations were associated to deregulation of major signaling pathways, *i.e.* the p53, RTK-Ras-MAPK, RTK-phosphoinositide 3-kinase (PI3K)-PTEN-Akt-mTOR, and the pRB (retinoblastoma) pathways[32-35].

The TCGA data was also used as a base to molecularly cluster GBM into four distinct subtypes: Classical (EGFR amplification), Neural (neuron markers), Proneural (PDGFRα, and IDH1 mutations) and Mesenchymal (NF1 loss)[36]. Since then, the advances in genotyping, molecular characterization and clinical findings on GBM have generated a more complex subclassification aimed at improving treatment decisions to target individual tumors. For instance, all diffusely infiltrative gliomas (astrocytic, *i.e.* GBM and oligodendroglial) have recently been regrouped[2].

To explain this genetic and phenotypic heterogeneity, two different models of cancer cell evolution are still being discussed, the first being clonal selection, by which tumor cells acquire genetic alterations over time and undergo a selection process that favors the ones resisting to their microenvironment. The second is the cancer stem cell (CSC) model, according to which a population of tumorigenic $CD133^+$ and $CD44^+$ cells, found in the hypoxic niches of GBM[37], self-renew[38], and resist chemotherapy[39] and radiotherapy[40]. The characteristics of this cell subpopulation would account for tumor cell replenishing by differentiation of the CSCs as well as recurrence after treatment.

Treatment challenges

The current standard of care of GBM is still maximal surgical resection of the tumor, followed by radiotherapy with concomitant and adjuvant chemotherapy using the alkylating agent Temozolomide (TMZ)[41].

Importantly, patients whose tumors show epigenetic silencing (methylation) of the O6-methylguanine–DNA methyl transferase (MGMT) are predicted to have a favorable treatment response to TMZ[42] due to impaired DNA repairing machinery after guanine alkylation. This is especially relevant in elderly patients, where the tumor MGMT status in some cases will decide whether the patient should be offered radiotherapy or TMZ as monotherapy.

Nevertheless, the very infiltrative and heterogenic nature of GBM makes complete surgical resection almost impossible to achieve in practice and relapse occurs in almost all cases. However, GBM rarely spreads outside of the brain, explained partly by the short life expectancy of GBM patients, or the normally tightly regulated endothelial cell junctions of the brain, *i.e.* the blood brain barrier (BBB)[43].

Interestingly, it was recently shown that astrocytomas (including GBM) could form a network of interconnected tumor cells through long microtubes. These structures allowed cells to communicate, invade and confer increased radiotherapy resistance as compared to isolated cells[44]. The information exchanged through this system and its relevance in tumor development remain to be elucidated.

Due to the cell heterogeneity and identified genetic aberrations, a large panel of therapeutic approaches have been considered, including anti-angiogenic treatment (bevacizumab)[45], tumor vaccination against EGFRvIII antigen (rindopepimut)[46], small molecules (RTK inhibitors), but so far all have been disappointing with lack of survival effect and primary or acquired resistance. Although several potential immunotherapy-based therapies are currently under evaluation, including PD-L1 blockade[47] and CAR-T cells[48], further biological understanding of the disease progression is thus still highly needed for GBM target identification.

In summary, GBM patients have a poor prognosis, but commonly display an acceptable performance status at the time of recurrence/progression. This makes them strong candidates for innovative intervention studies, and GBM biology a highly clinically relevant and interesting research subject. Indeed, it encompasses most of the clinical challenges encountered in tumor treatment. First, its largely unknown causes and intracerebral location, which represents a great challenge for screening and early diagnosis, surgical procedures but also therapeutic targeting. Also, deciphering the complex mutational and signaling profile, which drives aggressiveness, infiltration, angiogenesis, drug-resistance but also stemness properties. Then, the specific stromal and matrix brain environment that affects tumor progression, cell-cell communication and infiltration. Finally, and maybe most importantly, its heterogeneity and adaptive capacities. The progression of GBM is associated with large necrotic and hypoxic areas observed in patient tumors. This translates the ability of malignant cells to adapt and thrive in an oxygen and nutrient poor microenvironment to escape treatment while allowing disease progression. In these conditions, efficient therapeutic target selection is a real challenge.

The understanding of the adaptive mechanisms within this particular microenvironment could pave the way for novel targeting approaches, not only for GBM but also in the management of metastases originating from major tumor types, including breast, lung, and malignant melanoma.

The Tumor Microenvironment

A very simplistic view of a solid tumor would be to consider it as a bulk of oncogene driven cancer cells dividing and expanding as a homogenous, well-defined organ.

To fully understand the complexity of tumor biology, it is important to consider the interactions cancer cells can have with each other, but also with the different stromal cells, *i.e.* fibroblasts, adipocytes endothelial cells, pericytes, and a variety of immune cells that are recruited to and embedded in the extracellular matrix[49].

This so-called tumor microenvironment (TME) is essential in the acquisition of the hallmarks of cancer most comprehensively described by Hanahan and Weinberg[50]. In addition, the TME displays unique physico-chemical parameters, namely hypoxia (deprived O_2) and acidosis (low pH), which have a major impact on tumor aggressiveness *(Figure 3)*.

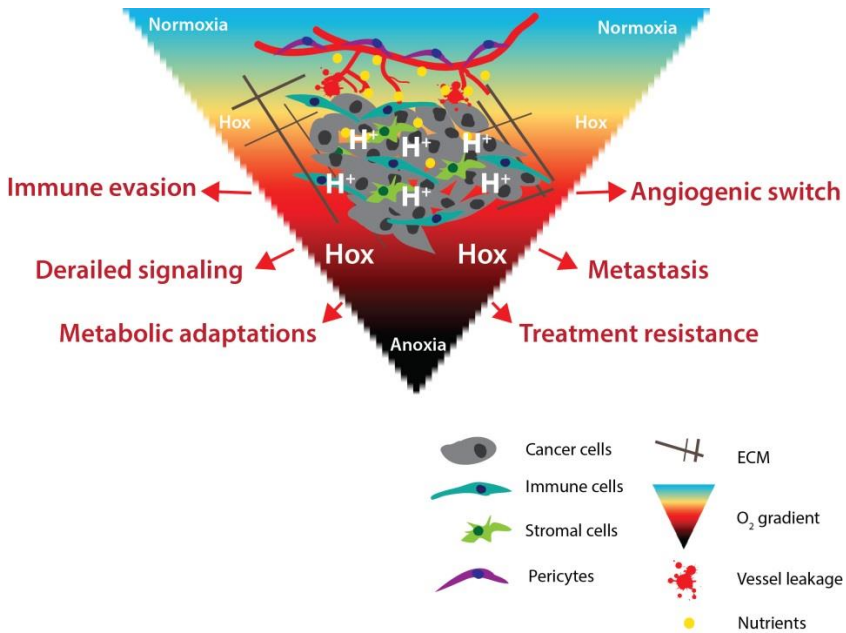

Figure 3: Overview of the TME
Hox: Hypoxia, ECM : Extracellular matrix, H^+: Acidosis regions

A common theme of the present study was to investigate cell adaptive mechanisms in the hostile TME. In this section, I will go into more details regarding the origins and roles of the different TME-associated conditions and components. These conditions are closely associated to cell metabolism changes with a special focus on lipid metabolism for the purpose of this thesis work.

Hypoxia

Uncontrolled cancer cell proliferation quickly leads to insufficient blood perfusion of the tumor regions located far from the vessel network, resulting in oxygen and nutrient scarcity. This situation of reduced O_2 availability compared to surrounding normal tissue is called hypoxia.

Chronic hypoxia arises from limited oxygen diffusion. Several studies based on diffusion models[51,52] or *in situ* measurements[53] indicated that the O_2 tension in tumors drops to near-zero values as close as 150–200 µm away from capillaries. It is conceptually important to keep in mind that this situation is not an on/off system but that a gradient of decreasing O_2 tension is established from the perivascular areas to the necrotic core, which represents the most obvious consequence of chronic hypoxia.

A situation somewhat closer to an on/off mechanism is during acute (or ischemic) hypoxia, occurring as a result of the disorganized, thrombotic, leaky vasculature of solid tumors. Thus, the blood flow is not continuous and O_2 levels can quickly drop or be restored leading to reoxygenation, and a phenomenon of cycling hypoxia, which is further discussed in **Paper I** and **II** of this thesis work.

The O_2 values to define hypoxia (chronic or acute) are relative, and tissue and situation-dependent. In the cancer context, studies by Vaupel *et al.* notably, measured pO2 across different tumor types *in situ* and confirmed reduced oxygen tension in tumors *vs.* normal tissue, but also established the presence of tissue areas with $pO_2 \leq 2.5$ mm Hg as characteristic of malignancies, hence defined as hypoxic[54].

Functionally, hypoxia has been linked to increased tumor aggressiveness through angiogenesis[55], immune evasion[56], coagulation[57], stemness[58], genomic instability[59] and metastasis by triggering epithelial to mesenchymal transition (EMT)[60,61]. Importantly, cycling hypoxia has been shown to further enhance these effects compared to hypoxia alone[62,63]. In the clinical setting, the volume of hypoxic regions has been correlated with shorter time to progression and decreased survival in GBM[64]. In addition, low O_2 concentration leads to decreased formation of free radicals, which confers resistance to radiotherapy[65].

The major signaling pathway involved in the hypoxic response is through the HIF (hypoxia-inducible factor). Initial studies on the erythropoietin (EPO) gene by Semenza *et al.*[66] identified specific hypoxia responsive elements (HRE, 5'–RCGTG-3') in the gene sequence, that was actively transcribed upon HIF protein binding under hypoxia. More than 100 genes can be activated as part of the HIF mediated response, and are widely involved in pro-tumorigenic processes[67,68]. Three analogs of HIF (HIF-1, 2, and 3) have been identified, with HIF-1 and HIF-2 being the most investigated so far. All three are heterodimeric, made of a stable β-subunit, and an oxygen-sensitive α-subunit. Under normoxia, the α-subunit is actively targeted for degradation by proline hydroxylation by prolyl hydroxylases (PHDs), recognized by the pVHL-E3 ubiquitin ligase complex[69]. In parallel, HIFs-α are prevented to bind to cotranscription factors (p300/CBP) by asparagine residue hydroxylation by asparaginyl hydroxylase factor–inhibiting HIF (FIH)[70]. Under hypoxic conditions, the activity of these enzymes is impaired and α-subunits can be stabilized. HIF-1 and HIF-2 differential regulation is still an active subject of research, but in general, HIF-1α seems to be more responsive to acute hypoxia (<24h) and degraded faster under chronic hypoxic conditions (48-72h) while HIF-2α is stabilized in milder, long term hypoxic conditions[71,72]. HIF-2 regulation is particularly relevant in the cancer stem cell perivascular niche context for instance[73]. Because of the relevance of the HIF pathway in cancer progression, several HIF inhibitors have been developed[74], which either reduce HIF-1α translation or its association with cotranscription factors for instance. The complexity of HIF pathway is a real challenge for compound validation, nevertheless promising drugs are in early clinical evaluation (*i.e.* PX-478[75]).

Importantly, the cellular responses to hypoxia are not exclusively HIF-mediated; work by Wood *et al.*[76] compared the hypoxic gene signature of HIF-1α wild-type and defective CHO cells and found that several genes of the established HIF-1α response were also upregulated in the absence of HIF-1α. Further, several groups[77,78] provided evidence for HIF-independent angiogenic activation mechanisms under hypoxic/ischemic stress for instance. The PI3K/Akt/mTOR pathways[79], or the unfolded protein response (UPR)[80,81] are also able to induce HIF-independent hypoxic responses that have particular implications for metabolic adaptations to the TME, discussed in the following sections.

Last but not least, O_2 deprivation has a major impact on cell bioenergetics and metabolism. Oxygen is essential for efficient ATP production as the final acceptor of electrons during oxidative phosphorylation (OxPhos) in the mitochondria. In the absence of oxygen, glucose is mostly used for glycolysis only and pyruvate further metabolized into lactate. This process is inefficient (2 mol ATP/mol glucose produced *vs.* 36 mol ATP/mol glucose for OxPhos) but confers an advantage to cells able to trigger elevated glycolytic rates under hypoxic conditions. Cancer cells have a very high energy demand for proliferation, and, as discussed

previously, alternate between cycles of hypoxia and reoxygenation, which eventually leads to selective proliferation of clones that have elevated glycolysis independently of the O_2 status. This characteristic of tumor cells is known as the Warburg effect[82].

The high glucose demand of malignant tumors has been used in the clinic for imaging, tracing the uptake of the radiolabeled glucose analog ^{18}F-fluorodeoxyglucose by positron electron tomography (FDG-PET), and can be of diagnostic and prognostic value in oncology. To image the hypoxic regions *in vivo*, the 18F-fluoromisonidazole (FMISO) tracer, reduced in low oxygen conditions, where it can accumulate is mostly used[83]. Compounds from the same family (nitroimidazoles) can also be used in pre-clinical studies (*i.e.* Pimonidazole).

The next section will present a direct effect of the metabolic switch to glycolysis, induced by hypoxia, which in turn is one of the hallmarks of the TME, acidosis.

Acidosis

Cancer cells secrete high amounts of lactic acid, the end product of glycolysis, into the extracellular environment, which dissociates into lactate + H^+. In addition, the pentose phosphate pathway (PPP), essential for nucleotide synthesis and NADPH production, is elevated in proliferating cancer cells[84]. For each mole of glucose-6-phosphate entering the PPP, one mole of CO_2 will be produced. This leads to a shift of the equilibrium between CO_2 and bicarbonate to bicarbonate and protons ($CO_2 + H_2O \rightarrow HCO_3^- + H^+$).

These two different mechanisms (lactic acidosis and hypercapnia) will lead to decrease of the extracellular pH[85,86]. Magnetic resonance spectroscopy measurements of tumor extracellular pH showed pH levels down to 6.1, while intracellular pH was generally just above pH 7[87]. These results indicate that tumor cells manage to maintain intracellular pH homeostasis. While CO_2 diffuses through the cell membrane bilayer, active transport of protons and acids through specific transporters and pumps is needed to perform this regulation.

Na^+/H^+ antiport exchangers (*e.g.* NHE1) are involved in proton extrusion to the extracellular environment and monocarboxylate transporters (MCTs) actively transport lactate and H^+[88,89]. Both MCT1 and MCT4 have been found to be upregulated by hypoxia[90,91]. Interestingly, different MCT isoforms show directional specificity in different microenvironments; MCT4 and MCT1 will actively export lactate and H^+ in hypoxic tissues, while MCT1 is also found in

actively proliferating cells in normoxic environments, including in endothelial cells[92], where it imports extracellular lactate to fuel the TCA cycle for ATP production[93].

Carbonic Anhydrases

Another important player of intracellular pH regulation is the carbonic anhydrase (CA) family[94]. CAs are membrane associated zinc metalloenzymes, which cooperate to catalyse the reaction $CO_2 + H_2O \rightarrow HCO_3^- + H^+$ in the extracellular compartment (CAIX and CAXII) or cytosol (CAII). The bicarbonate ions produced are re-imported into the cells by bicarbonate transporters (SLC4 family), the second component of this regulation machinery, where they will buffer intracellular protons *(Figure 4)*.

Among the 16 different CA isoforms in mammals[94], CAIX, and to a lesser extent CAXII, are strongly tumor-associated and upregulated by hypoxia in solid tumors[95-98]. CAIX also shows higher activity than CAXII[99]. Its expression is associated with poor prognosis, treatment resistance[100] and tumor growth *in vivo* and *in vitro*[101-103] making CAIX a very attractive marker and target of the hypoxic and acidic microenvironment. Originally, CAIX was identified as a 54/58 kDa membrane dimeric protein using the M75 monoclonal antibody (mAb)[104], later found to recognize motifs of the PG domain of CAIX[105]. Further characterization and mechanistic studies of CAIX is the focus of **Paper IV** of this study.

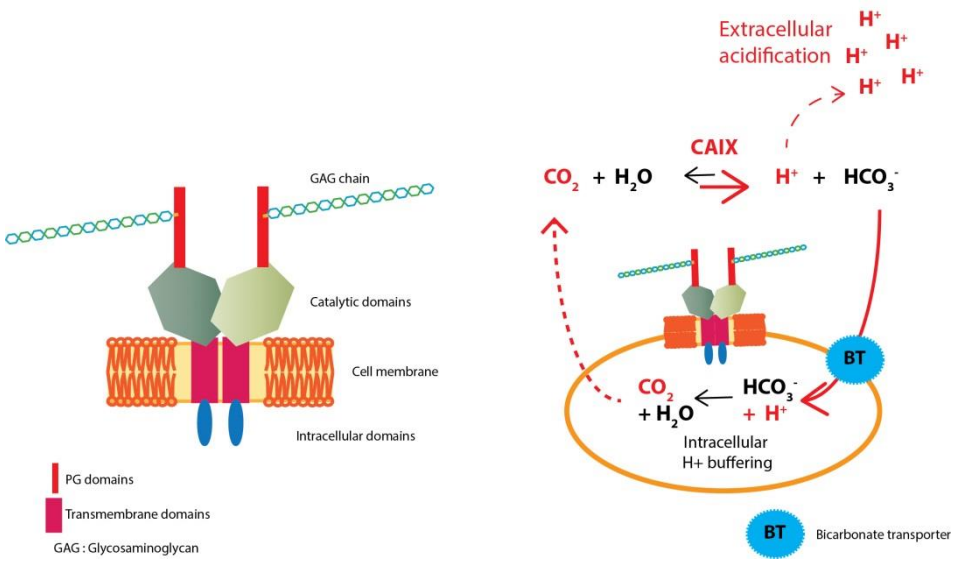

Figure 4 : CAIX structure (left) and catalytic activity (right)

Beyond hypoxia, acidosis is thus another element of strong selective pressure applied on tumor cells in the TME. Even though the kinetics and spatial distribution of hypoxia and acidosis do not completely overlap, *i.e*. tumor regions can experience one or both stresses, the functional effects of hypoxia and acidosis identified so far point in similar directions and lead to increased cell motility and metastasis[106], stemness[107], ECM remodeling and invasion[108], and genomic instability[109]. At the signaling level, short term extracellular acidosis has been shown to activate the p38 and ERK1/2 mitogen-activated protein-kinase (MAPK) pathway as well as downstream CREB transcription factor phosphorylation[110].

Importantly, similarly to hypoxia, acidosis is also a consequence of impaired tumor vasculature preventing the diffusion of cytotoxic metabolites and acids, which accumulate in the extracellular space. Angiogenesis, the formation of new blood vessels, which is one of the hallmarks of cancer and of the adaptive response to the TME will be discussed next.

Angiogenesis

To overcome oxygen and nutrient deprivation, tumor cells, and to an even larger extent, tumor cells under hypoxia and acidic stress secrete pro-angiogenic factors to co-opt preexisting vessels and recruit endothelial progenitor cells to build new vasculature. VEGF-A is the master regulator of endothelial cell activation and angiogenesis, and its expression is robustly hypoxia-induced[111,112]. In addition, glioma cells have been shown to upregulate the expression of VEGF-A at low pH and hypoxia *in vivo* in an independent manner[113,114]. Other major pro-angiogenic factors include FGF-2, HB-EGF, IL-1α, IL-8, TNF-α, TGF-β and angiopoietins.

The recruitment of pericytes will enable better stabilization and maturation of the new vascular network. Platelet derived growth factor subunit B (PDGFB), angiopoietin 1 (ANGPT1), and MMPs are known factors involved in pericyte recruitment. ANGPT1 produced by pericytes will subsequently stabilize ECs through TIE2 receptor tyrosine kinase binding. In the brain, the pericytes play a major role in maintaining the BBB integrity[115]. Nevertheless, the newly formed vessels are dysfunctional, with aberrant morphology, excessive branching and leakiness[116].

Beyond the well-established role of hypoxia and low pH in increased tumor cell secretion of pro-angiogenic, soluble factors, previous studies from our lab showed an important role of secreted extracellular vesicles (EVs) in the hypoxic GBM

microenvironment for EC activation and angiogenesis[117]. Besides, ECs are also able to adapt to hypoxia and maintain a high level of proliferation through enhanced glycolytic activity[118]. Most innate immune cells of the TME also exert pro-angiogenic activity through angiogenic factors and cytokine release, which will be further discussed in the next section.

The ECM composition is of importance in the angiogenic processes, as ECs must degrade the basement membrane through MMP secretion and recruitment of fibroblasts that actively remodel the matrix composition. The ECM is also a reservoir for matrix protein-binding angiogenic factors. VEGF-A and FGF-2 can be sequestered by HSPGs for instance and released under proteolytic activity or by the action of heparanase[119-121]. Several matrix proteins have been found to exert pro-angiogenic activity as such, for instance tenascin C, fibronectin and perlecan[122].

Given the importance of angiogenesis in tumor development, already postulated in 1971 by Folkman[123], anti-angiogenic therapy has been a very attractive field of research. Anti-VEGF-A monoclonal antibody (Bevacizumab) was first FDA approved for cancer treatment in 2004 and later in 2009 for recurrent GBM[124]. However, the redundancy of angiogenic factors, resistance to the treatment[125] as well as severe side effects (bleeding, thrombosis) have led to limited clinical benefit for most patients. Worse, in some cases bevacizumab treatment may aggravate the hypoxic status of tumors, eventually rendering them more aggressive after treatment arrest[126]. Other strategies such as decoy soluble receptors for FGF-2, ANGPTs, and VEGF-A are being studied, with one compound against VEGF-A recently FDA approved for metastatic colorectal cancer[127]. Most other FDA approved compounds against angiogenesis are multi-RTK inhibitors (*i.e.* sorafenib[128], sunitinib[129]) targeting the activity of VEGFR and PDGFR isoforms. The advantage of multi-RTK inhibitors is their broad spectrum of action that can circumvent angiogenic pathway redundancy. Another promising strategy to overcome resistance lies in the development of combination therapies of anti-angiogenic agents with agents targeting the hypoxic/acidic response, *i.e.* TH-302 alkylating agent[130], and clinical trials are ongoing[131]. Combinations with CAIX inhibition have been investigated but have not proved successful so far.

Immune cells of the TME

Cancer cells of the TME are immunogenic and actively recruit immune cells[132,133].

However, there is large evidence that under microenvironmental stress conditions, the phenotype of the immune cells is switched towards activation of

immunosuppressive and tumor growth promoting roles[134-136]. Innate immune cells, *e.g.* myeloid derived suppressor cells (MDSCs), tumor associated macrophages (TAMs), dendritic cells, and neutrophils, are for instance associated with tumor progression, and hypoxia reinforces their pro-inflammatory functions while inhibiting the T-cell mediated adaptive immune system[137]. Interestingly, acidosis has been shown to have similar immunosuppressive effects[138,139].

Hypoxia triggers the differentiation of MDSCs into TAMs[140] and TAMs are associated with poor prognosis[141], VEGF overexpression and angiogenesis[142], ECM remodeling and metastasis[143]. TAMs are usually M2-polarized macrophages (pro-tumorigenic) according to the classical M1/M2 phenotype definition[144], and both hypoxia and acidosis have been shown to induce M2 polarization[145,146]. The M1 phenotype (anti-tumorigenic) is driven by interferon-γ, lipopolysaccharide (LPS), GM-CSF while M2 differentiation depends on M-CSF (CSF-1), IL-13, and IL-4 stimulation. However, heterogeneous populations exist between these two phenotypes[147], and GM-CSF levels for instance have been correlated to both pro- and anti-tumorigenic effects[148]. In gliomas, where macrophages and microglia (regrouped under the term GAMs, by analogy with TAMs) can occupy as much as 30% of the tumor bulk and are believed to mostly resemble the M2 subtype[149], CSF1 has been shown to promote tumor progression without inducing M2 polarization of GAMs[150]. In another study, Gabrusiewicz *et al.*[151] profiled GBM infiltrating GAMs and found in majority MDSCs and resident microglia, and intriguingly showed that recruited macrophages were primarily resembling the M0 ("inactivated") phenotype. These findings highlight the complexity of immune cell regulation in the TME and the vast plasticity of TAMs.

Several leading-edge therapies aimed at alleviating immunosuppression are currently under very active development and are being implemented in the clinic, including cancer vaccines and checkpoint inhibitors such as the anti–CTLA-4 (ipilimumab) and anti-PD-L1 (pembrolizumab) monoclonal antibodies[152]. Interestingly, similar to anti-angiogenic treatments, hypoxic adaptation impairs treatment benefits, for instance through hypoxic upregulation of PD-L1 expression[153]. Hence, combined strategies aimed at reducing hypoxia-induced immunosuppression during checkpoint inhibition may be key. One such approach has been evidenced by Chafe *et al.*[154], showing that CAIX was needed for G-CSF mediated MDSC recruitment by hypoxic breast cancer cells, making CAIX an interesting target for combination therapy.

All in all, like tumor cells and other TME associated cells, immune cells display strong adaptive mechanisms under the influence of hypoxia and acidosis leading to cancer cell survival and tumor progression. In the next part, I will briefly introduce

major metabolic adaptations of cancer cells, and how they affect disease progression.

Metabolic adaptations in the hypoxic TME

The deregulation of cellular energetics is one of the emerging hallmarks of cancer[50]. To cope with their high energy and "building blocks" demand, cancer cells actively upregulate several nutrient uptake and biomolecule synthesis pathways. For instance, glucose transporters (GLUTs), amino acid uptake and lipid synthesis are upregulated[155]. These pathways are mostly under the control of PI3K-Akt-mTOR axis, one of the major signaling pathways activated in GBM, and the c-Myc oncogene for glutamine anaplerosis[156- 158].

These main characteristics of cancer cell metabolism for sustained proliferation are just one side of their inherent plasticity and resource use optimization *(Figure 5)*.

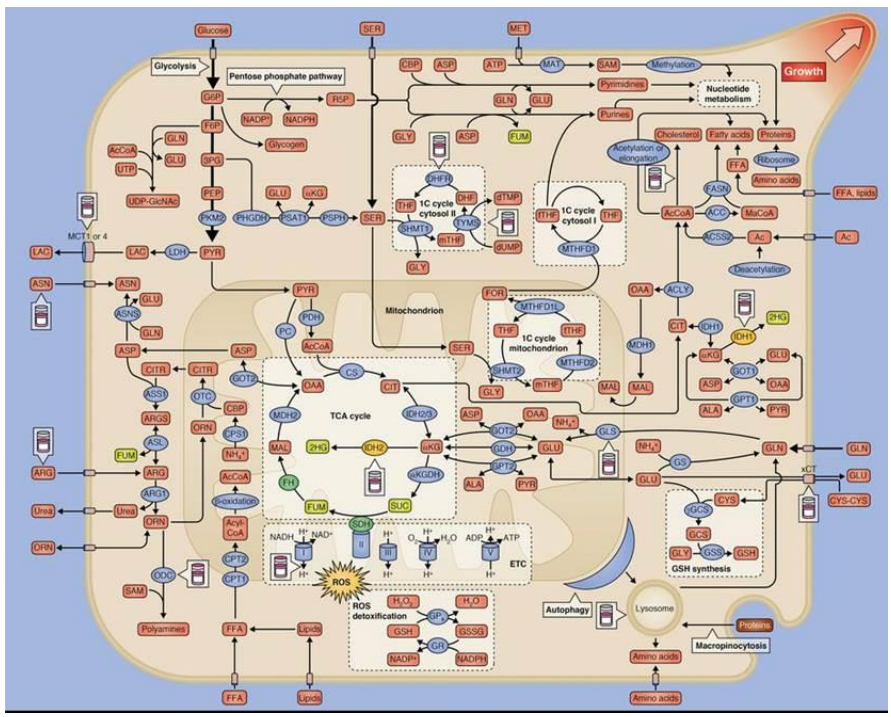

Figure 5 : Overview of the metabolic network of cancer cells

Indeed, under microenvironmental stress conditions, further level of regulation occurs. In addition, for similar reasons to the fact that hypoxia is not an on/off mechanism, the glycolytic phenotype and other metabolic phenotypes are not homogenous within the tumor and largely depend on nutrient availability, oxygenation and pH conditions[159].

This heterogeneity also leads to metabolic symbiosis between tumor compartments under different stress conditions, as introduced in the Acidosis section regarding the roles of MCTs in different microenvironments. These metabolic exchanges of resources can be homocellular (between similar cell types) or heterocellular (*e.g.* between adipocytes and cancer cells)[160,161].

Cancer cells can then either be seen as "picky" eaters[162] but most likely rather "opportunistic" eaters[163]. In the light of the previous work from our lab on extracellular lipid vesicle uptake[164] and the present studies in **Paper I** and **II**, the next focus will be on lipid and lipoprotein dependent mechanisms.

Evidence for extracellular lipid contribution to cancer

It has become evident that obesity and dysregulated lipid metabolism is associated with increased risk of cancer-associated mortality. A prospective study on 900.000 US residents estimated that around 20% of cancer deaths in women could be attributed to overweight and obesity[165], and excess bodyweight was associated to higher risk of a large range of malignancies in a meta-analysis of more than 280.000 incident cases[166].

Actively dividing cells require lipids at different levels, as energy source, components for membrane remodelling, or active signaling molecules for instance.

Lipids can either be *de novo* synthetised or acquired from extracellular sources. Cancer cells overexpress several enzymes involved in fatty acid and cholesterol synthesis pathways, mainly through the PI3K-Akt-mTORC1-SREBP axis (sterol regulatory element binding protein) master regulation[167- 169].

However, accumulating evidence indicates that uptake of extracellular lipids and lipoproteins can be an important mechanism of the malignant cell phenotype. Systemic lipid levels have been associated with increased tumor aggressiveness and enhanced metastasis in a hyperlipidemic mouse model[170], and tumors may actively recruit lipids from adipose tissue for sustained proliferation[171]. In the same line, SREBP targeting as therapeutic strategy was exclusively efficient in triggering apoptosis and alleviating GBM growth in lipoprotein deficient conditions, both in normoxia and hypoxia[172,173]. These findings suggest that

exogenous lipoproteins can sustain tumor growth when lipogenesis is impaired. Kamphorst *et al.*[174] showed that under hypoxic conditions, RAS transformed cells could bypass lipogenesis by scavenging extracellular fatty acids. At the pathophysiological level, observations on the decrease of total cholesterol levels in cancer patients[175] and lipid mobilization during cachexia[176] may also reflect the contributions of extracellular lipids to advanced disease progression.

Altogether, these studies point at a direct role of exogenous lipid availability on tumor growth rather than obesity-induced inflammation or hormonal changes.

Moreover, another hallmark of lipid metabolism in cancer cells is the detection of prominent intracellular lipid storage under the form of lipid droplets (LDs), as seen for instance by NMR spectroscopy[177], which has been linked to poor clinical outcome in breast cancer[178]. In glioma, Tugnoli *et al.*[179], could correlate vascular proliferation and tumor grade with the presence of cholesteryl esters (CE) and triacylglycerol (TAG). A study by Yue *et al.*[180] provided evidence for increased LD storage in PTEN-deleted prostate cancer *in vivo* that was dependent on exogenous lipoprotein uptake.

The mechanisms and functional roles of these phenotypes are still largely unknown. Based on previous work by our group on heparan sulfate PGs (HSPGs) as surface receptors involved in particle uptake and signal transduction[181], and the fact that HSPGs have been shown to mediate lipoprotein uptake in non-malignant cells[182-185], in **Paper I** we investigated their potential roles in tumor cell metabolic adaptive mechanisms to hypoxia and acidosis. The next sections will detail the characteristics of the two main lipid particle types at the core of the present study: Lipoproteins and intracellular LDs.

Lipoproteins

Definition – Structure - Biogenesis

Lipoproteins are the main lipid carrier particles in the blood. The main lipoprotein classes, High-Density Lipoprotein (HDL), Low-Density Lipoprotein (LDL), Very Low-Density Lipoproteins (VLDL) and chylomicrons differ in their origins and lipid composition, but are all surrounded by a single phospholipid membrane and specific patterns of apolipoproteins.

Dietary lipids (triacylglycerols (TAGs), phospholipids and cholesteryl esters (CEs)) are hydrolyzed by lipases in the intestinal lumen and repackaged in the endoplasmic reticulum of enterocytes into large TAG-rich chylomicrons (~1 µm diameter, surrounded by apolipoprotein (Apo) B-48, ApoA, ApoC and ApoE) for

release into the systemic circulation. After lipolysis at peripheral tissues, chylomicron remnants are taken up by the liver.

VLDL (~70 nm diameter, ApoB-100, ApoC, and ApoE-containing) are synthetized from liver fatty acids, free cholesterols and CEs. VLDL binds lipoprotein lipase (LPL) releasing fatty acids for peripheral tissues. As it loses TAGs, VLDL is enriched in CEs and loses ApoE to become LDL (~25 nm diameter, ApoB-100-containing). VLDL also gives rise to cholesterol-rich HDL particles (~10 nm, containing ApoE and ApoA).

The lipoproteins are transported mostly through blood circulation but can also be found in the lymph[186] as well as in cerebrospinal fluid for some types, with local synthesis in the CNS (ApoE, ApoJ-containing particles)[187]. In the case of undisrupted BBB, systemic lipoproteins are usually not allowed to enter into the brain, but reports have shown HDL[188] and LDL[189] transcytosis occurring through brain endothelial cells.

Uptake mechanisms

Lipoproteins are classically taken up in peripheral tissues by cell surface receptors that can either mediate whole particle uptake (*i.e.* LDLR, LRPs, scavenger receptor type A) or selective lipid exchange at the cell surface (*i.e.* scavenger receptor type B1 (SR-B1), or CD36). LDLR binds VLDL and LDL through ApoE and ApoB100, whereas LRP1 binds VLDL through ApoE but not LDL. VLDLR is structurally very similar to LDLR and binds ApoE-containing lipoproteins. Additionally, LPL binding to LRP1 and VLDLR will selectively release fatty acids from TAG-rich lipoproteins [190,191].

Scavenger receptors also play an important role in native lipoprotein uptake; SR-B1 binds LDL and HDL and mediates the selective uptake of cholesterol from these particles[192-194]. The closely related CD36 receptor also binds native lipoproteins with high affinity[195], and is involved in both cholesterol and free fatty acid uptake[196]. Importantly, CD36 has recently been suggested as a key target for metastasis initiating cells of oral squamous cell carcinoma through increased fatty acid internalization[197].

In addition to these receptors, cooperative interactions between PGs, lipoprotein receptors and lipoproteins for internalization are well established[198,184]. ApoB-100 and ApoE bind PGs[199-201] and the heparin and PG binding sites have been identified in each apolipoprotein[202-204]. In both cases, the interaction is mediated by electrostatic interactions between the negatively charged PG and clusters of basic amino acids of the apolipoproteins. The details of these cooperative models are not fully elucidated, but one can easily speculate based on the atherosclerosis model

that PG GAG chains sequester and concentrate lipoproteins in the vicinity of lipoprotein receptors, and that this cooperation would be of particular relevance during nutrient scarcity. LPL also binds HSPG and represents another level at which GAG chains are involved in lipoprotein clearance[205].

Other mechanisms of interactions have also been studied, in which releasing the interaction between lipoprotein receptor and HSPG resulted in unmasking of lipoprotein binding sites on HSPG and increased lipoprotein receptor-independent internalization through HSPG[206]. Indeed, evidence for the role of HSPG as independent endocytic receptors of TAG-rich lipoproteins is accumulating[207].

Lipoproteins and cancer

Several interesting studies have begun to unravel the contribution of lipoproteins to tumor progression. In a GBM model, the common EGFRvIII mutation has been found to enhance lipoprotein uptake through SREBP-mediated upregulation of LDLR[208]. This dependence on LDLR mediated cholesterol uptake has also been shown in pancreatic cancer[209] and breast cancer[210]. Cholesterol uptake from HDL and LDL enhanced proliferation in lymphoblastic leukemia and was associated to LD storage[211]. LDL cholesterol has also been shown to promote colorectal cancer progression through reactive oxygen species (ROS) and MAPK signaling[212]. Interestingly, in addition, a recent study by Huang *et al.*[213] provided evidence for a role of tumor-induced increase in circulating LDL and VLDL in tumor growth.

Notably, apart from their role in lipoprotein internalization, classical lipoprotein receptors (LDLR, VLDLR, LRP1) are also indirect signal transducers through association of their cytoplasmic tails with intracellular scaffolds. They can be phosphorylated to trigger endocytosis or recruit adaptors (PID, PDZ domain proteins) to mediate MAPK activation for instance[214]. In this scenario, lipoprotein receptors could be activated not only by lipoproteins but by a wide variety of extracellular ligands, and orchestrate a cross-talk between nutrient recruitment and down-stream signaling events.

Beyond adipocytes, lipid metabolism symbiotic mechanisms in the TME are largely unknown. However, one interesting observation regarding the structure and mechanistic details of lipoprotein particle uptake is their shared properties with EVs.

Similarities with extracellular vesicles

EVs are important mediators of cell-cell communication in the TME and enhanced tumor aggressiveness, especially in the context of stress adaptation[117].

Contrary to lipoproteins, EVs are surrounded by a bilayer membrane, but their size and density largely overlap, conferring them LDL-like behavior, which makes their discrimination a real challenge with current isolation techniques[215].

In addition, EVs have been shown to carry most apolipoproteins[216] and be able to transfer a variety of cargoes, including lipids, signaling molecules and miRNAs[217].

Importantly, EVs have also been shown to depend on HSPG for their internalisation, as well as carry GAG chains themselves[164], suggesting possible interactions between the two particles, potentially affecting their internalization[218].

In the TME, the EV effects are not only mediated by their protein content but also by their lipid cargo. Cancer associated fibroblasts transfer substantial amount of lipids and protein through ectosomes that support tumor cell growth[219]. In addition, hypoxic prostate cancer cells have been shown to upregulate TAG synthesis and transfer through EVs resulting in increased tumor growth in post-hypoxic phase[220]. Exosomes (*stricto sensu*) have a specific lipid enrichment profile compared to cells and are usually enriched in cholesterol, sphingomyelins, glycosphingolipids[221] but this composition is variable depending on cell type, experimental conditions and the type of EV studied, indicating that the extracellular lipid cargo available within the heterogenous TME is very diverse.

Downstream of lipoprotein and EV uptake, the internalized proteins are bound to lysosomal degradation and CEs and TAG can be stored under the form of LDs.

Lipid droplets

Definition – Structure - Formation

LDs are intracellular organelles involved in the storage of neutral lipids. They are gaining increasing interest as evolutionary conserved structures, also present in prokaryotes[222] under different forms and compositions. Their biology is more complex than their name would suggest, and they are involved in several pathological conditions including obesity, inflammation, viral infection, atherosclerosis and cancer[223-227].

LDs are composed of a core of TAGs and CEs surrounded by a single phospholipid membrane decorated with structural and functional proteins (perilipin family, Fsp27 (CIDEC), DGAT, caveolin-1, lipases, among others), which regulate their packaging and lipolysis. From their lipid and protein composition, LDs are thought to originate from the ER[228], and are often found in close proximity to it. Their size can vary up to ~10 µm for non-adipocyte cells and to whole cell size for adipocyte LDs[229].

Importantly, lipids stored in the LDs are mobilized when needed. They can be hydrolysed to free cholesterol by cholesterol hydrolases and to fatty acids by the TAG lipases (Adipose Triglyceride Lipase (ATGL), Hormone Sensitive Lipases (HSL) and Monoglyceride Lipases (MGL)) or digested by autophagy[230,231].

Lipid droplets in cancer

Following the findings of increased LD storage in high grade patient tumors, LDs have also been gaining strong interest in cancer biology.

LD formation has been shown to depend on various factors in different cancer types, but invariably associates with tumor aggressiveness. LD formation has been associated to SREBP-1 mediated upregulation of lipogenic enzymes, increased extracellular lipid uptake, and hormone receptor status in breast and prostate cancer[232].

Our studies (**Paper I** and **II)** and others have shown increased LD content in hypoxic regions of GBM[233] and a study by Bensaad *et al.*[234] suggested increased, HIF1-dependent fatty acid uptake as a mechanism behind these observations. In addition, hypoxia-driven overexpression of LD coat proteins (*i.e.* PLIN-2[235], HIG2[236]) facilitate LD formation, and fatty acid β-oxidation is inhibited in hypoxia[237], which further contributes to the lipid storing phenotype.

LDs act functionally as reservoirs for membrane building blocks and fatty acids for mitochondrial β-oxidation during the proliferative phase, but also as substrates of pro-tumorigenic and inflammatory factors such as cyclooxygenase 2 (COX2)[238]. The storage of TAG in LDs in hypoxia may also prevent the formation of toxic ROS that are produced by an excess of intracellular palmitate[234,239].

Interestingly, a study from Mitra et al.[240] identified that circulating tumor cells isolated from metastatic prostate cancer patients were all lipid-loaded and that this phenomenon correlated with increased LD formation *in vitro* in metastatic *vs.* non-transformed cells. Based on these findings, the authors suggested that LD may serve as a biomarker of metastatic cells, which introduces another interesting aspect of the lipid-loaded phenotype.

Therapeutic considerations

Pathways that select for tumor cells that successfully adapt to microenvironmental stress, further resulting in resistance to conventional cancer therapies, also represent a potential Achilles' heel of the cancer cell machinery and thus may offer alternative treatment targets of cancer.

Strategies aimed at blocking cholesterol esterification (*i.e.* avasimibe targeting SOAT/ACAT) or fatty acid synthesis (*i.e.* C75 targeting FASN) are attractive as many compounds are readily available. Interestingly, SOAT1 is highly expressed in GBM and targeting of SOAT1 with avasimibe suppressed GBM growth[241]. Avasimibe is unavailable for human use; however, mitotane (Lysodren), which has been used for decades to treat patients with adrenocortical carcinoma was recently shown to act through inhibition of SOAT1[242]. These compounds have never been tested against GBM in the clinical setting. Nevertheless, several studies point to an anti-cancer effect of statins[243,244], which motivates further evaluation of these strategies.

On the contrary, the increased extracellular lipid avidity could be exploited therapeutically for drug delivery. Several groups are developing lipoprotein-binding compounds or engineered lipoprotein-like nanoparticles that could be used for specific tumor targeting[245-247]. These strategies are conceptually interesting, but further understanding of the specific adaptive mechanisms of the TME are essential to dissect out the mechanisms and hence improve the specificity of the designs.

To conclude the above sections, environment sensing, macromolecular exchange and communication are essential for cancer cell survival under TME stress conditions. These processes are mainly regulated through controlled exchange of material and signal integration between the extracellular compartment and the cells. An overview of the mechanisms of endocytosis will be given in the next section.

Endocytic mechanisms

Endocytosis encompasses all the energy-dependent processes aimed at internalizing extracellular cargo within intracellular membrane vesicles. The endocytic mechanisms have different degrees of cargo selectivity but constitute hubs for signaling transduction of relevance for the adaptive response of cancer cells in the TME.

Cancer cell endocytosis pathways

Macropinocytosis

Macropinocytosis is the unselective internalization of relatively large pericellular fluid volumes (0.2-10 µm)[248] through actin cytoskeleton remodelling leading to membrane ruffling. The protrusions will then collapse and fuse with the membrane, creating a vesicle, the macropinosome. The internalized macropinosomes can then be recycled to the plasma membrane or directed to late endosomes and lysosomes for degradation. CSF-1 or EGF stimulation are known to induce macropinocytosis[249], which is dependent on PI3K activity[250] and Rac1 Rho-GTPase activation[251]. Macropinocytosis is involved in macrophage LDL uptake leading to foam cell formation[252] and represents a major nutrient protein uptake route in the upstream RAS-transformed tumor cells[253]. *In vitro* macropinocytosis can be followed by fluid phase markers such as dextran and inhibited with wortmannin (PI3K inhibitor).

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) was originally studied for its major role in LDL uptake through LDLR[254]. Clathrin-coated pits are formed at the membrane by clathrin triskelion assembly with adaptor proteins (AP2)[255], mostly in regions of the membrane enriched in phosphatidylinositol-4,5-bisphosphate (PtdIns4,5P2)[256]. The GTPase dynamin will then enable closure and formation of the clathrin coated vesicles (120 nm)[257] and can thus be targeted to prevent endocytosis[258]. The

vesicles are then intracellularly sorted to early endosomes for membrane recycling or maturation to late endosomes where receptors targeted for degradation are ubiquitinated, and recognized by the ESCRT machinery[259]. CME has an important role in receptor mediated signaling, either sustaining signaling activity by receptor recycling to the membrane or signal downregulation by directing receptors for lysosomal degradation[260]. CME is also the major constitutive entry pathway for transferrin receptor 1[261], and its ligand transferrin is commonly used for *in vitro* studies of CME.

Caveolin-mediated endocytosis

This endocytic pathway relies on the formation of caveolae, *i.e.* invaginations of the cell membrane stabilized by oligomers of caveolin proteins[262]. In addition to caveolins (mostly caveolin-1), cavin proteins (mostly cavin-1) are critical for caveolae structures (60 nm)[263]. Similar to CME, caveolae internalization is dynamin-dependent and can be triggered by receptor signaling activation, *i.e.* Src kinase for the gp60 albumin receptor[264,265]. Caveolae associate with membrane regions enriched in cholesterol and sphingolipids (lipid rafts), and caveolin-1 binds cholesterol[266]. Lipid rafts are components of the cell membrane organization, and cluster proteins in the membrane laterally[267]. As such, they are important platforms for signaling transduction. They can be isolated by their resistance to cold Triton X-100 detergent treatment[268], and are enriched in glycophosphatidylinositol (GPI)-anchored proteins[269]. Lipid rafts stain positive for the GM1 ganglioside marker Cholera Toxin subunit B (CTxB).

Thus, caveolin-mediated endocytosis is sensitive to cholesterol depletion by *i.e.* methyl-β-cyclodextrin, even though caveolin knockdown approaches are more specific to perturb this pathway. Importantly, surface recruitment of caveolin-1 can also mediate effects that are not directly related to caveolae endocytosis. For instance, Shvets *et al.*[270] have demonstrated that caveolins can reorganize the cell membrane proteome by excluding proteins out of caveolae-ruffling regions. Caveolae are important for lipid homeostasis, but the mechanisms are pleiotropic, involving *e.g.* fatty acid trafficking and lipid droplet formation, and the systemic effects observed are not fully understood[271]. Interestingly though, caveolin-1 is needed for uptake and transport of albumin in ECs[272] and Frank *et al.*[273] demonstrated a role for caveolin-1 in LDL transcytosis in endothelial cells as well as increased HDL-cholesterol levels in a caveolin-1 deficient mouse model.

Clathrin- and caveolin-independent endocytosis

These pathways are relatively less well-defined and diverse; however, they are associated with lipid rafts and their dynamin and Rho GTPase dependence are commonly used for subclassification

The clathrin independent carrier pathway (CLIC) is a dynamin independent pathway regulated by the cdc42 Rho GTPase family. It is involved in GPI-anchored protein internalization[274], and forms long tubular structures called GPI-anchored protein enriched compartments (GEECs). Their sorting to endosomes is PI3K and Rab5 dependent[275]. Other pinocytic pathways (~90 nm vesicles) include the Arf6 dependent pathway (dynamin independent)[276], flotilin dependent pathway (dynamin independent)[277] and the RhoA/Rac1 regulated IL2 receptor pathway (dynamin dependent)[278].

Targeting endocytosis through antibody-drug conjugates

In cancer, the endocytic pathways can be affected through the control of oncogenic events, membrane composition, and signaling activity that are further modified by the microenvironmental conditions. This leads to increased aggressiveness, motility and eventually metastasis[279].

In the context of targeted therapy through endocytic pathways, antibody drug conjugates (ADCs) have gained considerable interest. ADCs are usually made of three important parts, the monoclonal antibody recognizing the surface antigen of interest, fused to a cytotoxic payload via a linker region, which can be cleaved or not depending on the ADC design[280] *(Figure 6)*. The cleavage strategies include low pH labile constructs, or specific proteolytic cleavage in lysosomes, as well as non-specific proteolytic degradation of the antibody in the case of non-cleavable linkers. To date only two ADCs remain FDA approved, Brentuximab (Adcetris) targeting CD30 in lymphomas[281] and Trastuzumab-DM1 (Kadcyla, T-DM1) against HER2 in metastatic breast cancer[282]. In both cases the payload is a tubulin disrupting agent (either auristatin or maytansinoids)[283] causing cell cycle arrest and cell death upon release. Over 40 ADCs were in clinical trials in 2016[284]. The challenges associated with ADC development are to find antigens that are specifically expressed on the target cells while minimally in other tissues, that the antigen is internalized efficiently and that the linker/payload chemistry used enables efficient drug release during endocytosis and intracellular trafficking, while being stable in the circulation. In addition, ADC penetration in the tissues can be improved through the use of smaller antibody fragments, aptamers or scaffolds (affibodies)[285]. The findings of **Paper III** and **IV** provide further

understanding of the tumor biology and endocytic mechanisms in the TME that can help designing innovative ADC targeting strategies.

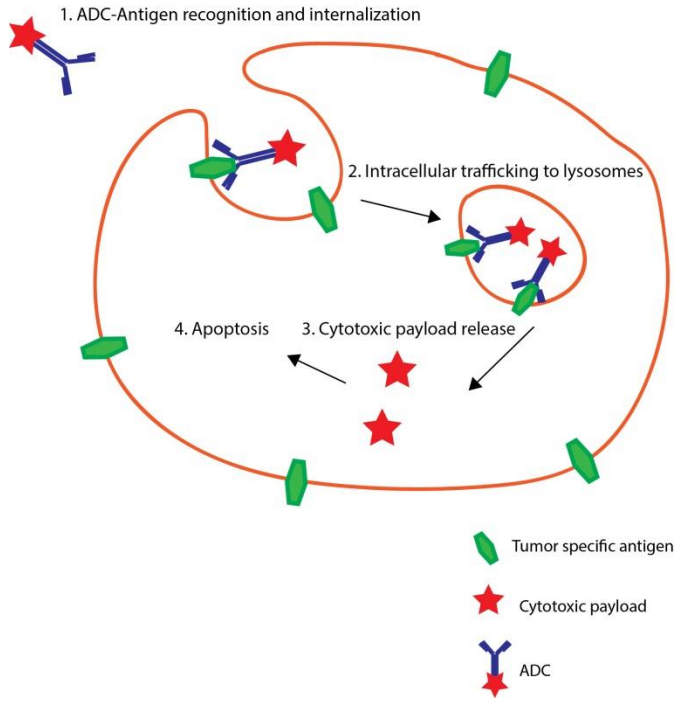

Figure 6: General ADC targeting mechanism

The aim of **Paper III** was to identify specific, hypoxia-induced, internalized proteins, and **Paper IV** reveals a novel role of GAG conjugation in cell surface protein internalization. Among the many relevant cell surface associated receptors, able to mediate cargo internalization and signal transduction, we have found that the PG class is of major importance.

Proteoglycans

General definition

PGs are a group of glycosylated proteins that have been studied originally from mucus and cartilage. They are well conserved throughout evolution, and are produced by virtually all mammalian cells. PGs have been identified intracellularly (serglycin), associated to the cell membrane (*e.g.* glypicans and syndecans), the basement membrane (*e.g.* collagen XVIII, perlecan) or widely extracellular (*e.g.* aggrecan, versican and small leucine-rich proteoglycans (SLRPs)).

Contrary to the short *N*- or *O*-linked, branched sugar chains of glycoproteins, PGs are characterized by their long unbranched chains of negatively charged GAG polysaccharides covalently linked to a core protein. The number and length of GAG chains, their disaccharide unit structure, sulfation pattern, core protein type and their cell of origin are major sources of diversity of the PGs.

This diversity explains that beyond their role as structural ECM and adhesion proteins, PGs can also bind a large variety of ligands (*e.g.* antithrombin III, HGF, FGFs, TGF-β, ApoE)[286], act as receptors and co-receptors for internalization, and play a major role in signal transduction. PGs are essential to cellular functions involved in normal development, as evidenced by PG mutations leading to Simpson-Golabi-Behmel syndrome or Hereditary multiple exostoses, but are also key players in pathologies such as inflammation, atherosclerosis, Alzheimer's disease and cancer.

GAG structure and synthesis

PGs are made of two principal components, a core protein linked to a GAG chain composed of 10-200 repeating disaccharide units of hexosamine and uronic acid (or galactose for keratan sulfate). PGs can carry from a single to more than 100 GAG chains (*i.e.* aggrecan). Some PGs can exist with or without GAG

substitution, and are named "part-time" PGs. **Paper IV** of the present study is based on this particular feature of PGs.

Four main GAG types have been identified: Heparan sulfate(HS)/Heparin, Chondroitin sulfate/Dermatan Sulfate (CS/DS), Hyaluronan (HA), and Keratan sulfate (KS) with specific disaccharide units *(Table 1)*. A single PG can contain several GAG types.

Table 1: Disaccharide units of GAGs

GAG type	Hexosamine	Uronic acid
HS	GlcNAc	GlcA/ IdoA
Heparin	GlcNAc	GlcA/ IdoA
CS	GalNAc	GlcA
DS	GalNAc	GlcA/ IdoA
KS	GlcNAc	None (Galactose)
HA	GlcNAc	GlcA

GalNAc : N-acetylgalactosamine, GlcNAc : N-acetylglucosamine, GlcA : Glucuronic acid, IdoA : Iduronic acid

The PG core protein is synthesized in the ER and GAG chains are initiated, polymerized and modified post-translationally. HS/CS synthesis starts in the late ER, with the xylosyltransferase-mediated transfer of an UDP (Uridine Diphosphate)-xylose to the serine of a serine-glycine motif of the core protein. In the Golgi, galactosyltranferases and glucuronyltransferase will sequentially add two galactoses and GlcA, respectively, to form a tetrasaccharide linker used as primer for disaccharide polymerization[287]. HS is further synthetized through the transfer of GlcNAc to this linker, CS/DS with GalNAc and subsequent polymerization with their specific disaccharide units by EXT1/EXT2 and GalNAcT-II/ GlcAT-II enzymes, respectively[288,289].

Contrary to proteins, the synthesis of GAG chains is non-templated, and the factors influencing HS or CS priming are still unknown. For instance, it is possible to induce HS polymerization on a core protein usually bearing CS chains, as long as the HS enzyme machinery is present[290]. Nevertheless, in some HSPGs (glypican-1) the globular domain of the core protein influences the presence of HS[291].

GAG Modifications

Firstly, in HS chains, GlcNAc is N-deacetylated and N-sulfated to N-sulfoglucosamine (GlcNS) by NDST enzymes[292], which use 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as the major sulfate donor[293].

As shown in *Table 1*, glucuronic acid (GlcA) of HS and CS chains can be epimerized to iduronic acid (IdoA), which is highly abundant in heparin and DS, by C5-GlcA epimerases. Subsequently, HS can be 2-*O* (on IdoA and GlcA), 3-*O* (GlcNAc) or 6-*O* (GlcNAc*)* -sulfated by sulfotransferases. Similarly, CS/DS chains can be 2-*O* (on IdoA and GlcA), 4-*O* (GalNAc) or 6-*O* (GalNAc) -sulfated.

As these modifications mostly occur close to GlcNS residues, negatively charged clusters along the chains are created that will have implications for ligand binding activities[294-296], and the resulting "heparanome" of HS is the subject of extensive analysis[297,298]. Interestingly, Stanford *et al.*[299] compared different HS sulfation mutant mice for triglyceride-rich lipoprotein clearance and found that in the liver, clearance was specifically dependent on 6-*O* sulfation but not 2-O sulfation. This highlights that specific sulfation patterns may have even more functional significance than overall sulfation levels.

Once on the cell surface, the sulfation status of HS chains can be further edited by the action of Sulf1 and Sulf2 endosulfatases, which remove the 6-O sulfate groups. This step has been involved in the repression of VEGF, FGF, and SDF-1 binding[300] and HSPG-mediated FGF signaling for instance[301]. Sulf2 is also involved in impaired triglyceride-rich lipoprotein clearance[302].

Finally, HS chains can be cleaved into short residues by heparanase on the cell surface. Heparanase secretion has been linked to angiogenesis and a variety of pro-tumorigenic effects[303,304]. Interestingly, heparanase and its proheparanase precursor form bind syndecans and have been shown to regulate exosome biogenesis[305].

Paper I and **IV** of this thesis work study mechanisms associated with cell surface PGs in the TME. The next chapters will focus on details of this family of PGs.

Cell surface proteoglycans

Cell surface PGs are usually classified into two main categories, syndecans and glypicans, which contain mostly HS chains *(Figure 7).*

Syndecans

Syndecans (SDCs) are type I transmembrane proteins usually linked to HS in the distal region of their ectodomain but can in some cases contain CS. The SDC family comprises 4 members of 20-40 kDa. SDC4 is found ubiquitously, SDC3 is mostly found in neurons[306] and muscle tissue, SDC2 on endothelial cells and

fibroblasts[307,308] and SDC1 on endothelial and epithelial cells[309]. The cytoplasmic tail contains two conserved domains C1 and C2 and a variable domain V specific to each SDC. The intracellular domains are involved with cytoskeleton dynamics and signal transduction[310]. C2 is for instance the binding site for PDZ proteins[311]. Importantly, SDC ectodomain can be shed by MMPs/ADAM[312], thus liberating soluble ligands/GAG complexes.

Glypicans

Glypicans (GPC) are GPI-anchored PGs (60-70kDa). Six GPCs have been identified in mammals[313]. The protein is globular, which is thought to impact its exclusive HS conjugation[291]. The HS insertion sites are located at the C-terminal region, meaning that GAG chains of GPCs sit close to the membrane. The GPI anchor can be released by cleavage by Notum[314] and the core protein can be cleaved by furin convertases. GPCs have been originally involved in morphogen/Wnt signaling[315] and cleavage by furin is needed for Wnt5A association with GPC3[316]. Indeed, the GPCs lack intracellular domains and mediate their signaling transduction through co-receptors, *i.e.* Frizzled[317]. GPCs have been found enriched in membrane rafts regions, which is consistent with their GPI anchor[318,319].

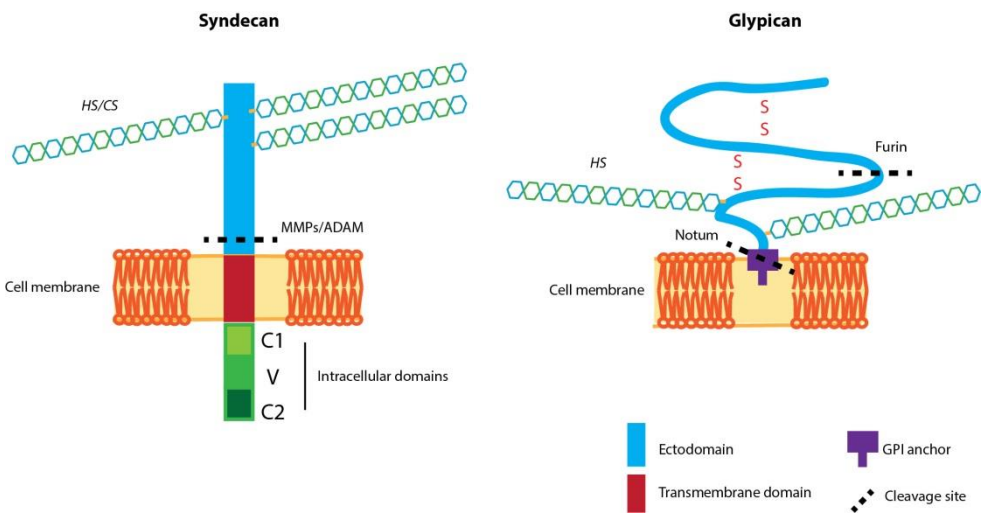

Figure 7: Schematic representation of syndecan and glypican structures.

Because of their membrane location, cell surface PGs are in the front line to mediate adaptations of cancer cells to their microenvironment, their ectodomain

and GAG chains can be affected by nutrient/ligand availability and they can act as endocytic receptors, signaling co-receptors, or mediate cell adhesion[181,320].

Signaling versatility

PGs play a key role in facilitating interactions between ligands and their receptors. The example of growth factors is probably the most studied, with for instance FGF-2 binding and presentation to FGF receptors and downstream ERK activation[321]. Upon ligand binding, HSPGs can cluster into lipid raft regions, which act as hubs for signaling activation[322,323]. Also. as high capacity receptors, PGs can bind and concentrate ligands for high affinity receptor presentation or allow their release through PG shedding or HS cleavage by heparanase[324,325]. In addition, PGs can mediate direct association between the ECM and the intracellular signaling machinery. SDC4 binds both integrins in focal adhesion and α-actinin intracellularly for instance[326]. In the same context, further level of signaling regulation by HSPGs lies in their ability to recruit and orchestrate large protein complexes such as syntenin. Syntenin in turn mediates the recycling of syndecan-FGF-FGFR containing endosomes, resulting in enhanced signaling receptor surface availability and increased cell spreading[327].

Altogether, this indicates that PGs are highly relevant therapeutic targets in cancer. Findings of the present study provide more or less directly new roles for PG-mediated cancer cell adaptation to the pro-tumorigenic microenvironment and suggest new potential therapeutic opportunities.

Targeting and current therapeutic status

The most established clinical use of GAG or GAG derivatives is indicated for patients with venous thromboembolism, based on the anticoagulant properties of heparin and low molecular weight heparins (LMWH)[328] In the oncology field, including in malignant glioma, increasing evidence argues for a beneficial effect of heparin/LMWH treatment on patient outcome but conclusive clinical studies are still needed[329,330].

Owing to their role in angiogenesis and metastasis, sulfatase and heparanase inhibitors have been developed[331]. The most clinically advanced anti-heparanase compound, PI-88, has been tested in a phase III clinical trial for hepatitis-C virus related hepatocellular carcinoma (PATRON/NCT01402908). Heparanase inhibiting suramin has also been evaluated in clinical trials for its antiangiogenic role, but has not found a role in the clinical setting[332].

Importantly, anti-HS antibodies and their specificity are valuable assets for mechanistic studies[333,334] and further developments could pave the way for blocking or delivery approaches. Interestingly, a conserved CS binding domain recognized by the malaria parasite protein VAR2CSA, has been found enriched in several malignant cells and its targeting using a VAR2CSA-toxin fusion construct reduced cell growth and metastasis *in vivo*[335]. Several approaches to perturb the PG machinery are available for experimental, functional studies, and are detailed in the Methods section.

The clinical implementation of GAG targeting therapies also relies on the identification of disease specific structural motifs. The profile of GAG is modified in several cancer types including GBM, *e.g.* overexpression of SDC1[336], GPC1[337], CSPG4/NG2[338] and CD44[339]. Recently, profiling of metastatic clear cell renal cell carcinoma identified a specific GAG profile marked by CS upregulation and HS downregulation that could be used as a non-invasive prognostic biomarker in urine and plasma[340]. The structural complexity of GAGs poses a great challenge for high-throughput analyses of complex samples, but recent development of mass spectrometry multiplex analyses may be close to clinical use in the management of mucopolysaccharidoses, *i.e.* disorders with defect GAG degradation[341].

Methods

This section gives a more detailed background and description of techniques and methods of relevant interest that were used in the thesis work. Further procedure details and information can be found in the material and methods sections of the publications attached.

Confocal laser scanning fluorescence microscopy

Fluorescence microscopy enables the visualization of details of samples (tissues, cells…) which are fluorescently labelled, either with a free dye *i.e.* Hoechst for nuclei, or conjugated to a primary or secondary antibody for the protein of interest. Fluorescence emitted by the sample after excitation by a filtered light source (usually a mercury lamp or a LED source) is filtered and seen through the objective or a camera.

Contrary to widefield fluorescence microscopy, which can give high background staining for thick specimens and reduce the level of details observed, confocal laser scanning microscopy enables high resolution and allows optical sectioning of the sample. To obtain the image, a focused laser source is moved across the sample (scanning) and the fluorescent signal obtained is detected after passing through a pinhole aperture, which only allows focused light (hence confocal) to be detected by a photomultiplier (PMT) and form the image pixel by pixel. The optical sectioning allows to perform e.g. colocalization experiments, 3D reconstruction, and maximum intensity projection images and can be used for live cell imaging.

For neutral lipid imaging (TAGs, CEs of LDs), several standard diffusible dyes are available such as Oil Red O, Nile Red, and the more stable and more LD-specific Bodipy® and LipidTox™. For lipoprotein particles, fluorescent red membrane lipophilic marker DiI (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate) is mostly used.

Super-resolution microscopy

The lateral resolution in optical microscopy is limited to ~200 nm because of light diffraction. Super-resolution microscopy enables to overcome this limitation by different techniques (PALM, STORM, STED…), hence improving the visualization of subcellular details with down to ~10 nm lateral resolution but requiring specific fluorophores in most cases. More specifically, the Airyscan (Zeiss) system (used in **Paper IV**) is made of a specific array detector consisting of 32 elements, each acting as a pinhole. This allows superior light detection than the single pinhole standard configuration, hence decreasing the signal-to-noise ratio while improving the resolution ~1.7x in all directions (140 nm laterally). Importantly, samples can be prepared in the same way as conventional confocal fluorescence microscopy, and the technique is thus compatible with live imaging.

Semi-quantification of microscopy images

Microscopy images can only be analysed semi-quantitatively, *i.e.* comparing different conditions arbitrarily. It is therefore very important to keep acquisition parameters identical within each experiment, including magnification, aperture, laser gain, digital gain, averaging, and scanning speed as well as using only linear post-acquisition modifications in a standardized manner on all pictures from the same experiment.

Semi-quantification of fluorescence images was performed in ImageJ for standard area or intensity measurements of stained structures from defined regions of interests, *i.e.* field of view, cells, plasma membrane *vs.* cytoplasm. In addition, Cell Profiler software (Broad Institute) was used for measurement of LD/vesicle number and intensity. This software creates automated modules and very precisely identifies objects (cells, nuclei, organelles) by applying the most appropriate thresholding and declumping methods for the application of interest and performs specific counting and normalization of these objects.

Transmission electron microscopy

Electron microscopy works similarly to light microscopy (especially transmission electron microscopy) but uses an electron beam to illuminate the sample and electromagnetic lenses instead of mirrors. The ~100.000x shorter wavelength of electrons enables resolutions down to ~0.1 nm. Electron micrographs, obtained on a fluorescent screen or detected by a CCD camera, can thus enable to see

subcellular structures in great details *i.e.* the cell lipid membrane bilayer or the fine structure of organelles.

The drawback with electron microscopy is the sample preparation, usually requiring harsh fixatives and labor-intensive embedding and sectioning to maintain biological structures. For the imaging of lipid structures and lipid droplets (LDs), osmium tetroxide is an excellent contrast agent (used in **Paper I**). Immunogold staining (secondary antibodies conjugated to nm-sized gold particles) can also be used to detect specific proteins.

Flow cytometry

Flow cytometry is a laser-based technique used to analyze the characteristics of single-cells on a large number of cells. Cells (usually fluorescently labelled) are injected in a fluidics system and pass through a laser beam that renders information on their size, granularity and the fluorescence intensity of interest.

For binding or uptake studies, a labelled antibody or particle (*e.g.* DiI-LDL) is incubated with the cells, either on EDTA-detached cells on ice or with adherent cells at 37°C, respectively, and cells are subsequently washed and resuspended in a non-clumping buffer (*i.e*. PBS/BSA/EDTA) for flow cytometry analysis.

Immunoprecipitation

Immunoprecipitation (IP) is used to purify and enrich (pulldown) a specific protein from a complex protein mix. The protein mix is pre-cleared against the plain beads to remove unspecific antigen binding, and incubated with an antibody (IgG) against the protein of interest before being captured by high IgG affinity protein A- or G-conjugated beads. The captured protein can then be further washed and processed for immunoblotting. The controls normally include total lysate before IP (input) and IP without primary antibody.

Immunoblotting

Western blot

Western blotting is a widely used method for protein detection and analysis. The samples are prepared in order to solubilize and denature the protein of interest and separated by SDS-PolyAcrylamide-Gel Electrophoresis (PAGE). The proteins are then transferred (blotted) onto a membrane (nitrocellulose or PVDF) for immunodetection by primary antibody followed by secondary HRP-coupled detection antibody. The specific protein signal is revealed usually by chemiluminescence and can be quantified by densitometry.

Arrays

Protein arrays are membrane-based semi-quantitative immunoassays working according to the same principle as sandwich ELISAs. Briefly the membranes are pre-probed with capture antibodies for a specific array of proteins (*i.e.* angiogenesis proteins, kinases...) onto which the samples of interest are incubated and detected using chemiluminescence (as explained above in the Western blot section). The great advantage of arrays is of course to alleviate the need for multiple western blots (usually 50-100 different proteins tested per array), saving both time and sample.

Equal amounts of each sample condition are added on each membrane for comparison. The quantification of the signal is also based on densitometry, whereby each duplicate signals from each antibody is background corrected, averaged and normalized to the average intensity of the reference spots of each membrane. This can be done manually on ImageJ or by using specific template tools (*i.e.* Protein Array Analyzer for ImageJ by Gilles Carpentier), which have been developed for accurate measurements.

Detergent-free cell membrane fractionation

To study the distribution of caveolin-rich domains in cell membranes, a detergent-free fractionation method, based on buoyancy separation by sucrose ultracentrifugation was developed[342,343] (used in **Paper IV**). Briefly, the cells are scraped in a sodium carbonate buffer and membranes disrupted by sonication before being placed at the bottom of a discontinuous sucrose gradient (40% - 35%

- 5% sucrose) and centrifuged for 3 h at 175.000x g. The proteins from each fraction can then be precipitated (chloroform/methanol) and analyzed by WB.

Caveolin-rich fractions are then expected to be enriched at the interface of the 35%-5% gradient, specifically from most other cellular proteins due to their buoyancy. The advantage of a detergent-free method (contrary to disruptive Triton X-100 treatments) is to enable to retain all proteins associated with caveolae, which is particularly important for instance for co-distribution studies.

Homotypic Aggregation/Spheroid formation assay

In the context of metastasis, in the hematogenous/circulating setting, a critical step is the ability of cells to survive and aggregate during their circulation and at the metastatic site.

Tumor cell ability to form coherent multicellular aggregates can be evaluated using homotypic aggregation/ spheroid formation assay[344]. The cells are grown under conditions of interest to be compared, detached and resuspended in growth medium as a hanging drop (30 µL) under the lid of a cell culture plate to initiate cell aggregation. The spheroid formation can then be imaged at early and later time-points and the resulting aggregate/spheroid area quantified. Viability of the cells can also be assessed and cells able to form tight spheroids can be embedded in cutting compound for further ICC analyses.

Antibody-drug conjugate cytotoxicity assay

To pre-screen the efficacy of ADC target candidates while avoiding the expensive and time-consuming linking chemistry of the primary antibody to the payload, the standard experiment consists in using a secondary-ADC to a monoclonal antibody recognizing the tumor antigen target candidate. The tumor cells are incubated with the anti-tumor antigen target antibody with or without different concentrations of the secondary ADC. After 2-4 days the cell viability is assessed by the addition of MTS compound (used in **Paper III** and **IV**), which is reduced only by viable/proliferating cells to produce a soluble colored product that can be detected by colorimetry. The toxicity of the secondary antibody alone (no anti-target primary antibody) is controlled in parallel.

The IC50 (the ADC concentration needed to obtain 50% decrease in viability relative to 100% viability vehicle control) can be calculated using a dose-response

non-linear fitting model with a curve fitting software (*i.e.* GraphPad, GraphPad Software) and compared between groups using the extra sum-of-squares F-test.

Proteoglycan function and analysis

Mutant cell lines

Mutant cells of GAG synthesis have been established in the widely used Chinese Hamster Ovary (CHO) background by the Esko group[345] and represent valuable tools for PG-dependent cell mechanistic studies. For the present studies, the following mutant cell lines have been used:

Table 2 : CHO cell lines mutated for the GAG biosynthesis pathway

Cell line	Enzyme mutated	Phenotype
pgsA (CHO-745)	Xylosyltransferase (XT-1)	GAG deficient
pgsD (CHO-677)	N-acetylglucosaminyl/ glucuronosyltransferase (EXT-1)	HS deficient
pgsE (CHO-606)	N-deacetylase/N-sulfotransferase-1 (NDST-1)	Undersulfated HS
pgsF (CHO-F17)	HS 2-O-sulfotransferase (HS2ST)	2-*O*-sulfation-deficient HS

Enzymatic treatments

Several enzymes can be used to study the role of PG-GAG chains in the *in vitro* setting. Heparinases I (cleaves highly sulfated HS chains), II (cleaves high and low sulfation HS chains) and III (cleaves low sulfated chains), if used in combination can very efficiently digest cell surface HS chains into disaccharides. Chondroitinase ABC lyase digests chondroitin 4-sulfate, chondroitin 6-sulfate, and also DS.

Antibodies

Several anti-HS antibodies have been developed, each having different degrees of specificity, *e.g.* anti-HS AO4BO8[346] or the 3G10 antibody[347], which recognizes a HS neo-epitope after digestion by heparin lyase III (heparitinase I). These

antibodies have been widely used for uptake, binding, tissue staining, or signaling activation assays. Interestingly, the anti-3G10 antibody, owing to its specificity, can be used for WB on cell-surface HS-digested cells to detect the overall expression of HSPG core proteins (SDCs and GPCs).

Lipoprotein preparation

Lipoproteins (commercially obtained from Intracel and AlfaAesar) were purified from fresh human plasma by ultracentrifugation, sequential high-speed flotation in KBr (1.21-1.063 g/mL for HDL, 1.063-1.019 g/mL for LDL and 1.006-0.95g/mL for VLDL). Fluorescent labelling of lipoproteins was done using DiI (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate), followed by re-floatation and dialysis. The identity and purity of preparations was verified by agarose gel electrophoresis and Sudan Red stain for lipids.

Cholesterol/Triglyceride assays

Cellular lipids were extracted from sonicated cells using a chloroform: isopropanol:NP-40 (7:11:0.1) mixture, followed by centrifugation and 50°C air-drying of the organic phase. Alternatively, LDs were isolated from cells by floatation using a gradient centrifugation isolation kit and lipids extracted as described above (**Paper I**). Cholesterol and triglyceride levels were measured by fluorometric assays. Briefly, the technique is based on the fluorescent detection of the product (hydrogen peroxide) of the enzymatic action of cholesterol esterase and oxidase on cholesteryl esters (for total cholesterol) and lipase followed by phosphorylation and oxidation (for triglycerides). For cholesterol, omitting the cholesterol esterase reaction will quantify free cholesterol. The ratio of cholesteryl esters is then calculated by subtracting the free cholesterol concentration to the total cholesterol. Fluorescent signals are read by a spectrophotometer and plotted against a standard concentration curve.

Laser Capture Microdissection

Tissue sections from biopsies or animal experiments are a valuable tool to understand the genetic or protein expression of cells *in situ*. Laser capture microdissection allows defining areas of interest of a tissue under microscopic

control and automatically dissect out this area through laser cutting and catapulting (see picture below). Sections are mounted on specific membrane slides for that purpose and special care has to be taken during sample preparation to preserve the quality of the DNA, RNA or proteins.

Immunohistochemistry/Cresyl violet-stained slides can be used to identify the areas of interests in the tissue. The cut sections can be further processed for gene or protein analysis (**Paper I**).

Array-Based Gene Expression Analysis

Gene arrays are used to analyze and compare the gene expression (RNA population) between different samples. The RNA purified from the sample is converted to cDNA followed by *in vitro* transcription to cRNA. In the BeadChip (Illumina) technique, which was used here (**Paper I, II, III**), beads are coated with oligonucleotide probes (~47000 transcripts) and distributed at the surface of a chip for hybridization (see picture below) with the biotin-labeled cRNA of the sample. Complementary hybridization is then detected by fluorescence array scanning.

Usually, downstream analysis of the raw data renders relatively extensive datasets of gene hits differentially expressed between samples. One way to get an overview of the response associated to the gene expression is to perform a functional annotation of the upregulated genes to identify the biological processes behind the complex data (**Paper II**).

Gene Ontology (GO) annotation is particularly useful to define the enriched functions of the gene list of interest. This method classifies and relates genes according to three main aspects: their Molecular Function, the Cellular component where they are active and larger Biological Processes in which the genes are involved. Different online platforms are available to perform this type of analysis (*e.g.* ConsensusPathDB, Panther, GOrilla, David).

Reverse-transcriptase qPCR

To validate the gene profile obtained from a gene array experiment, the RNA expression of specific hits of the sample can be tested by RT-qPCR. The RNA (usually purified total RNA) is reverse-transcribed to cDNA. The resulting cDNA amount is quantified by qPCR, which is carried out according to the usual PCR scheme, *i.e.* denaturation, annealing, elongation, but in the presence of a fluorescent dsDNA binding dye (**Paper I**) or specific reporter probe. The

fluorescent product is detected at each cycle to monitor the amplification of the DNA in real-time. The cycle number (quantification cycle, Cq) needed to reach the fluorescence threshold set is associated to each target. The quantification of gene expression is made either absolute (compared to a standard curve of known concentration for copy number) or relative to a calibrator (delta Ct method for fold change).

Mass spectrometry

Mass Spectrometry (MS) is now the golden standard for proteomic analysis. The technique is based on the analysis of the m/z (mass over charge) ratio of peptides obtained from a more or less complex protein mixture to identify and quantify the corresponding protein expression.

LC-MS/MS

LC-MS/MS, high performance liquid chromatography coupled to MS/MS (tandem mass spectrometry) is the most commonly used setup to perform the analysis of complex peptide mixtures ("Shotgun" proteomics).

First, proteins are isolated from the source, precipitated, reduced, alkylated and peptides obtained by trypsin digestion. Then, the peptides are separated according to their charge and hydrophobicity by HPLC (acetonitrile elution on C18 columns), before being injected (ionized) in line in the mass spectrometer for m/z analysis.

As peptides are large molecules, the information obtained from one MS analysis (full scan, MS1) may not be specific enough for protein identification. In MS/MS scans, precursor ions of interest (from the MS1 scan) are selected by a quadrupole filter (in the case of the Q-Exactive Plus mass spectrometer (ThermoFisher) that was used in this work, **Paper I** and **III**) before being further dissociated into daughter ions in a higher-energy collisional dissociation (HCD) cell and analyzed in the Orbitrap mass analyzer. The spectra obtained can be further analyzed with downstream softwares (Proteome Discoverer (Thermo)) and online search engines (Mascot, Sequest) interrogating sequence databases (UniProtKB) are then used to identify the protein content of the samples based on the occurrence of unique peptides associated to a specific protein in the analysis.

For label-free relative quantification of the protein content (MS1 filtering analysis), the Skyline software was used. Briefly, ion chromatograms corresponding to the peptides of interest from the MS1 analysis were extracted.

The abundance of the unique peptides, and hence corresponding proteins, was determined by integrating the three most intense ion peaks for each peptide. The areas obtained were then compared between the samples for relative quantification.

MIMS

Membrane-inlet mass spectrometry is a specific type of MS that uses gases as input analytes. In brief, a chamber containing the gas mixture to be analyzed is connected via a gas-porous membrane and a cooling trap to a mass spectrometer that will analyze the gas composition *(Figure 8)*. In **Paper IV**, this technique was used to study the carbonic anhydrase activity: CO_2+H_2O -> HCO_3^- + H^+ by following the decay of ^{18}O-labelled CO_2 from the sample chamber.

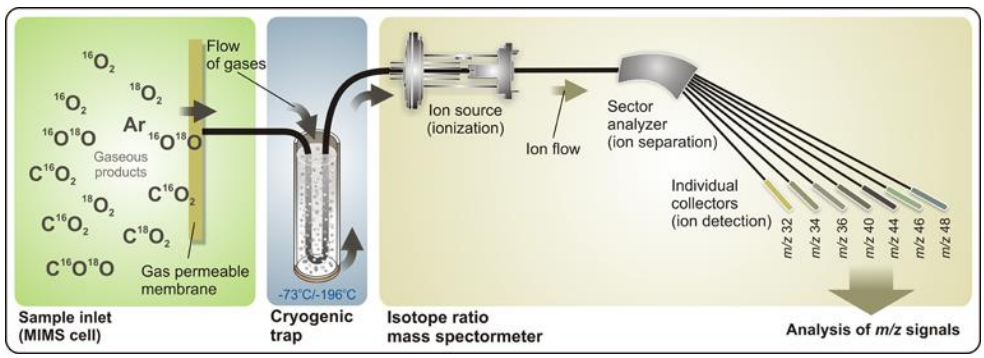

Figure 8: MIMS setup

Proximity extension assay

Proximity extension assay (PEA) is a multiplex immunoassay used to detect protein levels in complex biological samples with high specificity and sensitivity.

Specific pairs of oligonucleotide-labelled antibodies are designed to bind to a target protein of interested in the sample. Only matching pairs will hybridize and oligonucleotide sequences be extended by DNA polymerase. The resulting DNA reporter sequence (unique for each target) is amplified and the signal measured by qPCR *(Figure 9)*.

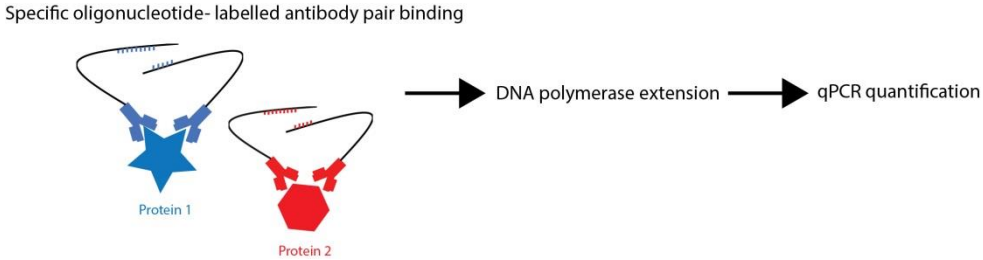

Figure 9: Workflow of the Proximity Extension Assay

After QC and analysis, normalized protein expression values (NPX) are obtained for each protein (biomarker) tested and can be compared between the samples.

Cell surface / Endocytosed proteome encoding

In **Paper III**, a method for the visualization, isolation, identification and quantification of cell surface and endocytosed proteins from cultured cells in hypoxia *vs.* normoxia was developed. The outline of the procedure is presented after *(Figure 10)*.

This method is based on the reversible labelling of the surface proteome (by the membrane impermeable and cleavable biotin moiety, EZ-Link Sulfo-NHS-SS-Biotin, ThermoFisher) followed or not by endocytosis.

In the case of endocytosis study, the remaining surface labelling after internalization was reduced with MesNa (Sodium 2-mercaptoethanesulfonate) followed by iodoacetamide alkylation (blocking) to visualize and isolate specifically internalized biotinylated proteins.

Surface or internalized proteome could then be analyzed by flow cytometry or confocal fluorescence microscopy using streptavidin conjugated fluorophores. In addition, LC-MS/MS analysis of the proteome(s) could be performed following streptavidin column purification. As further optimization, for endocytosed proteome studies, an extra streptavidin blocking step for any remaining biotinylated surface protein was then added prior to purification.

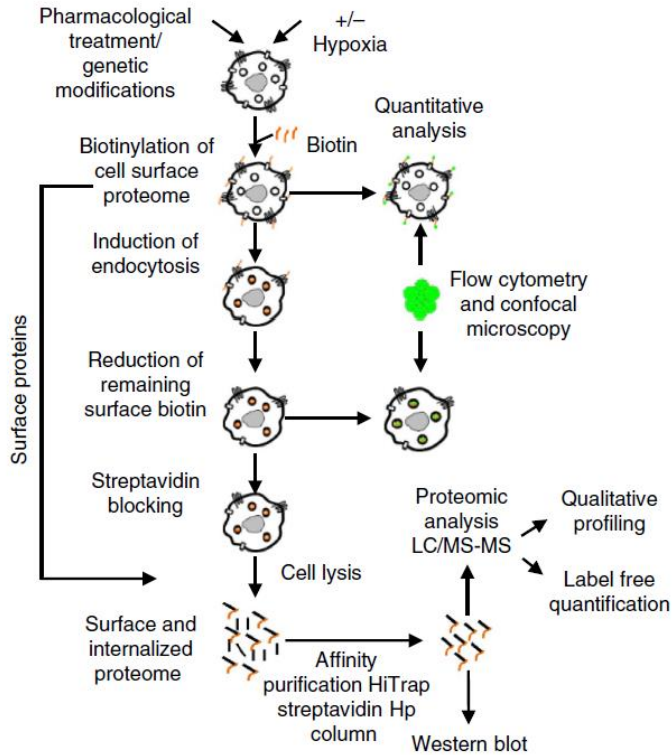

Figure 10: Schematic outline of the cell-surface and endocytosed proteome labelling for proteomic analaysis

From Bourseau-Guilmain et al. Hypoxia regulates global membrane protein endocytosis through caveolin-1 in cancer cells. Nature Communications 7, Article number: 11371 (2016) doi:10.1038/ncomms11371. Licensed under a Creative Commons Attribution 4.0 International License. (https://creativecommons.org/licenses/by/4.0/)

Aims and findings of the present investigation

The aim of this thesis work was to gain better understanding of cancer cell adaptive mechanisms in the tumor microenvironment under hypoxic and acidic stress, with a focus on PG-dependent uptake mechanisms and cell surface protein internalization as potential therapeutic targets.

Paper I

The first paper, *Metastasis Stimulation by Hypoxia and Acidosis-Induced Extracellular Lipid Uptake Is Mediated by Proteoglycan-Dependent Endocytosis* aimed at identifying the mechanistic details and functional roles of the adaptive lipoprotein uptake and lipid accumulation under microenvironmental stress.

We find that cancer cells adapt to stress through increased recruitment of lipoproteins, resulting in a lipid storing phenotype, and enhanced tumor forming capacity. The lipoprotein uptake pathway involving PGs is identified as a potentially targetable driver of tumor development.

Paper II

Paper II, *Effects of extracellular lipids on immune cell recruitment and pro-angiogenic signaling under tumor microenvironmental stress in glioma,* is a direct follow up of the findings from Paper I and aimed at elucidating the functional effects of microenvironmental stress–induced LD loading of cancer cells during the post-hypoxic phase in glioma.

Preliminary results from this study suggest that increased cancer cell LD loading and systemic lipid availability promotes glioma development, and that lipid loading reinforces the hypoxic and post-hypoxic signaling response.

Paper III

The aim of paper III, *Hypoxia regulates global membrane protein endocytosis through caveolin-1 in cancer cells*, was to investigate the effect of hypoxia on global cell proteome internalization, and to develop a method to identify cell surface protein targets for hypoxia-specific ADC targeting.

We find that stress conditions (hypoxia) typical of malignant tumors modulate membrane proteome turnover in cancer cells, providing opportunities for tumor specific drug delivery. As proof of principle, an ADC targeted at one of the identified tumor antigens was shown to selectively kill hypoxic cancer cells. Future studies aim at translating these findings into new treatment strategies of cancer.

Paper IV

Paper IV, *Tumor antigen glycosaminoglycan modification regulates antibody-drug conjugate delivery and cytotoxicity*, aimed at characterizing and dissecting the role of the GAG modification of the CAIX protein on its activity and internalizing capacity.

We provide first evidence that antibody-mediated cytotoxin delivery is regulated by GAG modification of the hypoxia-induced, tumor antigen CAIX. Pharmacological or genetic inhibition of glycosylation potentiated the cytotoxic activity of an anti-CAIX ADC targeted at hypoxic tumor cells.

Results and Discussion

Paper I

Metastasis Stimulation by Hypoxia and Acidosis-Induced Extracellular Lipid Uptake Is Mediated by Proteoglycan-Dependent Endocytosis

Key results

- Hypoxia and acidosis induce a reversible LD-loaded phenotype in cancer cells in the presence of extracellular lipids.
- LD-loaded cells exhibit increased spheroid forming capacity and hematogenous lung metastasis *in vivo*.
- Hypoxia and acidosis increase lipoprotein uptake.
- Hypoxia upregulates gene and protein expression of VLDLR and SR-B1 but global HSPG core proteins and GAG sulfation are not affected.
- Intact HSPGs are needed for stress-induced lipoprotein uptake.
- LDL and VLDL-induced ERK1/2 activation in hypoxia is HSPG-dependent.
- Stress induced LD loading depends on HSPG.

Discussion

Cancer cells acquire a pro-tumorigenic LD-loaded phenotype in TME conditions

In this study, we first provided further evidence for a role of lipid storage under the form of LDs as an adaptive mechanism to both hypoxia and acidosis stress conditions *in vivo* in GBM patient tumors and *in vitro* in different cancer cell lines. Importantly, this storage was dependent on the presence of extracellular lipids, and reversible as LDs were consumed when the cells were placed in reoxygenation. This is an important result as early reports linking LD levels with tumor grade were often based on NMR measurements of necrotic areas, which led to assuming

that LD accumulation was an end stage in cancer cells preceding necrosis[348]. Since then, we and others[234,349,350] have shown that hypoxia and acidosis induced LD formation, through different mechanisms, is reversible and associates with pro-tumorigenic and metastatic potential[220,351]. All these observations may not be mutually exclusive; indeed, one can envision a situation in which tumor cells in intermediate to severe hypoxic conditions could take advantage of a nearby "lipid" pool created by necrotic cores and store large amounts of LDs. Cycling hypoxic conditions in this intermediate area would then allow these cells to use their lipid stores for further proliferation and invasion.

In our study, we found LDs to be enriched mostly in CE. This suggests that consumption of these lipids could facilitate membrane turnover associated with proliferation, or have an impact on lipid raft-mediated signaling transduction[352]. In addition, synthesis of signaling lipid precursors such as arachidonic acid[353] could be enhanced. We further demonstrated increased spheroid/homotypic cell aggregation *in vitro* and metastasis *in vivo*. Intriguingly, analysis of circulating tumor cells (CTCs) from prostate cancer[240] showed that they contained high intracellular levels of lipids, and it is tempting to speculate that CTCs escape from hypoxic/acidic tumor areas fuelled with lipids. One can obviously think about the potential contribution of intracellular lipids, especially cholesterol, in the secretion of EVs and downstream effects on cancer progression. Some evidence along this line has already been given and warrants more understanding[220]. These aspects will be discussed further in relation to **Paper II**, in which we sought to investigate the role of the LD-loaded phenotype in reoxygenation conditions.

HSPGs mediate TME stress-induced lipoprotein uptake and are required for the LD-loaded phenotype

A major finding of this paper is the HSPG-dependent increased uptake of lipoproteins under hypoxia and acidosis. We found increased expression of VLDLR and SR-B1 at the gene and protein level at the hypoxic time-points of increased uptake. This is in accordance with previous studies on hypoxic VLDLR expression in other cell systems[354,355] whereas evidence for SR-B1 hypoxic upregulation was still lacking to our knowledge. Surprisingly, however, even though we could nicely show the HSPG dependence of this mechanism, no apparent effect of hypoxia was seen on the PG core protein composition or overall HS sulfation levels. In addition, binding and uptake of the AO4B08 anti-HSPG internalizing antibody were unchanged. Altogether, these results suggested that direct HSPG mediated internalization, potentially through SDC-1[207], could not explain these effects. One way to explain this result comes from the role of HSPGs as initial, high capacity lipoprotein receptors completed by secondary internalization events[185,206,356], through LPL-mediated attachment[357,358], and whole

particle or selective CE uptake[359] from hypoxia-responsive lipoprotein receptors (*i.e.* VLDLR, SR-B1). An alternative model would be a co-receptor complex of HSPG and lipoprotein receptor that follows a common internalization route with the ligand.

LDL and VLDL triggered prolonged ERK1/2 phosphorylation in hypoxic conditions in an HSPG-dependent manner. ERK signaling has pleiotropic roles in cancer depending on the context of activation and cell type, including growth, migration, cycling, and survival[360]. In the context of atherosclerosis, lipoproteins have been shown to activate MAPK signaling pathways[361]. Specifically, LDL can induce MAPK in vascular smooth muscle cells through calcium/calmodulin, phosphokinase C and MEK[362]. ERK1/2 activation has previously been associated with HSPG mediated endocytosis of lipoparticles *i.e.* in the EV context[164]. HSPG can present ligands and facilitate RTK activation on the cell surface[363]. Presentation of lipoproteins to SR-B1 receptor for instance could be involved in cholesterol signaling and the observed downstream MAPK activation[361,364]. Finally, another level of HSPG relevance in this context was shown with the dependence on HSPGs for the acquisition of the hypoxic and acidosis induced LD phenotype associated with increased metastasis.

These findings place HSPGs in a central role for cancer cell adaptation under hypoxia and acidosis, through mechanisms leading to the acquisition of a pro-tumorigenic phenotype. Further studies aimed at dissecting out the exact mechanisms involved at the downstream lipoprotein receptor level are needed, but clearly pilot studies evaluating the effect of heparin treatment would be of great interest in this context. Besides, Sarduy *et al.*[365] demonstrated the effect of a promising anti-CS GAG antibody inhibiting interaction between LDL and CS, showing an anti-atherogenic effect in $ApoE^{-/-}$ mice. The development of this type of specific anti-GAG antibodies to attenuate adaptive responses in the TME can only be encouraged.

Paper II

Effects of extracellular lipids on immune cell recruitment and pro-angiogenic signaling under tumor microenvironmental stress in glioma.

Key results

- Hypoxia and acidosis-induced LD phenotype of glioma cells increase macrophage recruitment and decrease survival in an immunodeficient orthotopic mouse model.
- Hypoxic LDL preloading of GL261 glioma cells results in increased tumor aggressiveness, increased macrophage recruitment and vascular density in a hyperlipidemic orthotopic mouse model.
- Hypoxic LDL preloading reinforces the hypoxic pro-tumorigenic gene and protein signature.
- Hypoxic LDL loading does not enhance HIF stabilization.

Discussion

In this study, we sought to evaluate the functional effects of the LD-loading under stress conditions *in vitro* and *in vivo*.

The LD-loaded phenotype associates with increased tumor aggressiveness in sustained hyperlipidemic conditions

Using the orthotopic GL261 mouse glioma model, we found that hypoxic LDL-preloading was associated with increased F4/80^{+} macrophage density in tumors in immunodeficient mice. Moreover, acidosis-selected GBM cells also displayed a reversible LD-loaded phenotype, which was associated with decreased survival in immunodeficient mice orthotopically. Further experiments are needed to dissect out the relative role of acidosis selection and the specific effect of the LD-phenotype in this context. As we expect the LDs to be relatively rapidly consumed after injection (within 24-48 h, **Paper I**), we next sought to investigate the combined effect of hypoxic LD-preloading with systemic lipid availability in the hyperlipidemic ApoE$^{-/-}$ mouse model under high fat diet. Interestingly, we found that mice injected with LD-loaded cells had significantly shorter survival in the ApoE$^{-/-}$ background compared to wild-type. More importantly, within the ApoE$^{-/-}$ background, mice receiving hypoxia LD-preloaded cells developed more aggressive tumors than non-loaded cells. Histologically, this was associated with increased macrophage recruitment in the ApoE$^{-/-}$ group *vs.* wild-type, which was

further enhanced in the ApoE$^{-/-}$ +LD loading group. In addition, vascular density, as measured by pericyte and endothelial cell coverage, was significantly higher in the ApoE$^{-/-}$ groups *vs.* wild-type. These findings indicate a role for the lipid loading phenotype in immune cell recruitment, even though increased tumor aggressiveness (tumor size or survival) did not reach significance (P = 0.141 and 0.175 respectively) in the immunodeficient model. In the ApoE$^{-/-}$ model, a potential limitation for the interpretation could be the pleiotropic effects of systemic hyperlipidemia, particularly the influence of pro-inflammatory cytokines and adipokines on carcinogenesis in general[366]. Nevertheless, in the ApoE$^{-/-}$ background, the effects of combined tumor cell LD loading and systemic hyperlipidemia are intriguing and may suggest an important role of LDs in the "priming" of the TME, including immune and vascular cell recruitment that drives tumor aggressiveness. A more in-depth profiling of the macrophage phenotype recruited will be needed to corroborate the conclusions and improve the mechanistic understanding of these observations.

LD loading reinforces the pro-tumorigenic, pro-angiogenic cell response in hypoxic and post-hypoxic conditions.

To gain more insight into the role of LD loading in hypoxic cancer cells, we performed gene and protein analysis of the glioma cells in hypoxic and post-hypoxic conditions in the presence or absence of LDL. Interestingly, several genes of the hypoxic response were further upregulated by LDL under hypoxia, associated *e.g.* with cell proliferation (ACTG2, CDK5RAP2) and stromal remodeling (LOXL4). Gene ontology analysis of the hypoxic gene hits further upregulated by LD loading were members of the anion and carbohydrate derivative binding and cargo and scavenger receptor activity, which interestingly encompasses the mechanism suggested in **Paper I**. In addition, the LDL response was enriched for biological processes involving MAPK signaling. During the reoxygenation phase, genes associated with cell cycling were upregulated following LD preloading. GM-CSF (CSF2 gene) was upregulated at both time-points of reoxygenation (6 and 48h). The role of GM-CSF is cancer-type dependent and double edged, evoking anti-tumor immune response through *e,g.* activation of dendritic cells[367], while being pro-tumorigenic and associated with poor prognosis in several cancer types, including GBM[368,369]. Specifically, GM-CSF also has pro-angiogenic effects through STAT-3 signaling activation[370].

We next employed the Olink multiplex platform (see Proximity extension assay above) to profile the proteome of conditioned medium from glioma cells in the different conditions. Interestingly, LD loading enhanced the secretion of key, pro-angiogenic factors (VEGF, HGF), MMP3, and Vimentin, which is part of the intracellular intermediate filament network and involved in LD biogenesis[371] but can also trigger axon growth through IGFR1 when secreted[372]. We then validated

the increased VEGF and HGF secretion by LD loading by ELISA as well as CAIX and Vimentin by immunoblotting. Interestingly, Tan *et al.*[373] recently found a role for HDL in rescuing diabetes-induced angiogenesis impairment, which was associated with SR-B1-mediated uptake and downstream HIF stabilization leading to enhanced VEGF-A production. Intriguingly, even though the identified proteins upregulated by LD loading are well established HIF targets, we did not find increased HIF stabilization during LD loading, either at early or later time-points.

Altogether, these results suggest that LD loading elicits a pro-angiogenic and immune response under hypoxic and post-hypoxic conditions *in vitro* and *in vivo*. Further functional studies *in vitro* will be required to determine the effect of the secretome of LD-loaded glioma cells on primarily endothelial cell activation and immune cell migration. In addition, and in the light of the elegant study by Schlaepfer[220], showing the role of lipogenesis in EV lipid composition, the involvement of EVs in this process is a nice avenue for future research. The main findings and mechanisms studied in Paper I and II are presented below *(Figure 11)*:

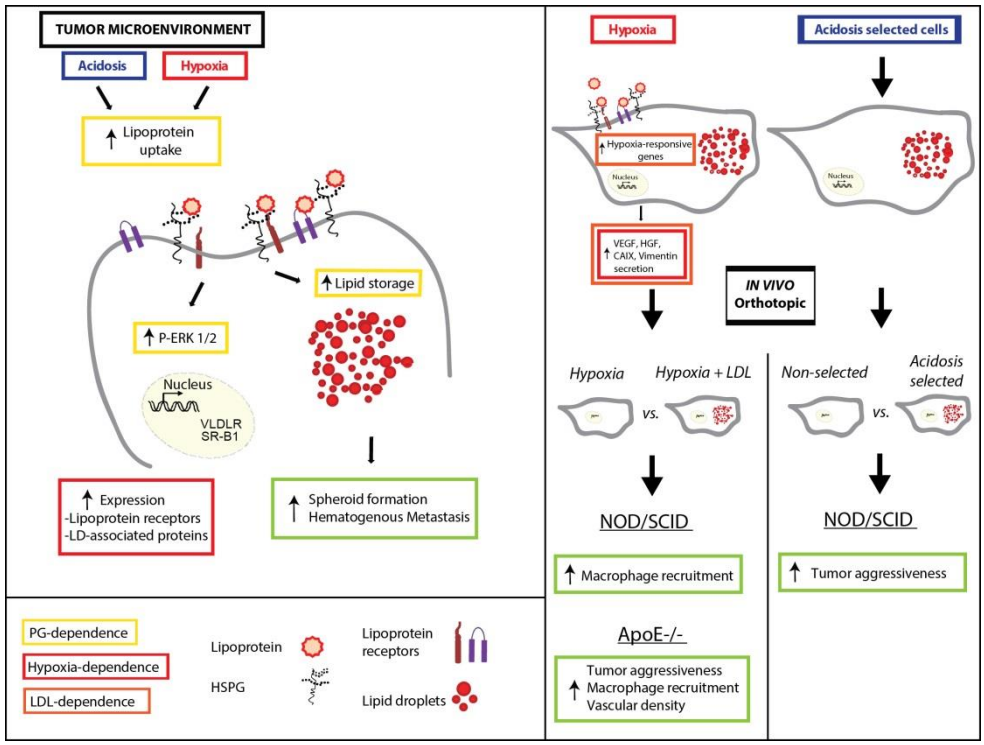

Figure 11 : Schematic figure summarizing the main mechanisms studied in Paper I (left panel) and Paper II (right panel)

Paper III

Hypoxia regulates global membrane protein endocytosis through caveolin-1 in cancer cells

Key results

- The global cancer cell-surface and internalized proteome are down-regulated by hypoxia.
- Major endocytic pathways are differentially regulated by hypoxia.
- Caveolin-1 expression is upregulated by hypoxia and negatively regulates cell-surface proteome internalization.
- Hypoxia downregulates cell-surface internalization by a dynamin-dependent mechanism.
- The hypoxic effect on surface proteome internalization is associated with caveolin-1 redistribution to the cell periphery in an HIF1-α independent manner.
- Several cell-surface proteins are actively internalized in hypoxia *vs.* normoxia and constitute interesting candidates for ADC targeting of the TME niche. CAIX was targeted as proof of concept.

Discussion

Hypoxia downregulates the cancer cell surface and internalized proteome through caveolin-1

The tightly regulated endocytic processes of normal cells are altered in tumors and may play an important role in their aggressiveness, through oncogenic mutations of p53[374] and Ras[375]), RTK signaling aberrations (*i.e.* EGFRvIII[376], Met[377]), or abnormal integrin recycling driving cell migration[378]. Here, in **Paper III**, the dynamics of the global cell surface proteome and its internalization under hypoxia were studied using a surface protein biotinylation method. Our results indicate a decrease in the global hypoxia-internalized secretome, negatively regulated by caveolin-1 in a dynamin-dependent manner. Interestingly, although necessary for caveolae formation and the uptake of various ligands and receptors (*i.e.* HER-2[379], albumin-paclitaxel nanoparticle[380]), caveolin-1 (22 kDa) has been shown to have a stabilizing and negative role in the endocytosis of several membrane raft-associated proteins, including in a dynamin-dependent manner[381]. Consistent with this idea, we found that acute hypoxia (2 h) triggered caveolin-1 relocation to the plasma membrane, and this occurred independently of HIF. The details of

caveolin-1 relocation under hypoxia constitute an interesting focus for further studies. Wickström *et al.*[382] found for instance a role for integrin β1 and integrin-linked kinase signaling at the plasma membrane in regulating the trafficking of caveolin-1 along microtubules. How this mechanism or similar trafficking events are affected by hypoxia would be worth investigating. Further, caveolin-1 can be palmitoylated, *i.e.* conjugated with palmitic acid, which promotes its association with and phosphorylation by Src kinase at Tyr14[383,384]. Caveolin-1 phosphorylation in turn impacts caveolae release[385] and endocytosis following receptor-ligand activation[386]. Hypoxia could influence caveolin-1 palmitoylation or phosphorylation and downstream trafficking, and this is presently under active investigation in our group.

Hijacking the hypoxia-internalized proteome for ADC targeting

Interestingly, we later showed that several surface proteins were overexpressed and more efficiently internalized in hypoxic conditions. These proteins included several proteins known to be part of the classical hypoxic response (*e.g.* CAIX, MCT4, GLUT transporters), integrins, RTKs as well as the SR-B1 receptor found to be increased on the surface and internalized at hypoxia. Although at a relatively late time-point of hypoxia (20 h), where we found no induction of lipoprotein uptake, this finding is interesting in the context of the increased HSPG-mediated lipoprotein uptake in **Paper I**, and may further strengthen a role for HSPG/SR-B1 cooperative mechanisms in lipoprotein uptake. More importantly, the proteins found to be hypoxia upregulated and more internalized under hypoxia constitute valuable candidates as ADC targets. Indeed, we could show that an anti-CAIX ADC was efficiently taken up in hypoxic cells in a caveolin-1-regulated manner, and that this resulted in specific cell killing of hypoxic cells. Further studies, including *in vivo* effects would be of great interest. This is of particular importance considering that since the approval in 2013 of T-DM1 ADC against HER2 expressing breast tumors[387], several clinical trials failed early, mostly due to insufficient target tumor expression or specificity[388]. Here, the high hypoxic expression and internalization of CAIX and other potential candidate proteins provide a stronger rationale to select for patients with highly resistant, hypoxic tumors.

Paper IV

Tumor antigen glycosaminoglycan modification regulates antibody-drug conjugate delivery and cytotoxicity

Key results

- CAIX is a part-time PG bearing CS or HS chains.
- CAIX GAG substitution is associated with increased cell aggregation.
- CAIX GAG substitution negatively regulates its internalization by increased association with pH sensitive caveolin-1 rich lipid domains.
- GAG depletion enhances the anti-CAIX ADC cytotoxic effect.

Discussion

CAIX GAG modification regulates its internalization under TME conditions through caveolin-1

Because of its central role in tumoral pH regulation and hypoxic overexpression, CAIX is both an interesting biomarker candidate and relevant therapeutic target. Here, we identified CAIX as a part-time PG, carrying a single chain of either CS or HS GAG at serine 54. CAIX GAG modification did not impact its dimerization, catalytic activity, or the cell proliferation rate. However, the GAG chain of CAIX participates in cell-cell homotypic aggregation. Along this line, we found that CAIX GAG depletion triggered its increased internalization.

At the endocytic level, we next found that CAIX co-localized with raft region markers and that GAG depletion was associated with decreased association with caveolin-1. Interestingly, acidosis had the same effect on CAIX re-localization in the membrane.

GAG modification as a fine-tuner of ADC delivery in the TME

Under hypoxic conditions, CAIX is highly internalized and thus constitutes a valid candidate for ADC delivery (**Paper III**). In this context, GAG modification of CAIX protein provides yet another level of regulation and specificity by negatively regulating its internalization. In addition, the acidosis-induced re-localization of CAIX is a nice example of how tumor microenvironmental conditions constitute both a challenge but also offer specific targeting possibilities.

Accordingly, CAIX GAG depletion, either genetically or pharmacologically by PNP-xyloside priming enhanced cell killing by anti-CAIX ADC targeting. *In vivo* experiments confirming the ADC effect would be of great interest, either following injection of wild-type *vs*. GAG-deficient CAIX cancer cells or using GAG-depleting agents together with ADC injection on wild-type cells.

Conclusions and Future perspectives

In this thesis, several adaptive mechanisms of tumor cells to their hypoxic and acidic microenvironment have been elucidated. The integration of signals originating from the oncogenic drive and direct signaling activation resulting from the TME parameters shape the cancer cells into specific and selective phenotypes in a dynamic manner.

At the metabolic adaptation level, we propose that signaling activation and endocytic mechanisms through PGs and GAG substitution represent interesting and relevant targets owing to their important roles in the plasticity of cancer cells under TME selection pressure. A major challenge, however, is that the current toolbox for *in vivo* targeting of PGs and GAGs is too unspecific, usually targeting HS or CS expressed also in normal tissue. The identification of tumor-specific GAG binding domains and their antibody targeting could pave the way for efficient blocking or delivery strategies. Strikingly, only 4% of the Database of Anti-Glycan Reagent are anti-GAG antibodies, highlighting the need for further efforts in this direction[389]. PG binding sites have been identified in ApoB100[204], ApoB48[390], ApoE[391] among others, but have not been fully implemented to my knowledge for blocking strategies, despite promising results of anti-GAG antibodies specifically blocking LDL binding[365]. Another useful anti-PG antibody is the M75 anti-CAIX[104]. As exemplified by the studies in **Paper III** and **IV**, in-depth understanding of the GAG-dependent trafficking and internalization of CAIX suggest targeted therapeutic approaches directed at this surface antigen. These results call for evaluation of the effects *in vivo* and further development of anti-CAIX ADC as well as targeting other identified hypoxia regulated proteins in **Paper III**. Besides, CD44 for instance is known to exist with or without GAG substitution, which regulates downstream signaling activation and metastasis[392], further highlighting the functional relevance of surface protein GAG targeting. Interestingly CD44 has been found enriched at the surface by hypoxia but downregulated following endocytosis compared to normoxic conditions (**Paper III**); how CD44 GAG modification may modify its internalization is also an interesting open question.

Nevertheless, altogether the density of GAG in the ECM and potentially redundant and/or autonomous mechanisms of internalization could complicate blocking approaches *in vivo.*

Interestingly, from the present studies, several links can be drawn to the hyperlipidemic, hypoxic and acidic situation found in atherosclerosis[393], which prompted us to investigate *in vivo* tumor effects in a model of systemic hyperlipidemia. The effects of hypoxia, acidosis and HSPG retention of lipoproteins in the arterial wall are key drivers of the atherosclerotic disease[394,395]. The effects of lipoprotein modifications (*i.e.* acetylation, oxidation) were not studied here but their role on tumor progression in the TME would represent an interesting area of research.

Besides, lipoproteins are structurally very similar to exosome EVs. How these two closely related particle types share and compete for the same internalization pathway could have important functional consequences, given the broad range of pro-tumorigenic cargoes carried in EVs[396]. Finally, EVs and lipoprotein mimetic particles are being developed as drug delivery vehicles due to their stability, half-life, and targeting capabilities. Interestingly, lipoprotein-like particles have been shown to transfer lipids to EVs intracellularly following SR-B1-mediated uptake further highlighting the possible interplay between the two particle types[397]. In these scenarios, HSPGs are expected to play a key role, which warrants further studies to gain detailed understanding of their internalization and intracellular sorting. The analysis of tumor-specific antigen glycosylation patterns, or concomitant interventional modulation of the cancer cell's glycan epitopes (by enzymatic edition for instance[398]) to ADC delivery, represent powerful tools for the design of novel PG-based targeted therapeutics.

Popular science summary

Cancer cells (the single units of the tumor bulk) are not the only components of a tumor. Several other cell types help them perform their functions, by ensuring the tumor architecture, its immune surveillance, its blood supply, all of this within a dense network of scaffold proteins. This is the tumor microenvironment. Moreover, as tumors grow, they quickly lack blood supply from the pre-existing vasculature meaning that they will find themselves in a stress environment with decreased oxygen (called hypoxic) and scarce nutrients. To continue proliferating in these conditions, they have to modify the pathways they use produce energy by "fermenting" glucose (glycolysis) instead of using the normal respiration needing oxygen. The drawback of this switch is that it produces acids. In low oxygen and acidic conditions, cancer cells can adapt to survive these conditions in which normal cells would die, to become more aggressive, spread more easily (metastasis) and resist treatments better.

The aim of this thesis work was to study the mechanisms by which cells adapt under these conditions to try and identify new treatment opportunities exploiting these mechanisms. We focused on the role of a specific type of proteins found on the surface of cells, called proteoglycans, which have the particularity to bear sugar chains that can bind many particles, including lipoproteins, the main fat carriers in the blood. In **Paper I**, we found that in hypoxia and acidic conditions, the cells internalized more lipoproteins and stored them into fat droplets. We showed that proteoglycans were needed for this. Further, fat-loaded cells could form tumor spheres better than non-loaded cells and metastasized more when injected in mice. In **Paper II**, we were interested in knowing more about the role of the fat storage the cells made under stress conditions once they were in better conditions and could proliferate. We found that these cells made more aggressive tumors when injected in mice. They also recruited more immune cells and triggered the formation of blood vessels which participate in the progression of the tumor. We could identify *in vitro* (in cells grown in the lab) molecules that fat-loaded cells produced in higher quantities that can explain the observations in the mice tumors. In **Paper III**, we studied how hypoxia modified the amount of proteins at the surface of cancer cells, and above all the number of proteins from the surface that the cells could internalize. We found that in hypoxia the cells generally have less protein on their surface but most importantly they also

internalize less proteins than non-stressed cells. We identified that caveolin-1, a protein that is found inside the cells blocked the internalization of the cell surface proteins in hypoxia. Interestingly several proteins were in the contrary more internalized during hypoxia, including one important protein for the survival of cancer cells in acidic conditions (because it pumps out toxic acids produced within the cells) called CAIX. One type of therapy that is very promising against cancer is called ADC, antibody-drug conjugate, it is made of a drug attached to an antibody that will recognize a specific protein on the surface of the cells, be internalized in the cells, liberate the drug inside the cell and kill the cell. We found that because CAIX is more internalized in hypoxia, when using an ADC to target CAIX, the cells were killed more efficiently. This means that the other proteins that were more internalized in hypoxia could also potentially be used for specific targeting of hypoxic tumor cells. In the **Paper IV**, we studied the structure of CAIX and found that CAIX was also a proteoglycan that could bear long sugar chains (called GAGs, glycosaminoglycans). We found that the GAG chain of CAIX reduced its internalization because CAIX bearing a GAG chain was preferentially associated with caveolin-1 (which blocks internalization) in the cell membrane compared to "naked" CAIX. Finally, when removing the GAG chain of CAIX, it was more efficiently internalized and make ADC treatment more effective. This is important for ADC therapeutics in general, because it means that other ADC target proteins that have GAGs could be simultaneously treated with the ADC and a drug that removes the GAG chain to make ADC treatments more effective and also more specific to the resistant hypoxic cancer cells.

From these studies, we could show further that proteoglycans are very important players in the context of the tumor microenvironment and we suggest new ways that could be used to develop novel therapeutic approaches.

Résumé simplifié

Les cellules cancéreuses (les plus petites unités vivantes qui composent les tumeurs) ne sont pas les seules composantes d'une tumeur. Plusieurs autres types cellulaires se trouvent au sein des tumeurs, et font partie intégrante de leur développement. Certaines assurent par exemple l'architecture de la tumeur, d'autres la surveillance immunitaire, l'approvisionnement sanguin et tout cela au sein d'un réseau de protéines de structure (collagènes…). Cet ensemble s'appelle le microenvironnement tumoral. Lors de leur croissance, les tumeurs vont rapidement être insuffisamment irriguées par les vaisseaux sanguins préexistants et se retrouver dans un environnement pauvre en oxygène (hypoxique) et en nutriments. Pour continuer à proliférer dans cette situation, les cellules cancéreuses sont obligées de modifier la façon dont elles produisent leur énergie à partir du glucose, en le «fermentant» (glycolyse) au lieu de respirer (une réaction qui a besoin d'oxygène) comme la plupart des cellules. L'inconvénient de la glycolyse est notamment la production d'acides toxiques qui doivent être évacués, rendant par conséquent leur environnement acide (acidose). Sous l'effet de l'hypoxie et de l'acidose, les cellules cancéreuses sont capables de s'adapter pour survivre et devenir ensuite plus agressives, elles acquièrent la faculté de se propager plus facilement (métastase) et sont plus résistantes aux traitements.

Le but de ce travail de thèse était d'étudier les mécanismes d'adaptation des cellules dans ces conditions afin d'identifier de nouvelles opportunités thérapeutiques. Nous nous sommes concentrés sur le rôle d'un type de protéine de la surface cellulaire, les protéoglycanes. Ces protéines ont la particularité de porter des chaînes de sucres (glycosaminoglycanes, GAGs) qui permettent de fixer différentes particules, dont les lipoprotéines, les principaux transporteurs de lipides dans le sang. Dans l'article I, nous avons montré que les cellules cancéreuses internalisaient les lipoprotéines en plus grande quantité en conditions d'hypoxie et d'acidose, et qu'elles se chargent en gouttelettes lipidiques. Nous avons montré que ceci dépend de la présence des protéoglycanes à la surface des cellules. Nous avons également mis en évidence que les cellules chargées de lipides dans ces conditions métastasent plus facilement. Dans l'article II, nous avons voulu connaître plus en détails le rôle du stockage de lipides observé en hypoxie et acidose une fois que les cellules avaient de nouveau la capacité de proliférer. Nous avons observé que ces cellules forment des tumeurs plus agressives. Elles

recrutent davantage de cellules immunitaires et entraînent la formation d'un plus grand nombre de vaisseaux sanguins qui participent à la progression de la tumeur. Nous avons ensuite identifié *in vitro* (dans des cellules cultivées en laboratoire) des molécules que les cellules chargées de lipides produisent en plus grande quantité et qui peuvent expliquer les observations faites dans les tumeurs de souris. Dans l'article III, nous avons étudié de manière plus globale comment l'hypoxie modifie la quantité de protéines à la surface des cellules et surtout la quantité de protéines internalisées. Nous avons montré qu'en hypoxie, les cellules ont généralement moins de protéines à leur surface mais internalisent surtout beaucoup moins de ces protéines que les cellules en conditions normales. Ceci est lié à la présence d'une protéine intracellulaire, la cavéoline 1, qui bloque l'internalisation des protéines de surface en hypoxie. Nous avons noté qu'au contraire de la tendance globale, de nombreuses protéines étaient davantage internalisées en hypoxie, notamment une protéine importante pour la survie des cellules en acidose, CAIX (qui permet de pomper les acides toxiques en dehors de la cellule). Un type de thérapie prometteuse contre le cancer utilise des ADC (anticorps couplés à des toxines), qui reconnaissent des protéines spécifiques de la surface des cellules, sont internalisés, libèrent leur toxine et tuent la cellule. Nous avons montré que parce que CAIX est davantage internalisée dans les cellules hypoxiques, l'efficacité des ADC contre CAIX est accrue. Ce résultat est intéressant car il suggère que les autres protéines qui sont plus internalisées en hypoxie constituent aussi des cibles intéressantes pour un traitement ADC spécifique aux cellules hypoxiques. Dans l'article IV, nous avons étudié plus en détails la structure de la protéine CAIX et avons identifié CAIX comme étant un protéoglycane avec une chaîne de GAG. Lorsqu'il porte une chaîne de GAG, CAIX est associé préférentiellement avec la cavéoline-1 dans la membrane de cellules, ce qui bloque son internalisation. En supprimant la chaîne de GAG, CAIX est internalisée plus facilement et l'efficacité d'un traitement contre CAIX accrue. Ce résultat est aussi important parce qu'il montre que l'efficacité spécifique des traitements ADC sur les cellules hypoxiques peut être améliorée lorsqu'ils ciblent des protéoglycanes, en appliquant simultanément des traitements qui suppriment les chaines de GAGs.

Ces études démontrent de nouveaux rôles pour les protéoglycanes dans les adaptations des cellules cancéreuses à leur microenvironnement et ouvrent la voie à de nouvelles approches thérapeutiques.

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