CHARACTERIZATION OF THE MECHANISMS BY WHICH CARBONIC ANHYDRASE IX FACILITATES TUMOUR GROWTH AND METASTASIS

by

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Abstract

The presence of hypoxic microenvironments in solid tumours is a marker of poor prognosis in numerous cancer types, including breast cancer; the second leading cause of cancer-related death.

Hypoxia results in an adaptive response in tumour cells through the activation of the transcription factor Hypoxia Inducible Factor-1α (HIF-1α), which stimulates the expression of a large number of genes that contribute to tumour progression. One of the most prominently activated genes is Carbonic Anhydrase IX (CAIX), which facilitates the acidification of the extracellular space and cell invasion by producing protons. Moreover, it assists in keeping the intracellular space neutral through the generation of bicarbonate, which is shuttled into the cytoplasm by bicarbonate transporters, ultimately favouring cell survival. CAIX facilitates breast tumour growth and metastasis; however the exact mechanism remains unknown. The overexpression of CAIX in hypoxic solid tumours, its limited expression in normal tissue and the presence of an extracellular catalytic domain makes this protein an excellent therapeutic target.

The intent of this thesis was to unveil the mechanisms by which CAIX facilitates tumour progression, and to characterize novel small molecule inhibitors and antibodies targeting CAIX.

It was found that the intracellular (IC) domain of CAIX regulates its catalytic activity, which is required for cell survival, cell invasion and metastasis. The extracellular proteoglycan-like (PG-like) domain of CAIX does not regulate CAIX catalytic activity; however it does modulate cell migration, invasion and metastasis. I identified a role of CAIX in promoting tumour cell invasion through interaction with membrane-bound matrix metalloprotease-14 (MMP-14) and localization in invadopodia. The IC domain of CAIX mediates this interaction and CAIX enzymatic activity appears to regulate the ability of MMP-14 to degrade type I collagen during cell invasion.

From the pool of anti-CAIX inhibitors and antibodies characterized in this thesis, the inhibitor U-104 was excellent at blocking CAIX enzymatic activity and has entered
phase I clinical trials. Likewise, anti-CAIX antibody MM-26 blocked 50% of CAIX activity and induced cell death *in vitro*.

The work described here provides new insight into the mechanism of CAIX-mediated tumour invasion and metastasis and has identified two new therapeutic strategies for targeting CAIX.
Preface

This thesis is presented in six chapters. All of the experiments described in Chapter 3 were designed by Dr. Shoukat Dedhar, Dr. Paul McDonald and myself and carried out by myself.

The flow enrichment experiments described in Chapter 4 were performed by the Flow Cytometry Core Facility (FCCF) housed in the Terry Fox Laboratory of the B.C. Cancer Research Center (BCCRC). Dr. Shawn Chafe and Dr. Eiko Kawamura helped me with the set up of the Flow Cytometer for membrane-bound CAIX analysis and Cell Cycle analysis, respectively (Chapter 4, Figures 4.4 and 4.13). Dr. Shawn Chafe generated the 4T1 knockdown cell lines (4T1shCAIX, Chapter 4, Figure 4.2) that I utilized for the overexpression of WT huCAIX and mutant forms. Dr. Mykola Maydan generated the CAIX constructs (Chapter 4, Figure 4.1). Dr. Shawn Chafe also generated the CAIX knockdown in MDA-MB-231 LM2-4, BxPC-3 and Pk-8 cell lines (Chapter 5, Figure 5.1). Mass spectrometry experiments described in Chapter 4 were performed in Dr. Leonard Foster’s laboratory at the Centre for High-Throughput Biology (CHiBi) in UBC.

Dr. Yuanmei Lou, Jordan Gillespie and Christina Ostlund performed the in vivo experiments described in Chapter 5. All animal studies and procedures were performed in accordance with protocols approved by the Institution Animal Care Committee at the BC Cancer Research Centre and University of British Columbia (Vancouver, BC, Canada) as per protocol number: A14-0058 and under the project’s title: “Targeting Carbonic Anhydrase IX and hypoxia for the diagnosis and treatment of aggressive breast cancer”.

Dr. Kevin Bennewith and Nancy E. LePard carried out the clonogenic experiments from the spontaneous metastasis assay (Figure 5.1) and Dr. Shawn Chafe and myself performed the clonogenic experiments from the experimental metastasis assay (Figure 5.2).

The real names of the novel antibodies developed by SignalChem Lifesciences Corporation and described in Chapter 3 have been hidden for patenting reasons and alternative names are used instead.
All other experiments were designed by Dr. Shoukat Dedhar and myself and carried out by me.
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List of Abbreviations

aa  amino acid
Ab  antibody
ADCC  antibody-dependent cell-mediated cytotoxicity
AE  anion exchanger
AMF  autocrine motility factor
ARP2/3  actin related proteins 2/3
ATP  adenosine triphosphate
AZA  acetazolamide
BCCRC  BC Cancer Research Centre
BM  basement membrane
BSA  bovine serum albumin
CA  catalytic
CAI/II/IX  Carbonic Anhydrase I/II/IX
CBP  CREB binding protein
CCD  charge coupled device
CDC  complement-dependent cytotoxicity
CHiBi  Centre for High-Throughput Biology
CNBr  cyanogen bromide
COX-2  cyclooxygenase-2
CTAD  C-terminal activation domain
DCIS  ductal carcinoma in situ
DKK1  Dickkopf-related protein 1
DMEM  Dulbecco’s modified Eagle medium
DMSO  dimethyl sulfoxide
DOCK1  Dedicator of cytokinesis 1
EC  extracellular
ECM  extracellular matrix
EGF  epidermal growth factor
ELISA  enzyme-linked immunoabsorbent assay
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ER-</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FCCF</td>
<td>Flow Cytometry Core Facility</td>
</tr>
<tr>
<td>FIH</td>
<td>factor inhibiting HIF</td>
</tr>
<tr>
<td>FITC-CAI</td>
<td>fluorescein isothiocyanate-Carbonic Anhydrase Inhibitor</td>
</tr>
<tr>
<td>GEFs</td>
<td>guanine-nucleotide exchange factors</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GLUT-1/-4</td>
<td>glucose transporter-1/-4</td>
</tr>
<tr>
<td>Gt IgG</td>
<td>goat IgG</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>H</td>
<td>hypoxia</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF-1</td>
<td>hypoxia-inducible factor-1</td>
</tr>
<tr>
<td>HK</td>
<td>hexokinase</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia response element</td>
</tr>
<tr>
<td>huCAIX</td>
<td>human CAIX</td>
</tr>
<tr>
<td>IC</td>
<td>intracellular</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton(s)</td>
</tr>
<tr>
<td>LCIS</td>
<td>lobular carcinoma \textit{in situ}</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LOX</td>
<td>lysyl oxidase</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>max</td>
<td>maximum</td>
</tr>
<tr>
<td>MCT</td>
<td>monocarboxylate transporter</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>MDR</td>
<td>multidrug resistance protein</td>
</tr>
<tr>
<td>min</td>
<td>minimum</td>
</tr>
<tr>
<td>MLC</td>
<td>myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>myosin light chain phosphatase</td>
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<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
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<tr>
<td>moCAIX</td>
<td>mouse CAIX</td>
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<tr>
<td>MoIgG</td>
<td>mouse IgG</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>MT-MMP</td>
<td>membrane-bound MMP</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>N</td>
<td>normoxia</td>
</tr>
<tr>
<td>N-WASP</td>
<td>neural Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>NBC</td>
<td>sodium bicarbonate co-transporter</td>
</tr>
<tr>
<td>NE AA</td>
<td>non-essential amino acids</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NHE</td>
<td>sodium-proton exchanger</td>
</tr>
<tr>
<td>ODDDD</td>
<td>oxygen-dependent degradation domains</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAR4</td>
<td>PRKC apoptosis WT1 regulator protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDAC</td>
<td>pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PDH</td>
<td>pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PDK</td>
<td>pyruvate dehydrogenase kinase</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PG</td>
<td>proteoglycan</td>
</tr>
<tr>
<td>PHD</td>
<td>prolyl hydroxylases</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
</tbody>
</table>
PIPs  phosphoinositides
PKA  protein kinase A
PR-  progesterone receptor
PTEN  phosphatase and tensin homolog
PVDF  polyvinylidene fluoride
Rb IgG  rabbit IgG
rCAIX  recombinant CAIX
RNAi  RNA interference
RPMI  Roswell Park Memorial Institute medium
RPPA  Reverse Phase Protein Array
RT  room temperature
s  second(s)
SDS  sodium dodecyl sulfate
shNS  shRNA non-silencing
shRNA  small hairpin RNA
SP1  specificity protein 1 transcription factor
SPR  Surface Plasmon Resonance
TGF-β  transforming growth factor-β
TIMP  tissue inhibitor of metalloproteinase
Tks-4/-5  adaptor protein Tyr kinase substrate with four/five SH3 domains
TM  transmembrane
TUNEL  terminal deoxynucleotidyl transferase dUTP nick end labeling
uPA  urokinase plasminogen activator
uPAR  uPA receptor
UVic  University of Victoria
V  volts
VEGF  vascular endothelial growth factor
VHL  von Hippel-Lindau tumour suppressor
WB  Western blot
Acknowledgements

I would like to express my gratitude to my supervisor Dr. Shoukat Dedhar, for the opportunity of training in his lab and for his mentorship throughout my graduate studies.

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To my mom and dad
Chapter 1. Introduction

1.1 Breast cancer

Breast cancer is a heterogeneous disease both clinically and biologically (van de Vijver et al., 2002) characterized by a distant pattern of metastasis involving regional lymph nodes, bone marrow, brain, lung and liver (Colditz, Baer, Heather, & Tamimi, 2006; Dewan et al., 2006).

1.1.1 Classification

Over 95% of breast cancers originate from the epithelial portion of the mammary gland and are therefore classified as adenocarcinomas. Two main classifications of breast malignancies exist: *in situ* (non-invasive) and invasive cancers. Both are morphologically similar, but *in situ* cancers remain confined to the duct (ductal carcinoma *in situ*: DCIS) or the lobule (lobular carcinoma *in situ*: LCIS) and do not infiltrate to the surrounding stroma like invasive cancers (Colditz et al., 2006). Around 80% of invasive cancers are infiltrating ductal carcinomas while infiltrating lobular carcinomas represent 10% of invasive cancers. Other types of breast cancer exist such as medullary, adenoid cystic, mucinous and tubular and they usually have an excellent prognosis (Colditz et al., 2006).

Breast cancer can also be classified in terms of the molecular genetic characteristics of the tumours. Tumours that express both estrogen and progesterone receptors (ER- and PR-positive) tend to be more differentiated. Patients with this type of tumours tend to have better prognosis and respond better to hormonal or anti-estrogen therapies (Colditz et al., 2006; Rosen, 2001). 20% of breast cancers display amplification of the HER2/neu protooncogene, which associates with poorer prognosis and poorer response to chemotherapy. HER2/neu also predicts response to anti-HER2 antibody Herceptin therapy which has been shown to improve response to chemotherapy in these types of tumours (Colditz et al., 2006; Pegram, Pauletti, & Slamon, 1998; Schnitt,
Triple negative breast tumours do not express HER2/neu nor the estrogen and progesterone receptors (i.e. they are HER2, ER, PR). This type of cancer accounts for 10-17% of all breast carcinomas and it is more aggressive and more resistant to therapy than the other molecular subtypes with most of the deaths occurring in the first 5 years following therapy (Dent et al., 2007).

1.1.2 Risk factors

Exposure to hormones such as estrogens plays an important role in breast cancer etiology. Therefore early menarche and late menopause are considered risk factors for breast cancer, together with nulliparity or late age of first pregnancy, obesity, high-fat diet, chronic use of hormone replacement therapy and family history of breast cancer (germline mutations of brca1, brca2 and p53) (Colditz et al., 2006; Dent et al., 2007; Ruddon, 2007).

1.1.3 Epidemiology

Breast cancer is the most common type of cancer among Canadian women and it is the second leading cause of cancer-related death both in Canada and the United States. Breast cancer in men is quite uncommon (Breastcancer.org, 2014; CSC, 2014; StatisticsCanada, 2010, 2014). It is estimated that there will be 24,400 new cases of breast cancer in women in 2014 in Canada, and only 210 new cases in men. Therefore the incidence rate for every 100,000 people is of 99 for women and 0.9 for men. Breast cancer has an estimated death rate for 2014 of 18 and 0.3 in women and men, respectively, for every 100,000 cases. The estimated five-year relative survival (for 2006-2008) is of 88% for females and 80% for men. However, five-year survival rates tend to be lower for triple-negative breast cancer (Breastcancer.org, 2014). The mortality rate for breast cancer is still very high, ranking second in the distribution of cancer-related deaths in women and surpassed only by lung cancer. Breast cancer accounts for 13.8% of all cancer-related deaths (CSC, 2014; StatisticsCanada, 2010, 2014).
1.1.4 Models for studying breast cancer

The mouse 4T1 breast tumour model of triple negative breast cancer has several characteristics that make it a good animal model for the study of human breast cancer (Pulaski & Ostrand-Rosenberg, 2001). 4T1 cells are one out of five tumour subpopulation lines that were isolated from a single mammary tumour that arose spontaneously in a Balb/cfC3H mouse (Dexter et al., 1978). 4T1 is a 6-thioguanine-resistant variant selected from the original 410.4 line without mutagen treatment. 4T1 cells form hypoxic tumours upon injection in the mammary gland of mice and were found to metastasize spontaneously through the haematogenous and lymphatic routes first to the lungs (within 7-14 days) and then to the liver (at around day 21) (Aslakson & Miller, 1992; Dexter et al., 1978). They also form metastasis in the brain and bones (Pulaski & Ostrand-Rosenberg, 2001). Interestingly, 67NR is another line derived from the same tumour that when injected in the mammary gland of mice forms well-vascularized tumours that are not hypoxic and do not present metastatic abilities (Aslakson & Miller, 1992; Dexter et al., 1978).

The fact that 4T1 cells can be injected orthotopically (i.e. in the mammary gland) in immunocompetent mice where they grow a tumour that later metastasizes spontaneously to the same organs as the ones observed in human breast cancer makes this model a suitable one for the study of human breast cancer. In addition to this, these cells are resistant to 6-thioguanine, which allows for precise quantification of the metastatic cells in distant organs (Pulaski & Ostrand-Rosenberg, 2001).

The human MDA-MB-231 tumorigenic breast cancer cell line was derived from a metastatic site (pleural effusion) of a 51-year old female. This model of triple negative breast cancer forms poorly differentiated adenocarcinomas (grade III) when injected in nude mice (ATCC, 2014b). The highly metastatic variant of the human adenocarcinoma MDA-MB-231, LM2-4 was obtained through surgical removal of the primary orthotopically transplanted tumour in mice followed by two rounds of metastasis selection (Munoz et al., 2006).
1.2 Pancreatic cancer

Cancer of the pancreas is currently an important medical and public health problem, especially due to the lack of early diagnosis, its aggressive behaviour and resistance to current therapy options (Anderson, Mack, Thomas, & Silverman, 2006). This type of cancer rapidly disseminates to the lymphatic system and distant organs such as the liver, lungs and peritoneal cavity, which accounts for its lethality (Hezel, Kimmelman, Stanger, Bardeesy, & Depinho, 2006).

1.2.1 Classification

About 95% of pancreatic cancers arise from the exocrine side of the pancreas, which is composed of acinar cells that produce and discharge enzymatic secretions into the pancreatic ductal system. The other 5% is composed of endocrine tumours arising from the islets of Langerhans (Anderson et al., 2006). Cancers of the exocrine pancreas, or adenocarcinomas are thought to arise from ductal cells, and display an infiltrative growth pattern that extends beyond the gross tumour (Bardeesy & DePinho, 2002).

Mutated K-RAS oncogene is present in >90% of pancreatic ductal adenocarcinomas and represents the earliest and most frequent genetic alteration (Kanda et al., 2012; J. P. Morris, Wang, & Hebrok, 2010).

The uncommon islet cell tumours of the endocrine pancreas are often a feature of the hereditary syndrome, multiple endocrine neoplasia type I. Other rare types of pancreatic cancers exist such as sarcomas of the pancreas (Anderson et al., 2006; O’Meara, 2002).

1.2.2 Epidemiology

Pancreatic cancer is the fourth most common cause of cancer-related death in both men and women in Canada (CCS, 2014) and the United States (Siegel, Ma, Zou, & Jemal, 2014). The incidence rate of pancreatic cancer for women between 1992 and 2005 remained stable (8.49 and 8.48 per 100,000 people, respectively) and a 10% decrease
in the incidence in men was observed (from 11.1 in 1992 to 9.89 in 2005 per 100,000 people) (Flook & van Zanten, 2009).

Approximately 99% of pancreatic cancer cases occur in individuals older than 50 years of age. Risk factors associated with pancreatic cancer are smoking and family history of pancreatic cancer (Flook & van Zanten, 2009).

Pancreatic cancer has the poorest 5-year survival rate among all major malignancies with 6% of the individuals surviving 5 years after diagnosis (Pancreatic Cancer Canada, 2011). Median survival is about 3 months after diagnosis. It is estimated that this type of cancer accounts for 5% of all cancer deaths (Siegel et al., 2014). The total number of annual pancreatic cancer cases in Canada is expected to more than double from 2636 in 2006 to 5619 in 2031 (Flook & van Zanten, 2009).

1.2.3 Models for studying pancreatic cancer

Several pancreatic cancer cell lines have been derived and are available for the study of pancreatic cancer in vitro. BxPC-3 is a pancreatic cancer human cell line derived from a 61-year old female with primary adenocarcinoma of the pancreas. Cells can form tumours within 21 days of subcutaneous injection in nude mice (ATCC, 2014a). PK-8 is another pancreatic cancer cell line originally established by Tachibana’s group (Kobari et al., 1986). It was derived from a xenografted tumour in athymic nude mice (Balb/c nu/nu) from liver metastasis of carcinoma of the head of the pancreas. Tumours are developed after 2 weeks of subcutaneous implantation in athymic nude mice (Kobari et al., 1986).

1.3 Hallmarks of cancer

Hanahan and Weinberg have proposed that cancer cells need to acquire certain biological capabilities during their development as tumour cells. The hallmarks of cancer proposed by them include: 1) the ability to sustain proliferative signalling, 2) the
ability to evade growth suppressors, 3) the ability to evade cell death, 4) the ability to become immortal, 5) the ability to induce angiogenesis and 6) the ability to activate invasion and metastasis (Hanahan & Weinberg, 2000). Two emerging hallmarks were proposed in 2011: i) the ability of cells to reprogram their metabolism (in the absence of oxygen and nutrients) and ii) the ability to evade the immune response. Genomic instability generates the genetic diversity required by the tumour. In the same way, it is becoming more recognized that the tumour microenvironment plays an important role in almost every single step of tumour progression and metastasis (Quail & Joyce, 2013).

Inflammation has been proposed to enhance tumourigenesis and cancer progression since it supplies growth factors, survival factors, pro-angiogenic factors and enzymes that remodel the extracellular matrix (ECM) (Hanahan & Weinberg, 2000, 2011).

1.3.1 Cell migration, invasion and metastasis

Migration and invasion are important processes for the tumour cell since they allow the cell to move within the tissue and eventually metastasize to distant organs. Whereas migration is a complex process comprising the movement of the cells only, invasion requires the penetration of the basement membrane and stroma. Invasion encompasses adhesion of the cells, degradation of the ECM and migration (Friedl & Wolf, 2003).

A tumour cell needs to successfully complete a number of steps in order to get from the primary tumour to a distant organ. These steps have been called the “invasion-metastasis cascade” and include: 1) the induction of epithelial to mesenchymal transition (EMT), 2) local invasion through the extracellular matrix and stromal cell layers, 3) intravasation into lymphatic and blood vessels, 4) survival in the vasculature, 5) arrest at distant organ sites, 6) extravasation into parenchyma of distant organs, 7) survival and formation of micrometastasis and 8) re-activation of proliferative programs to generate macrometastasis. The important role of stromal cells during this invasion-metastasis cascade is now very well recognized (Valastyan & Weinberg, 2011).
1.3.1.1 EMT

Epithelial-mesenchymal transition refers to the ability of epithelial cells to partially or fully transition into a mesenchymal phenotype (Lamouille, Xu, & Derynck, 2014). EMT is integral to development and is also a very important step during the invasion process since it marks the transition from collective to single-cell migration (Friedl & Wolf, 2003; Lamouille et al., 2014). During EMT, epithelial cell-cell junctions are dissolved (due to decreased claudin and occludin expression and consequent cleavage and degradation of E-cadherin), the apical-basal polarity is lost, the cytoskeleton undergoes reorganization, and there is downregulation of the epithelial gene expression signature and activation of mesenchymal genes. As a consequence, cells undergo changes in cell shape, protrusions are formed and cells become more motile. In many cases, cells acquire the ability to degrade the ECM (Figure 1.1) (Lamouille et al., 2014).

Figure 1.1. Epithelial-mesenchymal transition. Epithelial cancer cells (left) have the ability to partially (middle) or fully (right) transition into a mesenchymal phenotype. Characteristics of each phenotype and effectors of this process are indicated. EMT: epithelial-mesenchymal transition, ECM: extracellular matrix. Modified from (Kalluri & Weinberg, 2009).
1.3.1.2 Different types of cell migration

During the migratory process, cells modify their shape and stiffness to interact with the ECM (Friedl & Wolf, 2003). Tumour cells can migrate and invade as individual cells: ‘individual cell migration’ or as multicellular units: ‘collective invasion’ (Figure 1.2) (Valastyan & Weinberg, 2011).

1.3.1.2.1 Collective migration

Two variants of collective migration have been observed in vivo. The first one resembles steps during embryogenesis, where sheets and strands of cells invade locally while maintaining contact (Bell & Waizbard, 1986; Davidson & Keller, 1999). In the second one some clusters of cells detach from the sheets or strands and move as a functional unit along paths of least resistance (Figure 1.2) (Friedl et al., 1995; Nabeshima, Inoue, Shimao, Kataoka, & Koono, 1999). Interestingly, collective migration has been observed in cancers of epithelial origin too (Nabeshima et al., 1999). Cell-cell adhesion and cell-cell communication through cadherins and gap junctions, respectively are characteristics of collective migration (Friedl & Wolf, 2003). During this type of migration the cells at the front are highly motile and are the ones that generate migratory traction through formation of pseudopods. It has been observed that these cells can undergo total or partial EMT. The cells behind are just dragged passively (Friedl et al., 1995). The leading cells interact with integrins and require protease-mediated degradation of the ECM in order to generate tracks (Nabeshima et al., 2000). A tight coordination of cytoskeletal activity should exist during collective invasion (Madhavan et al., 2001).

1.3.1.2.2 Individual migration

Two interconvertible programs of individual cell migration have been described. The first one is protease-, stress-fiber- and integrin-dependent and is called mesenchymal migration. This type of movement has been observed in cancers of mesenchymal origin such as fibrosarcomas (Wolf et al., 2003) but epithelial cancer cells can also adopt a mesenchymal migratory phenotype through EMT (Moll, Mitze, Frixen, & Birchmeier,
The second type of individual cell migration is protease-, stress-fiber- and integrin-independent, but RHO/ROCK-dependent and is called amoeboid migration (Figure 1.2) (Friedl & Wolf, 2003; Valastyan & Weinberg, 2011). This form of migration is present in very motile cells such as neutrophils, dendritic cells and lymphocytes that glide through the substrate (Friedl & Wolf, 2003; Lämmermann & Sixt, 2009) and has also been observed in lymphomas and small-cell lung carcinomas (Rintoul & Sethi, 2001; Verschueren, De Baetselier, & Bereiter-Hahn, 1991).

Mesenchymal cells move via the five-step migration cycle described below (Figure 1.3) (Friedl & Wolf, 2003). These cells display a fibroblast-like morphology and their migration is dependent on integrin-mediated adhesion. Traction forces are also generated on both cell poles (Friedl & Wolf, 2003; Pals & De Gortier, 2010). Integrins, proteases and membrane-type matrix metalloproteases (MT-MMPs) have been found at fibre binding sites where they execute proteolysis of the ECM (d’Ortho et al., 1998; Sameni, Moin, & Sloane, 2001; Wolf et al., 2003). During mesenchymal integrin-mediated migration, adhesions need to form and turn over rapidly, which together with their polarization are going to dictate cell speed and directional persistence (Huttenlocher & Horwitz, 2011). Adhesion is important because it generates traction by linking the actinmyosin filaments to the ECM (Huttenlocher & Horwitz, 2011; Izzard & Lochner, 1976).

On the other hand, during individual amoeboid cell migration there is very little integrin-mediated adhesion to the substrate and there have been some reports of it being completely integrin-independent (Figure 1.2) (Lämmermann et al., 2008). This movement is driven by short and weak interactions with the substrate generated by cortical filamentous actin. Therefore, cells that undergo amoeboid migration do not degrade their ECM barriers. Cells are usually highly deformable because of their lack of focal contacts, which allows them to move faster than during other types of cell migration (Friedl & Wolf, 2003; Pals & De Gortier, 2010). Conditions such as abrogation of pericellular proteolysis, disruption of integrin-mediated adhesion and a confined
environment have been shown to induce a switch from mesenchymal to amoeboid migration (Lämmermann et al., 2008; Liu et al., 2015; Wolf et al., 2003).

![Diagram](image)

**Figure 1.2. Different types of cell migration.** Individual cancer cells can move independently by adapting a ‘mesenchymal’ phenotype or by undergoing the protease-independent ‘amoeboid’ cell migration. On the other hand, ‘multicellular strands’ or ‘sheets’ that do not detach are able to invade local areas. Some ‘clusters’ may detach and move to more distant tissues. The plasticity of cancer cells allows them to interconvert between different migration programmes (indicated by black arrows). EMT: epithelial-mesenchymal transition, ECM: extracellular matrix. Modified from (Friedl & Wolf, 2003).

A five-step model of individual mesenchymal cell migration in 2D has been established over the past 40 years (Figure 1.3) (Friedl & Wolf, 2003). Individual mesenchymal cell migration through tissues involves a multistep process of protrusion, adhesion to the substrate, stabilization at leading edge, proteolysis, cell body translocation and release of adhesions and detachment of the cell's trailing edge (Abercrombie, Heaysman, &
Pegrum, 1971; Huttenlocher & Horwitz, 2011; Ridley et al., 2003). Importantly, when cells move in 3D they need to also remodel the ECM (see section 1.3.1.4).

The first step during mesenchymal cell migration involves the protrusion of the leading edge where actin polymerization plays a big role. The neural Wiscott-Aldrich syndrome adaptor protein (N-WASP) binds to phosphoinositides (PIPs) on the inner side of the plasma membrane. PIPs bind and activate guanine-nucleotide exchange factors (GEFs) that regulate the activity of small GTPases RAC, RHO, and CDC42. The binding of N-WASP to PIPs and to CDC42 activates N-WASP and induces the recruitment of the actin-nucleating ARP2/3 complex. The subunits 2 and 3 of this complex resemble the structure of monomeric actin and this allows them to serve as nucleation sites for new actin filaments. RHO GTPase promotes actin stress fibre formation whereas CDC42 and RAC participate in filopodia and lamellipodia formation, respectively (Friedl & Wolf, 2003; Kaibuchi, Kuroda, & Amano, 1999; Rohatgi et al., 1999). The second step involves interaction with the ECM and formation of focal contacts. Clustered integrins initiate intracellular signalling which prompts the recruitment of actin-binding proteins such as talin, vinculin and paxillin to focal contacts (Friedl & Wolf, 2003; Miyamoto et al., 1995).

During the third step proteases are recruited and proteolysis of the ECM takes place. Soluble gelatinases like MMP-2 and MMP-9 as well as membrane-bound collagenases like MMP-14 have been found to be particularly important in cancer progression (Friedl & Wolf, 2003; Ohuchi et al., 1997). The fourth step involves cell contraction, which occurs when active myosin II binds to actin filaments (Friedl & Wolf, 2003; Katoh et al., 2001). This is regulated by the Ca²⁺- and calmodulin-dependent myosin light-chain kinase (MLCK) which phosphorylates myosin light chain (MLC) activating myosin II. RHO/ROCK also phosphorylates and activates myosin II. On the other side, this process is enforced by the RHO/ROCK-mediated inactivation of the MLC phosphatase (MLCPTase) (Friedl & Wolf, 2003; Kamm & Stull, 2001). Finally, the disassembly of focal contacts allows the detachment of the trailing edge. Several mechanisms are involved in this process. For example, cleavage of focal contact components such as integrins mediated by membrane-bound sheddases (Friedl & Wolf, 2003; Moss & Lambert, 2002).
Figure 1.3. The five-step model of individual cell migration and the role of pH. Cell migration is a multistep process of (1) pseudopod protrusion, (2) adhesion to the substrate, (3) proteolysis, (4) actomyosin contraction and (5) translocation and detachment of trailing edge. See main text for details on each step. A slightly alkaline intracellular pH (green star) is required for the de novo assembly of actin filaments (step 1 and 2), the function of GTPase CDC42 (step 1) and the regulation of new focal adhesions (step 2). Intracellular pH regulation by NHE-1 modulates cell polarity and directional migration. A local acidic extracellular pH favours ECM proteolysis (step 3, blue star), while a local acidic intracellular pH regulates actomyosin contraction (step 4). The steps where CAIX could be playing a role are indicated with a green ellipse. ECM: extracellular matrix. MLCK: myosin light-chain kinase. MLCPtase: myosin light-chain phosphatase. PIPs: phosphoinositides. Modified from (Friedl & Wolf, 2003).
1.3.1.3 pH and migration

Dysregulated pH often observed in cancer cells can promote cell migration and invasion through different mechanisms (Webb, Chimenti, Jacobson, & Barber, 2011). Indications of this come from the observation that the migration of human melanoma cells is optimal at pH 7.0 but suboptimal at higher or lower pH values. Maximal adhesion was observed at extracellular pH values of 6.6-6.8 (Stock et al., 2005).

pH regulates many processes that underlie cell migration such as cell volume, cytoskeletal dynamics and reorganization and cell adhesion (Stock & Schwab, 2009).

The sodium-proton exchanger NHE-1, an important component of the pH homeostasis in the cell that is also heavily implicated in tumourigenesis, promotes cell polarity and directional migration by interacting with the cytoskeleton and by mediating its ion-transport activity (Denker & Barber, 2002; Stock & Schwab, 2009). The extrusion of protons and concomitant intrusion of sodium ions mediated by NHE-1 leads to water influx resulting in osmotic swelling, which favours cell migration and induces membrane swelling (Charras, Yarrow, Horton, Mahadevan, & Mitchison, 2005; Saadoun, Papadopoulos, Hara-Chikuma, & Verkman, 2005; Webb et al., 2011).

An intracellular pH higher than 7.2 increases the de novo assembly of actin filaments, due to the pH-dependent activities of actin-binding proteins such as talin (Srivastava et al., 2008; Webb et al., 2011). In the same way, it has been observed that the function of GTPase CDC42, which determines the polarity of migrating cells, requires a slightly alkalized intracellular pH (Figure 1.3) (Frantz, Karydis, Nalbant, Hahn, & Barber, 2007). The regulation of cell-substrate adhesion also requires an alkaline intracellular pH and an acidic extracellular pH (Figure 1.3). Actin filament binding by talin, a focal adhesion-associated protein stabilizes cell-substrate adhesions (Srivastava et al., 2008). The mechanism of pH-sensitive actin-binding by talin has been elucidated recently. The protonation of histidines induces changes in the conformation and dynamics of the actin-binding site in talin (Stock & Schwab, 2009). Focal adhesion turnover is also regulated by pH, since the aforementioned protein talin shows decreased binding to
actin filaments at alkaline pH values, which results in increased migration (Srivastava et al., 2008; Stock et al., 2008). Finally, the disassembly of adhesions and the retraction of the trailing edge in a migratory cell are modulated by myosin II and the RHOA/ROCK pathway, both sensitive to pH. Myosin II is activated only at the trailing edge of the cell due to the local low intracellular pH and high Ca^{2+} concentrations (Figure 1.3) (Ridley et al., 2003).

1.3.1.4 The role of MMPs in tumour cell invasion

Matrix metalloproteases (MMPs) belong to the family of zinc-dependent endopeptidases (Gross & Lapiere, 1962). The 23 MMPs found in humans are classified based on architectural features (Kessenbrock, Plaks, & Werb, 2010). The general structure commonly found in MMPs consists of three domains, the pro-peptide, the catalytic domain and the hemopexin-like C-terminal domain, which is linked to the catalytic domain via a flexible hinge region (Kessenbrock et al., 2010; Sternlicht & Werb, 2001).

MMPs can also be divided into secreted (MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, -13, -19, -20, -21, -22, -27, -28) and membrane-bound proteinases (MMP-14, -15, -16, -17, -23, -25) (Kessenbrock et al., 2010).

MMPs are initially expressed as zymogens (enzymatically inactive pro-forms) due to the interaction between a cysteine residue in the pro-domain with the zinc ion in the catalytic site (Kessenbrock et al., 2010). The cysteine switch occurs when the pro-domain is removed by proteolysis mediated by furin (intracellularly) or by other MMPs or serine proteases (extracellularly) (Kessenbrock et al., 2010; Sternlicht & Werb, 2001). For example, one monomer of MMP-14 forms a complex with tissue inhibitor of metalloproteinase 2 (TIMP-2) while the other monomer of MMP-14 cleaves the pro-form (zymogen) of soluble MMP-2 (72 kDa) to the intermediate form (68 kDa), a step that is necessary for complete activation by auto-catalytic cleavage of MMP-2 (Overall et al., 2000).
The activity of MMPs can be regulated in different ways such as compartmentalization, regulation of gene expression, conversion from zymogen to active enzyme, and the presence of physiological inhibitors such as TIMPs (Kessenbrock et al., 2010).

MMPs play an important role in various physiological processes outside of tumour progression. They participate in tissue remodelling and organ development (Kessenbrock et al., 2010; Page-McCaw, Ewald, & Werb, 2007) and in the regulation of inflammatory processes (Kessenbrock et al., 2010; W. C. Parks, Wilson, & López-Boado, 2004).

During cancer progression MMPs and cathepsins have been found to be very important for the local invasion process since they contribute to the loss of the basement membrane (BM) barrier through active proteolysis. It has been observed that cancer cells have enriched MMP activity (Kessenbrock et al., 2010; Valastyan & Weinberg, 2011). MMPs (in particular soluble MMP-1 and -2) were also found to be promoting breast carcinoma intravasation in synergy with cyclooxygenase-2 (COX-2) and epiuregulin (Gupta et al., 2007). Extravasation represents a greater barrier for tumour cells since the microvessels in distant normal tissues are expected to be intact and functional. It has been proposed that the primary tumour can secrete factors such as angiopoietin-like-4, epiuregulin, COX-2, MMP-2 and MMP-1, which then disrupt the vascular endothelial cell-cell junctions to facilitate extravasation into distant tissues (Gupta et al., 2007; Valastyan & Weinberg, 2011). Soluble MMP-9 has been found to be important for modifying the local microenvironment during micrometastasis formation. MMP-9 is secreted by hematopoietic progenitor cells and its activation results in the stimulation of integrins and liberation of molecules from the ECM like vascular endothelial growth factor (VEGF) (Psaila & Lyden, 2009).

**1.3.1.5 pH and invasion**

It has been proposed that extracellular tumour acidosis is a pro-invasion factor since it facilitates local invasion of tumour cells as well as metastasis formation (Calorini, Peppicelli, & Bianchini, 2012). Different studies have provided evidence to support this
theory. In an interesting study, the presence of peritumoural acid gradients in vivo was confirmed by utilizing pH-sensitive fluorescent probes and dorsal window chambers in mice. Degradation of the ECM was observed in the region immediately next to the tumour edge and coincided with zones exposed to the acidic microenvironment (R. A. Gatenby, Gawlinski, Gmitro, Kaylor, & Gillies, 2006). In a different study, transient alkalinization by systemic administration of sodium bicarbonate to mice bearing MDA-MB-231 mammary xenografts resulted in reduction of cathepsin and MMP activity as measured in vivo by utilizing enzyme activatable fluorescence agents, suggesting that the buffering of tumour extracellular pH affects optimal conditions for these enzymes. The exact mechanism involved was not investigated (Robey & Nesbit, 2013).

Furthermore, other studies have shown that pre-treatment of melanoma cells with acidic extracellular pH upregulates the expression of several proteolytic enzymes (MMPs and cathepsins) and pro-angiogenic factors (VEGF-A and interleukin (IL)-8), and prompts the development of metastasis in vivo (Martínez-Zaguilán et al., 1996; Rofstad, Mathiesen, Kindem, & Galappathi, 2006). It was found that low extracellular pH promotes angiogenesis through increased transcription of VEGF (Fukumura et al., 2001) and IL-8 (L. Xu & Fidler, 2000). Acidosis increases the transcriptional activation of VEGF through increased binding of transcription factors NF-κB and AP-1 to the IL-8 promoter (L. Xu & Fidler, 2000). A later study suggested that an acidic extracellular pH activates phospholipase C and increases binding of NF-κB to the MMP-9 promoter (Kato et al., 2005). Acidic extracellular pH also induces the redistribution of cathepsin B-containing vesicles to the cell membrane and enhances secretion of this protease in malignant cells (Rozhin, Sameni, Ziegler, & Sloane, 1994). Interestingly, the activation of the serine protease pro urokinase-type plasminogen activator (pro-uPA) by cathepsin L was found to be more effective at acidic pH values (Goretzki et al., 1992).

1.3.1.6 Invadopodia and tumour cell invasion

It is becoming more recognized that invasive cells develop structures called invadopodia. Invadopodia (podosomes in normal cells) are cell protrusions composed of an actin-rich core surrounded by many adhesion and scaffolding proteins (Linder &
Kopp, 2005; Murphy & Courtneidge, 2011). Actin nucleators, polymerization activators, actin-binding proteins, actin-crosslinking proteins, kinases and small GTPases all congregate at the invadopodia (Gimona, Buccione, Courtneidge, & Linder, 2008; Murphy & Courtneidge, 2011). Essential players of the invadopodia include the actin regulators cortactin and N-WASP, the Tyr kinase Src, adaptor protein Tyr kinase substrate with four SH3 domains (Tks-4), adaptor protein Tyr kinase substrate with five SH3 domains (Tks-5) and the transmembrane MMP-14 (also known as MT1-MMP) (Gimona et al., 2008; Murphy & Courtneidge, 2011). Importantly, mature invadopodia will have recruited and activated multiple proteases including MMP-14, MMP-9 and MMP-2, the ADAM family of sheddases, cathepsins and serine proteases such as urokinase plasminogen activator surface receptor (uPAR) (Linder, 2007; Murphy & Courtneidge, 2011; Stylli, Kaye, & Lock, 2008).

It is believed that podosomes and invadopodia coordinate the degradation of the ECM with cell motility. In support of this, podosomes are found in normal cell types involved in tissue remodelling like osteoclasts, which participate in bone resorption (Burgstaller & Gimona, 2005), while the presence of invadopodia has been correlated with invasion and metastasis (Murphy & Courtneidge, 2011).

It is important to separate invadopodia from other types of cellular protrusions or adhesive structures, such as filopodia, lamellipodia and focal adhesions, although it has been observed that all these structures share many common proteins, in particular the ones that regulate actin polymerization. However, their lipid composition, localization and morphology is what distinguishes one from the other (Murphy & Courtneidge, 2011). Invadopodia, unlike other types of cell protrusions, are found ventrally under the nucleus, are sites of strong proteolysis, usually last for a few hours and they protrude farther than podosomes of normal cells (Murphy & Courtneidge, 2011; Weaver, 2008).

The process underlying the formation of invadopodia is just beginning to be understood (Figure 1.4). During initial steps, focal adhesions are dissolved and Src phosphorylates Tks-5 originating the re-localization of Tks-5 to regions containing PIPs. Tks-5 displays
a PIP-binding domain (PX domain). This switch is stimulated by growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor-β (TGF-β). Tks-5 might form the scaffold that recruits cortactin in precursors of invadopodia (Murphy & Courtneidge, 2011; Oser et al., 2009). Initially, cortactin sequesters cofilin, inhibiting cofilin’s actin severing activity (Magalhaes et al., 2011). The actin-binding protein talin recruits the moesin-NHE-1 complex to the invadopodia (Beaty et al., 2014). The Src-mediated phosphorylation of cortactin induces the binding of cortactin to NHE-1. This, together with the local increase in pH caused by NHE-1 facilitates the liberation of cofilin, which severs actin to form the free barbed ends required to elongate actin filaments (Magalhaes et al., 2011; Murphy & Courtneidge, 2011). The assembly of the invadopodia also requires the recruitment of actin-binding proteins such as ARP2/3 and N-WASP, which support actin nucleation and polymerization (Murphy & Courtneidge, 2011; Yamaguchi et al., 2005). Tks-5 forms a complex with N-WASP and cortactin, while cortactin also associates with N-WASP (Murphy & Courtneidge, 2011). Dephosphorylation of cortactin inhibits the severing activity of cofilin allowing the stabilization of invadopodia (Magalhaes et al., 2011; Murphy & Courtneidge, 2011). A mature invadopodia is capable of degrading the ECM through the coordinated secretion of soluble MMP-2 and MMP-9 and the presentation of MMP-14 at the tip of the protruding structure (Buschman et al., 2009; Emily S Clark, Whigham, Yarbrough, & Weaver, 2007; Murphy & Courtneidge, 2011). MMP-14 is expressed at basal levels at the cell’s plasma membrane and it undergoes endocytosis and recycling from late endosomes (Remacle, Murphy, & Roghi, 2003). It is believed that the origin of invadopodial MMP-14 is from late endosomes or from intracellular stores of the secretory pathway, and that Tks-4 is involved in tethering MMP-14 at the invadopodia membranes (Bravo-Cordero et al., 2007; Buschman et al., 2009; Remacle et al., 2003).
Figure 1.4. The steps of invadopodia formation. (1) Initiation: TGF-β, PDGF and EGF stimulate the formation of invadopodia. Src kinase phosphorylates Tks-5, which re-localizes to regions containing PIPs. Talin recruits NHE-1. (2) Assembly: Actin-binding proteins such as N-WASP and Arp2/3 are recruited. Phosphorylation of cortactin induces recruitment of NHE-1, which increases the local pH and liberates cofilin. Cofilin severes actin filaments. (3) Maturation: the degradation of the ECM is facilitated through the secretion of MMP-2, -9 and the presentation of MMP-14 to the tip of the cell protrusion. Dephosphorylation of cortactin causes binding to and inactivation of cofilin. The potential role of CAIX in contributing to the acidification of the extracellular space and facilitating ECM degradation is indicated with a green ellipse. ECM: extracellular matrix. PIPs: phosphoinosites. Modified from: (Murphy & Courtneidge, 2011).
Only a few studies have investigated the role of podosomes during embryonic development. Deletion of the podosome and invadopodia associated protein Tks-4 originates a phenotype consistent with defects in neural crest derived cells (Iqbal et al., 2010; Murphy & Courtneidge, 2011). The Tks-5 protein has also been implicated in neural crest cell migration (Murphy & Courtneidge, 2011), while MMP-14, ADAM19 and collagen seem to be implicated in cell movement during gastrulation (Coyle, Latimer, & Jessen, 2008; McCusker, Cousin, Neuner, & Alfandari, 2009).

On the other hand, there is growing evidence describing the role of invadopodia proteins in cancer cell invasion and metastasis. Recent reports have utilized intravital tumour imaging to visualize invadopodia structures in fibrosarcoma, melanoma, breast and bladder cancer models in vivo (Leong et al., 2014). In a different study, MMP-14 was shown to be absolutely required for cancer cell invasion through 3D gels of native type I collagen and through the stromal barrier of mammary gland explants (Sabeh, Shimizu-Hirot a, & Weiss, 2009). In vivo the inhibition of MMP-14 impairs tumour growth and invasion while overexpression of this collagenase promotes tumourigenesis (Hotary et al., 2003). MMP-14 expression is also associated with poor prognosis in a variety of human cancers including breast, lung and colorectal cancers (Têt u et al., 2006; Y.-Z. Wang et al., 2014; Yang, Gao, Rao, & Shen, 2013), while the levels of invadopodia-associated protein cortactin correlate with aggressiveness in squamous cell carcinoma and in head and neck carcinoma (E S Clark et al., 2009; Murphy & Courtneidge, 2011).

Hypoxia is another major factor that stimulates invasiveness of the tumour cell (Pouysségur, Dayan, & Mazure, 2006) and will be described in more detail in the next section.
1.4 Hypoxia, HIF-1α and pH homeostasis

1.4.1 Hypoxia and the hypoxia-inducible factor-1α (HIF-1α)

It is now generally recognized that the vast majority of solid tumours develop hypoxic areas (Fyles et al., 2006; Kimbro & Simons, 2006). Hypoxia refers to low oxygen levels and though it is loosely defined, it often refers to pO₂ (partial pressure of oxygen) values of less than 5-10 mmHg (Chaudary & Hill, 2007). Hypoxia develops as a result of excessive tumour proliferation - which causes tumours to outgrow their blood supply - and poor or abnormal vasculature, which might refer to irregular architecture, blind ends and leakiness, among others. The amount and the size of hypoxic regions within a tumour may vary (from <1% to >50%) (Bennewith & Dedhar, 2011) and tumour cells might be exposed to persistent (chronic) or varying (acute) hypoxia (Boyle & Travers, 2006; Chaudary & Hill, 2007). However, it has been observed that tumours with larger areas of hypoxia are more likely to metastasize and the extent of hypoxia in tumours is a marker of poor prognosis in several solid cancers including lung, cervical, pancreatic, and breast cancer (Hockel et al., 1996; Schindl et al., 2002; Sun et al., 2007; Q. Wang et al., 2014). Likewise, increased hypoxia-inducible factor-1α (HIF-1α) and VEGF expression in patients with untreated prostate cancer was found to be a predictor of a shorter time to treatment failure (Vergis et al., 2008).

Hypoxic tumours are also more resistant to radiotherapy and chemotherapy (Bertout, Patel, & Simon, 2008). Hypoxia-mediated resistance to therapy involves both direct and indirect mechanisms. For example, radiotherapy requires cellular O₂ to react with the free radicals produced in order to generate a peroxy radical that chemically modifies and damages the DNA (Gray, Conger, Eber, Hornsey, & Scott, 1953; Multhoff, Radons, & Vaupel, 2014). Hypoxic cells also upregulate the multidrug resistance protein (MDR1), an ABCB1 transporter that extrudes drugs (J. Chen et al., 2014; Comerford et al., 2002). Furthermore, abnormal vasculature caused by hypoxia-induced expression of VEGF limits drug diffusion and delivery (Mésange et al., 2014). Hypoxic cells also display a slower cell cycle, elevated DNA repair, reduced apoptosis and survival, all of which reduce the efficacy of radio and chemotherapies (Mayer & Vaupel, 2013).
Hypoxia induces a HIF-1α-mediated cascade that results in the nuclear translocation of HIF-1α and subsequent activation of hypoxia-regulated genes. HIF is a transcription factor participating in the activation and/or repression of a number of genes involved in maintaining cell homeostasis and tumour progression (C. Brahimi-Horn & Pouysségur, 2006). HIF is a heterodimeric complex, composed of one of three oxygen-regulated α-subunits and one of two constitutively expressed β-subunits (M. C. Brahimi-Horn & Pouysségur, 2009).

Under normal oxygen levels HIF-1α is hydroxylated in two proline residues by prolyl-hydroxylases (PHD proteins) that require oxygen, 2-OG and Fe^{2+} as co-factors (Pouysségur et al., 2006). The residues modified are P402 and/or P564 and they are part of the oxygen-dependent degradation domain (ODDD) of HIF-1α. This modification is recognized by the tumour-suppressor protein von Hippel-Lindau (VHL), which is a component of an E3 ubiquitin ligase complex and ubiquitinates HIF-1α targeting it to proteasomal degradation (Pouysségur et al., 2006). In addition to this, the factor inhibiting HIF-1 (FIH) is another type of oxygen sensor controlling the hypoxic response. FIH also hydroxylates HIF-1α this time on an asparagine residue (N803) located in the most carboxy-terminal transcripational activation domain (C-TAD). The consequence of this modification is transcriptional repression through the impairment of the interaction of the C-TAD with transcriptional co-activator p300/CBP (Pouysségur et al., 2006). In hypoxia, there is less oxygen therefore PHD and FIH enzymes are not as active and the levels of HIF-1α are stabilized and/or HIF-1α-mediated transcription is not repressed (Pouysségur et al., 2006; Schofield & Ratcliffe, 2004; Semenza, 2004). Studies performed in vitro have shown that HIF-1α gets activated at a cut-off value of 1.5% oxygen (10 mmHg) (Pouysségur et al., 2006).

Stable HIF-1α dimerizes with HIF-1β and translocates to the nucleus (M. C. Brahimi-Horn & Pouysségur, 2009). This heterodimer helps in the transcription of several tens of genes containing site-specific HREs (hypoxia-response elements). These genes are involved in different pathways such as erythropoiesis, angiogenesis, glycolytic
metabolism, pH homeostasis, autophagy, migration and invasion (Semenza & Wang, 1992; Semenza, 2003).

Tumour cells switch their metabolism to engage the glycolytic pathway and HIF-1α stimulates this by activating expression of glucose transporter 1 (GLUT-1) and glycolytic enzymes such as pyruvate dehydrogenase kinase 1 (PDK1), hexokinase 2 (HK2) and lactate dehydrogenase A (LDH-A). PDK1 phosphorylates and deactivates pyruvate dehydrogenase (PDH), which is the enzyme that transforms pyruvate into acetyl-CoA (Chiche, Brahimi-Horn, & Pouysségur, 2010) (Figure 1.5). All these modifications allow for more efficient intake of glucose and rapid conversion into pyruvate. Although glycolysis produces less energy than oxidative phosphorylation it has been proposed that the metabolic intermediates generated can be used for the production of lipids, amino acids and nucleotides (M. C. Brahimi-Horn & Pouysségur, 2007; Ebbesen et al., 2009; R. a Gatenby & Gillies, 2004; Semenza, 2003).

Some of the genes induced by HIF-1 have the potential to promote tumour invasion and metastasis since they are involved in extracellular matrix remodelling, generation of new blood vessels, degradation of the basement membrane and regulation of cell migration. These include MMP-2, cathepsin D, VEGF, angiopoietin 2 (Ang2), enzymes belonging to the lysyl oxidase family (LOX), the receptor tyrosine kinase c-Met and the cytokine receptor CXCR4, as well as keratins 14, 18 and 19, fibronectin, vimentin and uPAR (Pouysségur et al., 2006; Semenza, 2003). The membrane-bound MMP-14 has also been found to be upregulated in hypoxia, under the control of the HIF-2α subunit (Petrella, Lohi, & Brinckerhoff, 2005).

Inhibiting HIF-1α expression or activity seemed like a great approach to enforce tumour regression and important effort was put into developing small molecule inhibitors of HIF-1α (Kong et al., 2005). However, the pleiotropic action of HIF and the presence of hypoxic areas in certain organs of the body like the normal retina and testes is a major concern. Therefore, therapies are now directed at HIF-1α gene products that are important for tumour invasion and/or metabolism (Pouysségur et al., 2006). Section 1.5
introduces one of the HIF-1α-upregulated genes as an excellent candidate for cancer targeted therapy.

**1.4.2 pH homeostasis in the tumour cell**

An important consequence of engaging the glycolytic metabolism is that cells need to cope with the huge production of lactate, CO₂ and protons, which acidify the intracellular space (Chiche, Brahimi-Horn, et al., 2010). Maintenance of a neutral intracellular pH is crucial for the survival of the cell since a difference of 0.1-0.2 pH units might have disastrous consequences for important cell processes such as cell proliferation, migration, ATP production, protein synthesis and caspase-mediated apoptosis (Chambard & Pouyssegur, 1986; Pouysségur et al., 2006; Roos & Boron, 1981). Despite the vast production of lactate, CO₂ and protons it has been observed that the intracellular pH of tumours lies between 7.0-7.4 (similar to normal cells) while the extracellular pH is more acidic, with values between 6.0 and 7.0 being reported (Gillies, Raghunand, Karczmar, & Bhujwalla, 2002; Vaupel, Kallinowski, & Okunieff, 1990). Tumour cells maintain a proper intracellular pH by activating systems like Na⁺-H⁺ exchanger-1 (NHE-1), anion exchangers (AE) and Na⁺-HCO₃⁻ co-transporters (NBC), as well as the HIF-1-mediated intracellular pH regulating systems such as the monocarboxylate transporter 4 (MCT4), Carbonic Anhydrase IX and Carbonic Anhydrase XII (CAIX and CAXII) (Figure 1.5) (Chiche, Brahimi-Horn, et al., 2010; S. K. Parks, Chiche, & Pouyssegur, 2011).

NHE-1 is one of the most important proteins involved in pH regulation and is almost ubiquitous throughout the body (Counillon & Pouysségur, 2000). NHE-1 participates in the extrusion of H⁺ contributing to the acidification of extracellular pH and neutralization of intracellular pH. Metabolism-generated CO₂ diffuses freely across the plasma membrane where it is converted to HCO₃⁻ and protons by the activity of CAIX/CAXII. The protons remain in the extracellular space acidifying it while AEs together with NBCs have been proposed to transport HCO₃⁻ inside the tumour cell. MCT4 is responsible for extruding both lactate and H⁺. The bicarbonate that enters the
cell can react with protons in the cytoplasm, increasing the cellular production of CO$_2$ (Figure 1.5) (Chiche, Brahimi-Horn, et al., 2010; S. K. Parks et al., 2011).

It has been proposed that during the somatic evolution of breast carcinogenesis hypoxic and glycolytic breast tumour cells are nutrient-deprived and need to cope with acidosis. The HIF-1-mediated induction of the glucose transporter-1 (GLUT-1) and CAIX may pose an adaptive advantage and may be a selection force in the development of invasive ductal carcinoma (C.-L. Chen, Chu, Su, Huang, & Lee, 2010).
**Figure 1.5. Intracellular pH regulation in the tumour cell.** HIF-1α stimulates the glycolytic switch by activating the expression of glucose transporter GLUT1 and glycolytic enzymes such as hexokinase 2 (HK2), lactate dehydrogenase (LDH) and pyruvate dehydrogenase kinase (PDK) an inhibitor of pyruvate dehydrogenase (PDH), therefore inhibiting mitochondrial uptake of pyruvate. Tumour cells activate the sodium-proton exchanger-1 (NHE-1), bicarbonate transporters such as anion exchangers (AE) and sodium-bicarbonate co-transporters (NBC), the monocarboxylate transporter 1 (MCT1) and Carbonic Anhydrase II (CAII). HIF-1α upregulates MCT4, Carbonic Anhydrase IX (CAIX) and Carbonic Anhydrase XII (CAXII). As a result of the activity of these enzymes and transporters the extracellular pH is decreased, which favours migration and invasion, and this is accompanied by neutralization of intracellular pH, favouring the survival of the cancer cell. Modified from (Chiche, Brahimi-Horn, et al., 2010).
Even though all the proteins mentioned here are important for pH homeostasis of the tumour cell, I decided to investigate CAIX in more detail; as there was no previous systematic study of the role of each domain of CAIX in the different steps of tumour progression. Furthermore, the exact mechanism of how CAIX facilitates tumour growth and metastasis was still unclear.

1.5 CAIX

Carbonic anhydrases (E.C. 4.2.1.1) are ubiquitous in nature and are a family of metalloenzymes that catalyze the reversible hydration of CO$_2$ into HCO$_3^-$ and H$^+$ (H$_2$O + CO$_2$ $\rightleftarrows$ HCO$_3^-$ + H$^+$ with an apparent pK$_a$ of 6.2 ± 0.2) (Li, Tu, Wang, Silverman, & Frost, 2011). There are five different Carbonic Anhydrase families (α, β, γ, δ and ε) with no significant amino acid sequence similarity; therefore they are thought to be an example of convergent evolution. The α-class family, which CAIX belongs to, is only found in vertebrates and is the only class found in mammals (McKenna, 2014).

CAIX was first identified by Pastorekova's group in 1992 in the cervix carcinoma HeLa cell line and was initially called the “MN protein”. They also observed for the first time that CAIX was induced in high density (i.e. mildly hypoxic) cell cultures (J Pastorek et al., 1994).

1.5.1 CAIX structure

CAIX is a 54/58 kDa N-glycosylated transmembrane protein, formed of 459 amino acid (aa) residues (De Simone & Supuran, 2010). There is only one single copy of the gene coding for CAIX in the human genome. The protein coding sequence of CAIX is composed of 11 exons that cover ~11 kb in the p12–p13 region of chromosome 9 (Nakagawa et al., 1998; Opavský et al., 1996). The mature protein is composed of four distinct domains, the catalytic (CA) and the proteoglycan-like (PG-like) domains (257 aa and 59 aa long, respectively) are located extracellularly, and they are linked to a small intracellular (IC) domain (25 aa long) through the transmembrane (TM) segment (20 aa
long) (Figure 1.6) (J Pastorek et al., 1994). There is also an N-terminal signal peptide (37 aa long) that gets truncated upon protein maturation. Most type I membrane proteins destined towards the secretory pathway display a signal peptide. This includes proteins inserted in cellular membranes like CAIX.

**Figure 1.6. Representation of CAIX in the cell membrane.** CAIX exists as a dimer in the cell membrane. The proteoglycan-like (PG-like) and catalytic (CA) domains are facing the extracellular space and are linked to the intracellular (IC) domain through the transmembrane (TM) domain. Amino acid sequence of the IC domain is shown at the bottom, where potentially phosphorylated residues are highlighted in red. Modified from (Vicenzo, Mika, & Di, 2009).
The proteoglycan-like domain was given that name due to its similarity to the keratan sulfate-binding domain of a large proteoglycan aggrecan (De Simone & Supuran, 2010). CAIX is the only Carbonic Anhydrase isoform containing the PG-like domain. Proteoglycans are highly glycosylated proteins that interact via their multiple binding domains with several structural molecules in the ECM, binding together collagen, laminin, fibronectin and hyaluronic acid. They can also mediate binding of cells to the ECM or mediate cell adhesion by reorganizing actin filaments of the cytoskeleton (Ruddon, 2007; Ruoslahti, 1989).

CAIX is co-translationally N-glycosylated on asparagine N346 residue within the catalytic domain. The glycan added is N-acetylglucosamine with attached high mannose-type glycan structures. The PG-like domain also contains an N-linked glycosylation site (Asn) with attached high mannose-type glycan structures and an O-linked glycosylation site (Thr) with attached keratan sulfate-like oligosaccharides (Chiche, Ilc, Brahimi-Horn, & Pouysségur, 2010; Hilvo et al., 2008).

The duplets of human CAIX observed by Western blot at 54/58 kDa have previously been investigated. However, both forms were sensitive to N-glycosidase and EndoH suggesting that one is not the de-glycosylated (i.e. lacking the N-glycosylations) version of the other (Li, Wang, et al., 2011). The presence of O-glycosylation in both forms of the protein was not investigated. Further work is needed to determine if the second band represents a truncated form of CAIX or if other post-translational modifications are taking place.

The crystal structure of the catalytic domain of human CAIX has been solved and this domain has shown similarities with other α-Carbonic Anhydrases for which the crystal structure has also been obtained (De Simone & Supuran, 2010; Hilvo et al., 2008). A ten-stranded antiparallel β-sheet forms the core of the molecule and an intramolecular disulfide bond is present between Cys23 and Cys203. This bond is important for the orientation of the polypeptide loop containing Thr199, which supports efficient catalysis by properly orientating the Zn\(^{2+}\) coordinated nucleophile through a hydrogen-bond interaction (De Simone & Supuran, 2010; Hilvo et al., 2008). The active site of
CAIX is located in a large conical cavity that extents from the surface to the center of the protein with the Zn$^{2+}$ ion located at the very bottom of this cavity and coordinated by three histidine residues. The active site of the enzyme is highly conserved among all Carbonic Anhydrase isoforms (De Simone & Supuran, 2010; Hilvo et al., 2008).

The dimeric nature of CAIX was confirmed by the same crystallography studies where it was found that a Cys41-mediated intermolecular disulfide bond stabilizes two adjacent monomers. Two hydrogen bonds and several van der Waals interactions contribute further to the stabilization of the dimer (De Simone & Supuran, 2010; Hilvo et al., 2008). Importantly, the active sites of the dimer are exposed to the extracellular (EC) space, which would be a requirement for efficient CO$_2$ hydration. The N-terminal regions of both monomers are facing the extracellular space while the C-terminal regions are located intracellularly facilitating the anchoring to the cell membrane (De Simone & Supuran, 2010; Hilvo et al., 2008). The extracellular position of the PG-like domain, at the border of the active site suggests a role for this domain in regulating catalytic domain-mediated hydrolysis and possibly also, cell interactions. The PG-like domain of CAIX is abundant in negatively-charged amino acids and it has been proposed that this domain could interact with positively-charged residues delimiting the active site and found in CAIX but not in other isoforms, therefore controlling access to substrate or even participating in the proton-transfer reaction (De Simone & Supuran, 2010; Hilvo et al., 2008).

1.5.2 CAIX expression and tissue distribution

All human isoforms belong to the α-class family and while CAI, CAII, CAIII, CAVII and CAXIII locate in the cytosol, CAIX together with CAIV, CAXII and CAXIV are membrane-bound. There is one isoform that is secreted into saliva and milk (CAVI) and two that locate to the mitochondria (CA VA and CA VB). Only the CAII isoform has an almost ubiquitous expression throughout the body, and CA VB and CAXIII are also widely distributed. The other isoforms have different expression patterns in different tissues (Frost, 2014). CAIX distribution in normal tissue is restricted to the gastrointestinal tract (specifically to the pancreas, biliary tree, stomach, small intestine and the seminal
duct epithelia) (Potter & Harris, 2004; Saarnio et al., 1998) but its expression has been shown to be up-regulated in the hypoxic zones of a number of solid tumours such as breast (particularly in basal-like or triple negative breast cancers), (Chia et al., 2001; Tan et al., 2009), pancreatic (Couvelard et al., 2005), colon (Korkeila et al., 2009), lung (Swinson et al., 2003), brain (Nordfors et al., 2010), bladder (Hoskin, Sibtain, Daley, & Wilson, 2003), uterine cervix (Loncaster et al., 2001) and head and neck carcinomas (Koukourakis et al., 2006), where it is also associated with poor prognosis (Couvelard et al., 2005; Ilie et al., 2010; Korkeila et al., 2009; Loncaster et al., 2001; Lou et al., 2011). High expression levels of CAIX are also found in fetal lung and muscle, whereas the corresponding adult tissues do not express it (Ivanov et al., 2001). The expression of CAIX in the gastrointestinal tract seems to be limited to areas of high proliferation, such as the basolateral surfaces of duodenal and jejunal crypt epithelia (Potter & Harris, 2004; Saarnio et al., 1998). CAXII is the only other isoform that has also been found to be up-regulated in tumours, however its expression in invasive breast cancer has been found to be a biomarker of good prognosis (Watson et al., 2003).

CAIX is induced under hypoxia or high cell density through the HIF-1α pathway as described previously (Wykoff et al., 2000). CAIX is actually one of the most responsive genes to hypoxia, the reason being that the HRE for the ca9 gene is in close proximity to the 5’ transcription start site (P Swietach, Hulikova, Vaughan-Jones, & Harris, 2010). It is also known that the MAP kinase and PI3 kinase pathways signal to HIF-1 in an SP1-dependent manner, cooperating in the activation of ca9 transcription (Kaluz et al., 2002; Kopacek et al., 2005; Pastorekova, Ratcliffe, & Pastorek, 2008).

1.5.3 CAIX function

CAIX catalyzes the reversible hydration of CO₂ into HCO₃⁻ and H⁺ (H₂O + CO₂ ⇌ HCO₃⁻ + H⁺) and is among the most active Carbonic Anhydrase isoforms (Li, Tu, et al., 2011). CAIX catalysis occurs via de-protonation of a water molecule bound to Zn²⁺ in the active site of the enzyme (Boone, 2014). The kinetics parameters of the CO₂ hydration reaction catalyzed by CAIX extracellular domain (comprised of catalytic and PG-like domains) were obtained through an in vitro stopped flow assay at 25°C and pH 7.4. These
experiments revealed a turnover rate $k_{\text{cat}}$ of $1.1 \times 10^6 \text{s}^{-1}$ and a catalytic efficiency $k_{\text{cat}}/K_m$ of $1.5 \times 10^8 \text{M}^{-1} \times \text{s}^{-1}$ (Innocenti et al., 2009). It has also been observed that CAIX activity in intact cells increases at more acidic pH values due to an increase in the dehydration reaction (Li, Tu, et al., 2011).

In normal tissue CAIX expression is restricted to areas of high proliferative activity (the basolateral surface of the enterocytes of duodenum and jejunum), which has led to the proposal that CAIX might play a role in regulating cell proliferation (Potter & Harris, 2004; Saarnio et al., 1998). CAIX staining was most intense in the crypts, and it is known that stem cells in the crypts are a source for rapid renewal of the epithelium (Gordon, 1989). Interestingly, our laboratory has shown that CAIX is required for the maintenance of cancer stem cells stemness properties (Lock et al., 2013). In the same way, some more recent studies have confirmed a role of CAIX in regulating cell proliferation of tumour cells in vitro (see section 1.5.6) (Chiche, Ilc, et al., 2010; Robertson, Potter, & Harris, 2004). Genetic disruption of CAIX expression by normal cells in mice resulted in gastric hyperplasia of the glandular epithelium further suggesting that CAIX plays a role in regulating cell proliferation. Importantly, the mice developed normally and CAIX deficiency did not promote tumuorigenicity (Gut et al., 2002).

The localization of CAIX at the basolateral membrane may also support a role in the transport of bicarbonate or hydrogen ions and in the maintenance of intracellular pH (Potter & Harris, 2004). The basolateral surfaces are locations where bicarbonate and hydrogen ions are extruded into the lumen, these cells might be exposed to extreme pH values and CAIX could have a protective role (Potter & Harris, 2004). CAIX-mediated production of bicarbonate can contribute to intracellular pH neutralization once inside the cell (Geirnaert et al., 2014).

The role of CAIX in cancer cells is slightly more understood. It has been proposed that since CAIX displays an extracellular catalytic domain the $H^+$ ions generated contribute to the acidification of this milieu while the $\text{HCO}_3^-$ ions are transported back into the cytosol by means of different bicarbonate transporters such as AEs and NBCs, where
they buffer protons, neutralizing the intracellular pH (see Figure 1.5) (Chiche, Brahimi-Horn, et al., 2010). This produces another pool of CO$_2$ that leaves the cell and re-enters a new round of hydration (Helmlinger, Sckell, Dellian, Forbes, & Jain, 2002; Pastorekova et al., 2008). It has been observed that the enzymatic activity of CAIX is insensitive to high lactate concentrations but is inhibited by bicarbonate suggesting that CAIX preferentially catalyzes the CO$_2$ hydration arm of the reaction (Innocenti, Vullo, Scozzafava, Casey, & Supuran, 2005; Pastorekova et al., 2008; Pastoreková & Zavada, 2004). The proof of concept that CAIX is able to acidify the extracellular pH in hypoxia was established by Pastorekova’s group and they also demonstrated that the catalytic domain of CAIX is mediating this function (Svastová et al., 2004).

Low extracellular pH is often associated with tumourigenic transformation, breakdown of the extracellular matrix, migration and invasion (S. K. Parks et al., 2011) (see section 1.3.1.5). The current theory states that CAIX in cancer cells is ultimately facilitating cell invasion and metastasis through the acidification of the extracellular space and subsequent degradation of the ECM. Likewise, since the maintenance of pH homeostasis by tumour cells is critical for cell survival (a change in the intracellular/extracellular pH ratio of 0.1-0.2 pH units can have terrible consequences for many biological processes), it has been proposed that CAIX promotes cell survival by facilitating the neutralization of intracellular pH (S. K. Parks et al., 2011). The HCO$_3^-$ ions generated by CAIX catalysis might also be contributing to cell growth and proliferation since they can be used as building blocks for the synthesis of pyrimidine nucleotides (De Simone & Supuran, 2010; Pouysségur et al., 2006; Svastová et al., 2004; Pawel Swietach et al., 2008).

**1.5.4 Proposed role of each domain in the regulation of CAIX enzymatic activity**

There has been some interest in determining if domains other than the catalytic domain of CAIX might play a role in regulating its Carbonic Anhydrase activity (Innocenti et al., 2009). Initial kinetic experiments with purified CA domain or CA+PG-like domains have suggested that the PG-like domain improves the catalytic efficiency of CAIX at more acidic pH values (optimal activity at pH 6.49) typical of hypoxic solid tumours, facilitating a process that would normally be disadvantaged in acidic pHs. In contrast,
other highly active Carbonic Anhydrases (such as CAI and CAII, which lack the PG-like domain) displayed maximal activities around pH 7.0, suggesting that the PG-like domain is an evolutionary feature of CAIX (De Simone & Supuran, 2010; Innocenti et al., 2009). However, experiments where they overexpress ΔCA- and ΔPG-CAIX in MDCK cells (see Figure 1.6) proved that the deletion of the PG-like domain had no effect on the acidification of the extracellular pH caused by hypoxic cell cultures (Svastová et al., 2004). In terms of the involvement of the IC domain in this function, the Pastorekova group performed mutational analysis of basic amino acids (4 or 5 arginine and lysine residues) within the IC domain and observed impairment in the extracellular pH acidification mediated by MDCK cells in hypoxia (Hulikova et al., 2009). In a recent study, the phosphorylation of the T443 residue, and the simultaneous dephosphorylation of the S448 residue (both within the IC domain) were required for hypoxic MDCK cells-mediated acidification of the extracellular pH and for binding of the FITC-Carbonic Anhydrase Inhibitor (FITC-CAI) (Ditte et al., 2011) which has been previously shown to only bind active CAIX (Dubois et al., 2007; Lou et al., 2011).

1.5.5 CAIX role in the regulation of intracellular pH

In a series of interesting experiments where spheroids of HCT116 cells (colon carcinoma) or RT112 cells (bladder carcinoma) were transfected with CAIX and fluorescent dyes were used to image both intracellular and extracellular pH, it was found that only in the presence of CAIX a neutral intracellular pH is achieved and that CAIX also contributes to generate an acidic extracellular pH (Pawel Swietach et al., 2008; Pawel Swietach, Patiar, Supuran, Harris, & Vaughan-Jones, 2009). A different group has also reported that both CAIX and CAXII are required for the maintenance of a neutral intracellular pH in spheroids of LS174T cells (Chiche et al., 2009).

1.5.6 CAIX role in cell survival and proliferation

Although it has been proposed that CAIX might be contributing to the survival of tumour cells, not many groups have been able to directly probe it. In one previous study
from our laboratory, increased cell death in hypoxic 4T1 cells was observed when CAIX was depleted by shRNA (Lou et al., 2011).

A few studies have investigated the role of CAIX in tumour cell growth and proliferation in vitro. Depletion of CAIX expression in MDA-MB-231 and MDA-MB-468 breast cancer cells by RNAi reduced both cell survival in clonogenic assays and cell proliferation assessed by growth curves (Robertson et al., 2004). The proliferation index of CAIX-silenced LS174T colon carcinoma spheroids was also diminished compared to control cells (Chiche, llc, et al., 2010).

1.5.7 CAIX role in cell adhesion, migration and invasion

Treatment of immobilized purified CAIX with monoclonal antibody M75 which binds to the PG-like domain of the protein abrogated its capacity to facilitate the attachment of cells suggesting that the PG-like domain of CAIX has an important role in mediating cell adhesion (Zavadova & Zavada, 2005). There is one report that states that cells that express CAIX display an increased rate of adhesion and spreading and that deletion of the PG-like domain or treatment with M75 antibody (which targets the PG-like domain of CAIX) abrogates these functions (Csaderova et al., 2013). Other studies have shown that CAIX reduces cell adhesion and cell-cell contacts mediated by E-cadherin through competitive interaction of CAIX with β-catenin (Švastová et al., 2003).

In an initial study by Svastova et al., 2012 the overexpression of CAIX increased the migration rates of MDCK cells but the deletion of the CA domain or treatment with broad Carbonic Anhydrase inhibitor acetazolamide did not completely abolish cell migration suggesting that other domains might be involved, for example the IC domain of CAIX could be modulating cell signalling pathways important for cell migration. CAIX was also found to localize to focal adhesions where it co-localizes with paxillin (Csaderova et al., 2013) and to re-localize to the lamellipodia upon stimulation with hepatocyte growth factor (HGF), where it co-localizes with components of the migratory machinery (AE2 and NBC) (Svastova et al., 2012). Likewise, increased cell migration, invasion and diminished focal adhesions and cell adhesion were observed
when CAIX was expressed in C33A cervical carcinoma cells (Shin et al., 2011). The depletion of CAIX in fibrosarcoma cells led to decreased MMP-3, MMP-9, integrin β3, type IV collagen and ROCK1 gene expression, as well as decreased cell invasion and migration (Radvak et al., 2013). In a study focusing on the IC domain of CAIX, they found that when the T443 residue mimics a phosphorylated amino acid and the S448 residue a non-phosphorylated one, there is a small increase in the migration rate of MDCK cells, as a consequence of increased CAIX activity (Ditte et al., 2011).

### 1.5.8 CAIX and cell signalling

The intracellular domain of CAIX has been implicated in mediating signal transduction. This domain contains three putative phosphorylation sites (T443, S448 and Y449; Figure 1.6) (Pastorekova et al., 2008), which may also regulate interactions between CAIX and other proteins. Dorai et al., demonstrated that the Y449 residue of CAIX gets phosphorylated in an EGF-dependent manner and that this phosphotyrosine interacts with PI3-kinase contributing to Akt activation creating a positive feedback loop (Dorai, Sawczuk, Pastorek, Wiernik, & Dutcher, 2005), however these observations have later been challenged by Frost’s group (Li, Wang, et al., 2011). Another study has suggested that T443, a putative cyclic AMP-dependent protein kinase A (PKA) phosphorylation site is involved in the regulation of CAIX extracellular activity and acidosis. They also showed that this regulation requires the simultaneous dephosphorylation of residue S448. No downstream pathways were investigated (Ditte et al., 2011).

### 1.5.9 CAIX binding partners

At the beginning of this project only a few proteins had been identified as potential binding partners of CAIX. β-catenin and Dickkopf-related protein 1 (DKK1) were identified through co-immunoprecipitation of endogenous proteins (Shin et al., 2011; Švastová et al., 2003), while the interaction of CAIX with AE1/2/3 and with NBCe1 has been observed by co-immunoprecipitation of exogenously expressed proteins (Morgan, Pastoreková, Stuart-Tilley, Alper, & Casey, 2007; Orlowski, De Giusti, Morgan, Aiello, & Alvarez, 2012). The catalytic domain of CAIX was required for the interaction with
AE1/2/3 and the extracellular loop of NBCe1 was required for the interaction with CAIX suggesting that the interaction of CAIX with these transporters occurs extracellularly. Insight into the significance of these interactions come from the observation that the presence of CAIX increased the transport activity of AE1/2/3 and NBCe1 (Morgan et al., 2007; Orlowski et al., 2012). Proximity ligation assays have confirmed the interaction of CAIX and AE2/NBCe1 in cancer cells (Svastova et al., 2012). The previous observations have led to the proposal that the complex of a bicarbonate transporter with a Carbonic Anhydrase enzyme forms a “Bicarbonate Transport Metabolon” (McMurtrie et al., 2004; Vince & Reithmeier, 1998). The metabolon concept was introduced first in 1985 and it proposes that temporary complexes are formed between sequential enzymes of a metabolic pathway. These complexes are held in close proximity by non-covalent interactions possibly through structural elements of the cell like membrane proteins or components of the cytoskeleton (Srere, 1985). An increase in the transmembrane bicarbonate flux would be the outcome of the interaction of bicarbonate transporters with Carbonic Anhydrases (McMurtrie et al., 2004). The presence of the metabolon would increase the rate of bicarbonate intake and further intracellular pH neutralization since the locally produced bicarbonate would immediately be transported to the cytosol (Pastorekova et al., 2008).

Early in 2013 the interactome of CAIX from hypoxic HEK293 cells was published. This study revealed that most of CAIX binding partners belong to the nuclear transport machinery suggesting that CAIX is also a membrane-bound signal transducer that undergoes nuclear translocation. However, neither β-catenin nor DKK1 were identified through the mass spectrometry analysis (Buanne et al., 2013).

1.5.10 CAIX role in tumour progression and metastasis

Previous reports from our laboratory have demonstrated that depletion of CAIX leads to tumour regression and inhibition of metastasis in murine and human breast cancer pre-clinical models. In the same way, pharmacological inhibition of CAIX activity in these models attenuated tumour growth and metastasis (Lou et al., 2011). Likewise, Pouyssegur’s group has reported that silencing of CAIX in LS174T colorectal cancer
xenografts leads to a 40% reduction in tumour volume, while silencing both CAIX and CAXII resulted in 85% reduction in tumour growth (Chiche et al., 2009).

1.5.11 CAIX as a therapeutic target

CAIX is an attractive target for anti-cancer therapy for a number of reasons. It is overexpressed by a wide number of hypoxic solid tumours whereas it has a very limited expression in adult normal tissue. Furthermore, its expression has been associated with poor prognosis in a wide variety of cancer types (Couvelard et al., 2005; Illie et al., 2010; Korkeila et al., 2009; Loncaster et al., 2001; Lou et al., 2011). Likewise, its cell surface localization and the extracellular localization of its catalytic domain facilitate efficient targeting by antibodies and small molecule inhibitors. Finally, it has been suggested that CAIX performs functions that are critical for tumour growth and metastasis, such as intratumoural pH regulation, survival, migration and invasion (Lou et al., 2011; McDonald, Winum, Supuran, & Dedhar, 2012; Pastoreková & Zavada, 2004). This has created interest in developing antibodies and small molecule inhibitors as major tools to target CAIX. An overview of these tools is given in the next two sections.

1.6 CAIX-specific small molecule inhibitors

The concept of specifically targeting cancer cells with the use of small molecule inhibitors has long been explored. There are several examples in the clinic of targeted small-molecule compounds that have been approved for cancer treatment, such as Imatinib (Glivec), which is used for the treatment of chronic myeloid leukemia and targets the fusion protein Bcr-Abl (Kobayashi, 2011).

1.6.1 CAIX inhibitors currently available

The group of Claudiu Supuran (University of Florence, Italy) has long been working in developing Carbonic Anhydrase inhibitors as potential therapeutic agents for cancer and for a wide range of other diseases such as Alzheimer's disease and osteoporosis.
There are an outstanding number of Carbonic Anhydrase inhibitors available that encompass several broad classes, such as acetazolamide (AZA), methazolamide and sulthiame among many others (Innocenti, Vullo, Scozzafava, & Supuran, 2005; Maresca et al., 2009; J. C. Morris et al., 2011; Pacchiano et al., 2011; Supuran, 2008).

An outstanding problem in the design of Carbonic Anhydrase inhibitors is related to the high number of isoforms of this protein present in the human body, their differential localization in many tissues and organs and the lack of selectivity of most of the inhibitors available (Supuran, 2008). However CAIX and CAXII are the only membrane-bound isoforms avid for sulfonamides and sulfamates, which makes it possible to design membrane-impermeant Carbonic Anhydrase inhibitors either by adding charged species, bulky entities or hydrophilic sugar moieties (Supuran, 2008).

In order to develop novel and selective inhibitors of CAIX, Supuran’s group synthesized a number of compounds based on ureido-sulfonamide and coumarin/thiocoumarin scaffolds and structure-activity relationships based on the X-ray structures of CAIX and CAII. The inhibitors obtained were tested for their inhibitory activity in vitro (Maresca & Supuran, 2010; Pacchiano et al., 2011; Thiry et al., 2009). Some of the coumarines and sulfonamides/sulfatames demonstrated particular promise in selectively inhibiting the membrane-bound Carbonic Anhydrases, CAIX and CAXII over the cytoplasmic forms CAI and CAII (Maresca & Supuran, 2010; Maresca et al., 2009; Pacchiano et al., 2011; Winum et al., 2003).

The mechanism of inhibition of Carbonic Anhydrase activity is different for each class. The sulfonamides inhibit CAIX by coordinating to the zinc ion within the active site, while coumarin-based inhibitors are considered suicide inhibitors since they bind irreversibly at the entrance of the active site cavity after undergoing hydrolysis to 2-hydroxycinnamic acids (Maresca et al., 2009; Pacchiano et al., 2011; Shareef, Udayakumar, Sinha, Saleem, & Griggs, 2013).

Acetazolamide, though being a sulfonamide, is truly a pan-Carbonic Anhydrase inhibitor and it is not an ideal compound for targeting CAIX due to its potential off-target effects,
like inhibition of cyclooxygenase 2, as well as low potency and lack of effectiveness in tumour models (Supuran, 2008).

1.6.1.1 Previous characterization of U-104, GC-205 and SLC-148, -149 and -150

CAIX inhibitors U-104 and GC-205 were selected as the lead compounds from each class (sulfonamides and glycosyl coumarins) for the evaluation of inhibition of CAIX activity from cells in this project.

SLC-148, -149 and -150 were later derived from U-104 by SignalChem Lifesciences Corporation through medicinal chemistry and were also evaluated here for their inhibition of CAIX activity in a cell-based assay. Previous work with U-104 and GC-205 inhibitors has involved characterization of their specificity and selectivity by utilizing a stopped-flow activity assay to determine the inhibition profile of CAI, CAII, CAIX and CAXII (Pacchiano et al., 2011; Touisni et al., 2011; Winum et al., 2003), and has also investigated their effect on cell survival and viability of cancer cells (Lou et al., 2011) and McDonald, PC and Chafe, SC; unpublished results). Furthermore, there has been a significant amount of work characterizing the effects of U-104 and GC-205 as inhibitors of tumour growth and metastasis in vivo. Administration of each of these inhibitors has proven useful in inhibiting primary tumour growth in human and mouse models of orthotopic, CAIX-positive breast cancer (Pacchiano et al., 2011; Touisni et al., 2011). In addition to this, these two inhibitors were efficacious in reducing metastasis in experimental pre-clinical models of breast cancer metastasis (Lou et al., 2011).

The in vitro characterization of these CAIX small molecule inhibitors is presented in Chapter 3.
1.7 Anti-CAIX monoclonal antibodies

1.7.1 Mechanisms of tumour cell killing by antibodies

Immunotherapy with monoclonal antibodies can exert its effects through a variety of mechanisms. Such therapeutics can mediate direct alterations in receptor or antigen function (i.e. agonist/antagonist functions), stimulate the immune system or deliver a specific drug pre-conjugated to the antibody (Scott, Wolchok, & Old, 2012).

Monoclonal antibodies can kill tumour cells directly by receptor agonist or antagonist activity (e.g. blocking receptor dimerization, downstream signalling and ultimately leading to reduced proliferation and apoptosis). Enzyme-binding antibodies can lead to neutralization, impairment of signalling and cell death. Antibodies that are able to undergo cell receptor-mediated internalization can be conjugated and used to specifically deliver a payload (e.g. drugs, toxins, radioisotopes) inside the cell and have been called antibody-drug conjugates (ADCs). ADCs that do not internalize can be engineered to deliver a payload extracellularly, for example, to inhibit two membrane enzymes (Scott et al., 2012). Furthermore, some antibodies, after binding to their target stimulate the host immune system by means of complement-dependent cytotoxicity (CDC), induction of macrophage-mediated phagocytosis, induction of antibody-dependent cell cytotoxicity (ADCC), or by the regulation of T cell function (Scott et al., 2012). Finally, monoclonal antibodies can induce vascular and stromal cell ablation by vasculature receptor antagonism or stromal cell inhibition (Scott et al., 2012).

The Fc (fragment, crystallizable) region of an antibody is important for activating CDC and ADCC. Since a greater therapeutic effect is observed when the antibodies exert biological effector functions such as ADCC, more emphasis is being placed on optimizing Fcγ receptors, for example by removal of core fucose residues from N-glycans (Scott et al., 2012). This has been found to increase binding to Fcγ receptors resulting in enhanced ADCC response (Scott et al., 2012).
This work focuses on the characterization of a subtype of the first class of therapeutic antibodies, the enzyme-binding function-blocking ones. In the case of CAIX, these antibodies bind to the active site of the enzyme disrupting its catalytic activity, and since CAIX is proposed to facilitate cell survival they are expected to ultimately induce cell death (McDonald et al., 2012).

1.7.2 Pros and cons of antibodies as cancer therapy

Same as with small molecule inhibitors, investigators have utilized monoclonal antibodies for targeting cancer cells for decades. There are several examples in the clinic of successful application of the immunotherapy approach. Monoclonal antibody Herceptin (which targets receptor tyrosine-protein kinase ERBB2 and is used for treatment of breast, colon, lung, ovarian and prostate tumours) abrogates tumour cell signalling and induces ADCC, while Rituximab (targeting B-lymphocyte antigen CD20 and used for treatment of non-Hodgkin’s lymphoma) also induces ADCC (Scott et al., 2012). In the past 18 years more than 12 antibodies have been approved by the FDA for the treatment of solid tumours and haematological malignancies (Scott et al., 2012).

Antibodies are huge molecules (the IgG molecule has a molecular weight of around 150 kDa) and therefore it is argued that they will not penetrate well into the hypoxic areas of solid tumours, especially since the vasculature is commonly compromised. However data from our laboratory in collaboration with Dr. Bernard’s laboratory (BCCRC, Vancouver, Canada) using an ¹¹¹Indium-labeled anti-CAIX monoclonal antibody have demonstrated tumour specific localization of this antibody by CAIX-positive xenografts in vivo (unpublished data). Monoclonal antibodies might also work well against circulating tumour cells. Antibodies are highly specific and have not been associated with the development of stem-cell related resistance, they often have milder toxicities than other approaches, they have a longer half-life in circulation (several days) and they have proved to exert good synergistic effects with chemotherapeutic agents (Imai & Takaoka, 2006).
Different factors will determine the success of therapeutic monoclonal antibodies. The antigen target should be abundant, accessible and expressed homogenously on the surface of cancer cells. Expression of the antigen should be restricted to tumour cells only. Minimal antigen secretion will avoid binding of the antibody to its antigen in the circulation rather than in the tumour. Antibodies that do not internalize will enhance a greater ADCC or CDC response, whereas internalization is desirable when using certain types of ADCs (Scott et al., 2012). As mentioned earlier, many of these requirements are met by CAIX.

1.7.3 Anti-CAIX antibodies currently available

Recently, efforts have been made to develop antibodies that target the catalytic domain of CAIX and that can therefore possess function-blocking abilities. High-throughput screening technologies such as the use of phage display libraries have been used to identify these types of antibodies. Antibody MSC8 was shown to inhibit CAIX activity from cells and membrane fragments expressing CAIX and from tumour cell spheroids (Murri-Plesko et al., 2011; C. Xu et al., 2010), although its efficacy as anti-tumour agent has not been demonstrated (McDonald et al., 2012). MSC8 IgG inhibited CAIX activity in intact cells by 61% when used at 20 μg/ml whereas MSC8 Fab (fragment, antigen-binding) provided 48% inhibition at the same concentration, only when the antibody was present continuously in the solutions (Murri-Plesko et al., 2011).

VII/20, another anti-CAIX monoclonal antibody directed against the catalytic domain of CAIX and generated using hybridoma technology has been shown to bind to CAIX and undergo receptor-mediated internalization. It also inhibited tumour growth of colorectal cancer HT29 xenografts only when treatment was started immediately after cell inoculation (Zatovicova et al., 2010).

Up until 2009 the only anti-CAIX antibody used for immunotherapy was chimeric antibody cG250. Although this antibody targets the PG-like domain of CAIX and therefore does not exhibit function-blocking capacities, it has been demonstrated that it can induce ADCC. This antibody is marketed by WILEX AG under the trade name
RENCAREX® and is currently in Phase III clinical trial where it is used as an adjuvant therapy to reduce recurrence in surgically-treated renal cell carcinoma (RCC) patients in combination with IL-2 or interferon (IFN)-alpha. It was shown that treatment with this antibody leads to disease stabilization and increased 2-year survival rates (Ebbesen et al., 2009; Siebels et al., 2011).

1.7.4 Novel anti-CAIX antibodies

With the aim to develop anti-CAIX function-blocking antibodies that could be used as tools in cancer therapy, we initiated collaboration with different companies and research centres.

Anti-human CAIX antibodies developed by SignalChem Lifesciences were derived by immunization of mice with purified recombinant CAIX (rCAIX) encompassing the catalytic and PG-like domains. Standard hybridoma technology was utilized for this purpose. This method involves the repetitive immunization of animals with the purified target molecule. After a certain antibody titer is reached in serum, the animals are euthanized and the spleen cells, each of which makes only one type of antibody, are fused with myeloma cells. Myeloma cells are white blood cancer cells and are therefore immortal. When the two cell types are fused, the now immortal culture generates large amounts of a single type of antibody, best known as monoclonal antibody (National Research Council (US) Committee on Methods of Producing Monoclonal Antibodies & Antibodies, 1999; Nee, 2013).

SignalChem Lifesciences antibodies were screened for binding to CAIX by ELISA and validated by Western blot and flow cytometry. Their CAIX binding specificity was evaluated by surface plasmon resonance (SPR) against a panel of 40 native and denatured proteins. They were also tested for their inhibition of CAIX activity in an activity assay that utilizes a fluorescent esterase substrate that CAIX is able to cleave.
The anti-CAIX monoclonal antibodies developed by the Deeley Cancer Research Centre in conjunction with the University of Victoria (UVic) were also derived by standard hybridoma technology after immunizing mice with the catalytic domain of human CAIX.

Finally, KalGene Pharmaceuticals polyclonal anti-CAIX antibodies were developed in rabbits upon immunization with small overlapping peptides within the catalytic domain of recombinant human CAIX. They were able to derive monoclonal antibodies from their lead anti-CAIX antibody thanks to recent developments in rabbit hybridoma production. Very little characterization was performed on UVic-Deeley Cancer Research Centre and KalGene Pharmaceuticals antibodies prior to sending them to our laboratory.

The characterization of these antibodies is presented in Chapter 3.

1.8 Objectives and hypothesis

1.8.1. Determine the mechanism by which CAIX is facilitating tumour growth and metastasis

At the beginning of this project different studies had suggested that CAIX is somehow facilitating tumour growth and the development of metastasis. The reaction catalyzed by Carbonic Anhydrases is well known, but the exact mechanism of how CAIX, either through the catalysis of this reaction or through other means, was participating in tumour growth and metastasis was unknown. Therefore, I aimed to shed new light into the mechanism by overexpressing WT and different mutant forms of human CAIX in a well-studied model of murine breast cancer (4T1 cells). I aimed to utilize these novel cell lines to study the role of different domains (IC, PG-like) and of particular residues within the IC domain (T443, S448, Y449) in regulating CAIX activity, cell survival, proliferation, migration and invasion. I also aimed to utilize the mutants with the most interesting phenotype in vitro to study their impact in tumour growth and metastasis in vivo.
1.8.2. Characterize novel CAIX small molecule inhibitors and antibodies

One of my aims was to characterize CAIX small molecule inhibitors in vitro, utilizing an assay that directly measures Carbonic Anhydrase activity in the presence of the enzyme’s physiological substrate. Therefore, I collaborated with Dr. Claudiu Supuran (University of Florence, Italy) who in the past years has developed a significant number of Carbonic Anhydrase inhibitors and who provided the different CAIX inhibitors used in this thesis.

At the time of initiating this project only a couple antibodies directed against the PG-like domain of human CAIX were used for clinical detection (M75) or immunotherapy (cG250), however there was no report on the development of CAIX function-blocking antibodies. I aimed to characterize novel antibodies developed by different third parties against the catalytic domain of human CAIX in order to confirm their specificity and determine their function-blocking abilities. The most promising antibodies would be tested in vitro for the induction of cancer cell death.

1.8.3 Hypothesis

The mechanism by which CAIX facilitates the metastasis of breast tumours is through the regulation of extracellular pH. Therefore, any impairment of CAIX catalytic activity will affect CAIX metastatic function.
Chapter 2. Materials and Methods

2.1 Cell culture, antibodies and reagents

The 4T1 murine breast cancer cell line, the MDA-MB-231 human breast cancer cell line, the U-87 human glioblastoma cell line and the A549 human lung carcinoma cell line were obtained from the American Type Culture Collection (ATCC). The MDA-MB-231 LM2-4\text{luc} \textsuperscript{+} cells were provided by Dr. Robert Kerbel (University of Toronto, Canada). The murine breast cancer cell line 67NR was kindly provided by Dr. Fred Miller (Karmanos Cancer Institute, Detroit, MI). MDA-MB-231 and A549 cell lines were maintained in DMEM (Gibco\textregistered Life Technologies, Burlington, Ontario) supplemented with 10\% fetal bovine serum (FBS, Gibco\textregistered Life Technologies, Burlington, Ontario) while 4T1 and 67NR cell lines also required the addition of non-essential amino acids (1x NE AA). LM2-4\text{luc} \textsuperscript{+} and U-87 cell lines were kept in RPMI supplemented with 10\% FBS. For culture in normoxia, cells were incubated in a humidified incubator at 37\°C, 5\% CO\textsubscript{2}. For culture in hypoxia, cells were incubated at 37\°C, in an atmosphere of 1\% O\textsubscript{2}, 5\% CO\textsubscript{2}, 94\% N\textsubscript{2} in a humidified incubator inside a sealed anaerobic workstation. Cells were thawed from storage in liquid nitrogen every 2-3 months and subcultured for multiple passages in 100 mm plastic culture dishes. For harvesting, cells were washed twice with sterile 1x phosphate buffer saline (PBS) followed by the addition of TrypLE express (Gibco\textregistered Life Technologies, Burlington, Ontario) and incubation for 10-15 min at 37\°C in a humidified incubator. Trypsin was neutralized with media containing 10\% FBS and the cells were resuspended by gentle pipetting and split accordingly.

The CAIX antibodies (goat anti-mouse CAIX AF2344, goat anti anti-human CAIX AF2188, monoclonal mouse anti-human CAIX MAB2188) and the mouse Ig\textsubscript{G\textsubscript{2A}} (MAB003) isotype control were obtained from R&D Systems (Minneapolis, MN). Mouse and rabbit IgG isotype controls (sc-2025 and sc-2027, respectively) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-PDI (3501) and rabbit anti-\text{β}-tubulin (2146) antibodies were obtained from Cell Signaling Technology (Danvers,
Massachusetts). The mouse anti-β-actin (A5441) was obtained from Sigma-Aldrich Corporation (St. Louis, MO).

Purified recombinant human CAIX (extracellular domain, comprised by catalytic and PG-like domains, 2188-CA) and human CAII (full-length, 2184-CA) were obtained from R&D Systems (Minneapolis, MN), the purified catalytic (CA) and extracellular (EC) domains of human CAIX were custom-made by SignalChem Lifesciences Corporation (Richmond, Canada). The CAIX inhibitors U-104 and FITC-CAI were kindly provided by Dr. Claudiu Supuran (University of Florence, Italy). Broad Carbonic Anhydrase inhibitor acetazolamide (AZA, A6011) was obtained from Sigma-Aldrich Corporation (St. Louis, MO). All inhibitors were diluted in 100% DMSO.

**2.2 Generation of stable cell lines**

For stable depletion of mouse CAIX in 4T1 cells, shRNAmir constructs (Open Biosystems, Huntsville, AL) were transduced into cells using lentivirus as per manufacturer’s instructions. Transduced cells were selected using puromycin. Stable 4T1shCAIX clones were derived by fluorescent activated cell sorting (FACS) of GFP-positive cells (marker of mouse CAIX depletion). Re-introduction of WT huCAIX or of the different constructs was accomplished by plasmid transfection into 4T1shCAIX and 67NR parental cells using Lipofectamine 2000 as per manufacturer’s instructions. Zeocin was used for selection of successfully transfected cells. For enrichment of the CAIX-positive population FACS of double GFP-positive and CAIX-positive cells was performed. 20,000-100,000 cells were sorted by the Flow Cytometry Core Facility (FCCF) located in the Terry Fox Laboratory of the BC Cancer Research Centre (BCCRC) and expanded in selection media.
2.3 Analysis of protein expression

Cells were grown in normoxia or hypoxia for 72 h to induce CAIX expression, followed by lysis at 4°C in RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.5% NaDoc) or NP-40 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1% NP-40) buffer. Equal amounts of lysate (15-30 μg) or purified recombinant CAIX (0.5-2 μg) were re-suspended in sample buffer containing 2-mercaptoethanol, boiled for 5-10 min at 100°C and loaded on SDS-PAGE gels. For non-reducing and non-denaturing conditions, the sample buffer did not contain 2-mercaptoethanol, samples were not boiled and SDS was not added to the running buffer. Western blots were performed as described previously (Lou et al., 2008) using goat anti-mouse CAIX (1:500), goat anti-human CAIX (1:500) and mouse anti-β-actin (1:10,000) primary antibodies. The novel antibodies 359-1 and 360-1 were used at 0.2-0.5 μg/ml, rabbit monoclonal antibodies 4H1-4L1 and 16H1 were provided as cell culture supernatant and were used neat (undiluted). UVic-Deeleay antibodies 538-542 were used at 5 μg/ml, while MM-26 and MM-04 antibodies were used at 2 μg/ml. Secondary antibodies IRDye® 680LT and 800CW donkey anti-goat (926-32214), -mouse (926-32222) or -rabbit (926-32223) IgGs (Life Technologies, Gaithersburg, MD) were used at 1:10,000 for detection using the LI-COR Bioscience Odyssey Imaging System and HRP-conjugated secondary antibodies such as HRP goat anti-rabbit (1:10,000, 1-035-003, Medicorp, Cranford, NJ), HRP bovine anti-goat (1:4,000, sc-2350, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and HRP horse anti-mouse (1:3,000, 7076, Cell Signaling Technology, Danvers, Massachusetts) were used for detection by chemoluminescence. For competition experiments, the primary antibodies were pre-incubated with their corresponding blocking peptide at a concentration ratio of 1:100 (antibody:peptide), for 16 h at 4°C prior to overnight incubation of the membrane with the antibody-peptide complex.
2.4 Analysis of CAIX membrane localization

Cells were grown for 72 h in hypoxia prior to harvesting with 0.02% EDTA-PBS (30 min, 37°C) and blocking with mouse Fc block (1:1,000, 553141, BD Biosciences, Mississauga, ON) for 10 min on ice. The cells were washed and incubated with primary antibody (mouse anti-human CAIX or mouse IgG isotype control, 5 μg/ml) for 15 min on ice. After washing in PBS, cells were incubated with Alexa 647 anti-mouse IgG2A (1:250, A21241, Life Technologies, Gaithersburg, MD) for 15 min on ice, in the dark. Finally, cells were resuspended in Hank’s Balanced Salt Solution supplemented with 2% FBS and 10 μg/ml propidium iodide (PI). The proportion of cells with positive membrane staining of human CAIX was obtained by using a 4-colour cell analyzer in the Flow Cytometry Core Facility (FCCF) of the Terry Fox Laboratory (BCCRC).

2.5 Immunofluorescence

Cells were grown on top of glass coverslips for 72 h in normoxia or hypoxia after which they were fixed for 10 min at RT with 4% paraformaldehyde (PFA, Sigma-Aldrich Corporation, St. Louis, MO). Cells were then permeabilised (unless otherwise specified) with 0.2% Triton X-100/PBS for 5 min, RT and blocked with 10% serum (same host species as the secondary antibody used) for 30 min, at RT. Staining or simultaneous co-staining with primary antibodies was performed overnight at 4°C in a humidified chamber (goat anti-mouse CAIX (1:100), goat anti-human CAIX (1:100), novel antibodies 359-1 (5-10 μg/ml) and 538-542 (10-50 μg/ml)). For ER staining cells were permeabilised with ice cold MeOH (10 min, -20°C) instead of Triton X-100 prior to the addition of mouse anti-PDI antibody (1:100). The exact same amount of IgG isotype control was used in each case. AlexaFluor 488/594 conjugated chicken anti-rabbit (A2144), -mouse (A21201) or -goat (A21468) secondary antibodies (1:400; Life Technologies, Gaithersburg, MD) were added for 1 h at RT in the dark, in combination with Hoescht 33342 (1:5,000; B2261, Sigma-Aldrich Corporation, St. Louis, MO) for nuclei staining. Coverslips were mounted in ProLong Gold mounting media (P36930,
Life Technologies, Gaithersburg, MD). Slides were imaged with a Zeiss Observer.Z1 microscope or with a Leica LSM780 confocal microscope using a 63x objective and a CCD camera. Images were processed using Photoshop. For competition experiments, the primary antibodies were pre-incubated with recombinant CAIX/CAXII at a 1:1 ratio for 1 h at RT prior to overnight incubation of the coverslips with the antibody-peptide complex.

2.6 CAIX activity assay

2.6.1 *In vitro* activity assay (recombinant CAIX)

The catalytic activity of purified recombinant Carbonic Anhydrase IX was assessed by methods previously described (Wilbur and Anderson, 1948). Commercially available extracellular domain of Carbonic Anhydrase IX (R&D Systems, Minneapolis, MN), or the catalytic or extracellular domains of CAIX custom-made by SignalChem Lifesciences Corporation (Richmond, Canada) were diluted to 10 μg/ml in 0.8 ml cold 0.02 M Tris-HCl buffer pH 8.0. 0.2 ml of 100% CO₂-saturated H₂O was added to the well-stirred borosilicate tube (4°C) to initiate the reaction, which was monitored with a narrow pH microelectrode (1362095, Accumet Fisher Scientific) for the first 150 seconds. CO₂ hydration generates H⁺ ions that can be measured with the pH electrode. In initial assays, measurements of CAIX-mediated pH acidification were stopped after a drop of 2 pH units or after the pH reached a plateau. To determine the rate of spontaneous CO₂ hydration, measurements were performed on enzyme-free ('buffer') samples. The increase in hydration rate above the spontaneous rate is a measure of Carbonic Anhydrase activity. To test the inhibitory capacities of novel antibodies the enzyme was pre-incubated with these at the concentrations indicated for 50 min, at 4°C, in a well-stirred vessel, prior to the addition of 100% CO₂-saturated H₂O. Acetazolamide (AZA; 200 μM, 30 min, 4°C) was used as a positive control of CAIX inhibition. IgG isotype control was used as a negative control.
2.6.2 CAIX “In-cell” activity assay

This assay is a modification of the one described above, in which intact live cells were utilized instead of recombinant CAIX. Briefly, cells were trypsinized, counted, and resuspended in cold CO₂-free isotonic buffer (20 mM Hepes, pH 8.0, 130 mM NaCl, 5 mM KCl). A 0.8 ml aliquot of cell suspension (equal to 7.5 x 10⁵ cells) was added to a 4 ml well-stirred borosilicate tube cooled to 4°C. A narrow pH microelectrode (1362095, Accumet Fisher Scientific) was inserted to monitor solution pH. The reaction was initiated by addition of a 0.2 ml aliquot of 100% CO₂-saturated water (4°C). To determine the rate of spontaneous CO₂ hydration, measurements were performed on cell-free (‘buffer’) samples. The increase in hydration rate above the spontaneous rate is a measure of Carbonic Anhydrase activity. Background Carbonic Anhydrase activity in 4T1 cells expressing WT and mutant human CAIX was determined by using 4T1shCAIX cell preparations. Chemical inhibition of Carbonic Anhydrase IX activity was assessed after 30 min incubation of 67NR/WT huCAIX cells with the inhibitors U-104, GC-205, SLC-148, -149, -150 at concentrations indicated in the text (ranging from 1-200 μM), in well-stirred, borosilicate tubes (4°C). In initial experiments, acetazolamide (AZA; 200 μM, 30 min, 4°C) was used as a positive control of CAIX inhibition. Antibody-mediated inhibition of CAIX activity was assessed after 50 min incubation (4°C) of 67NR/WT huCAIX cells with MM-26 antibody (35.7 and 100 μg/ml), in well-stirred, borosilicate vessels.

2.6.3 Calculation of extent of inhibition

The time required for the buffer (or negative control) pH to drop from initial pH (usually pH 8.0) to the pH half-way between the maximum and minimum pH was analyzed and defined as minimum (0%) activity. 100% activity was defined as the time, in seconds, required to achieve the same pH drop in the presence of WT huCAIX-expressing cells alone. For example,

Initial pH = 8.0
Lowest pH value achieved in 150 s = 7.0
pH half-way through = 7.5
Time required to get to pH 7.5 in buffer sample = 0% activity
Time required to get to pH 7.5 in WT huCAIX-expressing cells sample = 100% activity

Percent activity was plotted against time and the equation derived from this standard curve was used to calculate percent inhibition in the presence of the inhibitors/antibodies/CAIX mutants, based on the time required for the pH in these reactions to drop from 8.0 to the pH half-way between the maximum and minimum pH.

When performing a statistical test on the reaction curves, the time point chosen was also the time required for the sample pH to drop from 8.0 (or initial pH) to the pH half-way between the maximum and minimum pH in the presence of rCAIX or in WT huCAIX-expressing cells.

2.7 Binding of FITC-CAI

Cells were grown for 48 h in hypoxia to induce CAIX expression, followed by incubation with FITC-CAI (10 μM, 16 h, hypoxia). Cells were washed twice with PBS to remove excess stain and immersed in Live Imaging Cell Solution (Life Technologies, Gaithersburg, MD) to maintain viability during the duration of the imaging. The entire wells were first observed under the fluorescent microscope and representative fields for each well were captured with a Zeiss Axiovert 40 CFL microscope using a 32x objective and a CCD camera. Images were processed using Photoshop.

2.8 Migration and invasion assays

Cells were cultured for 72 h in hypoxia to induce CAIX expression, followed by serum-starvation for 24 h in hypoxia. Cells were then trypsinized, counted and 5 x 10⁴ or 1 x 10⁵ cells were seeded onto either 8 μm uncoated transwells (Corning Inc., Corning, NY) or 8 μm transwells coated with Matrigel™ (BD Biosciences; Mississauga, ON). Media
supplemented with 10% FBS and 100 ng/ml recombinant mouse EGF (Sigma-Aldrich Corporation, St. Louis, MO) was used as a chemoattractant as described previously (Hwang et al., 2011). Cells were allowed to migrate/invoke for 24 h in hypoxia after which non-invasive cells were removed from the top chamber with a cotton swab. Membranes were fixed for 10 min with ice cold MeOH and the nuclei of invasive cells were stained with Hoescht 33342 (1:5,000, Sigma-Aldrich Corporation, St. Louis, MO) for 10 min at RT. The number of migratory and invasive cells was obtained from at least 5 different fields with a Zeiss Observer.Z1 microscope using a 10x objective and a CCD camera. Images were processed using Photoshop.

2.9 Cell cycle analysis

Equal numbers of cells (1.75 x 10^3 cells/cm^2) were seeded in triplicate and allowed to adhere overnight at 37°C, 5% CO_2 and were then serum-starved for another 24 h to attempt to synchronize the cell cycle by inducing quiescence (G_0/G_1). The next day, media supplemented with 10% FBS was added and the cells were transferred to hypoxia for 48 h. After this period, the conditioned media and the cells were harvested, mixed and pelleted. The pellet was washed in PBS prior to overnight fixation in ice cold 70% EtOH. The pellet was washed again in PBS prior to incubation with PI staining solution (0.1% Triton X-100, 10 μg/ml PI and 100 μg/ml DNAse-free RNAse), for 30 min, RT. The number of cells in each phase of the cell cycle (G_1, S, G_2) was obtained by flow cytometry analysis in the FCCF located in the Terry Fox Laboratory (BCCRC). The Dean-Jett-Fox algorithm was utilized for modeling the data.

2.10 Growth curves

Equal numbers of cells (1.75 x 10^3 cells/cm^2) were seeded in triplicate and grown for 72 h in hypoxia. After this period, cells were carefully trypsinized and clumps were dissociated by intense pipetting. The number of cells in each plate was calculated twice
with the help of a hematocytometer and the doubling time was calculated by the online software Doubling Time (Roth, 2006).

2.11 MTT assays

Equal numbers of cells (1.5 x 10³ cells/cm²) were seeded in 96-well plates in quadruplicate. After 12 h incubation in hypoxia and no serum (day 0, minimal or null proliferation) cells were switched to media supplemented with 10% FBS and allowed to grow for another 72 h in hypoxia (day 1, 2 and 3). MTT reagent (5 mg/ml, Sigma-Aldrich Corporation, St. Louis, MO) was added at day 0, 1, 2, and 3 (10 μl/well, 4 h, 37°C) followed by 100 μl/well of solubilisation reagent (10 mM HCl, 0.1% SDS, overnight, 37°C). The absorbance was read with a spectrophotometer at 570/630 nm. Values obtained from day 1, 2 and 3 were normalized to day 0 to account for proliferation.

2.12 Immunoaffinity purification followed by protein identification by mass spectrometry

2.12.1 Immunoprecipitation using CNBr beads

4T1sh/WT huCAIX and 4T1sh/ΔIC cells cultured in hypoxia for at least 72 h were lysed at 4°C in NP-40 buffer containing protease inhibitor cocktail, 1 mM Na₃VO₄, 2 mM NaF and 1 mM PMSF. 5 mg of protein were immunoprecipitated at 4°C overnight using 30 μg of monoclonal anti-human CAIX antibody covalently linked to CNBr-activated Sepharose 4B (GE Healthcare, Cleverland, OH) as described previously (Chafe & Mangroo, 2010). The resin was extensively washed with NP-40 buffer, resuspended in sample buffer with denaturant SDS and boiled at 100°C for 10 min to elute the proteins. The proteins in the supernatant were separated from the resin by centrifugation using a polyacrylamide column for 1 min, at 13,000 rpm. 2-mercaptoethanol was added to the
supernatant and eluates were boiled again. Half of the samples were sent to Dr. Leonard Foster’s laboratory (CHiBi, UBC) for CAIX-binding partners identification by mass spectrometry (MS) analysis while the other half of the samples were loaded on SDS-PAGE gels and Western blots of human CAIX (using goat anti-human CAIX, 1:500) were performed as described above. Alternatively, the gels were fixed overnight in fixing solution (50% MeOH, 12% HAc, 0.05% formalin (35% formaldehyde)), sensitized for 2 min with sensitizer solution (0.02% Na2S2O3) and stained for 20 min with silver nitrate (0.2% AgNO3, 0.076% formalin (35% formaldehyde)) for visualization of the bait and the enriched proteins in the immunoprecipitation (IP) lane. Gels were developed by constant agitation in the developer solution (6% Na2CO3, 0.05% formalin (35% formaldehyde), 0.0004% Na2S2O3) and this process was ended with the stopping solution (50% MeOH, 12% HAc) after bands were optimally visible.

Immunoprecipitates sent to the MS facility were suspended in SDS sample buffer and run on a short 10% SDS-PAGE gel. Proteins were visualized by colloidal Coomassie (Candiano et al., 2004) and digested out of the gel as described previously (Chan, Howes, & Foster, 2006). Peptide samples were purified by solid phase extraction on C-18 STAGE tips (Ishihama, Rappsilber, Andersen, & Mann, 2002), and each treatment was labeled by reductive dimethylation using formaldehyde isotopologues (Parker et al., 2012). The final product was purified again by C-18 STAGE tips as previously done and analyzed by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) as described previously (Kang et al., 2014).

2.12.2 Immunoprecipitation using A/G sepharose beads

For the characterization of novel antibodies human CAIX was immunoprecipitated from MDA-MB-231 and 67NR/WT huCAIX CHAPS-lysates. The antibodies were added and the protein-antibody complex was pulled out using A/G-coupled sepharose beads (GE Healthcare, Cleverland, OH). Briefly, the beads were washed with CHAPS buffer (30 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% CHAPS) and 1 mg of lysate was pre-cleared with 30 μl of bead slurry (20 min, 4°C). The supernatant was separated by centrifugation and 5 μg (novel 359-1, 539 and 541 antibodies) or 25 μg (MAB2188) of antibody was used to
immunoprecipitate human CAIX from the lysate (overnight at 4°C). The same amount of isotype specific IgG was used as negative control. The next day 50 μl of pre-washed A/G beads were added for 1 h at 4°C, followed by extensive washing with cold CHAPS buffer. The protein-antibody complex was eluted from the beads by heating or boiling samples in loading buffer with denaturant SDS at 100°C for 10 min. The proteins in the supernatant were separated from the beads by centrifugation for 1 min, at 13,000 rpm. 2-mercaptoethanol was added to the supernatant and eluates were boiled again. Samples were loaded on SDS-PAGE gels and Western blots of human CAIX were performed as described earlier.

2.13 Apoptosis assays

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) was used for the analysis of apoptosis following the manufacturer's instructions. Briefly, cells were incubated for 72 h in hypoxia prior to growing them on coverslips for 48 h in hypoxia and low serum conditions (0.1% FBS), fixed and stained with the TUNEL reaction (Roche, Basel, Switzerland). For experiments with MM-26 antibody, cells were incubated for 72 h in hypoxia in the presence of this antibody (100 μg/ml) or mouse IgG isotype control (100 μg/ml) prior to fixation and TUNEL staining. Quantification was performed by imaging 4 random fields with a Zeiss Observer.Z1 microscope using a 10x objective and a CCD camera.

2.14 Statistical analysis

Statistical analysis was performed using the Data Analysis ToolPack Excel software. P values were calculated using Student’s t-test; data with P values < 0.05 were considered significant.
Chapter 3. Characterization of Novel CAIX Inhibitors and Antibodies

3.1 Synopsis

In order to study CAIX enzymatic activity I developed in vitro activity assays and utilized them to test the inhibitory capacities of novel small molecule inhibitors of CAIX as well as novel anti-CAIX antibodies. These tools can eventually be developed as cancer therapeutics.

I therefore initiated collaboration with Dr. Claudiu Supuran (University of Florence, Italy) and SignalChem Lifesciences Corporation (Richmond, Canada) to further test and characterize novel CAIX inhibitors developed by them.

The novel CAIX inhibitors were tested for their inhibition of Carbonic Anhydrase activity in the In-cell Carbonic Anhydrase activity assay utilizing 67NR cells expressing human CAIX. Novel inhibitor U-104 was proven to be better at blocking CAIX activity than the broad Carbonic Anhydrase inhibitor acetazolamide (AZA), since four times less U-104 inhibitor is needed to provide the same levels of inhibition. Furthering this, U-104 was proven to be more specific than AZA by Dr. Claudiu Supuran’s laboratory (Pacchiano et al., 2011) since it preferentially inhibits CAIX and CAXII, while AZA is able to block the activity of at least ten Carbonic Anhydrase isoforms (Supuran, 2008). The novel glycosyl coumarine GC-205 was slightly less effective than AZA at blocking CAIX activity. When used at the same concentration as AZA, GC-205 only provided 80% inhibition of CAIX activity. However, GC-205 was also proven to be more specific than AZA since it was shown to have selectivity for the membrane-bound isoforms CAIX and CAXII versus the intracellular forms CAI and CAII (Touisni et al., 2011).

Three inhibitors were derived from U-104 by SignalChem Lifesciences (SLC-148, -149 and -150) and I tested their CAIX-inhibitory abilities. The three derivatives showed similar inhibition rates to U-104 using the same previous concentration of 50 μM. SLC-149 proved to be the best candidate since it provided the greatest inhibition of CAIX
activity and was further characterized in a dose-response assay. When compared with U-104, SLC-149 was shown to be a better inhibitor at concentrations of 1, 10 and 25 μM. U-104 was approved for Phase I clinical trial as of September 2014.

Antibodies which target the catalytic site of an enzyme can potentially have function-blocking capacities. Therefore, our laboratory started collaborating with KalGene Pharmaceuticals Inc. (Vancouver, Canada), the Deeley Cancer Research Centre-University of Victoria and SignalChem Lifesciences Corporation (Richmond, Canada) to develop novel anti-CAIX antibodies. Ten novel antibodies were sent by the third parties mentioned above, and were initially tested for their specificity by a combination of Western blot, immunocytochemistry, and immunoprecipitation techniques, as well as by competition experiments with blocking peptides when available. Specific antibodies were tested for their inhibition of CAIX activity.

Antibody 359-1 developed by KalGene Pharmaceuticals was shown to be highly specific against CAIX, yet their function-blocking capacities remained controversial. However, antibody 359-1 proved to be an outstanding tool for the detection of human CAIX in the research laboratory. Two of the antibodies developed by the Deeley Cancer Research Center and the University of Victoria (antibodies 541 and 542) were proven to have some notable inhibitory abilities; however their specificity against CAIX was inconclusive and requires further characterization. Finally, one of the antibodies developed by SignalChem Lifesciences (antibody MM-26) proved to surpass the ones tested, as in addition to being proven specific against CAIX (experiments performed by SignalChem Lifesciences, unpublished data), I was able to demonstrate that this antibody is also effective at blocking CAIX activity both in vitro (using recombinant CAIX), and from cells expressing human CAIX. Antibody MM-26 is the first CAIX function-blocking antibody proven to be able to induce the death of cancer cells cultured in hypoxia and low serum conditions. Antibody MM-26 has yet to be proven to engage a host immune response, and induce antibody-dependent cell-mediated cytotoxicity (ADCC), which, in turn, would increase its efficacy.
3.2 Inhibitory capacities of small molecules U-104 and GC-205

As discussed in Chapter 1, CAIX is an excellent therapeutic target of cancer. Small molecule inhibitors offer the advantage of being able to penetrate the hypoxic regions of solid tumours where CAIX is expressed.

The CAIX small molecule inhibitors U-104 and GC-205 were synthesised and developed in Claudiu Supuran’s laboratory (University of Florence, Italy). U-104 is an ureido-substituted benzenesulfonamide and GC-205 is a glycosyl coumarine. The mechanism of sulfonamide-based inhibition of CAIX is through coordination to the zinc ion within the active site, while coumarin-based molecules act as suicide inhibitors, undergoing hydrolysis and binding irreversibly at the entrance of the active site cavity (Pacchiano et al., 2011; Maresca et al., 2009). Both U-104 and GC-205 were found to be highly selective for the membrane-bound isoforms CAIX and CAXII over the cytosolic forms CAI and CAII as demonstrated by their inhibitory or affinity constants ($K_i$; Table 3.1) (Winum et al., 2003).

Table 3.1 Inhibitory constants of CAIX inhibitors U-104 and GC-205.

<table>
<thead>
<tr>
<th></th>
<th>$K_i$ CAI (nmol/L)</th>
<th>$K_i$ CAII (nmol/L)</th>
<th>$K_i$ CAIX (nmol/L)</th>
<th>$K_i$ CAXII (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-104</td>
<td>5,080</td>
<td>9,640</td>
<td>45.1</td>
<td>4.5</td>
</tr>
<tr>
<td>GC-205</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>204</td>
<td>184</td>
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</table>

$^1K_i$: inhibitory constant

In order to test the CAIX activity-blocking ability of these two inhibitors I implemented a new assay in our laboratory. The assay was first developed by Wilbur and Anderson in 1948 (Wilbur & Anderson, 1948) and it directly measures the activity of either purified recombinant Carbonic Anhydrases or Carbonic Anhydrases endogenously or exogenously expressed by cell lines.

The inhibitors were tested using the *In-cell* Carbonic Anhydrase activity assay; as described in Materials and Methods (Chapter 2, section 2.6.2). In this assay, the drop in
pH of Hepes buffer during the first 150 s after the addition of CO₂-saturated water was recorded. CO₂ is the physiological substrate for CAIX. The source of Carbonic Anhydrase enzymatic activity was obtained from murine breast cancer 67NR cells stably expressing WT human CAIX (67NR/WT huCAIX) and re-suspended in Hepes buffer. CAIX expressed on the surface of these cells showed to be active even under normoxic conditions. 67NR parental (67NR par) cells do not express CAIX and were therefore used as a negative control. Any observed Carbonic Anhydrase activity in these cells would represent other isoforms of the enzyme. The reaction obtained when cell-free buffer was added was considered the spontaneous rate of CO₂ hydration. For a positive control of CAIX inhibition, 67NR/WT huCAIX cells were pre-incubated with commercially available broad Carbonic Anhydrase inhibitor AZA (200 μM) for 30 min, 4°C, in a well-stirred vessel prior to the addition of CO₂-saturated water. To compare to the inhibition obtained with AZA, 67NR/WT huCAIX cells were pre-incubated with U-104 (50 μM) or GC-205 (200 μM) for 30 min, 4°C, in a well-stirred vessel prior to the addition of CO₂-saturated water. The concentrations used for this assay were chosen based on the maximal dissolvable concentration of these inhibitors in DMSO.

Figure 3.1 shows that there is little or no Carbonic Anhydrase activity in 67NR parental cells, since the activity curve is notably similar to the one of the spontaneous rate of hydration (buffer). However, 67NR/WT huCAIX cells provided a marked increase in activity, which can be inhibited by treatment with AZA (200 μM) to the levels found in 67NR parental cells (Figure 3.1B) or to almost spontaneous activity (buffer) levels (Figure 3.1A). Interestingly, treatment with novel inhibitor U-104 (50 μM; Figure 3.1A) was very effective at significantly blocking CAIX activity, since cells incubated with this inhibitor showed levels of activity similar to the spontaneous (buffer) group, indicating that 100% inhibition was achieved. The inhibition provided by U-104 closely resembles the inhibition provided by AZA, except that four times less inhibitor was needed to obtain similar levels of inhibition. Treatment with novel inhibitor GC-205 (200 μM) was slightly less effective than U-104 and than AZA, especially considering that four times more inhibitor was used than in U-104 assays (Figure 3.1B). Treatment with GC-205 (200 μM) provided a significant reduction of CAIX activity (around 80%) when
normalized to DMSO and buffer controls as described in Materials and Methods (Chapter 2, section 2.6.3).
Figure 3.1. Function-blocking abilities of novel CAIX inhibitors U-104 and GC-205.
Novel inhibitors were tested in the In-cell Carbonic Anhydrase activity assay. The CAIX enzymatic activity present in 67NR/WT huCAIX cells was blocked with U-104 (50 μM) (A) and GC-205 (200 μM) (B). The broad Carbonic Anhydrase inhibitor AZA (200 μM) was used as positive control of inhibition of activity. The experiment was repeated twice and one representative experiment with triplicates is shown. The vehicle (DMSO) was compared to U-104/GC-205 groups at the indicated time points. 67NR par: 67NR parental cells. Bars represent the mean ± s.e.m. of three replicates. *P<0.05, **P<0.01, ***P<0.001 by Student’s t-test.
3.3 Inhibitory capacities of U-104 derivatives SLC-148, SLC-149 and SLC-150

SignalChem Lifesciences worked in developing different derivatives of U-104 that could potentially have increased inhibitory capacities by medicinal chemistry. During this process chemical modifications are performed to improve binding of the CAIX inhibitor to the active site of the enzyme. The inhibitory or affinity constants (Ki) of derivatives SLC-148, -149 and -150 towards the extracellular Carbonic Anhydrase isoforms CAIX and CAXII can be found in Table 3.2.

<table>
<thead>
<tr>
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<th>Ki(^1) CAIX (nmol/L)</th>
<th>Ki CAXII (nmol/L)</th>
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</thead>
<tbody>
<tr>
<td>SLC-148</td>
<td>9.5</td>
<td>43.7</td>
</tr>
<tr>
<td>SLC-149</td>
<td>4.4</td>
<td>4.5</td>
</tr>
<tr>
<td>SLC-150</td>
<td>2038</td>
<td>2.3</td>
</tr>
</tbody>
</table>

\(^{1}\)Ki: inhibitory constant

The new inhibitors were tested alongside U-104 in the In-cell Carbonic Anhydrase activity assay. A standard concentration of 50 \(\mu\)M was established for this initial experiment since I previously verified that this concentration provides excellent inhibition of CAIX activity (Figure 3.1A).

The source of Carbonic Anhydrase activity was obtained from 67NR/WT huCAIX cells resuspended in Hepes buffer and pre-incubated with DMSO for 30 min, 4°C, in a well-stirred vessel prior to the addition of CO\(_2\)-saturated water. The reaction obtained when cell-free buffer was used, was considered the spontaneous rate of CO\(_2\) hydration. For a positive control of CAIX inhibition, 67NR/WT huCAIX cells were pre-incubated with U-104 (50 \(\mu\)M) for 30 min, 4°C, in a well-stirred vessel prior to the addition of CO\(_2\)-saturated water. To compare to the inhibition obtained with U-104; 67NR/WT huCAIX cells were pre-incubated with novel inhibitors SLC-148/SLC-149/SLC-150 (50 \(\mu\)M, Figure 3.2A, B and C, respectively) for 30 min, 4°C, in a well-stirred vessel prior to the addition of CO\(_2\)-saturated water. Figure 3.2 shows that all three new inhibitors provided...
significant blocking of CAIX activity and similar inhibition rates to U-104. When comparing the time it took for each of the inhibitors to get to the pH value half-way between the maximum and minimum pH achieved (i.e. pH 7.55) it was obtained that U-104 inhibited CAIX activity by around 80% (t = 56 s), while SLC-148 and -150 provided around 85% inhibition (t = 60 s), and SLC-149 provided around 93% inhibition (t = 65 s).
Figure 3.2. Function-blocking abilities of novel CAIX inhibitors SLC-148, -149 and -150. Novel inhibitors were tested in the In-cell Carbonic Anhydrase activity assay. The CAIX enzymatic activity present in 67NR/WT huCAIX cells was blocked with (A) SLC-148, (B) -149, and (C) -150 (50 μM). U-104 (50 μM) was used as positive control of inhibition of activity in each case. The experiment was repeated twice and one representative experiment with triplicates is shown. The vehicle (DMSO) was compared to SLC-148, -149, -150 groups at the indicated time points. Bars represent the mean ± s.e.m. of three replicates. ***P<0.001 by Student’s t-test.
Since SLC-149 provided greater inhibition of CAIX activity than U-104 it was further tested in a dose response in the *In-cell* Carbonic Anhydrase activity assay against U-104 at the following concentrations: 1, 10, 25 and 50 μM (Figure 3.3). Both inhibitors provided significant inhibition of CAIX activity at all concentrations tested. It is important to reiterate that neither of the inhibitors was able to go into solution at concentrations higher than 50 μM, which is why this concentration is the maximal one in the curves.
Figure 3.3. Dose-response of U-104 and SLC-149. SLC-149 was tested in the In-cell Carbonic Anhydrase activity assay side to side to U-104 at different concentrations (50, 25, 10 and 1 μM). The experiment was repeated twice and one representative experiment with triplicates is shown. The vehicle (DMSO) was compared to the SLC-149 (blue arrows) and U-104 (red arrows) groups at one time point only. Bars represent the mean ± s.e.m. of triplicates. *P<0.05, ***P<0.001 by Student’s t-test.
When comparing SLC-149 to U-104, the former was proven to be a superior inhibitor than U-104 at all the concentrations tested, except for 50 μM where the inhibition provided by both of them was similar. The inhibition rates obtained with each inhibitor at each concentration are summarized in Figure 3.4. The maximal and the only significant differential was obtained at 1 μM where SLC-149 provided 68% inhibition of CAIX activity while U-104 provided only 42% inhibition of CAIX activity. Since the aim of characterizing these inhibitors in a dose-response assay was to find the minimum effective concentration of each drug required to produce >80% inhibition of CAIX activity, concentrations lower than 1 μM were not tested.

Figure 3.4 Percent inhibition of CAIX activity by U-104 and SLC-149. The percent inhibition of CAIX activity obtained with each inhibitor at each concentration (50, 25, 10 and 1 μM) was calculated from data described in Figure 3.3 as specified in Materials and Methods (Chapter 2, section 2.6.3). The inhibition obtained with U-104 was compared to the inhibition obtained with SLC-149 at each concentration. Bars represent the mean ± s.e.m. of triplicates. *P<0.05 by Student’s t-test.
In summary, the ureido sulfonamide U-104 was found to be a more potent inhibitor than the glycosyl coumarine GC-205, while the U-104 derivative, SLC-149 provided the greatest inhibition of CAIX activity.

3.4 Specificity and function-blocking capacities of novel anti-CAIX polyclonal antibody 359-1 and its derivatives

Another promising approach for targeting CAIX is the use of function-blocking antibodies. These are expected to induce tumour cell death as a consequence of CAIX inhibition of activity, as discussed in Chapter 1, section 1.7.1.

KalGene Pharmaceuticals provided two polyclonal antibodies: rabbit anti-CAIX 359-1 and 360-1. These antibodies were developed in rabbits upon immunization with overlapping peptides within the catalytic domain of recombinant human CAIX and were later affinity-purified.

In order to test the blotting capacities of these two antibodies, different forms of recombinant CAIX (rCAIX; extracellular (EC) domain or catalytic (CA) domain), normoxic and hypoxic lysates of parental MDA-MB-231 cells (MDA; human breast cancer line), MDA-MB-231 LM2-4lect* cells (LM2-4, highly metastatic variant of MDA-MB-231 cells) and 4T1 cells (murine breast cancer line), as well as lysates of 67NR or 4T1shCAIX cells expressing exogenous human CAIX (67NR/WT and 4T1/WT) were loaded on SDS-PAGE gels, and Western blots were performed as described in Materials and Methods (Chapter 2, section 2.3). I used commercially available goat anti-human CAIX antibody (AF2188, R&D Systems) as a positive control (Figure 3.5A, top). Both novel antibodies recognized CAIX at some extent but there were some important differences among them. Antibody 359-1 recognized bands of the correct size for the catalytic and the extracellular domain of rCAIX (around 37 and 50 kDa respectively), and also for human CAIX exogenously expressed in 67NR and 4T1 cells (67NR/WT and 4T1/WT). This antibody detected one band of the right size (54 kDa) both in parental
MDA normoxic and hypoxic lysates (Figure 3.5A, bottom left), and in parental LM2-4 normoxic and hypoxic cell lysates although the band in hypoxia was more intense (Figure 3.5B, left). There were additional bands being selected by this antibody, which perhaps represented degraded protein. Polyclonal antibody 360-1 recognized both forms of rCAIX and of human CAIX exogenously expressed by 4T1/WT and 67NR/WT cells, although the band for human CAIX in 4T1/WT lysates was very weak. This antibody also seemed to be detecting mouse CAIX in parental 4T1 hypoxic lysates but did not detect human CAIX in parental MDA hypoxic lysates (Figure 3.5A, bottom right).

In order to demonstrate that these antibodies are specifically recognizing CAIX, I performed competition assays utilizing a blocking peptide (CAIX peptide used for immunization) and a non-blocking peptide (the immunization peptide for the other antibody either 359-1 or 360-1), both provided by KalGene Pharmaceuticals. After overnight pre-incubation with the peptides (at 4°C, 1:100 antibody:peptide concentration ratio), the antibody-peptide complex was then used for blotting. Upon pre-incubation with the blocking peptide, but not with the non-blocking peptide, both antibodies 359-1 and 360-1 almost completely lost their blotting abilities, which strongly demonstrates their specificity (Figure 3.5B).
Figure 3.5. Specificity of novel anti-CAIX antibodies 359-1 and 360-1. (A) The ability of antibodies 359-1 and 360-1 (0.5 μg/ml) to recognize purified rCAIX (catalytic: CA and extracellular: EC domain, comprised by catalytic and PG-like domains), exogenous human CAIX (4T1/WT and 67NR/WT cells) and endogenous mouse (4T1 cells) and human (MDA and LM2-4 cells) CAIX from the aforementioned cell lysates was assessed by Western blot. Commercial antibodies AF2188 and AF2344 were used as positive controls. (B) The specificity was assessed further by incubating each of these antibodies with their blocking peptide (p359-1 for 359-1 Ab) or with the blocking peptide of the opposite antibody (p360-1 for 359-1 Ab). moCAIX: mouse CAIX, huCAIX: human CAIX, Ab: antibody, N: normoxia, H: hypoxia, WT: WT huCAIX.
Since antibody 359-1 was proven to be best at recognizing human CAIX by Western blot, I selected this antibody for further characterization. To demonstrate that polyclonal antibody 359-1 is also able to immunoprecipitate (IP) CAIX from cell lysates, I performed immunoprecipitation of human CAIX from 67NR cells that express exogenous WT human CAIX (67NR/WT huCAIX) as well as from parental MDA-MB-231 cells pre-incubated in hypoxia, as described in Materials and Methods (Chapter 2, section 2.12.2). Commercially available mouse anti-human CAIX antibody (MAB2188, R&D Systems) was used as a positive control of the IP (Figure 3.6). Polyclonal antibody 359-1 was able to successfully immunoprecipitate endogenous human CAIX from hypoxic MDA-MB-231 cells, similar to the positive control. In the same way, polyclonal antibody 359-1 was successful at immunoprecipitating exogenous human CAIX from 67NR cells expressing it (67NR/WT huCAIX; Figure 3.6), demonstrating that this antibody is a great tool for this type of biochemistry assays.
Figure 3.6. Immunoprecipitation abilities of novel anti-CAIX antibody 359-1. Top: Endogenous and exogenously-expressed human CAIX (huCAIX) was immunoprecipitated (IP) from MDA-MB-231 (MDA) and 67NR/WT huCAIX (67NR/WT) cell lysates respectively. 25 μg of commercial antibody MAB2188 (or mouse IgG) or 5 μg of novel antibody 359-1 (or rabbit IgG) were used for the IP. The presence of human CAIX was detected by Western blot utilizing commercial antibody goat anti-human CAIX (AF2188). Bottom: inputs.

Another important application in the research laboratory and especially in the clinic is immunocyto(histo)chemistry, since it is quite commonly used for histological verification of cancer cases. Therefore I wanted to test if antibody 359-1 could be useful
for these purposes. Immunocytochemistry staining of parental 4T1 and parental MDA-MB-231 LM2-4\textsuperscript{4luc}\textsuperscript{+} (LM2-4\textsuperscript{4luc}\textsuperscript{+}) cells growing in hypoxia was carried out, as described in Materials and Methods (Chapter 2, section 2.5). Commercially available antibodies; goat anti-mouse CAIX (AF2344, R&D Systems) and goat anti-human CAIX (AF2188, R&D Systems) were used as positive controls. When 4T1 parental cells were incubated with commercial antibody, a clear membrane staining of mouse CAIX was observed. LM2-4\textsuperscript{4luc}\textsuperscript{+} are mesenchymal cells, however membrane staining of human CAIX can still be detected when using the commercial antibody. Unfortunately, 359-1 did not prove to be a satisfactory antibody for immunocytochemistry as little to no signal was observed when used at a concentration of 10 \(\mu\)g/ml (Figure 3.7), which might implicate that the protein needs to be denatured for the epitope to be exposed. Future experiments with histological samples; different fixation methods and different amounts of antibody might confirm the utility of antibody 359-1 for these purposes.
Figure 3.7. Immunofluorescence staining abilities of novel anti-CAIX antibody 359-1. Hypoxic 4T1 and MDA-MB-231 LM2-4^{luc+} (LM2-4^{luc+}) cells were fixed and stained with commercial antibody goat anti-mouse CAIX (AF2344; 2 μg/ml, top) and goat anti-human CAIX antibody (AF2188; 2 μg/ml, bottom), or with novel antibody 359-1 (10 μg/ml, top and bottom) to detect mouse and human CAIX (moCAIX, huCAIX). The nuclei were counter-stained with Hoescht 33342.
In order to assess if the novel antibody 359-1 was able to block the enzymatic functions of CAIX, the antibody was tested in the in vitro Carbonic Anhydrase activity assay as described in Materials and Methods (Chapter 2, section 2.6.1). The source of enzymatic activity was provided by commercially available recombinant human CAIX (10 μg/ml; extracellular (EC) domain, 2188-CA, R&D Systems). When buffer was run in the assay, the small activity detected corresponded to spontaneous hydration of carbon dioxide. However, when the enzyme was added, the now catalyzed reaction occurred significantly faster. The enzyme was pre-incubated with broad Carbonic Anhydrase inhibitor AZA (200 μM) or DMSO and antibody 359-1 or rabbit IgG (50 μg/ml each) for 50 min (4°C) in a well-stirred vessel. Upon incubation of rCAIX with AZA, the activity of Carbonic Anhydrase was completely blocked, bringing it back to levels of spontaneous activity (buffer). Upon incubation with antibody 359-1, a small blockage of CAIX activity was observed. However, the rabbit IgG control also showed to be blocking CAIX activity (when compared to ‘rCAIX + DMSO’ group) making it difficult to estimate the contribution of antibody 359-1 and to conclude that antibody 359-1 has function-blocking capacities (Figure 3.8). However, the blockage of CAIX activity observed with antibody 359-1 (around 25% inhibition), suggests that this antibody could indeed possess function-blocking abilities. Further experiments with alternative negative controls such as IgG isotypes from different commercial sources and that do not display non-specific binding might contribute to confirm the inhibitory capacities of antibody 359-1.
Figure 3.8. Function-blocking capacity of novel anti-CAIX antibody 359-1. The new antibody was tested in the in vitro Carbonic Anhydrase activity assay. The source of CAIX activity was provided by commercially available recombinant CAIX (rCAIX; 10 μg/ml). The enzyme was pre-incubated with antibody (Ab) 359-1 or rabbit IgG (Rb IgG; 50 μg/ml) for 50 min at 4°C. Acetazolamide (AZA; 200 μM) was used as a positive control of CAIX inhibition. The experiment was repeated twice and one representative experiment with triplicates is shown. Bars represent the mean ± s.e.m. of triplicates.

In an attempt to obtain monoclonal antibodies with potentially greater function-blocking abilities, KalGene Pharmaceuticals derived several monoclonal antibodies out of the polyclonal antibody 359-1.

Monoclonal antibodies 4H1-4L1 and 16H1 were tested by Western blot and were found to detect CAIX by this technique. Monoclonal antibody 4H1-4L1 was able to recognize rCAIX (catalytic and EC domain) and CAIX from 67NR/WT cell lysates, but not CAIX from hypoxic parental lysates, or from 4T1/WT cell lysates (Figure 3.9, middle). Monoclonal antibody 16H1 only recognized rCAIX (catalytic and EC domain) and CAIX from 4T1/WT cell lysates but again, it did not detect CAIX from hypoxic parental lysates or from 67NR/WT cell lysates (Figure 3.9, bottom). Commercially available goat anti-human CAIX antibody (AF2188, R&D Systems) and goat anti-mouse CAIX antibody
(AF2344, R&D Systems) were used as positive controls and were successful in recognizing CAIX from all of the samples used (Figure 3.9, top). Again, there were extra bands being detected by antibody 4H1-4L1 and by commercial antibody goat anti-human CAIX (AF2188, R&D Systems), which could represent degraded protein.
Figure 3.9. Specificity of novel anti-CAIX antibodies 4H1-4L1 and 16H1. The ability of these antibodies (undiluted supernatants) to recognize purified recombinant CAIX (rCAIX), exogenous human CAIX (4T1/WT and 67NR/WT) and endogenous mouse and human CAIX (4T1 and LM2-4\textsuperscript{Luc}\textsuperscript{+} respectively) from cell lysates was assessed by Western blot. Commercial antibody anti-human CAIX (AF2188) and anti-mouse CAIX antibody (AF2344) were used as positive controls. CA: catalytic domain, EC: extracellular domain, huCAIX: human CAIX, moCAIX: mouse CAIX, N: normoxia, H: hypoxia, WT: WT huCAIX.
Antibodies 4H1-4L1 and 16H1 could not be further characterized due to the project being ceased by the third party.

3.5 Specificity and function-blocking capacities of novel anti-CAIX monoclonal antibodies 538-542

The Deeley Cancer Research Centre and the University of Victoria derived five different anti-CAIX monoclonal antibodies, following standard methods for hybridoma cell production after immunization of mice with the catalytic domain of human CAIX. Monoclonal antibodies 538, 539, 540, 541 and 542 were all tested by Western blot as previously described with other antibodies. Briefly, rCAIX (extracellular domain or catalytic domain), 4T1/WT and 67NR/WT cell lysates and normoxic and hypoxic LM2-4luc + cell lysates were loaded on SDS-PAGE gels. After transfer onto PVDF membranes, these were incubated with 5 μg/ml of each of the novel antibodies. Commercially available goat anti-human CAIX antibody (AF2188, R&D Systems) was used as a positive control. While this antibody was able to recognize all forms of human CAIX, novel antibody 540 recognized both forms of rCAIX solely. Antibodies 538, 539, 540 and 541 detected exogenous human CAIX in 67NR lysates (67NR/WT) while none of them recognized human CAIX being expressed in hypoxic and parental LM2-4luc + lysates. Novel antibodies 538-542 recognized bands at around 75 kDa, while some were also detecting bands at around 20, 30 and 40 kDa, none of them corresponding to the molecular weight of human CAIX (Figure 3.10).
Figure 3.10. Specificity of novel anti-CAIX antibodies 538-542. The ability of antibodies 538-542 (5 μg/ml) to recognize purified recombinant CAIX (rCAIX; top), exogenous human CAIX (4T1/WT and 67NR/WT; middle) and endogenous human CAIX (LM2-4luc; bottom) from cell lysates was assessed by Western blot. Commercial antibody anti-human CAIX (AF2188) was used as positive control. Red arrows indicate possible human CAIX being detected by these antibodies. CA: catalytic domain, EC: extracellular domain, huCAIX: human CAIX, WT: WT huCAIX, N: normoxia, H: hypoxia.
Commonly, antibodies which do not work for Western blot often succeed in immunocytochemistry assays. The reason for this is that if the epitope of the antibody is on the surface of the intact protein, then the protein needs to remain unaltered in order for the antibody to recognize the epitope. Proteins subjected to SDS-PAGE followed by Western blots were being denatured, which might be impacting the ability of the antibodies to recognize their epitopes. I therefore performed immunofluorescence staining of 67NR/WT huCAIX cells incubated in hypoxia, an assay in which the conformation of the protein should remain intact. I tested the five monoclonal antibodies 538-542 at a wide range of concentrations (10-50 μg/ml) and used the commercially available goat anti-human CAIX antibody (AF2188, R&D Systems) at 2 μg/ml as a positive control. While the positive control was successful at staining human CAIX in this cell line, only monoclonal antibodies 539 and 541 showed signal when using them at 50 μg/ml, however the staining pattern did not resemble the cell membrane staining observed for CAIX when using the control antibody (Figure 3.11), suggesting that it represented non-specific staining. Although different fixation methods (4% PFA for 10 min at RT, MeOH and MeOH/Acetone, for 5 and 15 min each, at -20°C) and staining of live cells were tested with these antibodies, no significant change in the type of staining was observed (data not shown).
Figure 3.11. Immunofluorescence staining abilities of novel anti-CAIX antibodies 538-542. Hypoxic 67NR/WT huCAIX cells were fixed and stained with commercial antibody anti-human CAIX (AF2188; 2 μg/ml) or with novel antibodies 538-542 (50 μg/ml) to detect human CAIX (huCAIX). The nuclei were counter-stained with Hoescht 33342.
In order to investigate further the specificity of monoclonal antibodies 539 and 541 and their intracellular staining pattern; competition experiments were performed. Commercial antibody goat anti-human CAIX (AF2188, R&D Systems) and novel monoclonal antibodies 539 and 541 were pre-incubated either with commercially available rCAIX (EC domain, 2188-CA, R&D Systems) or with rCAII (full-length, 2348-CA, R&D Systems) at a 1:1 concentration ratio (1 h, RT). CAII is cytosolic and ubiquitously expressed, pre-incubation with rCAII could aid in determining if antibodies 539 and 541 are binding to CAII instead of CAIX. The pre-incubated antibody-enzyme complexes were then used to stain hypoxic A549 human lung carcinoma cells. rCAIX but not rCAII was able to completely block the staining of commercial antibody goat anti-human CAIX (AF2188, R&D Systems). However, neither rCAIX nor rCAII were able to block the staining of novel antibodies 539 and 541. In fact, an increase in signal was observed when antibody 539 was pre-incubated with rCAIX (Figure 3.12); the reason for this is unknown.
Figure 3.12. Specificity of antibodies 539 and 541 towards CAIX. Novel antibodies 539 and 541 were pre-incubated with commercially available recombinant CAIX (rCAIX; EC domain only) or rCAII (full-length) for 1 h, RT at a 1:1 concentration ratio, prior to fixation and immunostaining of hypoxic A549 cell cultures. Commercial antibody anti-human CAIX (AF2188) was utilized as a positive control of human CAIX (huCAIX) staining. The nuclei were counter-stained with Hoescht 33342.
In the same way, there was no observation of loss of signal when the antibodies were pre-incubated with the catalytic domain of rCAIX at a 1:1 concentration ratio (1 h, RT; Figure 3.13). Altogether, this suggests that the signal observed when staining with these antibodies may not represent true CAIX signal.

A549 Hypoxia

CAIX/Nuclei

Figure 3.13. **Specificity of antibodies 539 and 541 towards the catalytic domain of CAIX.** Novel antibodies 539 and 541 were pre-incubated with the catalytic domain of recombinant CAIX (rCAIX-CA) for 1 h, RT at a 1:1 concentration ratio, prior to fixation and immunostaining of hypoxic A549 cell cultures. Commercial antibody anti-human CAIX (AF2188) was utilized as a positive control of human CAIX (huCAIX) staining. The nuclei were counter-stained with Hoescht 33342.
To test if monoclonal antibodies 539 and 541 are able to immunoprecipitate CAIX from cell lysates, another technique in which the conformation of the protein remains intact; immunoprecipitation of CAIX from 67NR/WT huCAIX cells was performed as described previously. As previously shown, this particular cell line expresses abundant human CAIX, making immunoprecipitation experiments easier to perform. KalGene Pharmaceuticals antibody 359-1 was used as a positive control since it has been proven to be a good tool for immunoprecipitation assays (Figure 3.6). While antibody 359-1 successfully immunoprecipitated human CAIX from this cell line, none of the novel antibodies tested were able to immunoprecipitate CAIX and the protein was still present in the flow-through fraction (Figure 3.14).

Figure 3.14. Immunoprecipitation abilities of novel anti-CAIX antibodies 539 and 541. Human CAIX (huCAIX) exogenously expressed in 67NR/WT huCAIX (67NR/WT) cells was attempted to be immunoprecipitated (IP) by utilizing 5 μg of antibodies 539 and 541 respectively, or mouse IgG (MoIgG). IP with antibody 359-1 (5 μg) was used as a positive control. The presence of human CAIX was detected by Western blot by utilizing commercial antibody anti-human CAIX (AF2188). Ab: antibody, RblgG: rabbit IgG. Right: input.
Finally, to test if the new antibodies 538-542 have the ability to block the enzymatic function of CAIX; I tested them in the *in vitro* Carbonic Anhydrase activity assay as described previously. When buffer was run in the assay, the small activity detected corresponds to spontaneous hydration of carbon dioxide. When commercially available rCAIX (10 μg/ml; EC domain, 2188-CA, R&D Systems) is added, the now catalyzed reaction occurs significantly faster. The enzyme was pre-incubated with broad Carbonic Anhydrase inhibitor AZA (200 μM), DMSO, or with each of the novel antibodies (50 μg/ml, 5:1 antibody:enzyme concentration ratio) for 50 min (4°C), in a well-stirred vessel prior to addition to the activity assay. The same amount of mouse IgG was used as a negative control. Upon incubation of rCAIX with AZA, the activity of CAIX was completely blocked, bringing it back to close to levels of spontaneous activity (buffer). Novel monoclonal antibody 539 provided a moderate but significant blockage of CAIX activity (around 28%) while monoclonal antibodies 541 and 542 both significantly blocked the activity of CAIX by around 50% (Figure 3.15). The fact that novel antibodies 539, 541 and 542 were proven to have function-blocking capacities calls for further experiments to demonstrate their specificity towards CAIX.
3.6 Specificity and function-blocking capacities of novel anti-CAIX monoclonal antibody MM-26

SignalChem Lifesciences developed over 50 novel monoclonal antibodies against CAIX through immunization of mice with the extracellular domain (catalytic and PG-like domains) of recombinant human CAIX and standard hybridoma technology. These antibodies were screened by SignalChem Lifesciences for binding to CAIX by ELISA and the findings were validated by Western blot and flow cytometry. Their CAIX binding specificity was validated against a panel of 40 native and denatured proteins using
Reverse Phase Protein Array (RPPA) and their binding kinetics were determined by SPR. They were also tested for their inhibition of CAIX activity in an assay different to ours that utilizes a fluorescent esterase substrate. However, the substrate used in these initial studies is relatively generic and is not the physiologic substrate of CAIX in vivo.

I selected monoclonal antibody MM-26 for further testing since it was shown to be the strongest candidate, due to its high specificity and enhanced function-blocking capacity. Monoclonal antibody MM-04 was chosen as a negative control since its characterization was nearly identical to that of MM-26 (i.e. they were both able to detect CAIX by Western blot, flow cytometry, ELISA, SPR and RPPA), with the exception that it did not block CAIX activity in assays performed by SignalChem Lifesciences.

Initially, I wanted to determine if the difference in the ability of antibodies MM-26 and MM-04 to block CAIX enzymatic function lay in the domain of CAIX that each was targeting. I therefore performed an experiment in which two different forms of rCAIX available in the laboratory (commercially available EC domain (2188-CA, R&D Systems) or catalytic and EC domains custom-made by SignalChem Lifesciences) were loaded in SDS-PAGE gels and Western blots with each of these antibodies and the commercial antibody goat anti-human CAIX (AF2188, R&D Systems) were performed. While both the commercial antibody and antibody MM-26, recognized all forms of CAIX, antibody MM-04 only detected those forms of rCAIX that contain the PG-like domain (Figure 3.16). With this epitope mapping experiment, I was able to prove that while antibody MM-26 recognizes the catalytic domain of CAIX, the epitope of antibody MM-04 is mapped to the PG-like domain of CAIX. This is because the only difference between the catalytic and the EC domain of rCAIX is the presence of the PG-like domain in the second one. These findings explain why antibody MM-04 does not possess function-blocking capacities, and more importantly, they confirm that antibody MM-26 recognizes the catalytic domain of CAIX, explaining its ability to inhibit CAIX enzymatic function.
Figure 3.16. Epitope determination of anti-CAIX antibodies MM-26 and MM-04. Recombinant human CAIX (rCAIX) from R&D Systems (extracellular (EC) domain) or recombinant human CAIX custom-made by SignalChem Lifesciences (SC, catalytic (CA) and extracellular (EC) domains) were loaded on SDS-PAGE gels and Western blots were performed utilizing commercial antibody anti-human CAIX (AF2188, top) or the novel antibodies MM-26 and MM-04 to detect human CAIX (huCAIX, bottom left). Bottom right: A highly exposed film is shown. Arrows indicate rCAIX-CA domain. CA: catalytic domain, EC: extracellular domain = comprised by CA and PG-like domains.)
To confirm that novel monoclonal antibody MM-26 has the ability to block the enzymatic functions of CAIX, I tested it in the *in vitro* Carbonic Anhydrase activity assay as described previously. When buffer was run in the assay, the small activity detected corresponds to spontaneous hydration of carbon dioxide. The addition of commercially available rCAIX (10 μg/ml; 2188-CA, R&D Systems) resulted in a significantly faster reaction. The enzyme (10 μg/ml or 238 nM) was pre-incubated with antibody MM-26 at different molar ratios (0.1:1, 1:1 and 5:1 antibody:enzyme molar ratios) for 50 min (4°C) in a well-stirred vessel prior to the addition to the activity assay. CAIX enzyme pre-incubated with mouse IgG (same ratios as described above) or with antibody MM-04 at a 5:1 antibody:enzyme ratio were used as negative controls. Novel monoclonal antibody MM-26 significantly inhibited CAIX activity at all the molar ratios tested. Antibody MM-26, showed more than 50% inhibition of CAIX activity when used at low concentrations (0.1:1 antibody:enzyme molar ratio). When the molar ratio was increased to 1:1, this antibody provided around 64% inhibition of CAIX activity. Finally, when the 5:1 molar ratio was used, the antibody MM-26 achieved an excellent 100% inhibition of CAIX activity. In contrast, antibody MM-04 did not block CAIX activity at the highest molar ratio (5:1) (Figure 3.17).
Figure 3.17. Function-blocking ability of novel anti-CAIX antibody MM-26. Antibody MM-26 was tested in the in-vitro Carbonic Anhydrase activity assay. The CAIX enzymatic activity was provided by commercially available recombinant CAIX (rCAIX; 10 μg/ml or 238 nM). Antibody MM-26 or mouse IgG (MoIgG) were pre-incubated with the enzyme for 50 min, 4°C at different molar ratios (0.1:1, 1:1, 5:1), prior to the addition of CO₂-saturated water. The non-function-blocking antibody MM-04 was used as a negative control (molar ratio 5:1). The experiment was repeated twice and one representative experiment with triplicates is shown. The IgG group was compared to the MM-26 group at one time point only. Bars represent the mean ± s.e.m. of triplicates. *P<0.05 by Student’s t-test.

To translate the previous results to a more physiological setting; I examined the monoclonal antibody MM-26 using the In-cell Carbonic Anhydrase activity assay as described previously. The source of CAIX enzymatic activity in this assay was provided by 67NR/WT huCAIX cells. Again, when buffer was run in the assay, the small activity
detected corresponded to spontaneous hydration of carbon dioxide, however when cells were added, the now catalyzed reaction occurred significantly faster. The cells were pre-incubated with different amounts of antibody MM-26 for 50 min (4°C), in a well-stirred vessel prior to the addition to the activity assay. Same amounts of mouse IgG and antibody MM-04 were used as negative controls. As a positive control for inhibition of activity, cells were pre-incubated with CAIX inhibitor U-104 (50 μM). Novel anti-CAIX monoclonal antibody MM-26 was proven to be effective at blocking CAIX activity from cells, providing around 18% inhibition of activity when used at 37.5 μg/ml (Figure 3.18A, non-significant) and a significant inhibition of activity (around 50%) when used at 100 μg/ml (Figure 3.18B). When used at this same concentration, antibody MM-04 did not inhibit CAIX activity (Figure 3.18B). On the other hand, small molecule inhibitor U-104 provided 100% inhibition of activity proving to be a good positive control (Figure 3.18B).
Figure 3.18. In-cell CAIX function-blocking ability of novel anti-CAIX antibody MM-26. Antibody MM-26 was tested in the In-cell Carbonic Anhydrase activity assay. The CAIX enzymatic activity was provided by 67NR/WT huCAIX cells. Cells were pre-incubated with antibody (Ab) MM-26 or mouse IgG (MolG) at (A) 37.5 μg/ml or (B) 100 μg/ml for 50 min, 4°C prior to the addition of CO2-saturated water. In (B), the CAIX inhibitor U-104 (50 μM) was used as positive control of inhibition of activity and the non-function-blocking antibody MM-04 was used as a negative control. The experiment was repeated twice and one representative experiment with triplicates is shown. The IgG group was compared to the MM-26 group at one time point only. Bars represent the mean ± s.e.m. of triplicates. **P<0.01 by Student’s t-test.
The increased efficacy of antibody MM-26 in the *in vitro* activity assay might likely be due to the fact that only two purified proteins are present in this assay (CAIX and antibody MM-26), which increases their probability to bind each other, as opposed to the cell-based activity assay where there might be interference of other proteins and possibly competition for binding to CAIX.

Based on my previous results demonstrating that antibody MM-26 is highly efficient at blocking CAIX function, as well as former results from the laboratory showing that CAIX is required for cell survival (Lou et al., 2011), I wanted to investigate if the MM-26-mediated blockage of CAIX activity could result in cell death.

To assess antibody-mediated cell death I performed TUNEL assays on U-87 human glioblastoma cells growing in hypoxia, 0.1% serum and pre-incubated with monoclonal antibody MM-26 (100 μg/ml, 72 h) as described in Materials and Methods (Chapter 2, section 2.13). U-87 cells, unlike 67NR parental cells, express CAIX in hypoxia and are highly glycolytic, therefore serum deprivation inflicts a great strain in these cells and cell death can be observed more easily than in other cell lines. The TUNEL labelling kit (Roche) utilizes the enzyme terminal deoxynucleotidyl transferase (TdT) to detect single- and double-stranded DNA breaks that are present in early apoptotic cells. The enzyme catalyzes the addition of labelled-dUTP at free 3’-OH groups present in the DNA, amplifying the fluorescent signal which can be visualized by fluorescence microscopy (Roche Diagnostics, 2014).

Incubation with monoclonal antibody MM-26 produced a significant two-fold induction of cell death when compared to the mouse IgG control, which was observed by an increase in positive signal (red dots) when cells were imaged using an epifluorescence microscope (Figure 3.19). The findings presented here confirmed the hypothesis that inhibition of CAIX catalytic activity can ultimately induce cell death through alteration of the intracellular pH homeostasis. This is the first report of a CAIX-specific function-blocking antibody that is capable of inducing cell death *in vitro*, opening exciting possibilities for future anti-cancer therapies.
Figure 3.19. Anti-CAIX antibody MM-26-mediated induction of cell death. Hypoxic U-87 cells were incubated with antibody MM-26 or mouse IgG (MoIgG; 100 µg/ml) for 72 h under low serum conditions (0.1% FBS). The presence of apoptotic cells was detected by TUNEL assays. The nuclei (blue) were counter-stained with Hoescht 33342. Red dots represent TUNEL-positive cells. Quantification of the percent of positive cells in five different fields is shown at the bottom. The experiment was repeated twice and one representative experiment with triplicates is shown. Bars represent the mean ± s.e.m. of triplicates. *P<0.05 by Student’s t-test.

3.7 Discussion

3.7.1 Novel CAIX small molecule inhibitors

The two novel CAIX inhibitors tested were proven to be successful at blocking CAIX activity. Ureido sulfonamide U-104 was superior to the traditional and commercially available AZA at inhibiting CAIX activity. U-104 provided the same degree of inhibition of CAIX activity than AZA when used at a concentration four times smaller (50 µM). Moreover, U-104 was proven to be selective against CAIX and CAXII, whereas AZA is a broad inhibitor of Carbonic Anhydrases (Pacchiano et al., 2011; Supuran, 2008). Glycosyl coumarine GC-205 was slightly less effective than AZA, providing only 80% inhibition of CAIX activity when used at an equal concentration (200 µM). However, GC-
205 is more selective than AZA, since it only inhibits the membrane-bound Carbonic Anhydrases CAIX and CAXII (Touisni et al., 2011). In the initial assays; 67NR parental cells, which do not express endogenous CAIX, were used as a negative control of CAIX activity, therefore I was able to conclude that the gain in activity observed when using 67NR/WT huCAIX cells was a result of exogenous human CAIX. In the same way, one can conclude that the inhibition provided by U-104 and GC-205 was merely and specifically inhibition of CAIX activity, since the activity in 67NR/WT huCAIX cells treated with the new inhibitors was never significantly smaller than that found in 67NR parental cells alone.

Both U-104 and GC-205 have previously been tested in pre-clinical models, demonstrating potent inhibition of tumour growth (U-104, 38 mg/kg) and metastasis (both U-104 and GC-205 dosing at 19 and 15 mg/kg respectively). These effects were only observed in CAIX-positive tumours (Lou et al., 2011). Importantly, no side effects were observed in these studies suggesting that only tumour cells are being targeted. The studies performed in this thesis demonstrate that the mechanism of tumour growth and metastasis inhibition originated by U-104 inhibitor is through the blockage of CAIX activity and supports the hypothesis that CAIX in tumour cells facilitates tumour growth and metastasis. Further experiments have been performed to demonstrate that the U-104-mediated inhibition of CAIX activity in several cancer cell lines ultimately results in concentration-dependent impairment of cell viability and leads to an increase in cancer cell death (McDonald, PC and Chafe, SC; unpublished results).

In initial experiments with U-104 and GC-205 only the maximal dissolvable concentrations were tested (50 and 200 μM respectively). Since the maximal dissolvable concentration for GC-205 (200 μM) only provided 80% inhibition of activity when compared with U-104 and AZA, no other concentrations were tested. The finding of alternative vehicles for dissolving GC-205 will open the possibility of increasing the concentration of this inhibitor with a concomitant increase in the inhibition of CAIX activity. On the other hand, U-104 at the maximal dissolvable concentration (50 μM) already provided almost complete inhibition of activity. Therefore, this inhibitor was
tested at different concentrations in the activity assay. Reducing the concentration of U-104 by half also reduced its inhibitory abilities to 55% of the original, therefore 50 μM was the best concentration for this inhibitor.

Since U-104 has proved to be a strong CAIX inhibitor both in vitro and in vivo, SignalChem Lifesciences worked in developing potentially better derivatives of U-104 by medicinal chemistry. Derivatives SLC-148, -149 and -150 provided similar inhibition rates to U-104 when tested at the maximal dissolvable concentration of 50 μM, however SLC-149 proved to have increased inhibitory potential than U-104, especially when used at the very low concentration of 1 μM.

Although the SLC-149 inhibitor has not been tested in pre-clinical models, finding the minimum effective concentration in vitro is important for its development in vivo. In this regard, SLC-149 showed enhanced potential than U-104, since when half or five times less inhibitor was used there was only a minimum effect on the inhibitory abilities of SLC-149, suggesting an outstanding success of this inhibitor in future pre-clinical models.

Future plans for GC-205, SLC-148, -149 and -150 small molecule inhibitors include further characterization in vitro and in pre-clinical models in order to attempt to get approval for clinical trials. One important future experiment is to determine the ability of these inhibitors to block other Carbonic Anhydrase isoforms that have not been previously studied. U-104 has entered Phase I clinical trial as of September 2014, providing a new anti-cancer therapy.

3.7.2 Novel CAIX antibodies

3.7.2.1 KalGene Pharmaceuticals anti-CAIX antibodies

Two of the antibodies developed by KalGene Pharmaceuticals showed good specificity towards CAIX. Antibodies 359-1 and 360-1 were proven to be powerful tools for Western blot detection of both rCAIX and human CAIX exogenously expressed by different cell types. The band corresponding to CAIX in 67NR/WT huCAIX lysates was
noticeably more intense than that of 4T1/WT huCAIX lysates. This confirms previous observations with commercially available antibodies indicating that the expression of human CAIX in 67NR cells was more successful than in 4T1 cells. Antibody 360-1 was not able to recognize human CAIX in hypoxic MDA-MB-231 lysates and therefore, was not under further examination since my final goal was to find antibodies that can potentially be developed as cancer therapies. Interestingly, when MDA-MB-231 lysates were used, antibody 359-1 detected bands of the right size (54 kDa) both in normoxia and hypoxia, whereas the commercial antibody only detected human CAIX in hypoxic lysates raising up the possibility that the antibody was also detecting other protein(s), or more likely; other Carbonic Anhydrases. However, the only other Carbonic Anhydrase isoform of similar molecular weight is CAXIV and it is unknown and unlikely that this isoform is expressed in MDA-MB-231 cells, since CAXIV has not been found to be upregulated in cancer cells.

Likewise, when LM2-4luc+ lysates were used, antibody 359-1 detected a band of the right size (54 kDa) in normoxic and hypoxic lysates, however the band corresponding to hypoxic lysates was more intense. Moreover, when the antibody was pre-incubated with its blocking peptide (CAIX peptide used for immunization), the ability to detect all of these bands was almost completely abolished, indicating that this antibody is specifically recognizing CAIX. Importantly, from the same Western blot, it is confirmed that the epitope of this antibody is located in the catalytic domain of CAIX, since this antibody is able to detect the catalytic domain of rCAIX in the membrane. This is in agreement with the way these antibodies were developed, by immunizing rabbits with overlapping peptides within the catalytic domain of CAIX.

The rabbit monoclonal antibodies derived from 359-1 (4H1-4L1 and 16H1) did not work well in recognizing CAIX from parental cell lines in Western blot assays. It was evident, however that these antibodies had the ability to recognize purified rCAIX, but when human CAIX was expressed in 4T1 and 67NR cells; none of these antibodies recognized both. Further experiments are required, specifically with blocking peptides,
before further conclusions can be made regarding the specificity and selectivity of antibodies 4H1-4L1 and 16H1.

It is quite common that antibodies which work well in Western blot assays (reducing and denaturing conditions) are less successful in assays where the protein remains intact, such as immunocytochemistry and vice versa (Abcam, 2010). The reason for this is that if the epitope is in a region not easily accessible, then the protein needs to be denatured in order for the epitope to be exposed and recognized by the antibody, whereas if the epitope is on the surface of the intact protein, then the protein needs to remain unaltered in order for the antibody to recognize the epitope. This might be one possible reason for the failure of antibody 359-1 in immunocytochemistry assays. However, antibody 359-1 was able to immunoprecipitate CAIX both from parental cells and from cells overexpressing CAIX, an assay in which the protein is usually recognized in its native conformation by the antibody. Another possible explanation for the lack of signal in immunocytochemistry experiments is that the rabbit IgG isotype control used was not up to the desired standard, as it provided background signal even at very short exposures, which indicates that it might have been binding non-specifically to Fc receptors on the cell surface. All of the immunofluorescence images were taken at an exposure that would show no background signal in the IgG isotype control. There was no visible signal with antibody 359-1 at this exposure. A possible way to fix this predicament would be to perform an antibody/IgG titration assay and choose the concentration with the lowest signal-to-noise ratio. However, the limited amount of antibody available did not allow for this.

The same background problem was present when antibody 359-1 was tested in the in vitro Carbonic Anhydrase activity assay but surprisingly, not when the antibody was used for immunoprecipitation purposes where the IgG isotype control proved to be free of non-specific binding. The IgG isotype control itself provided a significant degree of inhibition in the in vitro Carbonic Anhydrase activity assay, possibly due to non-specific binding to Fc receptors. Even though antibody 359-1 provided some degree of inhibition, it is difficult to truly assess its inhibitory abilities when the inhibition
provided by the IgG isotype control was greater. The monoclonal antibodies that were derived from 359-1 were made with the intention that greater inhibition rates would be obtained. However I was unable to characterize antibody 359-1 or any of its derivatives any further, due to the limited amounts I received from KalGene Pharmaceuticals and to the project eventually being ceased by them. In conclusion, further experiments are required to demonstrate that antibody 359-1 possess function-blocking abilities, however this antibody has proven to be an outstanding tool for the biochemistry and pathology laboratories where it can be used for the detection and immunoprecipitation of human CAIX.

3.7.2.2 Deeley-UVic anti-CAIX antibodies

From Western blot and competition experiments, followed by immunocytochemistry assays, it could be suggested that the specificity of antibodies 538-542 (Deeley Cancer Center-University of Victoria) towards CAIX is inconclusive. Only antibody 540 recognized rCAIX as well as human CAIX exogenously expressed by 67NR cells, but not CAIX from parental cell lines. To generate these antibodies, mice were immunized with the catalytic domain of human CAIX and screened by ELISA against full-length human CAIX. Interestingly, in the in vitro Carbonic Anhydrase activity assay that utilizes purified recombinant CAIX, three of these antibodies demonstrated inhibitory abilities (antibodies 539, 541 and 542) but antibody 542 did not recognize any form of CAIX by Western blot, and antibodies 539 and 541 only seemed to be recognizing human CAIX exogenously expressed by 67NR cells. Further experiments demonstrating the specificity of these antibodies towards CAIX, such as ELISAs and in vitro function-blocking assays utilizing all the different purified Carbonic Anhydrase isoforms that are available will be helpful for developing them as anti-cancer therapies.

The other bands present in the blots when utilizing all of these antibodies may correspond to different Carbonic Anhydrases with a similar catalytic domain amino acid sequence, which may also help explain the function-blocking abilities observed by antibodies 539, 541 and 542. As mentioned earlier, these antibodies were generated upon immunization of mice with the catalytic domain of human CAIX and it has been
observed that there is considerable 3D similarity in the catalytic domain of several Carbonic Anhydrase isoforms, with their active sites almost being superimposable (Vicenzo et al., 2009). However, there is no Carbonic Anhydrase isoform with a molecular weight higher than 54/58 kDa making it difficult to explain the presence of a band around 75 kDa that is detected by all five antibodies. The second band around 45 kDa detected only by antibody 540 in cell lysates could potentially be CAXII since this protein is overexpressed in breast cancer and has a molecular weight of 44 kDa. However, this antibody did not show function-blocking activity. The third band observed between 25 and 37 kDa that is being recognized by antibodies 538, 539 and 542 (the last two with potential function-blocking capacities) might be CAI, CAII, CAIII, CAVII or CAXIII (each with a molecular weight of 29 kDa). CAII is almost ubiquitous and CAVII and CAXIII are also widely distributed. However, competition experiments using either purified rCAIX (EC domain), rCAII or the catalytic domain of rCAIX did not abolish the immunocytochemistry signal observed when staining with antibodies 539 and 541 suggesting that these antibodies are not recognizing CAII either. It remains to be determined if the band between 25 and 37 kDa detected by antibodies 538, 539 and 542 represents a different Carbonic Anhydrase isoform, which would explain the function-blocking abilities observed by antibodies 539 and 542. Although the inhibition data of antibodies 539, 541 and 542 was promising, not being able to absolutely demonstrate their specificity towards CAIX made it increasingly challenging to move forward with this particular project. The only other membrane protein that is overexpressed in several types of cancer is CAXII. Future experiments to investigate if these antibodies are actually blocking CAXII instead of CAIX are required.

3.7.2.3 SignalChem Lifesciences anti-CAIX antibody

Antibody MM-26 developed by SignalChem Lifesciences is so far the most promising one in terms of therapeutic purposes. SignalChem Lifesciences performed a very detailed characterization of this antibody and proved it to be specific against CAIX. This antibody scored high in ELISA, SPR, RPPA and flow cytometry assays performed by SignalChem Lifesciences.
Furthermore, the function-blocking data presented here demonstrates that this antibody displays potent inhibitory capacities of CAIX function. At 100 μg/ml I was able to demonstrate a 50% inhibition of CAIX activity by function-blocking experiments that utilized whole cells. At this concentration, I also observed a two fold increase in cell death, a functional and therapeutically relevant consequence of inhibiting CAIX activity. Reports with a different CAIX function-blocking antibody have demonstrated 50-60% inhibition of activity when used at 20 μg/ml, however the effect on cell death was not assessed (Murri-Plesko et al., 2011).

It is often argued that big molecules such as antibodies will have trouble penetrating the hypoxic areas of tumour cells. Although the in vivo antibody localization and distribution of antibody MM-26 has not been determined, data from our laboratory utilizing a different monoclonal anti-CAIX antibody has demonstrated tumour-specific localization of the antibody by CAIX-positive xenografts in vivo, reassuring the fact that anti-CAIX antibodies can access CAIX-positive tumour cells.

Since antibody MM-26 does not undergo internalization by cells (unpublished results by McDonald, PC and Firmino, N) it may be conjugated to a small molecule inhibitor that is released extracellularly upon binding of the antibody to its target, creating a dual target drug. Likewise, further studies will unveil if this antibody is able to induce immune effector functions such as ADCC and CDC. Therapy with this antibody could be more efficacious if it were used in combination with traditional chemotherapeutic agents. Future plans with this antibody include further characterization in vitro and in pre-clinical models and are discussed in detail in Chapter 6.

Further characterization of this function-blocking antibody is required to determine if it is able to recognize other Carbonic Anhydrases. This could easily be done by Western blot and in vitro function-blocking assays utilizing all the different purified recombinant Carbonic Anhydrase isoforms. Along these lines, SignalChem Lifesciences has demonstrated that antibody MM-26 specifically recognized CAIX out of 40 proteins tested in RPPA assays.
In conclusion, this is the first report of an anti-CAIX function-blocking antibody (MM-26) that is able to induce cell death in vitro opening up exciting opportunities for novel anti-cancer therapies. Function-blocking antibodies can also be used experimentally in vitro and in pre-clinical models to investigate the role of CAIX in cancer physiology. Likewise, antibodies MM-26 and MM-04 were proven excellent tools for biochemistry and pathology laboratories, since they are able to specifically recognize human CAIX.
Chapter 4. Characterization of CAIX Mutants

The results presented in this Chapter and Chapter 5 have been submitted for publication.

4.1 Synopsis

It has been suggested that CAIX plays a key role in facilitating tumour progression and metastasis. Proposed mechanisms to explain this function suggest that CAIX is favouring cell survival, cell migration and cell invasion. However, it is still not fully established how CAIX impacts the aforementioned processes. The most common theory states that the acidification of extracellular pH mediated by CAIX ultimately favours cell invasion, while the neutralization of intracellular pH indirectly influenced by CAIX-mediated generation of HCO$_3^-$ may favour cell survival within the tumour, since a neutral intracellular pH is a pre-requisite for ATP, DNA and protein synthesis, cell proliferation, enzymatic function and the overall functioning of the cell. In this Chapter, I introduce the different human CAIX mutants, which were generated and studied with the aim to determine the role of different domains (mainly proteoglycan-like (PG-like) and intracellular (IC) domains) and different residues within the IC domain of CAIX. The potentially phosphorylated residues T443, S448 and Y449 were mutated to alanine, and the IC and PG-like domains of CAIX were completely truncated. These constructs were successfully stably expressed in 4T1 cells depleted of endogenous CAIX (4T1shCAIX), and all the mutants displayed cell membrane localization and dimerization capacities similar to WT huCAIX, although the ΔIC-huCAIX mutant exhibited lower levels of membrane-bound CAIX. A decrease in CAIX activity was observed with any mutation occurring within the IC domain of CAIX, which correlated with increased cell death. The PG-like domain of CAIX did not appear to regulate CAIX activity, and its deletion did not affect cell survival. However, the PG-like domain seemed to regulate cell migration, potentially through its interactions with the extracellular matrix (ECM), while none of
the domains and residues studied seemed to be involved in regulating cell proliferation. In fact, I only detected a small decrease in cell proliferation when I completely depleted endogenous CAIX in 4T1 cells. However, CAIX seems to be crucial in regulating cell invasion, and this phenotype will be described in more detail in Chapter 5. Mass spectrometry (MS) was performed in an effort to investigate IC binding partners of human CAIX, which could provide insight into alternative mechanism(s) of CAIX-mediated cell survival and invasion. One interesting interacting protein previously involved in invadopodia and filopodia formation is Myosin-10, which opens a whole new research avenue for future projects.

4.2 Expression and localization of human CAIX mutants

The different human CAIX constructs were generated by Dr. Mykola Maydan in our laboratory. Figure 4.1 depicts a diagram of the different CAIX constructs utilized for this study. Previous reports have suggested a role of the intracellular (IC) and proteoglycan (PG)-like domains in modulating CAIX activity (Ditte et al., 2011; Hulikova et al., 2009; Innocenti et al., 2009). Therefore, in order to assess the contribution of these domains in regulating the enzymatic activity of CAIX, mutants lacking the entire extracellular PG-like domain (ΔPG-huCAIX) or the entire IC domain (ΔIC-huCAIX) were generated. At the time these mutants were generated; little was known about the involvement of the IC domain of CAIX in the regulation of signal transduction pathways. Therefore, it was decided to mutate three potentially phosphorylated residues within the IC domain of CAIX: Thr443, Ser448 and Tyr449, each to the non-phosphorylated residue alanine. The double mutant S448A+Y449A was generated based on preliminary observations of very little impairment of CAIX enzymatic activity after single point mutation of each of these residues (data not shown).
Figure 4.1 Diagram of human CAIX mutants. (A) The distinct domains in WT human CAIX (huCAIX) are depicted along with the different mutants. The first mutant consisted of a point mutation to alanine in residue T443 (T443A). The second mutant consisted of two point mutations each to alanine in residues S448 and Y449 (S448A + Y449A). A mutant lacking the entire intracellular (ΔIC) domain and a mutant lacking the PG-like (ΔPG) domain were also generated. All mutants contained the signal peptide (SP). CA: catalytic domain, TM: transmembrane domain, aa: amino acid. (B) Sequence of the IC domain of CAIX where residues T443, S448 and Y449 have been highlighted in red.

Previously, my experience with plasmid transfection of shRNA to generate CAIX knockdown had demonstrated that this knockdown is unstable and ultimately is lost after a few weeks in culture (data not shown). As this could potentially cause difficulty in the study, alternative ways of generating this knockdown were explored. The 4T1shCAIX cell line (4T1 cells with endogenous knockdown of mouse CAIX) was generated by lentiviral transduction by Dr. Shawn Chafe; a post-doctoral fellow in our
I first tested the knockdown generated by different shRNA (shCAIX) sequences (Figure 4.2A) and derived 4T1sh48299 (shCAIX) subclones by fluorescence-activated cell sorting (FACS) of GFP-positive cells (marker for mouse CAIX depletion). I chose the clone with the best knockdown of mouse CAIX (moCAIX; clone 48299 D5; Figure 4.2B) to stably express WT huCAIX and the different mutant forms. The overexpression was achieved by plasmid transfection of the CAIX constructs and further selection of transfected cells using Zeocin as described in Materials and Methods (Chapter 2, section 2.2).

**Figure 4.2. Knockdown of mouse CAIX in 4T1 cells.** (A) 4T1 cells transduced with different shRNA sequences (4T1shCAIX) were verified for knockdown of mouse CAIX (moCAIX) by WB. (B) 4T1 cells transduced with sh48299 sequence (4T1sh48299) were selected for single-cell cloning and the knockdown of mouse CAIX in clone D5 was verified by WB. NS: non-silencing, N: normoxia, H: hypoxia.
To confirm that the 4T1shCAIX cell line was successfully transfected with the different human CAIX constructs; I grew the cell lines for 72 h in hypoxia to allow up-regulation of any remaining mouse CAIX in these cells. 4T1 non-silencing (4T1shNS) control cell lines were included as a positive control of the up-regulation of mouse CAIX in hypoxia. After 72 h in hypoxia, the cells were lysed and lysates were resuspended in sample buffer in the presence, or absence of 2-mercaptoethanol (reducing and non-reducing conditions respectively). The non-reduced samples were loaded on SDS-PAGE gels, as opposed to native gels.

Figure 4.3 depicts the proper expression of WT huCAIX, S448A+Y449A-huCAIX, T443A-huCAIX, ΔIC-huCAIX and ΔPG-huCAIX in 4T1shCAIX cells. The commercial antibody goat anti-human CAIX (AF2188, R&D Systems) was used for the detection of human CAIX forms. This antibody does not cross-react with mouse CAIX, since it did not detect any band when 4T1shNS hypoxic lysates were used (Figure 4.3A and B). Under reducing conditions (Figure 4.3A), WT huCAIX, S448A+Y449A-huCAIX and T443A-huCAIX mutants were present each as a double band at around 54/58 kDa. This doublet has previously been observed in different cell lysates such as MDA-MB-231 and it has been proven that the 54 kDa form is not a 58 kDa form lacking the N-glycan modifications (Li, Wang, et al., 2011). To date, there is no clear explanation of what the second band represents. However, when the IC domain of CAIX was depleted, (ΔIC-huCAIX) the 58 kDa band representing CAIX was now present at a slightly lower molecular weight and existed as a singlet. Likewise, when the PG-like domain of CAIX was depleted (ΔPG-huCAIX) the protein was now present at approximately 30 kDa, as expected, and corresponding to previous observations (Svastová et al., 2004) and again existed as a singlet. When the same lysates were loaded and separated in a parallel gel and blotted with commercial antibody goat anti-mouse CAIX (AF2344, R&D Systems, Figure 4.3C) I only detected signal corresponding to mouse CAIX in 4T1shNS lysates, demonstrating that the cell lines overexpressing the different forms of human CAIX displayed an effective knock-down of endogenous CAIX. It is important to mention that housekeeping Western blots were performed regularly throughout my research to ensure the stable cell lines maintained the expression of human CAIX and the knockdown of mouse CAIX.
Figure 4.3B demonstrates that under non-reducing (i.e. 2-mercaptoethanol was not added and samples were not boiled) and partial non-denaturing conditions (i.e. SDS absent in running buffer, SDS-PAGE) the different mutant forms of CAIX were able to form dimers, similar to WT human CAIX. CAIX functions as a dimer in the plasma membrane, therefore it was important to prove that the mutations did not affect the ability of CAIX to dimerize. Likewise, since CAIX is a membrane-bound enzyme, it was crucial to demonstrate that the mutations did not affect the ability of the protein to localize to the cell membrane. Therefore I performed immunocytochemistry staining of CAIX in sub-confluent cells grown for 72 h in hypoxia. In order to differentiate better cell membrane staining from intracellular staining, cells were fixed and the permeabilisation step was avoided. Figure 4.3D clearly shows that WT huCAIX as well as all the mutants tested properly localize to the cell membrane.
Figure 4.3. Expression, dimerization and localization of human CAIX mutants. (A) Human CAIX mutants were stably expressed by plasmid transfection in 4T1shCAIX cells and the expression of human CAIX (huCAIX) was verified by WB. (B) The ability to form dimers in each of these mutants was verified by non-reducing SDS-PAGE followed by WB. (C) The knockdown of mouse CAIX (moCAIX) in these novel cell lines was verified by WB upon comparison with hypoxic 4T1shNS (non-silencing) cell lysates. (D) Immunofluorescence staining of human CAIX in hypoxic and non-permeabilised 4T1shCAIX cells expressing WT huCAIX and the mutant forms. The nuclei (blue) were counter-stained with Hoescht 33342.
Another way to test cell membrane localization of a protein is to perform analysis of live cells by flow cytometry. During this procedure, live cells were incubated with a primary antibody against human CAIX, followed by a fluorescent-labelled secondary antibody. The cells were also incubated with propidium iodide (PI), which only stains the nuclei of dead cells, since the dye is membrane-impermeant. In this way, information can be obtained on the actual proportion of live cells that are CAIX-positive. Moreover, this technique allows the detection of membrane-bound human CAIX. As it is shown in Figure 4.4A (black bars) in 4T1sh/WT huCAIX and 4T1sh/ΔPG cell lines, 50% or more of the live cells were positive for CAIX (i.e. they had CAIX present at the cell membrane). However in the other cell lines the proportion of CAIX-positive (i.e. membrane-bound CAIX-positive) cells was reduced, especially in the 4T1sh/ΔIC line where 26% of the cells expressed CAIX at the cell membrane. The proportion of PI-positive cells was high, potentially a consequence of excessive harvesting needed to achieve a single cell suspension of 4T1 cells.

With the aim to enrich the membrane-bound CAIX-positive fraction in all the novel stable cell lines, the Flow Cytometry Core Facility (FCCF) housed in the Terry Fox Laboratory, sorted between 20,000 and 100,000 cells of the GFP-positive (marker for mouse CAIX depletion) and human CAIX-positive population. I expanded these new cultures and tested them again by flow cytometry. With this methodology I was able to enrich the CAIX-positive (i.e. membrane-bound CAIX-positive) fraction in all the cell lines (Figure 4.4A). The degree of enrichment was different for each of the cell lines, ranging from approximately 20-40%. (Figure 4.4A and B). The enrichment of the CAIX-positive population was stable for at least 3 months. For all the subsequent experiments, working stocks were prepared from “enriched” frozen stocks every 2-3 months.
Figure 4.4. Percent of human CAIX present at the cell membrane. (A) The amount of membrane-bound human CAIX (huCAIX) in recently created stable cell lines (‘new cell line’) was analyzed by flow cytometry and again after enrichment with the membrane-bound CAIX-positive population (‘post-enrichment’). The percent of cells with membrane-bound CAIX was calculated from the PI-negative population (live cells, left quadrants). (B) Scatter plots of ‘post-enrichment’ samples are shown (x-axis: PI, y-axis: human CAIX). The PI-positive population is shown for reference. PI: propidium iodide.
Due to the observation that less than half of ΔIC-huCAIX localized to the cell membrane and because quite often, exogenously expressed proteins accumulate in the endoplasmic reticulum (ER), I co-stained 4T1sh/ΔIC cells for human CAIX and the ER marker PDI (protein disulphide isomerase). I utilized 4T1sh/WT huCAIX cells as a control of basal ER localization in order to differentiate between ER localization originated by the overexpression of human CAIX, and the one possibly caused by the deletion of the IC domain of human CAIX and further possible protein misfolding. I incubated the cells for 72 h in hypoxia prior to fixation and fluorescent co-staining of human CAIX and PDI. When I observed the cells under the confocal microscope, almost every single 4T1 cell expressing WT huCAIX displayed proper cell membrane staining, while very little human CAIX signal was localized to the ER. In 4T1sh/ΔIC cells I observed two different phenotypes. There were a population of cells which resembled the phenotype observed in 4T1sh/WT huCAIX cells (little human CAIX localized at the ER, the majority of it at the cell membrane), as well as a second population of cells that displayed no cell membrane localization of human CAIX and some portion of the human CAIX signal localized to the ER (Figure 4.5). The observation of the second phenotype suggests that without the IC domain of human CAIX the protein may not fold properly and is targeted for degradation. Another possibility is that the IC domain of CAIX is needed for recruitment to the cell membrane through an interaction with one or more binding partners, however this requires further investigation.
Figure 4.5. Co-staining of human CAIX and the ER. Immunofluorescence co-staining of human CAIX (huCAIX) and the endoplasmic reticulum (ER; anti-PDI antibody) in hypoxic and permeabilised 4T1sh/WT huCAIX and 4T1sh/ΔIC cell lines. The nuclei (blue) were counter-stained with Hoescht 33342. Slides were imaged with a confocal microscope. Arrows indicate areas of co-localization. The z=5 plane is shown.

The results observed here confirm flow cytometry data regarding the cell membrane localization of ΔIC-huCAIX and further explain the fate of ΔIC-huCAIX that could not reach the cell membrane.
4.3 Activity of human CAIX mutants

Since it has been suggested before that the activity of CAIX might be modulated by other domains on top of the catalytic one (Ditte et al., 2011; Hulikova et al., 2009; Innocenti et al., 2009), I hypothesized that mutations within the IC domain of CAIX might be impairing the activity of this enzyme when expressed in 4T1 cells.

In order to test my hypothesis, I utilized the in vitro and In-cell Carbonic Anhydrase activity assays (see Chapter 2, sections 2.6.1 and 2.6.2).

In the in vitro Carbonic Anhydrase activity assay, which utilizes purified recombinant CAIX (rCAIX), samples were compared to buffer alone, which indicates spontaneous hydration of CO₂. The PG-like domain of CAIX appeared to be important in regulating its enzymatic activity in these assays. I compared the rates of pH acidification of the extracellular (EC) domain of rCAIX (catalytic plus PG-like domain, R&D Systems), the catalytic (CA) domain of CAIX (SignalChem Lifesciences) and another EC domain of CAIX from a different source (SignalChem Lifesciences). The acidification rate of the catalytic domain of human CAIX was reduced in comparison to those of the EC domains from two different sources. When I increased the amount of purified catalytic domain used by five-fold, I obtained similar rates as the ones observed with the entire EC domain (Figure 4.6).
**Figure 4.6. Contribution of each domain to the activity of CAIX: in vitro assay.** The activities of two different forms of rCAIX (CA: catalytic domain and EC: catalytic + proteoglycan-like domains) from two sources (R&D Systems and SignalChem Lifesciences (SC)) were analyzed in the *in vitro* Carbonic Anhydrase activity assay. The experiment was repeated twice and one representative experiment with triplicates is shown. Bars represent the mean ± s.e.m of triplicates.

To measure CAIX activity in cell lines expressing recombinant CAIX constructs the *In-cell* Carbonic Anhydrase activity assay was utilized. Samples containing buffer (i.e. no cells) give an indication of the spontaneous hydration of CO₂, while samples containing 4T1shCAIX cells provide indication of background Carbonic Anhydrase activity. Samples containing 4T1sh/WT huCAIX cells represent maximal CAIX activity. The remaining groups were normalized to buffer (0% activity) and to 4T1sh/WT huCAIX (100% activity). In this assay I observed reduced CAIX activity in all cell lines expressing mutants within the IC domain of CAIX (4T1sh/T443A: around 75% active, 4T1sh/S448A+Y449A: approximately 70% active and 4T1sh/ΔIC: around 65% active) but the 4T1sh/ΔPG mutant cell line was as active as 4T1sh/WT huCAIX cells (Figure
4.7), which suggests only the IC domain of CAIX regulates the activity of the enzyme. It is important to mention that around twice as many 4T1sh/ΔIC cells were used in order to compensate for the amount of membrane-bound CAIX present in this particular cell line (see Figure 4.4). Likewise, the number of cells used for the other mutant lines was also adjusted in this particular assay according to data shown in Figure 4.4. I included a group of 4T1sh/WT huCAIX cells pre-incubated with CAIX inhibitor U-104 (described and characterized in Chapter 3) as a positive control of CAIX inhibition. Cells incubated with this inhibitor showed to be around 5% active.
Figure 4.7. Contribution of each domain to the activity of CAIX: In-cell assay. (A) Activity of the different mutants was analyzed using an In-cell Carbonic Anhydrase activity assay. U-104 (50 μM) was used as a positive control of inhibition. Dotted line represents half of the pH drop (See Chapter 2, section 2.6.3). (B) Relative Carbonic Anhydrase activity in each line (ΔpH/second). Bars represent the mean ± s.e.m. of three independent experiments. *P<0.05, **P<0.01, ***/###P<0.001 by Student's t-test.
Another way to measure CAIX activity is through the FITC-labelled Carbonic Anhydrase inhibitor; which has previously been shown to only bind active CAIX (FITC-CAI) (Dubois et al., 2007; Lou et al., 2011). In this particular assay I incubated hypoxic live cells with the FITC-CAI for 16 h in hypoxia (10 μM) prior to imaging with an epifluorescence microscope. I observed binding of the FITC-CAI in 4T1shNS cells, which express CAIX in hypoxia but not in cells depleted of endogenous CAIX (4T1shCAIX) (Figure 4.8). In the same way, 4T1sh/WT huCAIX cells showed fluorescent labelling in almost every cell, while 4T1 cells that expressed any of the IC domain mutations displayed very little or no signal at all (Figure 4.9), further confirming my previous observations proposing that the IC domain of CAIX is involved in regulating its activity. 4T1sh/ΔPG cells still showed full binding of FITC-CAI; suggesting that this domain does not regulate CAIX activity (Figure 4.9) and supporting previous data (Figure 4.7).

![Figure 4.8. CAIX activity in 4T1shNS and 4T1shCAIX cell lines: binding of FITC-CAI.](image)

Figure 4.8. CAIX activity in 4T1shNS and 4T1shCAIX cell lines: binding of FITC-CAI. CAIX activity in the indicated cell lines was assessed by binding of FITC-Carbonic Anhydrase Inhibitor (FITC-CAI) to live cells (both colour and grayscale images are shown). Phase contrast images are shown for reference of cell density.
Figure 4.9. Contribution of each domain to the activity of CAIX: binding of FITC-CAI. CAIX activity in cell lines expressing the different CAIX mutants was assessed by binding of FITC-Carbonic Anhydrase Inhibitor (FITC-CAI) to live cells (both colour and grayscale images are shown). Phase contrast images are shown for reference of cell density.
4.4 Cell survival and proliferation of human CAIX mutants

There have been several reports suggesting a role for CAIX in the survival of tumour cells (Chiche et al., 2009; Lou et al., 2011). As previously mentioned in Chapter 1, the proposed mechanism to explain these observations is that the reaction catalyzed by CAIX generates HCO$_3^-$, which ends up back inside the cell; indirectly contributing to the neutralization of intracellular pH. The maintenance of a relatively neutral pH in such an acidic environment is fundamental for the survival of the tumour cell.

So far, I have demonstrated that the IC mutants that I generated affect CAIX enzymatic function; therefore these mutants may influence the ability of CAIX to regulate cell survival as well. To test this, I analyzed the amount of cell death present in CAIX mutant cell lines and compared it to 4T1sh/WT huCAIX line. I grew all the lines for 48 h in hypoxia and under low serum conditions (0.1% FBS) to mimic conditions present in the tumour. I quantified cell death with the TUNEL labelling kit (Roche) as described in Materials and Methods (Chapter 2, section 2.13).

Using this tool, I detected some basal amount of cell death in 4T1sh cells expressing WT huCAIX (4T1sh/WT huCAIX) and a 2-fold increase in cell death in all the cell lines expressing the intracellular (IC) domain mutants (4T1sh/S448A+Y449A, 4T1sh/T443A and 4T1sh/ΔIC) but not in the 4T1sh cells expressing the ΔPG mutant (Figure 4.10). The data obtained suggest that the IC domain of CAIX is required for regulating cell survival possibly through the modulation of CAIX enzymatic activity.
Figure 4.10. The intracellular domain of CAIX regulates cell survival. (A) The indicated cell lines were grown for 48 h in hypoxia and low serum conditions (0.1% FBS) prior to fixation and incubation with the TUNEL reaction. Red dots represent TUNEL-positive (apoptotic) cells. Grayscale images of nuclei are shown for reference of total number of cells per field. (B) Quantification of the percent of positive cells was performed in nine different fields and normalized to basal cell-death levels observed in 4T1sh/WT huCAIX (4T1sh/WT) cell line. Bars represent the mean ± s.e.m. of three independent experiments. *P<0.05 by Student’s t-test.
Some authors have observed that intracellular pH is able to regulate growth factor-mediated ribosomal protein S6 phosphorylation, protein synthesis and subsequent DNA synthesis. They also described that when the intracellular pH goes below 7.1-7.2 the rate at which cells enter the S phase of the cell cycle decreases, although cytoplasmic alkalization alone is not sufficient to trigger DNA synthesis (Chambard & Pouyssegur, 1986). Later experiments along this line have demonstrated that an intracellular pH below 7.2 impairs the activity of cyclin dependent kinase 1 (CDK1)/cyclin B1 and this delays $G_2/M$ entry. The mechanism proposed is through sustained inhibitory phosphorylation of CDK1 and decreased expression of cyclin B1 (Putney & Barber, 2003). Similar observations of the regulation of CDK1/cyclin B1 activity by intracellular pH have been made in Xenopus oocytes ( Sellier et al., 2006).

Since CAIX may have a role in maintaining a functional intracellular pH, I therefore proceeded to investigate if any of the mutants may impact the proliferation of 4T1 cells.

The reduction of tetrazolium salts in MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay has been widely used to study cell proliferation and viability. Metabolically active cells reduce the tetrazolium salt; therefore rapidly dividing cells express high rates of MTT reduction. This reduction is accomplished by dehydrogenase enzymes culminating in the generation of a purple precipitate, which can be quantified by spectrometry. Hence, this assay can also give information on cell viability, since dead cells will not be able to reduce the tetrazolium salt.

Cells expressing the different constructs were adhered for 12 h in normoxia, with no serum; to minimize proliferation. The cells were then switched to full media (DMEM + 10% FBS + 1x NE AA) and placed in hypoxia for a following 24, 48 or 72 h. In order to account for proliferation only, the readings at 24, 48 and 72 h were normalized to readings obtained after the first 12 h of seeding the cells in no serum conditions. When I performed MTT assays of 4T1 cells growing in hypoxia for 24, 48 and 72 h a difference between 4T1 cells expressing the mutant forms of human CAIX and 4T1sh/WT huCAIX cells was not observed ( Figure 4.11), suggesting that these mutations are not affecting the proliferation/viability of 4T1 cells. However, results from TUNEL experiments have
indicated that the IC domain of human CAIX is required for the regulation of cell survival. Notably, TUNEL assays were performed in hypoxia and under low serum conditions to simulate physiological conditions of the tumour cell, therefore providing data which is more physiologically relevant.

![Graph showing cell viability and proliferation of 4T1 cells expressing human CAIX mutants.](image)

Figure 4.11. **Cell viability and proliferation of 4T1 cells expressing human CAIX mutants.** MTT assays were performed in cells growing for 24, 48 and 72 h in hypoxia. Quadruplicate readings were normalized to those obtained after 12 h in normoxia and no serum (prior to switching to hypoxia and media supplemented with serum). The average of three independent experiments is shown.

One relatively simple way to study cell proliferation is by performing growth curves of the different cell lines. In this case, I seeded the same amount of cells and grew them for 72 h in hypoxia. I was careful that all the cultures were still sub-confluent at the end of the experiment. After 72 h the number of cells in each plate was obtained and the doubling time was calculated using the online software Doubling Time (Roth, 2006). In this particular set of experiments I included the 4T1shCAIX cell line since its proliferation capacity had not yet been characterized *in vitro*. I observed a small but statistically significant increase in the doubling time of 4T1shCAIX cells compared to 4T1shNS cells (around 10% increase, Figure 4.12). As for all of the cell lines expressing the human CAIX mutants; I did not observe any difference in their doubling time when
compared to 4T1sh/WT huCAIX cells (Figure 4.12), suggesting that only the complete absence of CAIX had an effect on cell proliferation.

**Figure 4.12. Growth curves of 4T1 cells expressing human CAIX mutants.** Growth curves were performed by seeding equal numbers of cells in triplicate and allowing them to grow for 72 hours (h) in hypoxia, after which the number of cells was obtained and doubling time was calculated. (A) The effect of CAIX knockdown was studied (4T1shCAIX versus 4T1shNS: non-silencing). (B and C) The effect of mutating CAIX is compared to 4T1 cells expressing WT human CAIX (WT huCAIX). Bars represent the mean ± s.e.m. of three independent experiments. ***P<0.001 by Student’s t-test.
All of the assays previously performed measure cell proliferation but do not provide information regarding the different stages of the cell cycle. Thus, I performed cell cycle analysis by flow cytometry of the different 4T1 cell lines cultured for 72 h in hypoxia and stained with propidium iodide (PI, 10 μg/ml). When I compared the 4T1shCAIX cells to the 4T1shNS cells I saw a small but significant increase (3% increase) in the number of cells at G2/M phase. This increase was accompanied by a decrease in the G1 population of cells (4% decrease, Figure 4.13). Interestingly, other studies have observed that the arrest in metaphase I of starfish oocytes is maintained both by the MAP kinase pathway and by blocking the increase in intracellular pH. When the intracellular pH is increased, the arrest is overcome even in the presence of active MAP kinase (Harada, Oita, & Chiba, 2003). Likewise, an alkaline intracellular pH was shown to suppress the mitotic arrest triggered by an activated DNA damage checkpoint (Liao, Hu, Arno, & Panaretou, 2006; Park, Lyons, Ohtsubo, & Song, 2000; Webb et al., 2011; Zhao et al., 2008). The phenotype observed here requires further investigation to determine if the absence of CAIX originates cell cycle arrest.

The data presented here is congruent with my previous observation of an increase in the doubling time of 4T1shCAIX cells. However, I did not observe any significant difference in the number of cells expressing the CAIX mutants at any of the cell cycle phases when compared to 4T1sh/WT huCAIX cells (Figure 4.13), therefore suggesting once more that only the complete absence of CAIX may impact cell proliferation.
Figure 4.13. Cell cycle analysis of 4T1 cells expressing human CAIX mutants. The indicated cell lines were grown for 72 h in hypoxia prior to staining with propidium iodide (PI) and to performing cell cycle analysis by flow cytometry. (A) The effect of CAIX knockdown was studied (4T1shCAIX versus 4T1shNS: non-silencing). (B and C) The effect of mutating CAIX is compared to 4T1 cells expressing WT human CAIX (WT huCAIX). Bars represent the mean ± s.e.m. of three independent experiments. **P<0.01 by Student's t-test.
4.5 Cell migration of human CAIX mutants

Some reports have proposed that CAIX may also be involved in cell migration (Ditte et al., 2011; Shin et al., 2011; Svastova et al., 2012). Hence, I decided to investigate the effect of the different CAIX mutations on cell migration. For this purpose, I grew cells for 72 h in hypoxia prior to seeding them in uncoated transwell chambers and I allowed the cells to migrate for 18 h in hypoxia. Serum containing recombinant mouse epidermal growth factor (rEGF; 100 ng/ml) was used as a chemoattractant. The number of cells which crossed the membrane for each of the human CAIX-mutant cell lines was compared to the number of 4T1sh/WT huCAIX cells that managed to cross the membrane. I observed a significant decrease in the migration capacities of 4T1sh/T443A and 4T1sh/ΔPG cell lines suggesting that both the IC and the PG-like domain of CAIX are involved in cell migration (Figure 4.14). However, the 4T1sh/ΔIC cell line only showed a non-significant reduction in its migration capacities making it difficult to conclude that the IC domain of CAIX is indeed involved in cell migration. Therefore, further experiments are required to determine the role of the IC domain in the regulation of the migratory phenotype.
Figure 4.14. Contribution of each domain of CAIX to the regulation of cell migration. Hypoxic cultures of the indicated cell lines were allowed to migrate through a polycarbonate membrane (transwell) for 18 h in hypoxia. 10% FBS containing 100 ng/ml rEGF was used as a chemoattractant. Left: Representative images of the number of migratory cells (nuclei) per field for each cell line are shown for reference. Right: The number of cells that crossed the membrane for each cell line was obtained and normalized to the number of 4T1sh/WT huCAIX (4T1sh/WT) cells that migrated across the membrane. Bars represent the mean ± s.e.m. of three independent experiments. *P<0.05, **P<0.01 by Student’s t-test.
4.6 Intracellular binding partners of human CAIX.

As previously stated there have been reports suggesting a role of the IC domain in regulating CAIX activity and invasion (Ditte et al., 2011; Hulikova et al., 2009; Shin et al., 2011). In the same way, many of the experiments which were performed have suggested important roles for the IC domain of CAIX in modulating cell survival (this Chapter) and invasion (Chapter 5). One possible mechanism by which the IC domain of CAIX can contribute to cell survival and invasion is through the regulation of its own Carbonic Anhydrase activity, which was proven here to be true. Another possible mechanism is through the interaction of CAIX with other proteins.

Therefore, I performed mass spectrometry of immunoaffinity purified WT huCAIX and ΔIC-huCAIX to differentiate proteins binding to the IC domain of human CAIX from the total amount of proteins binding to human CAIX. The mass spectrometry portion was achieved through collaboration with Dr. Leonard Foster’s laboratory at the Centre for High-Throughput Biology (CHiBi, UBC).

In order to confirm that I was able to cleanly immunoprecipitate (IP) both forms of human CAIX from 4T1sh/WT huCAIX and 4T1sh/ΔIC cells, I immunoprecipitated the protein from these cell lysates using a monoclonal mouse anti-human CAIX antibody (MAB2188, R&D Systems) coupled to CNBr beads and separated the samples onto SDS-PAGE gels prior to performing silver staining (Figure 4.15A) or to immunoblotting with a polyclonal goat anti-human CAIX antibody (AF2188, R&D Systems; Figure 4.15B). The immunoprecipitation of human CAIX from both cell lines was successful and I observed enrichment in the number of proteins that co-immunoprecipitated with WT huCAIX versus ΔIC-huCAIX (Figure 4.15C). The IgG isotype control utilized during immunoaffinity purification of human CAIX did not show non-specific binding of CAIX (Figure 4.15B). Three independent samples prepared in the same way as described above were sent for mass spectrometry analysis. One representative gel containing the WT huCAIX and the ΔIC-huCAIX immunoprecipitation samples and Commasie-stained prior to mass spectrometry analysis is shown in Figure 4.15C (image provided by Dr. Foster).
Figure 4.15. Immunoprecipitation of human CAIX from 4T1sh/WT huCAIX and 4T1sh/ΔIC cell lines. (A) Human CAIX (huCAIX) was immunoprecipitated (IP) from 4T1sh/WT huCAIX cell lines and the amount of enriched proteins was detected by silver staining. (B) The IP of huCAIX from 4T1sh/WT huCAIX and 4T1sh/ΔIC cell lines was confirmed by WB of huCAIX prior to sending the samples to the mass spectrometry facility. (C) Coomasie staining of enriched proteins in each fraction: IgG, IP-WT huCAIX and IP-ΔIC-huCAIX provided by the mass spectrometry facility (CHiBi-UBC).
The multiconsensus hits obtained by three sets of mass spectrometry data analysis, i.e. potential binding partners of human CAIX, are listed in Table 4.1 together with their sequence coverage (ΣCoverage, i.e. the amount of the protein sequence matched) and relative abundance ratios (i.e. amount of protein pulled down by anti-CAIX antibody versus IgG isotype control).
Table 4.1 List of proteins identified by mass spectrometry as binding partners of human CAIX.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>%Coverage</th>
<th>α-CAIX/IgG WT</th>
<th>α-CAIX/IgG ΔIC</th>
<th>α-CAIX WT/ΔIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin-9</td>
<td>58.67</td>
<td>13.69</td>
<td>2.8</td>
<td>4.88</td>
</tr>
<tr>
<td>Myosin-10</td>
<td>43.57</td>
<td>17.51</td>
<td>2.92</td>
<td>5.99</td>
</tr>
<tr>
<td>Isoform 2 of Myosin-14</td>
<td>23.49</td>
<td>15.21</td>
<td>3.26</td>
<td>4.66</td>
</tr>
<tr>
<td>Actin cytoplasmic 2</td>
<td>70.13</td>
<td>10.95</td>
<td>3.92</td>
<td>2.79</td>
</tr>
<tr>
<td>Myosin-11</td>
<td>10.63</td>
<td>15.01</td>
<td>2.98</td>
<td>5.03</td>
</tr>
<tr>
<td>Tubulin beta-5 chain</td>
<td>49.77</td>
<td>21.5</td>
<td>7.83</td>
<td>2.74</td>
</tr>
<tr>
<td>Tubulin beta-4B chain</td>
<td>46.29</td>
<td>19.61</td>
<td>8.09</td>
<td>2.42</td>
</tr>
<tr>
<td>Unconventional myosin-VI</td>
<td>15.64</td>
<td>14.04</td>
<td>2.55</td>
<td>5.5</td>
</tr>
<tr>
<td>Tubulin alpha-1A chain</td>
<td>38.36</td>
<td>47.28</td>
<td>13.3</td>
<td>3.55</td>
</tr>
<tr>
<td>Tubulin alpha-1C chain</td>
<td>38.53</td>
<td>47.28</td>
<td>13.3</td>
<td>3.55</td>
</tr>
<tr>
<td>Tubulin beta-3 chain</td>
<td>32.44</td>
<td>23.17</td>
<td>8.12</td>
<td>2.85</td>
</tr>
<tr>
<td>Actin, alpha cardiac muscle</td>
<td>31.3</td>
<td>13.80</td>
<td>3.89</td>
<td>3.54</td>
</tr>
<tr>
<td>Tubulin beta-4A chain</td>
<td>36.71</td>
<td>22.04</td>
<td>7.91</td>
<td>2.78</td>
</tr>
<tr>
<td>Tubulin alpha-4A chain</td>
<td>31.92</td>
<td>44.25</td>
<td>14.1</td>
<td>3.13</td>
</tr>
<tr>
<td>Tubulin beta 6</td>
<td>29.53</td>
<td>18.31</td>
<td>8.09</td>
<td>2.26</td>
</tr>
<tr>
<td>Unconventional myosin-lb</td>
<td>12.52</td>
<td>13.71</td>
<td>3.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>43.84</td>
<td>8.92</td>
<td>2.06</td>
<td>4.33</td>
</tr>
<tr>
<td>Deducator of cytokinesis protein</td>
<td>4.86</td>
<td>15.08</td>
<td>4.88</td>
<td>3.09</td>
</tr>
<tr>
<td>Unconventional myosin-lc</td>
<td>8.58</td>
<td>17.27</td>
<td>4.06</td>
<td>4.25</td>
</tr>
<tr>
<td>Leucine-rich repeat and calponin homology domain-containing protein 3</td>
<td>14.02</td>
<td>15.32</td>
<td>4.89</td>
<td>3.13</td>
</tr>
<tr>
<td>Coronin, actin binding protein 1C</td>
<td>15.82</td>
<td>15.25</td>
<td>6.31</td>
<td>2.41</td>
</tr>
<tr>
<td>Elongation factor 1-alpha 1</td>
<td>14.29</td>
<td>6.83</td>
<td>2.77</td>
<td>2.46</td>
</tr>
<tr>
<td>ADP/ATP translocase 1</td>
<td>16.52</td>
<td>10.8</td>
<td>3.85</td>
<td>2.80</td>
</tr>
<tr>
<td>Isoform 2 of PRKC apoptosis WT1 regulator protein</td>
<td>3.46</td>
<td>13.86</td>
<td>3.36</td>
<td>4.12</td>
</tr>
<tr>
<td>60S ribosomal protein L31</td>
<td>11.2</td>
<td>5.91</td>
<td>2.56</td>
<td>2.30</td>
</tr>
</tbody>
</table>
Several of the intracellular binding partners of human CAIX identified by this method are involved in cell motility, therefore suggesting a mechanism for CAIX-modulated invasion (Chapter 5).

Tubulins were the most common proteins found interacting with CAIX. Tubulins are major components of microtubules (The UniProt Consortium, 2014). The next set of enriched proteins was comprised of different isoforms of Myosins. Myosin-9, and -10 appear to play a role in cytokinesis and cell shape. During cell spreading, they also play an important role in cytoskeleton reorganization, focal contacts formation and lamellipodial retraction (The UniProt Consortium, 2014). Unconventional Myosin-6 is a motor protein that functions in cell migration (The UniProt Consortium, 2014). The Dedicator of cytokinesis (DOCK1) protein functions as a guanine nucleotide exchange factor (GEF), and activates Rac1 and Rac3 RHO small GTPases (The UniProt Consortium, 2014). Interestingly, RHO GTPases are involved in cell adhesion and migration (Kaibuchi et al., 1999; Miyamoto et al., 1995). Different isoforms of actin, which participate in cell motility, were also identified (The UniProt Consortium, 2014). The finding of Isoform 2 of PRKC apoptosis WT1 regulator protein (PAR4) as binding partner of CAIX could potentially help explain the cell death phenotype reported in this Chapter, since this is a pro-apoptotic protein capable of inducing apoptosis in cancer cells (The UniProt Consortium, 2014).

The finding of Myosin-10 as a putative CAIX-binding partner is interesting, since this protein has previously been involved in invasion through the targeting of integrins to filopodia structures (Arjonen et al., 2014). However since Myosin-10 is a 250 kDa protein, I encountered significant experimental difficulty when trying to co-immunoprecipitate Myosin-10 with CAIX and detect the first one by Western blot. Hence, further work is required to confirm this particular interaction.
4.7 Discussion

4.7.1 Expression and localization

The results presented in the first section of this Chapter demonstrate that the generation of stable cell lines which expressed the different forms of human CAIX, was successful. All the human CAIX mutants were properly expressed in 4T1 cells and were able to form dimers similar to WT huCAIX, as demonstrated by non-reducing gel electrophoresis followed by Western blot. Although these were not strict non-denaturing conditions (i.e. the gels contained SDS), the purpose of visualizing the dimeric form of the protein was achieved. This confirms previous observations; both the transmembrane and the catalytic domain of CAIX are required for proper dimer formation (Hilvo et al., 2008). In the same way, all the human CAIX mutants properly localized to the cell membrane. Both cell membrane localization and dimerization are essential requirements for the enzymatic activity of CAIX. Nevertheless, one concern was that only 44% of ΔIC-huCAIX localized to the cell membrane, while in other cell lines the proportion of WT or mutant-huCAIX at the cell membrane ranged from 65-87%. This could have implications on the way results from different assays are interpreted, since the phenotype observed could be the consequence of having less membrane-bound CAIX rather than having less active membrane-bound CAIX. This will be discussed further in the next sections.

An interesting finding is the presence of a double-band observed when blotting for human CAIX. This double-band has been observed in different cell lines such as MDA-MB-231, PK-8, BxPC-3 ((Li et al., 2011) and our own observations) but is not observed in mouse 4T1 cells for example (Figure 4.2). However, different antibodies were used to detect mouse and human CAIX respectively and the observation of a single band in mouse 4T1 cells might reflect the inability of the antibody used to recognize both forms of the protein. In fact, CAIX is usually described as a 54/58 kDa protein, making reference to the double band often observed. Experiments have been performed and demonstrated that the 54 kDa protein is not a 58 kDa form lacking the N-glycan modifications (Li, Wang, et al., 2011), however other protein post-translational
modifications have not been investigated. Interestingly, when I delete either the IC or the PG-like domain of human CAIX one of the two bands is lost. The reason for this is unclear but may potentially be due to misfolding or degradation of these constructs.

My observations indicate that less than half of the ΔIC-huCAIX that does not reach the cell membrane is localized at the ER, the localization of the remaining was not investigated. There is one report that indicates that ΔIC-huCAIX localizes completely to the ER (Hulikova et al., 2009), however I did not observe absolute ER co-localization of this protein in the 4T1sh/ΔIC cell line. It is possible that without the IC domain of human CAIX the protein may encounter problems folding properly and therefore is transported into the cytoplasm for degradation. If the IC domain of CAIX was required for proper insertion in the plasma membrane as suggested by Hulikova et al., (2009) then none of the ΔIC-huCAIX should be observed at the plasma membrane and this is not the case. Degradation of the ΔIC-huCAIX mutant can be tested in future experiments by utilizing inhibitors of protein degradation during a time-course experiment followed by immunoblot of total cell extracts. This would clarify if the protein is being degraded by the degradation pathway that is blocked by the inhibitor. Cells expressing WT-huCAIX would be utilized to account for basal amounts of degradation.

Interestingly, during the flow cytometry assays I observed very high amounts of cell death, as indicated by the positive PI staining. One possibility for this is that extensive handling of the cells during the live staining procedure may have damaged them. 4T1 cells require long harvesting periods in order to obtain a single cell suspension; this can be one of the main causes of observing high levels of basal cell death. The positive PI signal detected may also represent a false positive, since it has been observed that PI can stain cytoplasmic RNA and lead to high false positive rates (up to 40%; (Rieger, Nelson, Konowalchuk, & Barreda, 2011)); especially since in these experiments cells were not treated with RNase A to decrease cytoplasmic RNA. Furthermore, MTT assays performed under similar conditions did not suggest differences in cell viability among the mutants, suggesting that handling and harvesting was the main cause of the high levels of basal cell death observed.
4.7.2 Activity

The activity assay that I implemented in the laboratory, which was originally developed by Wilbur and Anderson in 1948, provided important quantitative data of human CAIX activity. Through this assay I was able to demonstrate that mutations of particular residues within the IC domain of CAIX (T443A, S448A+Y449A) or the complete deletion of it (ΔIC) can cause between 25% and 35% reduction of CAIX activity. This reduction of CAIX activity was observed even after utilizing almost twice as many 4T1sh/ΔIC cells in order to compensate for the lower amount of membrane-bound CAIX present in this particular cell line. On the other hand, the FITC-labeled Carbonic Anhydrase Inhibitor (FITC-CAI), which has previously been shown to only bind active CAIX (Dubois et al., 2007; Lou et al., 2011) only provided qualitative data and it seems that any partial impairment of CAIX activity, as the one observed with 4T1sh/T443A, 4T1sh/S448A+Y449A and 4T1sh/ΔIC cell lines blocked the ability of the inhibitor to recognize and bind human CAIX, possibly because these mutations affect the conformation of the active site. Therefore, care should be taken when interpreting experiments that utilize this inhibitor solely because it could potentially lead to over/under-estimation of the amount of CAIX activity present in the cell lines under study. There is one report that states that mutating the T443 residue to alanine completely abolishes CAIX function. They based this conclusion on results from utilizing FITC-CAI solely (Ditte et al., 2011) and I obtained similar results when I incubated the 4T1sh/T443A cell line with the FITC-CAI inhibitor. However, the T443A-huCAIX mutant was shown to be around 75% active when I directly measured the ability of T443A-expressing cells to convert CO₂ to carbonic acid using the In-cell activity assay. Both, the In-cell and in vitro activity assays reported in this thesis, measure Carbonic Anhydrase activity directly by utilizing the physiological substrate of CAIX (CO₂) as opposed to the indirect measurement provided by FITC-CAI. The reason for the discrepancy between assays is unclear but emphasizes the need for multiple methods to make biochemical measurements.
Another report that studied different residues within the IC domain of CAIX (mainly arginines and lysines) found an impairment of CAIX-mediated extracellular pH acidification upon mutation of these residues (Hulikova et al., 2009) suggesting that the modifications performed within the intracellular domain of CAIX might be altering the conformation of extracellular catalytic domain, therefore impairing its enzymatic activity.

The assay utilizing the FITC-CAI and the *In-cell* Carbonic Anhydrase activity assay proved that the PG-like domain had no role in regulating CAIX activity. However, other reports that studied the kinetics of different forms of recombinant CAIX (catalytic versus extracellular domain) in a stopped-flow assay, suggest that the PG-like domain is indeed implicated in the regulation of CAIX activity (Innocenti et al., 2009). It is noteworthy to emphasize that experiments with recombinant CAIX utilized a form of the enzyme lacking the intracellular and transmembrane domains, and that this could account for the difference in results obtained when utilizing full-length CAIX expressed in cells.

A stopped-flow assay is similar to the *in vitro* Carbonic Anhydrase activity assay described in this Chapter and in Chapter 3, since it consists of a rapidly mixing device used to study fast reactions (McNaught & Wilkinson, 1997). Results from the *in vitro* Carbonic Anhydrase activity assay described here and from experiments utilizing the stopped-flow assay (Innocenti et al., 2009) appear to suggest that the PG-like domain is also involved in the regulation of CAIX activity. However, results from the Carbonic Anhydrase activity assay where whole cells are utilized indicate that the PG-like domain had no role in mediating CAIX activity. This highlights the importance of always performing experiments under physiological conditions, since very different results might be obtained with purified recombinant proteins than when utilizing cells and even more *in vivo*. The difference in results obtained from the *in vitro* and *In-cell* Carbonic Anhydrase activity assays likely lies in the fact that CAIX is present in the cellular context in the latter assay. It is clear however from this and other reports that
the catalytic domain of CAIX provides the bulk of Carbonic Anhydrase activity and that the IC domain of CAIX regulates this function.

There are different ways in which the IC domain of CAIX may regulate its Carbonic Anhydrase activity. Recent reports have proven that the T443 and Y449 residues undergo phosphorylation; the phosphorylation of T443 is mediated by cAMP-PKA and results in increased CAIX activity, while the phosphorylation of Y449 occurs upon binding of EGF to its receptor, however the kinase responsible for this modification has not been identified (Ditte et al., 2011; Dorai et al.; 2005). The potential phosphorylation of the S448 residue has not been investigated. Another potential mechanism for the regulation of CAIX enzymatic activity may be through interaction with other proteins. This was one of the main reasons for investigating potential binding partners of CAIX by mass spectrometry. The results obtained from immunoaffinity purification of CAIX followed by mass spectrometry need to be investigated further in order to determine if one or more of the intracellular binding partners of CAIX identified play a role in regulating CAIX function and will be discussed further in section 4.7.5.

**4.7.3 Cell survival and proliferation**

Results from TUNEL assays support the hypothesis that CAIX can indirectly modulate cell survival through the regulation of intracellular pH, and confirm previous observations of a role of CAIX in tumour cell survival (Lou et al., 2011). I observed twice as much cell death in all the cell lines expressing some form of IC domain mutation of human CAIX, which are also the cell lines with some degree of impaired CAIX activity. However the ΔPG-huCAIX mutant is still fully active and I did not observe any effect on cell survival. To my knowledge, this is the first report suggesting a role of the IC domain and particularly amino acid residues T443, S448 and Y449 of CAIX in regulating cell survival.

Although I only measured CAIX enzymatic activity and not changes in intracellular pH directly, it is expected that a less active enzyme generates less $\text{HCO}_3^-$ and therefore the neutralization of intracellular pH would be impaired. However, a direct measurement of
intracellular pH is required to confirm this hypothesis. The intracellular pH can be measured in intact cells by utilizing intracellular pH fluorescent probes such as BCECF and SNARFs or intracellular microelectrodes. However, this type of technology is not available in our laboratory.

The results from TUNEL assays do not seem to be biased by the fact that only half of ΔIC-huCAIX is present at the cell membrane (as indicated by flow cytometry analysis), since I observed similar amounts of cell death when only certain residues within the IC domain of human CAIX are mutated (T443, S448 and Y449) suggesting that the numbers obtained represent real values.

The basal amounts of cell death observed by TUNEL assays, which measure early apoptosis, were not as high as the ones observed by PI staining followed by flow cytometry analysis. PI dye can only penetrate the membrane of late apoptotic and necrotic cells since the integrity of the cell and nuclear membrane of these cells is compromised. These observations suggest that the increase in basal cell death observed by flow cytometry might have indeed been the result from a combination of cell harvesting and handling conditions and cytoplasmic RNA staining as mentioned before.

The main advantage of TUNEL assays is that one can assess apoptotic cell death on a cell-per-cell basis. Importantly, MTT experiments were not performed under the same conditions as TUNEL assays (i.e. low serum) and this might explain the discrepancy in the results. Furthermore, MTT assays do not strictly measure number of live cells but number of active mitochondria. Further investigation of the cell death phenotype observed with human CAIX mutants may be performed by utilizing other assays such as Annexin V/PI co-staining followed by flow cytometry analysis, which is useful for differentiating between viable, apoptotic and necrotic cells (Rieger et al., 2011). Blotting for PARP, caspase-3 and caspase-7 may shed more light on the type of cell death that is undergoing in these lines. Preliminary experiments (data not shown) as well as a previous report from the laboratory suggest that 4T1 cells do not undergo caspase-dependent cell death in the absence of CAIX (Lou et al., 2011), therefore investigation of other types of cell death that are caspase-independent should be pursued, especially
since single- and double-stranded DNA breaks like the ones detected by TUNEL assays, have been observed in other types of cell death such as necrosis (Didenko, Ngo, & Baskin, 2003).

The results shown here seem to indicate that only the complete absence of CAIX can impact cell proliferation. The mutations within the IC domain of human CAIX had no effect, suggesting that more than a small reduction in CAIX activity is needed. Consequently, one important future experiment would be to determine if the complete inhibition of CAIX activity with potent small molecules such as U-104 has any effect on cell proliferation. My results confirm previous observations done in MDA-MB-231 and MDA-MB-468 breast cancer cells and LS174T colon carcinoma cells where depletion of CAIX resulted in diminished cell proliferation (Chiche, Ilc, et al., 2010; Robertson et al., 2004) and support the hypothesis that the HCO$_3^-$ generated by CAIX-mediated catalysis might be used as building blocks for pyrimidine residues, favouring cell proliferation (De Simone & Supuran, 2010). In the absence of CAIX there is a decrease in the amount of bicarbonate available as building blocks, which in turn slows down the proliferation rate. One report that supports this hypothesis described decreased growth rates of cells growing in bicarbonate-free media and treated with Carbonic Anhydrase sulphonamide inhibitors, a phenotype that was reversed upon replenishing the media with nucleotide precursors (Chegwidden, Dodgson, & Spencer, 2000).

4.7.4 Migration

The PG-like domain of CAIX appears to regulate cell migration, however since the ΔPG-huCAIX mutant is still as active as WT huCAIX, the mechanism involved is likely different. Proteoglycans are major components of the extracellular matrix (ECM) where they form complexes with other PGs or with water, cations and/or collagen (Hammond, Khurana, Shridhar, & Dredge, 2014). In addition, membrane-bound proteoglycans interact weakly (through their sugar moieties) with components of the ECM including hyaluronic acid, fibronectin, collagen or elastin, enhancing adhesion in cooperation with integrins (Couchman, 2010; Theocharis, Skandalis, Tzanakakis, & Karamanos, 2010). Therefore, the PG-like domain of CAIX may be regulating cell migration through
interactions with the ECM. To my knowledge, this is the first report suggesting a role of the PG-like domain of CAIX in cell migration. Most of the previous studies have only concentrated on the effect of overexpressing CAIX on cell migration, and have observed that upon stimulation with HGF, CAIX re-localizes to lamellipodia structures where it co-localizes with the migratory machinery (AE2 and NBC) (Svastova et al., 2012).

My results seem to differ from those of Ditte et al. (2011) since they found that the phosphorylation of residue T443 with the simultaneous dephosphorylation of S448 is required for proper cell migration. I observed that the phosphorylation of T443 is the only apparent requirement for proper cell migration but the simultaneous dephosphorylation of S448 residue was not investigated. The role of the IC domain of CAIX in cell migration requires further investigation. For example the CAIX constructs (WT huCAIX, ΔIC-CAIX and the IC domain point mutants) could be expressed in other cancer cell lines, especially cell lines that display different migratory abilities and cell migration on different matrices could be assessed by live cell imaging. Alternative experiments could be aimed to investigate the activation status of components of the migratory machinery such as Src kinase and RHO, RAC and CDC42 GTPases.

4.7.5 Identification of binding proteins by immunoaffinity purification followed by mass spectrometry.

As mentioned throughout this Chapter, another way to explain many of the phenotypes observed with CAIX mutants is through the interaction of CAIX with other proteins, which may be affected or lost after mutating CAIX. However, only a few CAIX interacting partners have been identified and thoroughly studied including β-catenin and DKK-1 (Kim et al., 2012; Švastová et al., 2003), and only recently a report was published where components of the nuclear transport machinery were identified as binding partners of CAIX by mass spectrometry (MS) of CAIX immunoaffinity-purified from hypoxic HEK293 cells (Buanne et al., 2013).

CAIX-binding proteins detected by mass spectrometry and reported here do not overlap with results from the aforementioned group, but some of them could help understand
the survival and cell invasion phenotypes described here and in Chapter 5. In fact, cytoskeletal proteins could potentially play a role in modulating cell invasion. However, it is quite common to detect cytoskeletal proteins as binding partners of the batch protein by mass spectrometry, which is why it is important to confirm if they represent authentic results (Gingras, Gstaiger, Raught, & Aebersold, 2007). Additional experiments are needed to understand the significance of the interaction of CAIX with each of the proteins identified.

Initial validation of the results obtained by MS would involve co-immunoprecipitation of human CAIX and each of the potential binding partners from parental and hypoxic cell lysates as well as pull-down assays utilizing purified proteins in order to demonstrate a direct interaction. However, the latter approach will require the purification of full-length recombinant CAIX, which is often challenging for membrane proteins. Other assays such as the proximity ligation assay and Förster Resonance Energy Transfer (FRET), demonstrate interaction of two proteins in situ.

Validating the interaction of CAIX with one or more Myosin isoforms would represent an interesting finding since Myosins are involved in cell spreading and migration processes and recently Myosin-10 has been involved in the targeting of integrins to filopodia (Arjonen et al., 2014). Myosin-10 was also found to localize to invadopodia structures and to be important for ECM degradation at the invadopodia and for cell invasion, although the exact mechanism was not investigated (Cao et al., 2014). Since Myosin-10 was previously involved in protein targeting to migratory/invasive structures it is tempting to speculate that Myosin-10 might be mediating transport of CAIX to the invadopodia structures described in Chapter 5.

Preliminary assays were performed in an attempt to confirm the interaction of CAIX with Myosin-10. However since Myosin-10 is a 250 kDa protein, I encountered significant experimental difficulty when trying to detect Myosin-10 by Western blot. Further work and special electrophoresis and transfer conditions will be needed to achieve this confirmation. For example, the percent of polyacrylamide in the gel may
need to be adjusted and the transfer buffer may require SDS while methanol may need to be excluded.

In the same way, validating the interaction of CAIX with Isoform 2 of PRKC apoptosis WT1 regulator protein and investigating the implications of this interaction might be useful in broadening our understanding of the cell death phenotype observed with the intracellular domain-CAIX mutants.

Interestingly, MMP-14, a protein identified as binding partner of CAIX in Chapter 5 by co-immunoprecipitation assays, was not present in the list of proteins identified by MS. However, both CAIX and MMP-14 are membrane-bound proteins and it is recognized that the application of MS to study intact membrane protein complexes has remained a challenge. Therefore, the identification of further binding-partners of CAIX might have also been compromised during these studies. Membrane proteins are usually underrepresented in MS approaches due to their poor solubility and low abundance. Signals from abundant proteins like cytoskeletal, ribosomal and metabolic can obscure the ability to detect the membrane proteins of interest. In addition to this, there is also low recovery of hydrophobic peptides of membrane proteins after in gel digestion. Some approaches to address these problems include membrane protein enrichment, different solubilization treatments and extra digestion steps (Barrera, Di Bartolo, Booth, & Robinson, 2008).

In brief, the work described in this Chapter revealed that the IC domain of CAIX modulates its Carbonic Anhydrase activity and this seems to translate into the regulation of cell survival. The PG-like domain of CAIX did not appear to regulate CAIX activity nor cell survival but was found to be involved in the cell migration process.
Chapter 5. Hypoxia-induced Carbonic Anhydrase IX Regulates Invasion and Metastasis by Interacting with and Activating MMP-14 within Invadopodia

The results presented in this Chapter and Chapter 4 have been submitted for publication.

5.1 Synopsis

Tumour hypoxia promotes tumour cell invasion and metastasis. However, the molecular mechanism of hypoxia-induced invasion is poorly understood. Here we demonstrate that Carbonic Anhydrase IX (CAIX), a hypoxia-induced cell surface protein involved in pH regulation, is required for tumour cell invasion and metastasis. CAIX co-localizes with the membrane-bound matrix metalloprotease, MMP-14, within cortactin and Tks-5 positive invadopodia, and co-immunoprecipitation experiments demonstrate an interaction between hypoxia-induced CAIX and MMP-14 in several types of tumour cells. Depletion of CAIX by shRNA or pharmacologic inhibition of its catalytic activity results in the inhibition of invasion and degradation of type I collagen, a substrate of MMP-14. The intracellular domain of CAIX is required for interaction with MMP-14, and for stimulation of MMP-14-mediated matrix degradation and invasion. We have identified CAIX as a novel, hypoxia-induced component of invadopodia and we demonstrate that CAIX regulates MMP-14-mediated matrix degradation and invasion.

5.2 Introduction

Tumour invasion contributes significantly to the formation of metastases, which are responsible for tumour-associated mortality (Nguyen, Bos, & Massagué, 2009;
Valastyan & Weinberg, 2011; Vanharanta & Massagué, 2013). The mechanisms involved in tumour cell invasion are complex and are modulated by many genetic and microenvironmental factors. Tumour hypoxia is a significant component of most solid tumours, and it is known to promote epithelial-mesenchymal transition (EMT) (Jung, Fattet, & Yang, 2014), as well as tumour cell invasion (T. Wang et al., 2014).

The inhibition of oxidative phosphorylation in low oxygen tension is accompanied by increased glycolysis and accumulation of lactic acid, leading to acidic extracellular pH, which has been shown to activate proteases and stimulate local matrix degradation and tissue remodelling (Chambers & Matrisian, 1997; Estrella et al., 2013; Kessenbrock et al., 2010). Tumour cells adapt to hypoxia and acidosis by up-regulating NHE-1 and CAIX, a hypoxia-induced cell surface protein that regulates pH, allowing for the survival of tumour cells within hypoxic niches in solid tumours (McDonald et al., 2012; Neri & Supuran, 2011; S. K. Parks, Chiche, & Pouysségur, 2013; Supuran, 2008). Recent reports have demonstrated a critical role of CAIX in tumour growth and metastasis (Lou et al., 2011; S. K. Parks et al., 2013), and while CAIX has been suggested to play a role in tumour invasion (Jaromir Pastorek & Pastorekova, 2014), the molecular basis of CAIX-mediated invasion is poorly understood.

Tumour cell invasion is facilitated by the formation of invadopodia, which are actin-rich protrusions that are formed by metastatic tumour cells to degrade the extracellular matrix and facilitate the invasive stages of metastasis (Beaty & Condeelis, 2014; Gligorijevic et al., 2012; Yamaguchi, 2012). Invadopodia are formed initially as structures enriched in actin regulators such as cortactin, N-WASP, ARP2/3, coflin and Tks-5 (Oser et al., 2009; Yamaguchi et al., 2005). The maturation of invadopodia apparently involves talin-mediated recruitment of NHE-1, which drives coflin-independent actin polymerization; as well as recruitment/secretion of matrix metalloproteases such as MMP-2, MMP-9 and MMP-14 required for ECM breakdown (Beaty et al., 2014). MMP-14- and Tks-5-positive invadopodia have been shown to be required for tumour cell extravasation during metastasis (Leong et al., 2014).
Although tumour hypoxia stimulates tumour cell invasion, the role of hypoxia in invadopodia formation and maturation are poorly understood and only explored recently (Gould & Courtneidge, 2014; Lucien, Brochu-Gaudreau, Arsenault, Harper, & Dubois, 2011).

Here we demonstrate that CAIX function is critical for hypoxia-mediated tumour cell invasion and metastasis. We find that CAIX stabilizes MMP-14, and interacts with it through the intracellular domain. In addition, CAIX co-localizes with MMP-14 within cortactin- and Tks-5-positive, mature invadopodia. Furthermore, CAIX catalytic activity, which converts carbon dioxide to bicarbonate and hydrogen ions resulting in local acidification at the extracellular face of the plasma membrane, is required for MMP-14-mediated activity as determined by type-I collagen degradation and invasion through this matrix.

5.3 Materials and methods

5.3.1 Cell culture and antibodies

The 4T1 murine breast cancer cell line, MDA-MB-231 human breast cancer cell line and the BxPC-3 human pancreatic cell line were obtained from the American Type Culture Collection (ATCC). The MDA-MB-231 LM2-4luc+ (referred here as MDA-231 LM2-4) cells were provided by Dr. Robert Kerbel (University of Toronto, Canada). PK-8 cells were provided by Drs. Donald Yapp and Sylvia Ng (BC Cancer Research Centre, Vancouver, Canada). The MDA-MB-231 cell line was maintained in DMEM (Gibco® Life Technologies, Burlington, Ontario) supplemented with 10% fetal bovine serum (FBS; Gibco® Life Technologies, Burlington, Ontario) while the 4T1 cell line also required the addition of non-essential amino acids (1x NE AA). The PK-8, BxPC-3 an MDA-231 LM2-4luc+ cell lines were kept in RPMI supplemented with 10% FBS. For culture in normoxia, cells were incubated in a humidified incubator at 37°C, 5% CO₂. For culture in hypoxia, cells were incubated at 37°C in an atmosphere of 1% O₂, 5% CO₂, 94% N₂ in a humidified incubator inside a sealed anaerobic workstation.
The CAIX antibodies (goat anti-mouse CAIX AF2344, goat anti anti-human CAIX AF2188, monoclonal mouse anti-human CAIX MAB2188) and the mouse IgG2a (MAB003) isotype control were obtained from R&D Systems (Minneapolis, MN). The mouse monoclonal anti-cortactin (ab33333), mouse monoclonal function-blocking anti-MMP-14 (ab78738) and rabbit polyclonal anti-MMP-14 (ab51074) were obtained from Abcam (Cambridge, MA). The rabbit anti-Tks-5 (aka FISH; sc-30122) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse anti-β-actin (A5441) was obtained from Sigma-Aldrich Corporation (St. Louis, MO).

**5.3.2 Cloning of CAIX truncation and point mutants**

Full length WT human CA9 cDNA cloned into the pTREX-A (pcDNA4/TO/myc-His A; Life Technologies, Gaithersburg, MD) plasmid vector was a generous gift from Dr. Jacques Pouysségur (University of Nice, France). The construct encoding for the ΔIC mutant was generated by introduction of a stop codon after amino acid (aa) 433 by site-directed mutagenesis. The construct encoding for the ΔPG mutant was generated from two PCR products by amplification of the sequences corresponding to amino acids 1-37 and 130-459 using 2 sets of primers, followed by ligation and cloning of the product into the pcDNA4 vector. The CAIX intracellular tail single and double point mutants (T443A, S448A, Y449A and S448A+Y449A) were generated by site-directed mutagenesis. Plasmid DNAs were purified using a DNA purification kit (Qiagen Inc., Venlo, Netherlands) and constructs were sequenced to verify fidelity prior to transfection into cells.

**5.3.3 Generation of stable cell lines**

For stable depletion of human and mouse CAIX in MDA-MB-231 LM2-4Luc, BxPC-3, PK-8 and 4T1 cells, shRNAmir constructs (Open Biosystems, Huntsville, AL) were transduced into cells using lentivirus as per manufacturer’s instructions. Transduced cells were selected using puromycin. Stable 4T1shCAIX clones were derived by fluorescent activated cell sorting (FACS) of GFP-positive cells (marker of mouse CAIX depletion). Re-introduction of WT human (hu) CAIX or of the different constructs was
accomplished by plasmid transfection into 4T1shCAIX cells using Lipofectamine 2000 as per manufacturer’s instructions. Zeocin was used for selection of successfully transfected cells. For enrichment of the CAIX-positive population FACS of double GFP-positive and CAIX-positive cells was performed. 20,000-100,000 cells were sorted by the Flow Cytometry Core Facility (FCCF) located in the Terry Fox Laboratory of the BC Cancer Research Centre (BCCRC) and expanded in selection media.

5.3.4 Analysis of protein expression

Cells were grown in normoxia or hypoxia for 72 h to induce CAIX expression, followed by lysis at 4°C in RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.5% NaDoc) or NP-40 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1% NP-40) buffer. For treatment with CAIX inhibitor U-104, a final concentration of 50 μM was added to the media for the last 24 h in hypoxia. Equal amounts of lysate (15-30 μg) were loaded on SDS-PAGE gels. Western blots were performed as described previously (Lou et al., 2008) using goat anti-mouse CAIX (1:500), goat anti-human CAIX (1:500), rabbit anti-MMP-14 (1:500) and mouse anti-β-actin (1:10,000) primary antibodies.

5.3.5 Invasion assays

Cells were cultured for 72 h in hypoxia to induce CAIX expression, followed by serum-starvation for 24 h in hypoxia. Cells were then trypsinized, counted and 5 x 10^4 or 1 x 10^5 cells were seeded onto either 8 μm transwells coated with Matrigel™ (BD Biosciences; Mississauga, ON) or 3 μm transwells (Corning Inc., Corning, NY) coated with type I rat tail collagen (Life Technologies, Gaithersburg, MD; 2.25 mg/ml collagen; pH adjusted with NaOH and acetic acid as per manufacturer’s instructions). Media supplemented with 10% FBS and 100 ng/ml recombinant mouse EGF (Sigma-Aldrich Corporation, St. Louis, MO) was used as a chemoattractant. For treatment with CAIX inhibitor U-104 (50 μM) or function-blocking anti-MMP-14 antibody (20 μg/ml), cells were pre-incubated for 30 min at RT with rotation, followed immediately by transfer onto the transwell filters. Cells were allowed to invade for 24 h in hypoxia after which non-invasive cells were removed from the top chamber with a cotton swab. Membranes
were fixed for 10 min with ice cold MeOH (-20°C) and the nuclei of invasive cells were stained with Hoescht 33342 (1:5,000; Sigma-Aldrich Corporation, St. Louis, MO) for 10 min at RT. The number of invasive cells was obtained from at least 5 different fields at low magnification (10x) by using a Zeiss Observer.Z1 microscope and a CCD camera.

5.3.6 Mouse tumour models

All animal studies and procedures were performed in accordance with protocols approved by the Institution Animal Care Committee at the BC Cancer Research Centre and University of British Columbia (Vancouver, BC, Canada).

*Syngeneic orthotopic tumours and spontaneous metastasis.* 4T1shNS, 4T1shCAIX and 4T1sh/WT huCAIX cells (1 x 10^6 cells/animal) were implanted orthotopically into the fourth mammary fat pad of 7-9 week-old female BALB/c mice as described previously (Ahlskog, Dumelin, Trüssel, Mårlind, & Neri, 2009). At day 26 post-tumour cell inoculation the mice were euthanized by CO₂ asphyxiation, the lungs were resected and cells were dissociated by enzymatic digestion as described previously (Hamilton et al., 2014). Erythrocytes were lysed using AKC buffer (155 mM NH₄Cl, 10 mM KHCO₃ and 240 µM EDTA), cells were enumerated and aliquots of 2.5 x 10^3 to 1 x 10^6 cells were plated in media containing 60 µM 6-thioguanine (Sigma-Aldrich Corporation, St. Louis, MO) to allow the exclusive growth of 4T1 cells. Cells were incubated for 10 days (37°C, 5% CO₂) before staining colonies with malachite green for quantification. The total number of clonogenic tumour cells in the lungs was calculated by multiplying the proportion of colony-forming tumour cells by the total number of cells recovered from the lungs.

*Experimental metastasis assays.* 4T1shNS, 4T1shCAIX, 4T1sh/WT huCAIX, 4T1sh/ΔIC-CAIX and 4T1sh/ΔPG-CAIX cell lines were injected directly into the tail vein of 7-9 week-old female BALB/c mice (5 x 10^5 cells/animal). Mice were euthanized at 16 days post-injection (experimental end point for 4T1shNS group) and lungs were harvested for clonogenic assays as described above.
5.3.7 Gelatin zymography

Cells were cultured for 72 h in hypoxia to induce CAIX expression, passaged and plated at a density sufficient to achieve 70% confluency with a further 18 h of culture in hypoxia. Cells were then serum starved for another 24 h, and media was subsequently collected and concentrated using a 30 kDa cut-off centrifugal filter unit. Equal amounts of protein (10 μg) were diluted in sample buffer, loaded onto a gelatin gel (0.15% gelatin, 8% polyacrylamide, 0.4% SDS) and separated at 110V for 3 h. Gels were washed 3x with Triton X-100 buffer and activated for 16 – 48 h in activation buffer (10 mM Tris-HCl, pH 7.5; 1.25% Triton X-100 (v/v); 5 mM CaCl₂; 10 μM ZnCl₂). Finally, gels were washed extensively in dH₂O, stained with Coomasie blue and de-stained until bands were optimally visible. Densitometry was performed with Image J.

5.3.8 Collagen degradation

12-well plates were coated with type I rat tail collagen (Life Technologies, Carlsbad, CA) for 30 min at 37°C, 5% CO₂ in a humidified incubator as described for invasion assays. Cells that were cultured in hypoxia for 72 h and serum starved for 24 h were seeded onto collagen-coated wells (1 x 10⁴ cells/well) and allowed to degrade the collagen for 24 h in hypoxia. Cells were then trypsinized and washed away. The collagen was fixed with 4% PFA for 30 min, (RT) and stained with Coomasie blue for 2 h. Six random fields were imaged at low magnification (5x) by using a Zeiss Axiovert 40 CFL microscope and a CCD camera.

5.3.9 Invadopodia formation and imaging

Glass coverslips were coated with Oregon Green 488-gelatin (Life Technologies, Carlsbad, CA) or unlabelled gelatin as described previously (Artym, Yamada, & Mueller, 2009). Briefly, coverslips were coated with 50 μg/ml poly-L-lysine followed by 0.5% gluteraldehyde, and inverted on an 80 μl drop of gelatin for 10 min. Coated coverslips were then incubated with 5 mg/ml NaBH₄ and washed extensively with PBS. 5 x 10⁴ cells were seeded on the gelatin-coated coverslips and incubated for 6 h in hypoxia, after which they were fixed with ice cold MeOH for 10 min. For treatment with CAIX
inhibitor U-104, cell suspensions were pre-incubated in the presence of the inhibitor (50 μM) for 30 min (RT) on a rotator prior to being seeded on gelatin in the presence of U-104. Immunofluorescence staining was performed by incubating with the primary antibodies overnight at 4°C (goat anti-mouse CAIX (1:100), goat anti-human CAIX (1:100), mouse anti-MMP-14 (1:500), rabbit anti-Tks-5 (1:50) and mouse anti-cortactin (1:400)). The coverslips were then incubated with AlexaFluor 488/594/647 conjugated secondary antibodies (1:400; Life Technologies, Gaithersburg, MD) for 1 h, at RT. Cells were imaged with a Leica LSM780 confocal microscope using a 63x objective. Gelatin and CAIX were excited using the 488 nm line and the emission collected through a 500-550 nm band-pass filter. Cortactin and MMP-14 were excited using the 543 nm line and the emission collected through a 560 nm long-pass filter. CAIX and Tks-5 were excited using the 633 nm line and the resulting fluorescent light was observed using a 650 nm long-pass filter. Images were pseudo-coloured with the Carl Zeiss microscope and imaging software Zen. Images were processed using Photoshop.

5.3.10 Co-immunoprecipitation

Cells were cultured in normoxia or hypoxia for at least 72 h and were lysed at 4°C in NP-40 buffer containing protease inhibitor cocktail, 1 mM Na₃VO₄, 2 mM NaF and 1 mM PMSF. 1–3 mg of protein was immunoprecipitated at 4°C overnight using either 30 μg of monoclonal anti-human CAIX antibody or 15 μg of monoclonal anti-MMP-14 antibody covalently linked to CNBr-activated Sepharose 4B (GE Healthcare, Cleverland, OH) as described previously (Chafe & Mangroo, 2010). The resin was extensively washed with NP-40 buffer, resuspended in sample buffer and boiled at 100°C for 10 min under non-reducing conditions. The proteins in the supernatant were separated from the resin by centrifugation using a polyacrylamide column for 1 min, at 13,000 rpm. 2-mercaptoethanol was added to the supernatant and eluates were boiled again. All samples were loaded on SDS-PAGE gels and Western blots were performed as described above.
5.3.11 Statistical analysis

Statistical analysis was performed using the Data Analysis ToolPack Excel software. $P$ values were calculated using Student’s t-test; data with $P$ values < 0.05 were considered significant.

5.4 Results

5.4.1 CAIX is required for breast and pancreatic tumour cell invasion and metastasis

To investigate the role of CAIX in invasion, we initially utilized the 4T1 murine breast cancer model (Lou et al., 2008, 2011) in which we have demonstrated a critical role of CAIX in metastasis formation (Lou et al., 2011). The role of CAIX in tumour cell invasion was evaluated in 4T1 cells in which CAIX expression was stably knocked-down by shRNA expression (4T1shCAIX; see Chapter 4). As shown in Figure 5.1A, silencing of endogenous murine CAIX expression results in a significant decrease in 4T1 cell invasion through Matrigel™, which is rescued by expression of WT human CAIX (resistant to murine CAIX-specific shRNA). The effects on invasion in vitro, are mirrored by lung metastases formation from 4T1 tumours grown orthotopically in the mammary glands of Balb/c mice (Figure 5.1B). Analysis of Matrigel™ invasion of human breast (MDA-231 LM2-4) and pancreatic (BxPC-3 and PK-8) tumour cells also demonstrates significant inhibition of invasion by either shRNA-mediated silencing of CAIX (Figure 5.1C), or inhibition of its activity with a highly selective CAIX small molecule inhibitor, U-104 (characterized in Chapter 3) (Lou et al., 2011; Pacchiano et al., 2011) (Figure 5.1D). Under the conditions of these assays, no significant cell death was observed (data not shown).
Figure 5.1. CAIX is required for cell invasion and metastasis. (A) The invasive abilities of 4T1 cells expressing shNS (non-silencing), shCAIX or rescued with WT human CAIX (sh/WT huCAIX) were assessed using Matrigel™-coated transwell chambers. (B) Same cell lines as in A were injected orthotopically in BALB/c mice. The number of metastatic cells in lungs was obtained through a colony formation assay. (C) Invasive abilities of BxPC-3 and LM2-4 cells expressing shNS or shCAIX or (D) 4T1sh/WT huCAIX, LM2-4 and PK-8 cells incubated with CAIX inhibitor: U-104 (50 μM) were assessed by Matrigel™-coated transwell chambers. Bars represent the mean ± s.e.m. of three independent experiments. *P<0.05, ***P<0.001 by Student’s t-test.
5.4.2 The intracellular and proteoglycan-like domains of CAIX are required for tumour invasion and metastasis

We wanted to determine the molecular basis of CAIX-mediated invasion, and also to determine the critical domains of CAIX involved in this regulation. We therefore utilized the CAIX mutants generated and described in Chapter 4 (Figure 4.1 and 4.3). Analysis of Matrigel™ invasion demonstrates that deletion of both the IC and the PG-like domains results in decreased invasion (Figure 5.2A and B). In the same way, double point mutation of residues S448 and Y449 impacted the invasion of 4T1 cells (Figure 5.2A and B). To determine whether this decrease in invasion capability also resulted in decreased metastasis, we carried out experimental metastasis assays on the 4T1 cell lines expressing the different forms of CAIX. As shown in Figure 5.2C, lung metastases formation after tail vein injection (which examines the contribution of invasion during the extravasation and colonization steps of metastasis), was significantly lower in 4T1 cells depleted of CAIX (4T1shCAIX), and also much lower in cells expressing the IC and PG-like deleted mutants relative to those expressing WT human CAIX (4T1sh/WT). Interestingly, it was demonstrated in Chapter 4 that the deletion of intracellular domain or any point mutation within it also results in partial inhibition of CAIX activity, whereas deletion of the PG-like domain did not have any effect on CAIX activity as measured in the In-cell Carbonic Anhydrase activity assay (Figure 4.7).

The data presented here and in Chapter 4 demonstrate that while both the intracellular domain and the PG-like domain of CAIX contribute to CAIX-mediated tumour cell invasion and metastasis, only the intracellular domain of CAIX is capable of regulating the catalytic activity of the enzyme. Therefore, two different mechanisms for the regulation of invasion by CAIX are taking place, one that is CAIX enzymatic activity-dependent and one that is activity-independent. The extracellular PG-like domain of CAIX might be modulating invasion through mediating interactions with components of the ECM as described for other PGs (Couchman, 2010; Theocharis et al., 2010) and previously discussed in Chapter 4.
Figure 5.2. Intracellular (IC) and proteoglycan-like (PG-like) domains of CAIX regulate cell invasion. (A and B) Invasive capacities of the indicated cell lines were analyzed using Matrigel™-coated transwell chambers. (A) Representative fields, (B) quantification. (C) The indicated cell lines were injected intravenously in BALB/c mice. The number of metastatic cells in lungs was obtained through a colony formation assay. Bars represent the mean ± s.e.m. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 by Student’s t-test. WT: WT human (hu) CAIX.
5.4.3 CAIX regulates MMP-14-mediated type I collagen invasion and degradation

The catalytic reaction of CAIX in converting CO₂ to HCO₃⁻ simultaneously results in the production of hydrogen ions (McDonald et al., 2012; Neri & Supuran, 2011) making the immediate extracellular face of CAIX-expressing cells more acidic. The acidic extracellular microenvironment has been previously reported to enhance tumour cell invasion through the activation of proteases such as cathepsins and matrix metalloproteases (Estrella et al., 2013; R. a Gatenby & Gillies, 2008).

Since we have demonstrated that CAIX acidifies the extracellular pH (Figure 4.7), we therefore examined the effect of CAIX depletion on the expression and activities of MMPs such as MMP-2, -9 and -14 that have been implicated in tumour invasion. While there was only a small difference in the activity of soluble MMP-9 (data not shown), the activity of MMP-2 was greatly reduced in 4T1 cells depleted of endogenous CAIX (4T1shCAIX) or treated with CAIX inhibitor, U-104 (Figure 5.3A). Since MMP-2 is specifically activated through proteolytic cleavage by MMP-14 (Haage, Nam, Ge, & Schneider, 2014; Overall et al., 2000), this particular result also indicates decreased MMP-14 activity upon inhibition of CAIX expression and more importantly, upon inhibition of CAIX activity. The expression of the membrane-bound protease, MMP-14 (both pro- and active forms) was also significantly reduced in 4T1 tumour cells in which CAIX expression was depleted (4T1shCAIX). The expression of MMP-14 was rescued by the expression of WT human CAIX in 4T1shCAIX cells (Figure 5.3A), suggesting that CAIX may regulate both MMP-14 expression and activity.

Next we wanted to determine whether the effect seen on the protein levels and activity of MMP-14 could be translated to a decrease in the degradation of type I collagen, another measure of MMP-14 activity (Poincloux, Lizárraga, & Chavrier, 2009). As shown in Figure 5.3B, 4T1 cells expressing CAIX (4T1shNS) induce robust degradation of type I collagen and this was significantly inhibited in 4T1 cells depleted of endogenous CAIX (4T1shCAIX), but rescued by 4T1 cells expressing WT human CAIX.
Next we determined whether depletion of CAIX and concomitant decrease of MMP-14 activity also resulted in inhibition of invasion through type I rat tail collagen, a substrate of MMP-14. As shown in Figure 5.3C, CAIX depletion resulted in a significant decrease in invasion through type I collagen, and this inhibition was rescued by expression of WT human CAIX. In the same way, deletion of the intracellular domain also resulted in a significant decrease in invasion through type I collagen, whereas deletion of the PG domain did not have a significant effect on it (Figure 5.3C). In addition, treatment of 4T1sh/WT cells with the CAIX inhibitor, U-104, which did not affect MMP-14 or CAIX protein levels, also resulted in significant inhibition of invasion (Figure 5.3D), as did a specific, function-blocking monoclonal anti-MMP-14 antibody (Kaewprag, Umnajvijit, Ngamkham, & Ponglikitmongkol, 2013) (Figure 5.3D).

These data suggest that CAIX promotes invasion through type I collagen in an MMP-14-dependent manner and suggest that the CAIX-mediated extracellular pH acidification may regulate MMP-14 function.
Figure 5.3. CAIX regulates MMP-14-mediated degradation of type I collagen. (A) Left: MMP-2 activity in cells depleted of endogenous CAIX (shCAIX) or treated with CAIX inhibitor U-104 (50 μM) was detected by zymography. Densitometry is shown at the bottom. Right: mouse CAIX, human CAIX (moCAIX, huCAIX) and MMP-14 protein expression levels in 4T1shNS (non-silencing), shCAIX and 4T1sh/WT huCAIX hypoxic cells. The ability of the indicated cell lines to degrade (B) or invade (C) through type I rat tail collagen-coated transwell chambers was assessed. (D) Invasive abilities of 4T1sh/WT huCAIX cells incubated either with anti-MMP-14 antibody (20 μg/ml; left) or CAIX inhibitor U-104 (50 μM; right) were assessed through the same assay. Bars represent the mean ± s.e.m. of three independent experiments. *P<0.05, **P<0.01 by Student’s t-test.
5.4.4 CAIX co-localizes with MMP-14 in mature invadopodia

It is now widely accepted that invading tumour cells degrade the ECM, and especially collagen, by forming structures called invadopodia, which are actin-rich protrusions that are formed by metastatic tumour cells to degrade the ECM and facilitate metastasis (Beaty & Condeelis, 2014; Yamaguchi et al., 2005). Invadopodia initially form as precursors enriched in cortactin and F-actin and can be detected by staining for cortactin and Tks-5 (Beaty & Condeelis, 2014), a phosphatidylinositol 4,5-bisphosphate (PiP₂)-binding protein enriched in invadopodia (Bergman, Condeelis, & Gligorijevic, 2014; Murphy & Courtneidge, 2011). The invadopodia mature and degrade matrix through the recruitment and activation of proteases such as MMP-14, an established component of mature invadopodia (Beaty & Condeelis, 2014; Yamaguchi et al., 2005).

In light of our data demonstrating that CAIX regulates tumour cell invasion in an MMP-14-dependent manner, we wanted to determine whether hypoxia-induced CAIX was localized to invadopodia and whether CAIX associated with MMP-14 within invadopodia. We therefore carried out immunofluorescence confocal microscopy on hypoxia-treated cells growing on FITC-gelatin to detect mature invadopodia.

As shown in Figure 5.4A, parental 4T1 cells under hypoxia form mature, gelatin-degrading, Tks-5-positive invadopodia which contain MMP-14. CAIX also localizes to such invadopodia where it co-localizes with cortactin (Figure 5.4B), although CAIX and cortactin staining is also observed at the peripheral cortex of the cells. CAIX also co-localizes with Tks-5 and MMP-14 in invadopodia-like structures as shown in Figure 5.4C. We also examined the localization of MMP-14 under hypoxia in the CAIX depleted 4T1 cells (4T1shCAIX). As shown in Figure 5.4D, MMP-14 continues to localize to invadopodia in these cells, suggesting that CAIX does not regulate MMP-14 recruitment to invadopodia. Next, we wanted to determine whether exogenously expressed human CAIX also localized to mature invadopodia. As shown in Figure 5.4, human CAIX co-localizes with Tks-5 (Figure 5.4E) and MMP-14 (Figure 5.4F) at mature invadopodia in 4T1sh/WT cells.
Figure 5.4. CAIX co-localizes with MMP-14 in the invadopodia of 4T1 cells. 4T1 cells were grown on 488-labelled gelatin for 6 h (hypoxia) prior to fixing and staining for different markers of invadopodia as indicated. Co-localization of (A) Tks-5 and MMP-14 or (B) moCAIX and cortactin at the invadopodia of 4T1 cells. (C) Co-localization of moCAIX, Tks-5 and MMP-14 in 4T1 cells. (D) Localization of MMP-14 at the invadopodia in 4T1shCAIX cells. Co-localization of (E) huCAIX and Tks-5 or (F) huCAIX and MMP-14 at the invadopodia in 4T1sh/WT huCAIX cells. Arrows indicate co-localization of the different markers in invadopodia structures (Left scale bar: 10 μm, Right scale bar: 3 μm). moCAIX: mouse CAIX, huCAIX: human CAIX. See Figure 5.5 (legend) for further description of left, center and right panels in A-C, E and F.
The MDA-MB-231 human breast cancer cells are highly metastatic and are often used for studies of invadopodia (Bowden et al., 2006; Lin et al., 2014; Rey, Irodlle, Waharte, Lizarraga, & Chavrier, 2011). We therefore wanted to determine whether CAIX localizes to invadopodia in these cells under hypoxia. As shown in Figure 5.5, CAIX co-localizes with cortactin (Figure 5.5A), Tks-5 (Figure 5.5B) and MMP-14 (Figure 5.5C) at invadopodia in MDA-MB-231 cells, in addition to 4T1 cells.

**Figure 5.5. CAIX co-localizes with MMP-14 in the invadopodia of MDA-MB-231 cells.** Representative confocal images of endogenous human CAIX (huCAIX) showing its co-localization with (A) cortactin, (B) Tks-5 and (C) MMP-14 in invadopodia structures in MDA-MB-231 cells. Arrows indicate co-localization of the different markers in invadopodia (Left scale bar: 10 μm, Right scale bar: 3 μm). (A-C) **Left panels:** image of cells identifying a region of interest (ROI; white box) containing invadopodia. **Center panels:** high magnification images of the ROI with individual channels showing the indicated protein staining. **Right panels:** merged images. huCAIX: human CAIX.
5.4.5 CAIX and MMP-14 interact through the CAIX intracellular domain

The decrease in MMP-14 expression in CAIX depleted cells, and the co-localization of CAIX and MMP-14 in invadopodia structures, suggested that the two proteins might interact. To address this possibility, we first carried out co-immunoprecipitation analysis in the 4T1 cells expressing WT human CAIX and various CAIX mutants. As shown in Figure 5.6A, MMP-14 was readily co-immunoprecipitated with human CAIX in extracts from cells which express WT and PG-like domain-deleted CAIX. However, MMP-14 was not co-immunoprecipitated with human CAIX in cells expressing the IC domain-deleted CAIX. These data demonstrate that CAIX interacts with MMP-14, and that the IC domain of CAIX is required for this interaction.

Since 4T1 cells express exogenously transfected CAIX, we wanted to determine whether hypoxia-induced endogenous CAIX also interacted with MMP-14. We analyzed this in normoxia or hypoxia-treated MDA-MB-231 (Figure 5.6B), and pancreatic ductal adenocarcinoma (PDAC) PK-8 cells (Figure 5.6C). In both cases MMP-14 is readily co-immunoprecipitated with CAIX under hypoxic conditions, and in the case of PK-8 cells reciprocal immunoprecipitation of MMP-14 also pulled-down CAIX in hypoxia.
Figure 5.6. CAIX interacts with MMP-14 through its intracellular (IC) domain. (A) Endogenous MMP-14 co-immunoprecipitates with WT human CAIX (huCAIX) expressed in 4T1 cells but not with ΔIC-huCAIX, while the deletion of the PG-like domain has no effect on the interaction. (B) Endogenous MMP-14 co-immunoprecipitates with endogenous human CAIX in hypoxic MDA-MB-231 cells only. (C) Endogenous MMP-14 co-immunoprecipitates with endogenous human CAIX in hypoxic PK-8 cells only (top). Reciprocal co-immunoprecipitation in PK-8 cells (bottom). Right: inputs. N: normoxia, H: hypoxia.
The data in Figure 5.6A demonstrate that deletion of the IC domain abolishes the interaction of MMP-14 with CAIX. We therefore wanted to identify putative regions within the IC domain of CAIX involved in mediating the interaction of CAIX with MMP-14. The group of Pastorekova has recently shown that Thr443, a putative cyclic AMP-dependent PKA phosphorylation site can regulate the extracellular activity of CAIX and extracellular acidosis (Ditte et al., 2011). Their data also showed that this regulation simultaneously required dephosphorylation of Ser448. Likewise, it has been suggested that Tyr449 undergoes phosphorylation upon stimulation with EGF (Dorai et al., 2005). These data suggest that these closely aligned amino acid residues (Figure 1.6 and 4.1) may contribute to or regulate the interaction of CAIX with MMP-14. We therefore utilized 4T1sh cells stably expressing the following point mutations: T443A, S448A, Y449A and double mutant, S448A+Y449A (S+Y) and analyzed the interaction between CAIX and MMP-14 in extracts from cells expressing these mutants. Surprisingly, the interaction was significantly reduced in extracts from cells expressing the T443A and Y449A human CAIX mutants (Figure 5.7A and C), and was completely abolished in extracts from cells expressing the S448A and S448A+Y449A human CAIX mutants (Figure 5.7B and D). These data demonstrate a critical role of these putative phospho-amino acid residues within the intracellular domain of CAIX in the interaction of CAIX with MMP-14.

Collectively, these data demonstrate that CAIX, when induced by hypoxia, interacts with MMP-14, through its intracellular domain, and co-localizes with MMP-14 in mature invadopodia.
Figure 5.7. Residues S448, Y449 and T443 modulate the interaction of CAIX with MMP-14. Endogenous MMP-14 co-immunoprecipitates with WT human CAIX (huCAIX) expressed in 4T1 cells but the interaction is decreased in (A) T443A-huCAIX and (C) Y449A-huCAIX mutants, and completely lost in (B) S448A-huCAIX and (D) S448A+Y449A-huCAIX (S+Y) mutants. Right: inputs.
5.5 Discussion

Tumour invasion is an essential step during the process of metastasis. Invasion of tumour cells is an active process during intravasation, which requires degradation of the basement membrane, and it is also required during the extravasation step and during colonization at distant sites. The current paradigm posits that EMT of epithelial cells or of “stem” cells results in the expression of genes and activation of pathways that promote migration and invasion. A necessary step of invasion is the degradation of the ECM.

There is considerable evidence supporting that tumour hypoxia promotes both EMT and invasion (Hill, Marie-Egyptienne, & Hedley, 2009; Lendahl, Lee, Yang, & Poellinger, 2009; Lohse et al., 2014) and recent work has shown that cancer stem cells, which have undergone EMT also reside in hypoxic niches (Lock et al., 2013; Peitzsch, Perrin, Hill, Dubrovsk, & Kurth, 2014). However, the molecular basis of the connection between hypoxia and tumour invasion, especially the pathways involved in hypoxia-driven formation of structures such as pseudopodia and invadopodia, which coalesce the matrix degradation machinery, are poorly understood.

Hypoxia is accompanied by inhibition of oxidative phosphorylation, increased glycolysis and accumulation of lactic acid, leading to acidic extracellular pH, which has been shown to activate proteases and stimulate local matrix degradation and tissue remodelling (Chambers & Matrisian, 1997; Estrella et al., 2013; Kessenbrock et al., 2010). Tumour cells adapt to hypoxia and acidosis by upregulating cell surface, pH regulating NHE-1 and CAIX. NHE-1 has been shown to play a critical role in regulating invadopodia function by modulating intracellular pH (Brisson et al., 2013; Busco et al., 2010; Lucien et al., 2011), and it has recently been shown to be recruited to the invadopodia through the talin-moesin complex (Beaty et al., 2014).

However, the role of CAIX in hypoxia-mediated tumour cell invasion, or formation and/or function of invadopodia has not been investigated in detail. We and others have demonstrated a critical role of CAIX in tumour growth and metastasis (Lou et al., 2011;
S. K. Parks et al., 2013), and while CAIX has been suggested to play a role in tumour invasion (Jaromir Pastorek & Pastorekova, 2014), the molecular basis of CAIX-mediated invasion is poorly understood.

The results presented here suggest that the activity of CAIX is required for invasion since the least active mutants also displayed impaired invasion. There might be a need for a minimal decrease in CAIX activity (i.e. threshold) in order to see an effect on cell invasion since cells expressing the T443A mutant, which were slightly more active than the rest of the mutant lines did not show a significant decrease in its ability to invade. The PG-like domain also seems to be regulating cell invasion although the mechanism might be a different one, possibly through mediating interactions with the ECM and facilitating cell adhesion as discussed in Chapter 4.

The data presented here identify, for the first time, a mechanistic role of CAIX in promoting tumour cell invasion through interaction with MMP-14, localization in invadopodia and regulation of MMP-14-mediated type I collagen-degradation activity. MMP-14 specifically cleaves the 72 kDa pro-gelatinase A (MMP-2) generating a 68 kDa activation intermediate which is critical for full activation to the 66 kDa form by autocatalytic cleavage of MMP-2 (Overall et al., 2000). We observed reduced levels of active MMP-2 both in 4T1 cells with CAIX knockdown and 4T1 cells treated with CAIX inhibitor U-104, suggesting that CAIX enzymatic activity is important for activation of MMP-14. MMP-14 is capable of cleaving other substrates such as type I collagen, fibronectin, laminin and vitronectin, which are also cleaved by other MMPs, however MMP-14 is the only protease described so far to specifically cleave and activate MMP-2 (Barbolina & Stack, 2008).

Our data suggest that CAIX regulated invasion requires both the IC domain, and the extracellular PG-like domain. However, only the IC domain mediates the interaction with MMP-14 and regulates the catalytic activity of CAIX, which is also required for CAIX mediated invasion. Specifically, our data showing that type I collagen-degradation activity is dependent on both CAIX and MMP-14 activities, since this activity is inhibited by the CAIX specific inhibitor U-104 (Lou et al., 2011; Pacchiano et al., 2011), as well as
by a function blocking anti-MMP-14 monoclonal antibody, strongly suggests a regulatory role of CAIX towards MMP-14 activity.

Our data also point to a critical role of a short region within the IC domain of CAIX in mediating its interaction with MMP-14. Interestingly this region contains three putative phosphorylation residues, the mutations of which dramatically reduce or abolish interaction with MMP-14, suggesting possible regulation of interaction between these two proteins through phosphorylation-dephosphorylation events. It has been reported that PKA is the kinase responsible for phosphorylation of residue T443 (Ditte et al., 2011) and that residue Y449 undergoes phosphorylation upon stimulation with EGF, however the kinase responsible for the latter modification has not been identified (Dorai et al., 2005). Future experiments will utilize phospho-mimetic mutants to investigate further the role of these modifications on the interaction of CAIX with MMP-14.

Further studies are also required to investigate the functional role of CAIX/MMP-14 complexes in other parts of the cell as observed in Figure 5.4 and 5.5. CAIX has been previously found in lamellipodia structures and focal adhesions (Csaderova et al., 2013; Svastova et al., 2012), while the localization of MMP-14 in lamellipodia and pseudopodia has also been described (Kwiatkowska, Kijewska, Lipko, Hibner, & Kaminska, 2011; Yu et al., 2012).

The precise mechanisms involved in the regulation of MMP-14 activity by CAIX remain to be elucidated. Our data suggests that CAIX plays no role in the recruitment of MMP-14 to invadopodia, since MMP-14 is localized to invadopodia in CAIX-depleted cells, however this observation was not quantified and requires further investigation. Furthermore, our data clearly demonstrate that CAIX catalytic activity is required for the regulation of MMP-14 activity (activation of MMP-2 and degradation of type I collagen); therefore it is possible that the role of CAIX in invadopodia is to directly stimulate MMP-14 activity (Figure 5.8). One possible mechanism is through the release of hydrogen ions during the conversion of carbon dioxide to bicarbonate mediated by
CAIX, which would activate the closely associated MMP-14, although proof for this will require purification and structural analysis of the complex.

Figure 5.8. Model for CAIX-mediated regulation of MMP-14 activity. The model depicts the cooperative tasks of CAIX and MMP-14 in the invadopodium. The H⁺ generated by CAIX enzymatic activity are proposed to activate MMP-14, which in turn mediates degradation of collagen in the extracellular matrix (ECM). Phosphorylation of CAIX intracellular domain might modulate interaction with MMP-14. Alternatively, CAIX might be mediating the stabilization of membrane-bound MMP-14. Potential CAIX interactor identified by proteomics analysis Myosin10, might mediate targeting of CAIX to the invadopodium membrane. Likewise, the Dedicator of cytokinesis protein 1 (DOCK1), identified as a potential binding partner of CAIX functions as a guanine-nucleotide exchange factor (GEF) that activates RHO and RAC GTPases important for the formation of actin stress fibres and protrusive structures. Ctn: cortactin.

Interestingly, extracellular acidification has been postulated to activate proteases (Estrella et al., 2013; LeBleu et al., 2014), and specifically, MMP-14 showed increased
collagen I degradation activity *in vitro* at pH values < 7.0 (Gioia et al., 2010). Along these lines, treatment of tumour bearing mice with bicarbonate has been shown to decrease cathepsin and MMP activity within the tumours (Robey & Nesbit, 2013). Importantly, recent studies have linked expression of MMP-14 with cancer progression and metastasis, and poor prognosis in patients (Hauff et al., 2014; Jia et al., 2014; Macpherson et al., 2014; Rossé et al., 2014) possibly due to increased degradation of the ECM and increased invasion and metastasis.

This is the first demonstration of a functional interaction between two cell surface metallo-enzymes, and our data demonstrate that the interaction and co-localization of CAIX and MMP-14 within mature invadopodia results in the promotion of MMP-14-dependent matrix degradation and invasion.

Thus we provide novel mechanistic insights into hypoxia-driven invasion and identify a new protein complex for targeting tumour progression, especially since recent reports have demonstrated a critical role of invadopodia during the extravasation step of metastasis (Leong et al., 2014). In this regard, CAIX is a better target than NHE-1 since it is specifically induced in the hypoxic regions of solid tumours (Chia et al., 2001; Tan et al., 2009). Since CAIX specific inhibitors are in clinical trials, our findings identify tumour invasion as new target for these compounds.
Chapter 6. Conclusions and Future Directions

6.1 Novel CAIX antibodies and small molecules inhibitors

From the five novel CAIX inhibitors characterized during this work, it can be concluded that the two with the highest CAIX inhibition capacities were ureido-sulfonamide U-104 and its derivative SLC-149. Inhibitor U-104 has been extensively characterized in our laboratory by Dr. Paul McDonald and Dr. Shawn Chafe for its effects on cancer cell viability and cancer cell death, extracellular pH acidification and inhibition of tumour growth and metastasis in vivo. This inhibitor entered Phase I clinical trial (safety trials) in September of 2014.

Future experiments will now aim to facilitate the next class of inhibitors: glycosyl coumarines (GC-205) into clinical trials. However, this requires further in vitro characterization of this inhibitor as well as additional work in pre-clinical models. One important challenge with this inhibitor is finding an improved vehicle for animal models. The current vehicle does not allow the drug to be diluted at higher concentrations and it is not well tolerated by the mice. GC-205 and SLC-149 need to be tested in an extended number of cell lines for their effects on extracellular/intracellular pH (by utilizing pH dyes for example), cell death, cell proliferation, cell migration and invasion. They will also be tested in vivo in pre-clinical models alone or in combination with chemotherapeutic agents such as paclitaxel with the aim to determine optimal dosing for inhibition of tumour growth and metastasis, side effects and toxicity.

Some examples of tumour-specific targeting exist in the clinic, such as the use of anti-HER2 monoclonal antibody Herceptin used to treat HER2-positive breast cancers. In addition to this, a new strategy for tumour-specific targeting are dual target drugs; which either inhibit two enzymes, two receptors, or act on an enzyme and a receptor, (Guo, 2009) opening a vast research avenue for our CAIX inhibitors and antibodies and which could potentially increase their selectivity and effectiveness.
From all the antibodies tested, polyclonal antibody 359-1 from KalGene Pharmaceuticals proved to be specific against CAIX and a very good tool for Western blot and immunoprecipitation assays. However, this antibody did not show function-blocking activity and the project was discontinued by KalGene Pharmaceuticals due to lack of funding.

Similarly, I was unable to prove that the antibodies obtained from Deeley Cancer Centre and the University of Victoria were CAIX-specific, and although they did show some function-blocking activity it remains to be determined if they are indeed inhibiting CAIX. This project was unfortunately discontinued by the third-party, and consequently we are unable to access more antibodies.

Finally, the monoclonal antibody MM-26 developed by SignalChem Lifesciences and initially characterized by them for its CAIX-specificity was proven to block CAIX function in my activity assays using rCAIX and 67NR/WT huCAIX cells. Antibody MM-26 can achieve 100% inhibition of rCAIX catalytic activity when used at a 5:1 antibody:enzyme molar ratio, and 50% inhibition of human CAIX activity when CAIX is expressed in cells and the antibody is used at 100 μg/ml. I was able to prove that this antibody has the ability to induce a two-fold increase in the death of hypoxic cancer cells in vitro when used at 100 μg/ml.

Future plans with this antibody include mapping the binding epitope and improving its function-blocking capacities by creating single arm Fabs, since there might be steric hindrance caused by the large antibody molecule that is preventing further blockade of CAIX catalytic activity in cells. Mapping the binding epitope of this function-blocking antibody will provide information on the residues that are critical for the activity of the enzyme. In the same way, it is imperative to study the biological effects of the inhibition of CAIX catalytic activity in an extended number of normal and cancer cell lines, such as its effect on intracellular/extracellular pH, cell death, cell proliferation, cell migration and invasion. It is also relevant to study whether this antibody can mediate ADCC or CDC since these additional mechanisms would certainly increase its efficacy. During ADCC, the antibody functions as a flag that directs the immune system to kill cells that
display antibody binding. Analysis of the in vivo antibody localization and distribution is important in order to determine tumour-specific localization of this antibody. Interestingly, we have demonstrated in the laboratory the tumour-specific localization of another anti-CAIX monoclonal antibody by CAIX-positive xenografts in vivo suggesting that this immunotherapy approach for targeting CAIX may be successful. Lastly, this antibody will be tested in vivo in pre-clinical models for its potential inhibition of tumour growth and metastasis, alone or in combination with chemotherapeutic agents. Before approval for clinical trials, the antibody will require chimerization and humanization.

6.2 Characterization of CAIX mutants

The generation of stable cell lines expressing different forms of CAIX was challenging, especially because 4T1 cells express endogenous CAIX in hypoxia. The 4T1shCAIX cells that I received and that were generated by plasmid transfection of shRNA targeting CAIX would lose CAIX knockdown over time, likely due to release of the plasmid from the genome. Better results are usually achieved by transduction of shRNA mediated by lentivirus, therefore Dr. Shawn Chafe in our laboratory kindly assisted in generating the CAIX knockdown again by this method. I observed a more stable knockdown in this new cell line, and by generating a frozen stock I was able to carry out all of my experiments with this line. This line was used for expression of the mutants and I did not experience any issues regarding the loss of CAIX knockdown, even after plasmid transfection of human CAIX constructs. Thus, for any future work where stable cell lines are required the use of lentivirus transduction of shRNA will be chosen.

I demonstrated that all the human CAIX mutants (T443A, S448A+Y449A, ΔIC and ΔPG-huCAIX) are able to form dimers and to localize to the cell membrane. I only observed a significant reduction in cell membrane localization when the IC domain was absent, possibly suggesting that the protein is not folding properly and around 50% of it stays in the cytoplasm where it may be degraded. A previous report suggested that the IC
domain of CAIX is required for its cell membrane localization (Hulikova et al., 2009) however, I observed that half of ΔIC-huCAIX is present at the cell membrane. In the same way, less than half of cytoplasmic ΔIC-huCAIX co-localized with the ER creating the need to investigate the subcellular localization of the rest of this mutant protein. The use of inhibitors of protein degradation could help elucidate if ΔIC-huCAIX is undergoing degradation.

I also showed that the IC domain of CAIX regulates its Carbonic Anhydrase activity, but the PG-like domain did not seem to have any effect on CAIX activity when expressed in cells, suggesting an inside-out mechanism of regulation of activity. Interestingly enough, the cell lines expressing the IC domain mutants and that display impaired CAIX activity also showed an increase in cell death rates, supporting the hypothesis that CAIX can indirectly regulate cell survival through the modulation of pH. An important future experiment would be to measure the intracellular pH of 4T1 cells expressing the CAIX mutants that display impaired activity. Techniques such as the utilization of intracellular pH-sensitive dyes or the insertion of microelectrodes in intact cells have been developed. It is crucial to develop all the previous assays in our laboratory together with an activity assay in hypoxic conditions, as formerly described (Robertson et al., 2004), since our efforts to measure the activity of hypoxic parental cells were unsuccessful, and may potentially require the isolation of cell membranes (Robertson et al., 2004). In my experiments, cell death was assessed by TUNEL assays in hypoxic and serum-starved cells, coupled with media with full buffering capacities (i.e. in the presence of bicarbonate). This type of media has been suggested to mask the effects of CAIX, since the presence of bicarbonate in the media saturates the bicarbonate transporters, therefore not allowing room for CAIX-mediated regulation of pH (Chiche et al., 2009). Thus, future experiments will investigate the cell death phenotype in unbuffered (i.e. bicarbonate-free) media. Likewise, growing the cells in 3D spheroids might pose a higher strain in the regulation of pH by creating a larger diffusion gradient and might result in increased amounts of cell death. In the same way, future plans include studying the cell death phenotype in more detail, for example to determine if
the absence of CAIX is coupled to the induction of apoptosis or to a different type of cell death.

CAIX seems to be involved in regulating cell proliferation, however only the complete absence of CAIX produced a negative effect on cell proliferation. None of the mutants with impaired CAIX activity demonstrated reduced proliferation rates. These mutants were only 25-30% less active than WT human CAIX, which is why an important future experiment would be to test the effect of CAIX inhibitor U-104 on cell proliferation, which I have shown to be providing more than 90% inhibition of CAIX activity. This experiment will shed light on the mechanism of CAIX-mediated regulation of cell proliferation.

When I assessed the migratory abilities of the different mutants, it became evident that only the extracellular PG-like domain modulated cell migration. The mechanism that I propose for this process is through the interaction of the PG-like domain with the extracellular matrix, which may regulate cell adhesion and ECM remodelling. In the absence of the PG-like domain these processes would not take place and the cells would not migrate as efficiently. Supplementary experiments are needed to corroborate this hypothesis, where it would be investigated if the PG-like domain of CAIX directly interacts with components of the ECM such as collagen, laminin, fibronectin and proteoglycans. Live cell imaging of cells migrating along these matrices and investigation of the activation status of components of the migratory machinery such as Src kinase, RHO, RAC and CDC42 GTPases are important future assays that will shed light on the mechanism of CAIX-mediated migration. Antibodies that target the PG-like domain of CAIX, such as the commercial antibody M75 are convenient tools to study the role of the PG-like domain in ECM interactions. Cells expressing the ΔPG-huCAIX mutant or cells pre-incubated with antibody M75 can be used to study migration and attachment to different matrices or to purified heparan-sulfate proteoglycan.

The results from immunoaffinity purification of CAIX followed by binding-proteins identification by mass spectrometry opened a new research avenue, since many potential intracellular binding partners of human CAIX were identified and require
validation. Many of the proteins recognized can potentially explain the survival and migratory/invasive functions of CAIX such as PAR4 and DOCK1, respectively. One intriguing finding was the identification of Myosin-10 as one of the top-ranked hits. This protein was recently involved in filopodia and invadopodia formation, which could further explain the CAIX-mediated invasion process described in Chapter 5.

Finally, it would be interesting to elucidate the role of each of the two individual residues (S448 and Y449) in the phenotypes observed such as decreased survival and decreased invasion. However, initial experiments have shown very little effect of any of these mutations on CAIX enzymatic activity (data not shown). It is unknown if the S448 residue undergoes phosphorylation in vivo as well as the kinase responsible for this modification. Continuing in this direction, the laboratory will create phosphomimetics of T443 and S448 residues by mutating them to glutamate. The mutation of Y449 to glutamate may potentially not be useful to mimic phosphotyrosine, therefore a system that phosphorylates the protein might need to be created (Anthis et al., 2009). Phosphomimetics are useful when the original phosphorylation only creates a negative charge effect that affects the conformation of the protein, but when the phosphorylation is needed to recruit a binding partner, alternative techniques such as in vitro phosphorylation should be sought after. This would allow studying the role of phosphorylation on the interaction of CAIX and other proteins in vitro. The use of kinase inhibitors is another alternative to study the role of this modification in the cell system.

In brief, the stable cell lines that I generated allowed me to study different processes important for cancer cells and to determine that the IC domain of CAIX regulates its Carbonic Anhydrase activity, which ultimately modulates cell survival. The extracellular PG-like domain of CAIX was found to be involved in regulating cell migration.
6.3 Role of CAIX in cell invasion

The data presented in this thesis demonstrate that CAIX plays an important role in regulating cell invasion, since its presence and more importantly its activity was shown to be required for proper cell invasion in different cell lines. Both the IC and the PG-like domains of CAIX appear to be involved in this process, suggesting that two different mechanisms are taking place. The role of the IC domain of CAIX in modulating the invasion of cancer cells might be linked to its ability to also regulate CAIX catalytic activity. While the PG-like domain of CAIX, which did not play a role in regulating CAIX catalytic activity in cells, might actually be mediating invasion through interaction with molecules in the ECM. Both domains were also shown to be important for the extravasation and colonization steps during metastatic formation. By investigating the invasive phenotype further I was able to demonstrate that CAIX forms a protein complex with MMP-14 in the invadopodia membrane (since both proteins co-localized at the invadopodia of parental 4T1 and MDA-MB-231 cells) but also possibly in other migratory structures such as the lamellipodia. The presence of this complex was confirmed by immunoprecipitation experiments in parental MDA-MB-231 and PK-8 cell lines, and was only observed in hypoxia. The IC domain of CAIX, but not the PG-like domain, is required for the interaction with MMP-14.

This work also demonstrated that CAIX is required for MMP-14-mediated degradation of type I collagen, since degradation of this matrix was inhibited after genetic depletion of mouse CAIX and rescued by human CAIX expression in 4T1 cells. Invasion through this matrix was inhibited both when the CAIX inhibitor U-104 and the anti-MMP-14 function-blocking antibody were used; moreover an additive effect was observed when both reagents were used in combination (preliminary experiments, data not shown), suggesting that CAIX promotes invasion through type I collagen in an MMP-14-dependent manner and that CAIX is regulating MMP-14 function. It remains to be determined if CAIX directly activates MMP-14-mediated degradation of type I collagen, which can be achieved by modifying the current in vitro MMP-14 activity assay (Starr,
Dufour, Maier, & Overall, 2012) with the addition of recombinant CAIX and its physiological substrate, carbon dioxide.

A pragmatic future experiment would be developing an assay for measuring the extracellular pH of invading cells in vitro. This could be achieved with pH microelectrodes or membrane-impermeant pH fluorescent probes. The use of these probes together with intravital tumour imaging can help elucidate if peritumoural acidic gradients are also occurring in vivo. Development of these assays would allow us to measure the extracellular pH of the region immediately surrounding the outer membrane of an invading cell. This type of assays would confirm the hypothesis that the impaired catalytic activity observed in IC domain-CAIX mutants translates into deficient invasion, due to a compromise in the acidification of the extracellular pH. Similarly, it would be quite interesting to demonstrate that the PG-like domain of CAIX is indeed binding to components of the ECM.

The experiments I conducted verified that CAIX is important for both intravasation and extravasation processes; however the role of the IC and PG-like domains were only studied during cell extravasation. Determining if these domains also play a role in intravasation (e.g. by orthotopic injection of the cell lines and following spontaneous metastasis together with the monitoring of circulating tumour cells) will shed more light into the role of each domain during the metastatic process in vivo.

Another avenue worth investigating is determining whether the complex formed by CAIX and MMP-14 is only localized at the invadopodia, or can be found at other migratory structures such as the lamellipodia or focal adhesions, a possibility that has been suggested by my immunofluorescence images. The protocol for enrichment of invadopodia followed by immunoprecipitation developed by Dr. Susette C. Mueller (Georgetown University, Washington) could be used to further confirm that this complex is indeed formed at the invadopodia. During this experimental procedure, cells growing on top of gelatin are removed and the invadopodia structures that have protruded inside the gelatin are lysed separately allowing the investigator to differentiate between cell bodies and invadopodia structures. Co-staining of CAIX and
MMP-14 with lamellipodia and focal adhesion markers could prove useful in resolving if this complex is also formed at these structures.

This work has identified a new protein complex for targeting tumour progression creating the perfect opportunity for the development of dual target drugs. In this system a highly potent and selective drug against MMP-14 can be coordinated to an antibody directed against CAIX such as the novel and highly specific monoclonal antibody MM-26. The antibody does not need to internalize since the drug is released extracellularly. The system can be engineered so that the drug is not released until the antibody binds to its epitope creating a very powerful tool. The linker between the antibody and the drug could contain the cleavable sequence from the MMP-2 pro-enzyme, which only gets cleaved by MMP-14. In this way the drug would only be released upon binding of the antibody to CAIX and only in the instance of CAIX being in close proximity to MMP-14 in the cell membrane. This type of technology has been described for a different system (Ouyang, Lu, & Wang, 2014).

In conclusion, by performing the work described in Chapter 4 and 5 I was able to unveil two important mechanisms by which CAIX is facilitating tumour growth and metastasis, the first one being cell survival and the second one cell invasion. Both processes are related to the IC domain-mediated regulation of CAIX catalytic activity. However, the PG-like domain-mediated regulation of migration, invasion and metastasis seems to occur independently of CAIX enzymatic activity.

In summary, this thesis ratified the important role of CAIX during different steps of cancer progression (survival and metastasis) and described and characterized two novel tools for targeting CAIX, the CAIX small molecule inhibitor U-104, currently in Phase I clinical trial and the function-blocking anti-CAIX antibody MM-26. These new tools may eventually be developed as cancer therapeutics. This work also identified MMP-14 as part of a new protein complex (CAIX/MMP-14) important for tumour progression opening up the exciting possibility of utilizing dual target drugs for achieving a more specific and potent cancer treatment.
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