PhD Thesis
Su Chii Kong

pH Regulatory Transporters in Pancreatic Ductal Adenocarcinoma

Academic Advisor: Professor Stine Falsig Pedersen
Submitted: 31st August 2015
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PhD Thesis
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It could not have been possible to complete this doctoral journey of mine without the generosities and supports of the many people around me, to only some of whom it is possible to give particular mention here.

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Lastly, I thank all the “Super ions” in Marie Curie Initial Training Network IonTraC. It has been a great pleasure working together. And yes, someone among us will find a cure for PDAC one day.
Abstract (English)

The abnormal features of hypoxia and altered metabolisms in solid tumours lead to an increased glycolysis that is uncoupled from oxidative phosphorylation in the TCA cycle. Tumoural cells often exhibit dysregulated expressions and activities of various membrane pH regulatory transporters to cope with the elevated acid production from this glycolysis, as well as from cellular ATP hydrolysis, sequentially creating a favourable intracellular pH and hostile acidic tumour microenvironment, fortify the tumour cells with highly invasive, metastatic and drug resistant phenotype. In current work, we study the expressions and functional roles of these pH-regulating transporter in pancreatic ductal adenocarcinoma (PDAC), one of the deadliest human malignancies with an overall 5-year survival rate of only 6%.

Herein we focus on two pH-regulating transporter families, monocarboxylate transporters (MCTs) and V-ATPases. MCT isoforms 1 to -4 are the only proton-coupled isoforms transporting monocarboxylates such as L-lactate. We show that MCT1 and MCT4 are robustly expressed in all PDAC cell lines studied. These transporters were found localized on the plasma membrane of PDAC cells, colocalizing with MCT chaperone protein basigin. Lactate influx capacity was reduced upon siRNA-mediated silencing and pharmacological inhibition of MCT1 and/or MCT4. PDAC cell migration was not significantly affected by MCT1 inhibition with AR-C155858 and MCT1 silencing, yet was inhibited by the general MCT inhibitor 4-CIN. The migration of the fastest moving PDAC cells detected, BxPC-3, was also reduced after MCT4 knockdown. Both MCT inhibition with AR-C155858 and 4-CIN as well as MCT1 and MCT4 silencing reduce the PDAC cell invasiveness in the Boyden chamber assay. In addition, the silencing of MCT1 and/or MCT4 reduced the invasiveness of BxPC-3 cells in the spheroid outgrowth assays. We also observed that the silencing of MCT1 and MCT4 inhibited the proliferation of the studied PDAC cells, where MCT4 silencing exerts a bigger effect than MCT1 silencing.

V-ATPases are multi-subunit proton pumps whose involvement in the generation of tumour microenvironmental acidosis has recently emerged and have been implicated in the promotion of tumour aggressive phenotypes. In present study, substantial up-regulation of subunit a3 was detected in a panel of PDAC cells tested as compared to non-cancerous control HPDE. Silencing of subunit a3 inhibited the invasiveness in two PDAC cell lines studied yet increased their 2D cell motility on a tumour ECM-mimicking substratum. V-ATPases were also found to be associated with several cancer-related signaling pathways. V-ATPase inhibition with concanamycin A and a3 silencing up-regulated the HIF-1α and p21 protein level. Concanamycin A treatment in PDAC cells provoked an increase in p62, indicative of reduced autophagic flux; whereas silencing of a3 subunit did not. The underlying mechanisms leading to these observations remain to be elucidated. Finally, in BxPC-3 cells, cell proliferation was found to be decreased while apoptosis was increased with concanamycin A treatment, indicative of V-ATPases being involved in PDAC cell survival mechanisms as well.

Comprehending pH regulation in tumour cells might provide insights in preventing tumourigenesis by pH disruptions. Data presented in this thesis corroborate the roles of pH-regulating transporter proteins in PDAC progression and pave the way for future studies in search of specific therapeutic targets for this malignancy.
Mangel på ilt (hypoxi) og et ændret stofskifte, fører ofte til en øget glykolytisk respiration frem for en oxidative respiration i tumorceller. Tumorceller udviser ofte ændringer i reguleringen af ekspressionen og aktiviteten af forskellige pH-regulerende membrantransportører, for at modvirke den øgede syreproduktion, der opstår på grund af et øget glykolytisk stofskifte samt fra ATP hydrolyse i cellen. Den øgede syreudskillelse skaber et fordelagtigt intracellulært pH miljø samt et fjendtligt sult tumor mikromiljø, hvilket styrker tumorceller med en invasiv, metastatisk og lægemiddelresistent fenotype. I dette projekt, undersøger vi ekspressionen og funktionen af disse pH-regulerende transportører i bugspytkirtelkræft (pancreatic ductal adenocarcinoma, PDAC), en af de dødeligstede kræftformer blandt mennesker, og med en 5-års overlevelsesrate på kun 6%.

I dette projekt fokuserer vi på to pH-regulerende transportør familier, monocarboxylate transportører (MCT’s) og V-ATPaser. MCT isoform 1 og -4 er de eneste proton-koblede isoformer, der transporterer monocarboxylater så som L-laktat. Vi viser, at MCT1 og MCT4 er stærkt udtrykt i alle de undersøgte PDAC cellelinjer. Disse transportører blev fundet til at lokaliseres i plasmamembranen i PDAC celler og co-lokaliserede med det MCT associerede protein, basigin. Kapaciteten for laktat indstrømningen var reduceret ved siRNA-mediator nedregulering og farmakologisk inhibering af MCT1 og/eller MCT4. PDAC cellemigrering var ikke signifikant påvirket af MCT1 inhibering med AR-C155858 og MCT1 nedregulering med siRNA, men var hæmmet ved behandling med en generel MCT inhibitor, 4-CIN. Migrationen af de hurtigst bevægende PDAC celler, BxPC-3, var også reduceret efter MCT4 nedregulering. Inhibering af MCT med både AR-C155858 og 4-CIN, samt MCT1 og MCT4 nedregulering, reducerede invasionen af PDAC celler i Boyden Chamber Assays. Ydermere, reducerede nedreguleringen af MCT1 og/eller MCT4 invasionen af BxPC-3 celler i Spheroid Outgrowth Assays. Vi observerede også, at nedregulering af MCT1 og MCT4 inhiberede celleproliferation af de undersøgte PDAC celler, hvor nedreguleringen af MCT4 udover en større effect end nedregulering af MCT1.


Forståelsen af pH-regulering i tumorceller kan fører til indsigt i, hvordan man kan forebygge tumorgenesis ved pH-regulering. Data presenteret i denne afhandling understøtter de pH-regulerende transport proteiners rolle i PDAC udvikling og baner vejen for fremtidige undersøgelser, i et forsøg på at finde specifikke terapeutiske behandlingsmuligheder for denne dødelige sygdom.
實體瘤中的缺氧和代謝改變等異常特徵會導致糖酵解增加，有別於三羧酸循環中的有氧磷酸化。由於腫瘤細胞內糖酵解產酸量和三磷酸腺苷水解增加，細胞膜上的各種調節 pH 的轉運體的表達和活性失調，從而導致細胞內 pH 增高和細胞外的酸性腫瘤微環境，使腫瘤細胞呈現出高侵襲性，高代謝及抗藥性等特點。胰腺導管腺癌 (PDAC) 是人類致死率較高的惡性腫瘤之一，其 5 年存活率僅有 6%，本課題主要研究與胰腺導管腺癌相關的 pH 調節轉運體的表達和功能。

在此，我們主要研究兩種 pH 調節轉運體家族，單羧酸轉運體 (MCT) 和空泡型氫離子三磷酸腺苷酶 (vacuolar-type H+-ATPase, V-ATP 酶)。MCT 亞型 1 至亞型 4 是僅有的質子耦合亞型，主要轉運單羧酸如 L-乳酸。我們發現，MCT1 和 MCT4 在所有的胰腺導管腺癌細胞系中都是穩定高表達的。它們位於胰腺導管腺癌細胞的裂膜上，與 MCT 伴侶蛋白 basigin 共定位。當用小干擾 RNA 技術和抑製劑分別抑制 MCT1 和/或 MCT4 後，乳酸流入能力下降。MCT1 抑製劑 AR-C155858 和 MCT1 基因沉默對胰腺導管腺癌細胞遷移沒有明顯影響，但是 MCT 抑製劑 4-CIN 能夠抑制胰腺導管腺癌細胞遷移。BxPC-3 細胞是運動速度最快的胰腺導管腺癌細胞系，抑制 MCT4 基因能夠降低 BxPC-3 的運動速度。利用博伊登小室侵襲技術發現，MCT 抑製劑 AR-C155858 和 4-CIN 及 MCT1 和 MCT4 基因沉默均能降低胰腺導管腺癌細胞的侵襲。此外，利用球體向外生長方法發現，MCT1 和/或 MCT4 基因沉默會降低 BxPC-3 細胞的侵襲能力。我們也觀察到 MCT1 和 MCT4 基因沉默能抑制胰腺導管腺癌細胞的分裂。

V-ATP 酶是含有多個亞單位的質子泵，其參與腫瘤微環境酸化的發生，最近已出現，其在促進腫瘤侵襲方面發揮作用。在本研究中，與未癌性的人胰腺導管上皮細胞比較，a3 亞單位在胰腺導管腺癌細胞的表達大幅度上調。在兩種胰腺導管腺癌細胞中抑制 a3 亞單位的表達會抑制細胞的侵襲，但是會增加細胞在腫瘤性細胞基質上的活動度。V-ATP 酶也參與了多種腫瘤相關的信號通路。V-ATP 酶抑制劑 concanamycin A 和 a3 基因沉默均會上調低氧誘導因子 (HIF-1α) 和 p21 的蛋白表達。Concanamycin A 干預會導致胰腺導管腺癌細胞中 p62 表達增加，暗示著自噬通量的減少；而沉默 a3 亞單位則沒有這種現象的發生。導致這種現象發生的機制仍有待闡明。最後，Concanamycin A 會抑制胰腺癌 BxPC-3 細胞的細胞增殖和促進凋亡，意味著 V-ATP 酶也參與胰腺導管腺癌的存活機制。

解讀 pH 值在腫瘤細胞中的調控作用可以為我們通過 pH 干預預防腫瘤發生提供參考。

本論文中的數據證實了 pH 值調節轉運蛋白在胰腺導管腺癌的進展中發揮的作用，為未來治療腫瘤尋找特異性治療靶點的研究提供依據。
Preface

The overall aim of my PhD studies is to elucidate the expression levels and roles in pancreatic ductal adenocarcinoma (PDAC) of the membrane pH regulatory transporters, including the bicarbonate transporters, Na⁺/H⁺ exchangers (NHEs), monocarboxylates transporters (MCTs), and proton pump vacuolar-type H⁺ ATPases (V-ATPases), with particular focus on MCTs and V-ATPases and their functional roles in PDAC.

Current thesis is based on one review (Paper I) and two manuscripts (Paper II and III).


*contributed equally to the study.

In addition, during my PhD enrolment period, I have also contributed to the following paper, which is not included in this thesis.

Current thesis is organized into four chapters:

**Chapter 1** provides a general introduction to PDAC and the involvement of various membrane pH regulatory transporters in altered metabolisms of solid tumours. Bioinformatic analyses on expression levels of various pH regulatory transporters in pancreatic cancers has been discussed in Paper I, the chapter thus only describes the additional data, collected but not designated for publication, on the mRNA and protein expression levels of selected pH regulatory transporters analysed with quantitative RT-PCR, western blot and immunofluorescence analyses as described in appendices.

**Chapter 2** reviews the SLC16 family of monocarboxylate transporters (MCTs), followed by the summarization and discussion of data collected on the roles played by these transporters in PDAC corroborated by pharmacological inhibitors and siRNA knockdown approaches. Specifically, the expression and functional correlations of MCTs in lactate influx, migration, invasion of selected PDAC cell lines are conferred as reported in Paper II. Roles of MCTs in PDAC cell proliferation which is not included in the paper are also discussed in this chapter.

**Chapter 3** focuses on the transporter family of vacuolar-type H\(^+\)-adenosine triphosphatases (V-ATPases). The expressions of subunit \(a3\) and \(B2\) isoforms of V-ATPases are indicated. The chapter further summarizes and briefly discusses the data obtained on the functional roles of these transporters in PDAC cells, primarily emphasizes on subunit \(a3\) isoform. Functional roles described include the cell 2D-migration on ECM-mimicking substratum and invasion, as well as their involvements in several cancer-associated signalling pathways (Paper III).

The main findings and conclusions from this study are recapitulated in **Chapter 4**. The chapter further provides some future perspectives for this study.

The experimental work was mainly carried out in the Department of Biology, University of Copenhagen, Denmark during my 3 years PhD studies spanned from 1\(^{st}\) of September 2012 to 31\(^{st}\) of August 2015. Migration and proliferation assays involving the Incucyte®Zoom live cell imaging system were performed under the generosity of Prof. Dr. Luis A. Pardo during the 3+6-week secondment at the Max Planck Institute for Experimental Medicine in Göttingen, Germany. Additional experimental work on the V-ATPases-mediated PDAC cell migration and invasion was performed during a one-month secondment at the Hannover Medical School in Hannover, Germany under the kind supervision of Prof. Dr. Christian M. Stock.

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Su Chii, Kong

Copenhagen, 31\(^{st}\) August 2015
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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>4-CIN</td>
<td>α-cyano-4-hydroxycinnamate; CHC</td>
</tr>
<tr>
<td>AE</td>
<td>Anion exchanger</td>
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<tr>
<td>Akt</td>
<td>Protein kinase B</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
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<tr>
<td>CD147</td>
<td>Cluster of differentiation 147</td>
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<tr>
<td>ConA</td>
<td>Concanamycin A</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBDS</td>
<td>4,4-O-dibenzamidostilbene-2,2O-disulphonate</td>
</tr>
<tr>
<td>DIDS</td>
<td>4, 4-O-diiisothiocyanostilbene-2,20-disulphonate</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMMPRIN</td>
<td>Extracellular matrix metalloproteinase inducer</td>
</tr>
<tr>
<td>FdG-PET</td>
<td>18F-fluorodeoxyglucose positron emission tomography</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Glucose transporter 1</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor 1α</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response elements</td>
</tr>
<tr>
<td>Ki</td>
<td>Inhibitory constant</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LAMP-1</td>
<td>Lysosome associated membrane protein 1</td>
</tr>
<tr>
<td>LDH-A</td>
<td>Lactate dehydrogenase A</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NBC</td>
<td>Na⁺, HCO₃⁻ cotransporters (SLC4 protein family)</td>
</tr>
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<td>NHE</td>
<td>Na⁺/H⁺ exchanger (SLC9 protein family)</td>
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<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PanIN</td>
<td>Pancreatic intraepithelial Neoplasia</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(adenosine diphosphate-ribose) polymerase</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PED</td>
<td>Pancreatic Expression Database</td>
</tr>
<tr>
<td>PFK1</td>
<td>Phosphofructokinase 1</td>
</tr>
<tr>
<td>pH</td>
<td>Power of the concentration of the Hydrogen ion; negative logarithmic value of the Hydrogen ion (H⁺) concentration, -log₁₀[H⁺]</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>pH_e</td>
<td>Extracellular pH</td>
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<tr>
<td>pH_i</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>p-Rb</td>
<td>Phosphorylated retinoblastoma tumour suppressor protein</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small-interfering RNA</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
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<tr>
<td>TCIRG1</td>
<td>T-Cell Immune Regulator 1; ATP6V0A3</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar-type H⁺-adenosine triphosphatase</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Chapter 1
General Introduction
Chapter 1  General Introduction

1.1  Pancreatic Ductal Adenocarcinoma (PDAC)

PDAC is the most common type of pancreatic cancer accounting for 95% of cases and remains the most aggressive of human solid malignancies, usually detectable only when already inevitably fatal (Blum & Kloog 2014, Li et al 2004). The overall 5-year survival of PDAC is only 6% and accounts for 200,000 patient deaths annually worldwide, with about 40,000 deaths in USA alone and over 60,000 deaths in Europe (Jemal et al 2011, Siegel et al 2015). The dismal prognosis of this disease is due to early dissemination and rapid acquisition of drug resistance of PDAC (Ryan et al 2014). Currently the main conducive treatment for patients is surgical resection followed by radiation and/or chemotherapy with gemcitabine, a nucleotide analogue commonly applied for the treatment for advanced pancreatic cancer (Blum & Kloog 2014, Hidalgo 2010). However, more than 80% of PDAC patients present with disease that is not amenable to surgical intervention due to the disease’s highly infiltrative nature into pancreaticobiliary system and vital blood vessels nearby and further metastatic events at the time of diagnosis (Wolfgang et al 2013, Wray et al 2005). Indeed, metastatic disease burden is the main cause of mortality by PDAC (Yachida et al 2010, Yeo et al 2002).

Though some (~2-10%) PDACs occur with hereditary factors (Habbe et al 2006, Zalatnai 2006), most PDACs are associated with somatic mutations in a number of genes, including the small GTPases K-RAS with the universal frequency of >95% in human PDAC (Almoguera et al 1988), as well as tumour suppressor genes including p53, SMAD4/DPC4, P16\(^{inva}/CDKN2A, BRCA2, VHL,\) mismatch repair genes MLH1 and MSH2, and STK1 (Al Haddad & Adrian 2014, Caldas et al 1994, Hahn et al 1996, Scarpa et al 1993). Together, these gene alterations construct a complex mutational landscape affecting a variety of signalling pathways, such as DNA damage control, Hedgehog signalling, integrin signalling, and apoptosis (Jones et al 2008).

PDAC progress into malignant adenocarcinoma through a series of non-invasive, pre-neoplastic lesions arises in the exocrine part of the pancreas, with pancreatic intraepithelial neoplasias (PanINs) as the most common and best characterized putative precursors. Other less common precursor lesions include mucinous cystic neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN) (Hezel et al 2006). PanINs are classified into three histopathologic stages (PanIN-1 to -3) before evolving into the invasive PDAC, each stage correlates with successive
alterations of genes mentioned earlier (Figure 1.1). For example, early-stage PanINs already carry mutations in K-RAS along with loss or inactivation of P16$^{Ink4A}$ (Kanda et al 2012), and higher grade PanINs progress with further mutations in p53, SMAD4 or BRCA2 (Hong et al 2011).

A prominent feature of PDAC is the formation of a dense collagen-rich fibro-inflammatory stroma termed desmoplastic reaction or desmoplasia, which is known to contribute to disease progression and chemoresistance. This desmoplasia is a result of proliferation of activated pancreatic stellate cells (myofibroblast-like cells), numerous inflammatory/immune cells and increased deposition of extracellular matrix components including collagen type I, III, an IV, fibronectin, laminin, hyaluronan, and glycoprotein osteonectin (Apte et al 1998, Bachem et al 1998, Schober et al 2014). The resulting stroma, or tumour microenvironment, constitutes a dynamic signalling scaffold which is critically involved in the process of tumour formation, advancement, invasion and metastasis (Chu et al 2007, Mahadevan & Von Hoff 2007, Muerkost et al 2004).

1.2 Reprogrammed Metabolism in Tumours

Normal cells depend on mitochondrial oxidative phosphorylation (OXPHOS) to generate the energy necessary for cellular processes. Tumoural cells in contrary, poses reprogrammed metabolic preference to glycolysis at a rate of 1–64% in all cells to meet their energy demand depending on availability of oxygen and activation of different oncogenic pathways (Hanahan & Weinberg 2011, Zu & Guppy 2004). Inadequate oxygen delivery resulted from poor vasculature
and desmoplasia in tumours leads to hypoxia in certain regions of solid tumours and limits oxidative phosphorylation. Hypoxic tumour cells thus shift their metabolisms towards glycolysis, rapidly generating 2 moles of ATP per mole of glucose to fulfil their metabolic requirements. Glycolytic end-product pyruvate is further reduced to lactate by lactate dehydrogenase A (LDH-A), whose activity is induced in response to hypoxia-driven increase in hypoxia-inducible factor-1α (HIF-1α) activity and a variety of oncogenes, including \(c\)-\(myc\) (Shim et al 1997). In relevance to PDAC, a recent study showed that incubation of PDAC cell lines MIA PaCa-2 with excess glucose under hypoxic condition increased HIF-1α expression. This glucose-mediated HIF-1α elevation increased ATP contents and inhibited mitochondrial activities (Liu et al 2013). Nevertheless, this metabolism shift from glycolysis relative to OXPHOS in tumours is more than a simple adaptation to hypoxia (Gatenby & Gillies 2004). Otto Warburg first observed in the 1920s that tumour cells consume much larger quantities of glucose than do their normal counterparts and metabolize it primarily by glycolysis despite the presence of sufficient oxygen to support mitochondrial respiration (Warburg 1956). This phenomenon, termed aerobic glycolysis or Warburg effect, is repeatedly observed in tumours, and is currently widely used as a diagnostic marker through FdG-PET imaging techniques to detect malignant tumours (Gatenby & Gillies 2004). The metabolic switch of tumours from OXPHOS (38 ATP molecules generated per glucose) to much less efficient aerobic glycolysis (only 2 ATP molecules generated per glucose) is a result driven by several factors including gene reprogramming leading to the activation of oncogenes and inhibition of tumour suppressors, as well as mitochondrial DNA mutations (Dang 2012, Lopez-Lazaro 2008, Matoba et al 2006, Petros et al 2005, Shim et al 1997, Wang et al 2011). Importantly, this metabolic switch is also proposed as being attributable to the other diverse metabolic requirements beyond ATP during their rapid unbridled cell proliferation (Heiden et al 2009, Potter 1958). Glycolysis serves the key purpose for providing important precursors and glycolytic intermediates to fuel anabolic processes in the synthesis of essential macromolecules such as nucleic acids, lipids and various proteins required for cell proliferation (Lunt & Vander Heiden 2011). If glycolysis were to be constantly inhibited in the presence of oxygen, cell proliferation would cease. This could then explain why tumour cells and some non-transformed yet rapidly proliferating cells still activate glycolysis under aerobic conditions (Lopez-Lazaro 2008). Furthermore, despite its low efficiency in ATP yield per molecule of glucose, glycolysis has found to be a faster succession of reactions in generating ATP than ATP production in OXPHOS (Curi et al 1988), as long as glucose supplies are abundant, to meet the enormous ATP demand for the fast-growing tumour cells (Bui & Thompson 2006, Pfeiffer et al 2001).
1.3 Aberrant pH Profiles in the Tumour Microenvironment

Elevated production of lactic acid from anaerobic glycolysis, together with other proton sources such as ATP hydrolysis elsewhere in the cell, and carbonic acid, intracellular hydration of CO$_2$ upon aerobic glycolysis, could result in intracellular acid loading in cells and cause a drop in the intracellular pH ($\text{pH}_i$) (Gatenby & Gillies 2004, Helmlinger et al 2002, Yamagata et al 1998). This decline in $\text{pH}_i$ could be lethal to cells as growth factors below a certain $\text{pH}_i$ threshold (7.1–7.2) would fail to stimulate cell cycle progression (Lallemain et al 1984). Moreover, changes in $\text{pH}_i$ can affect the ionization state of acidic or basic amino acid residues that determine the conformation and later the activity of enzymes critical in various cellular biochemical processes. At the same time, it can also disturb the charge properties of the substrates and hence their affinity to the enzymes (Schulz & Munzel 2011). Earlier studies have also showed that reduction in $\text{pH}_i$ results in DNA fragmentation and degradation in cells and activation of endonuclease DNase II, leading to cell apoptosis (Barry & Eastman 1992, Park et al 1996). Tumour cells, especially of highly aggressive cancer cells, strategically overcome this by up-regulating the expression and/or activity of enzyme carbonic anhydrases (CAs) and a number of membrane pH regulatory transporters to extrude the excessive acid equivalents from cells, and subsequently acidify the extracellular milieu. Four major types of pH regulatory transporters have been identified in cells to be involved in this process, namely the bicarbonate transporters, Na$^+$/H$^+$ exchangers, monocarboxylate transporters and vacuolar-type ATPases (briefly discussed in Section 1.4). The increased activity of these transporters consequently creates an aberrant pH gradient typical of malignant tumour cells as characterized by a reversed pH gradient across the cell membrane of acidic extracellular pH ($\text{pH}_e$) and alkaline intracellular pH as compared to normal counterpart pH gradients. More specifically, tumour cells have been found to demonstrate alkaline $\text{pH}_i$ values of 7.12-7.7 and acidic $\text{pH}_e$ values of 6.2-6.9, compared to normal cells with $\text{pH}_e$ 7.3-7.4 and $\text{pH}_i$ 6.99-7.05, when studied in both in vitro cell-culture and in situ tumour spectroscopic studies using the $^{31}\text{P}$ isotope (Gillies et al 2002, Reshkin et al 2013b, Reshkin et al 2014). This reversed pH gradient created could bring about major functional consequences for the tumour cells and other cells in the tumour micromilieu during tumour progression.

Extracellular acidosis poses substantial cellular stresses in normal cells, leading to either necrotic or apoptotic, or both, cell death of normal cells via p53-dependent and caspase-3-dependent mechanisms (Park et al 1999, Williams et al 1999). In solid tumours however, acidic extracellular milieu created in tumour metabolic microenvironment due to the disordered acid extrusion mechanisms, provides a favourable environment for tumour survival and their transition towards
more aggressive phenotypes. As mentioned, acidic pH\textsubscript{e} could rapidly become cytostatic and/or lethal for most normal cells, including the cytotoxic T cells that normally function in mediating the immune response to tumour antigens (Lardner 2001). Also, the acidic tumour microenvironment has been found to facilitate the recruitment of immunosuppressive cells like myeloid-derived suppressor cells, further supporting the immune escape of tumour cells (Gabrilovich & Nagaraj 2009). Other studies reported acidic pH affect several events of invasiveness and metastatic cascade by inducing the up-regulation of angiogenic vascular endothelial growth factor (VEGF), proteolytic enzymes MMP-2 and MMP-9, carbonic anhydrase, interleukin-8 and cathepsin B (Fukumura et al 2001, Rofstad et al 2006, Shi et al 2000, Stock & Schwab 2009, Webb et al 1999, Xu & Fidler 2000). In addition, low pH\textsubscript{e} also contribute to drug resistance. Acidic extracellular space of solid tumours creates a physiological barrier for the cellular uptake of most chemotherapeutic drugs which behave as weak bases. These drugs are protonated in the acidic tumour microenvironment, and in the charged form, their membrane permeability is strongly reduced, resulting in great decrease of cellular uptake (Gerweck et al 2006, Pellegrini et al 2014, Raghunand & Gillies 2000, Raghunand et al 2003, Vukovic & Tannock 1997, Wojtkowiak et al 2011).

In addition to an acidic extracellular microenvironment, abnormal up-regulated expression and activities of various pH regulatory transporters also help maintaining the pH\textsubscript{i} of solid tumours in neutral or slightly alkaline range compared to normal tissues (Gallagher et al 2008, Gillies et al 2002). This elevated pH\textsubscript{i} has been suggested to be permissive for tumour cell growth even within an acidic environment and involved in natural tumourigenesis. Several studies acknowledged that increased pH\textsubscript{i} promotes growth-factor independent tumour cell proliferation by allowing cells to evade a number of cell cycle checkpoints (Kapus et al 1994, Moolenaar 1986, Putney & Barber 2003) and limiting apoptosis (Lagadic-Gossmann et al 2004, Matsuyama et al 2000). Increase pH\textsubscript{i} also stimulates glycolytic metabolism and inhibit gluconeogenesis by increasing the activity of several enzymes with alkaline optima such as phosphofructokinase 1 (PFK1) and lactate dehydrogenase (LDH-A), further drives higher level of aerobic glycolysis to satiate the high energy demand of tumour cells. Studies also found that activation of NHE1 (discussed below) in transformed NIH-3T3 cells and a subsequent increase in pH\textsubscript{i} is linked to malignant transformation and tumour formation in nude mice (Cardone et al 2005, Reshkin et al 2000a). Besides, increased pH\textsubscript{i} assists in the directed migration and invasion of tumour cells via several mechanisms involving GTPase CDC42, assembly of actin filaments, osmotic swelling and more (Frantz et al 2007, Khajah et al 2013, Koivusalo et al 2010, Webb et al 2011).
1.4 Membrane pH Regulatory Transporters

SLC solute carrier family is the second largest family of membrane proteins in the human genome that consists of vital membrane transporters involved in pH-regulating machineries (Figure 1.2). These includes anion exchangers (AEs) and Na⁺- HCO₃⁻ co-transporters (NBCs) of the SLC4 and SLC26 family which contribute to net HCO₃⁻ (or CO₃²⁻) influx into the cells, the SLC9 family of Na⁺/H⁺ exchangers (NHEs), the SLC16 family of H⁺-monocarboxylate transporters (MCTs) and the vacuolar-type H⁺-ATPases (V-ATPases) (Swietach et al 2014). Under healthy physiological circumstances, these transporters work in a co-ordinated fashion to regulate cell pH homeostasis for maintaining proper cell signalling and the metabolism function. During tumour progression, the expression and activity of these transporters are seen dysregulated and it is proposed that this contributes to the enhanced acid resistance for tumour cells to survive in the microenvironment where normal cells will perish (Gatenby & Gillies 2004, Gillies et al 2002). At the same time this helps to maintain a neutral to alkaline pH of tumour cells which facilitates cell proliferation and tumour growth as described earlier (Fang et al 2008, Gatenby & Gillies 2004, Huber et al 2010, Pouyssegur et al 1985).

**SLC4 Family of Anion Exchangers (AEs) and Na⁺- HCO₃⁻ Co-transporters (NBCs)**

In the SLC4 family, transporters could be functionally clustered further into several groups: electroneutral Cl⁻/HCO₃⁻ anion exchangers (AEs); electrogenic Na⁺-dependent HCO₃⁻ transporters NBCe1(SLC4A4) and NBCe2 (SLC4A5), of which one complete cycle of the transport activity of these transporters results in the cross-membranous movement of one or two net negative charges, thereby carrying electrical current and causing a shift in membrane potential ($V_m$) (Mount & Romero 2004, Romero et al 2013); electroneutral Na⁺-HCO₃⁻ co-transporters NBCn1(SLC4A7) and NBCn2 (SLC4A10); as well as electroneutral Na⁺-driven Cl⁻/HCO₃⁻ exchangers NDCBE (SLC4A8) (Romero et al 2013).
AE1, AE2 and AE3 belong to the Na⁺-independent Cl⁻/HCO₃⁻ anion exchangers encoded by the genes SLC4A1, SLC4A2 and SLC4A3, respectively. The three AEs mediate 1:1 electroneutral exchange of principally Cl⁻ and HCO₃⁻ and other monovalent anions such as HSO₄⁻ (Jennings 1976). Fu and group have previously established extensive studies to address the expressions and roles of these isoforms in human gastric cancer and colonic adenocarcinoma (Shen et al 2007, Suo et al 2012, Wang et al 2013, Wu et al 2010, Xu et al 2009, Yang et al 2008). AE2 has also been implicated in hepatocellular carcinoma by other studies (Hwang et al 2009, Wu et al 2006). Among the Na⁺-coupled HCO₃⁻ transporters, NBCn1 in particular has been demonstrated experimentally as the predominant acid extrusion mechanism in human breast carcinoma tissue at intracellular pH levels higher than 6.6 (Boedtkjer et al 2013). It has also been demonstrated that the strong up-regulation of NBCn1 upon N-terminally truncated ErbB2 receptor expression via Akt-, ERK-, Src- and Kruppel-like Factor 4-pathways contributes to pHᵢ regulation in breast cancer cells (Gorbatenko et al 2014b, Lauritzen et al 2010). A genome-wide transcriptomic analysis revealed that mRNA expression of NBCe1 is found significantly up-regulated in chronic myeloid leukemia stem cells relative to normal stem and progenitor cells (Gerber et al 2013). Subsequently, our data shows that the NBCe1 mRNA expression is highly up-regulated in the PDAC cell line BxPC-3 (Figure 1.3A). The Cl⁻/HCO₃⁻ exchangers NDCBE was suggested to act as a hybrid co-transporter/exchanger that co-transport Na⁺ and two HCO₃⁻ (or one Na⁺ plus one CO₃⁻, or one NaCO₃⁻ ion pair) into the cell in exchange for a single Cl⁻ (Romero et al 2004). Our current data shows an up-regulation in mRNA expression of NDCBE in all four PDAC cell lines applied (Figure 1.3A), pointing to an interesting specific therapeutic target in this cancer type.

**SLC9 Family of Na⁺/H⁺ Exchangers (NHEs)**

Na⁺/H⁺ exchangers (NHEs) regulate the pH homeostasis of cells by transporting out protons (H⁺) and exchange for a cation (Na⁺) to maintain intracellular electroneutrality. These transporters are encoded by the SLC9 gene family and nine isoforms (NHE1-9) have been identified in the human genome to date. NHE1 (SLC9A1) is the most extensively characterized isoform of these. NHE1 is ubiquitously expressed in in virtually all cells and tissues (Orlowski & Grinstein 2004, Sardet et al 1989) and is one of the major pH-regulating transporters involved in many physiological processes including cell migration. NHE1 is almost quiescent at a neutral pH in normal cells but is activated via increased affinity of the intracellular allosteric H⁺-binding site when the cell pHᵢ drops below a physiological resting set points of 6.9-7.1 pH units (Cardone et al 2005, Reshkin et al 2000b). Mitogen stimulation and oncogenic transformation lead to the hyperactivation of NHE1 and results in an elevation of pHᵢ and extracellular acidification beneficial for cell growth as
discussed in Section 1.3. Importantly, NHE1 has been implicated to play a vital role in tumour invasion and metastasis. Motile and invasive phenotype is a prerequisite for a cell to become metastatically competent. NHE1 has found to be polarized at the leading edge of the actin-rich plasma membrane protrusions which regulate cell motility, i.e. invadopodia. At site, NHE1 provides local pH regulation and promote cell cytoskeletal remodelling by the alkalization of pH, and invadopodial-dependent extracellular matrix proteolysis upon acidification of the extracellular peri-invadopodia nanospace, thereby facilitating tumour cell invasion (Busco et al 2010, Magalhaes et al 2011, Reshkin et al 2013a).

Numerous studies have provided evidence of the involvement of NHE1 in uncontrolled cell proliferation, motility and invasion of various cancer types such as breast cancer (Cardone et al 2007, Lauritzen et al 2012), melanoma (Stuwe et al 2007, Vahle et al 2014), non-small lung cancer (Provost et al 2012). Here we have examined several NHE isoforms in current bioinformative analysis and have found that NHE1 is up-regulated in PDAC in all three cancer genome databases (Paper I). Indeed, it has been suggested that the expression and activity of NHE1 correlate with the degree of aggressiveness of human PDAC cell lines (Cardone et al 2015, Pedersen & Stock 2013).

**SLC26 Family of Anion Exchangers**
The mammalian SLC26 family comprises of 11 isoforms, namely SLC26A1-SLC26A11, with SLC26A10 likely being a pseudogene. Along with the SLC4 family, the anion exchangers in the SLC26 family function as transporters of a variety of monovalent and divalent anions across the cell membranes, including chloride (Cl\(^-\)), sulphate (SO\(_4^{2-}\)), oxalate, iodide (I\(^-\)), formate, hydroxyl ion (OH\(^-\)), and bicarbonate (HCO\(_3^-\)) (Alper & Sharma 2013). SLC26A1 and SLC26A2 acts as selective sulphate transporters. SLC26A1 was suggested to function as SO\(_4^{2-}\)/HCO\(_3^-\) exchanger and probably responsible for SO\(_4^{2-}\) sulphation of proteoglycans in the liver (Markovich et al 1994, Quondamatteo et al 2006); while SLC26A2 functions as a SO\(_4^{2-}\)/Cl\(^-\) exchanger and involved in cartilage development and function (Forlino et al 2005, Satoh et al 1998). SLC26A3, SLC26A4, and SLC26A6 are coupled Cl\(^-\)/HCO\(_3^-\) exchangers (Mount & Romero 2004). SLC26A3 and SLC26A6 have been localised to the apical membrane of human pancreatic duct and mouse pancreatic duct and suggested to play a role in pancreatic HCO\(_3^-\) secretion (Greeley et al 2001, Song et al 2009). SLC26A7 and SLC26A9 function as selective Cl\(^-\) ion channels with minimal HCO\(_3^-\) permeability (Dorwart et al 2007, Kim et al 2005). Among others, SLC26A5 has been proposed to be a molecular motor rather than an anion transporter in mammals (Schaechinger & Oliver 2007). Interestingly, several isoforms in this family were found
down-regulated in various cancer types. While the roles of this down-regulation in cancer development is currently unknown, it is speculated that the down-regulation of these transporters may help in maintaining an elevated pH\text{\textsubscript{i}} in these cells, given their role in transporting Cl\textsuperscript{−}, HCO\textsubscript{3}{−}, and I\textsuperscript{−} (Gorbatenko et al 2014a).

As the major pH regulatory transporters investigated in our current work, more detailed Papers of \textit{SLC16 family of monocarboxylate transporters (MCTs)} and proton pump \textit{vacuolar-type ATPases (V-ATPases)} will be included in Chapter 2 and Chapter 3 in this thesis.

1.4.1 mRNA and Protein Expression Levels of pH Regulatory Transporters in PDAC

Alongside the bioinformatic analyses of pH regulatory transporter expression changes in PDAC as presented in Paper I, the mRNA expression levels of each genes were analyzed using quantitative RT-PCR as described in Appendix A, in selected human PDAC cell lines MIA PaCa-2, Panc-1, BxPC-3 and AsPC-1 and normalized to the mRNA expression level of control healthy immortalized human pancreatic ductal epithelial cell line, HPDE. ANOVA statistical analyses revealed a marked variation in the gene expression of different SLC families among the different PDAC cell lines. Part of the data on this work has been previously published in an abstract (Kong et al 2013). For clarity, here the gene expression changes are explained additionally as an expression fold change calculated by PDAC/control HPDE ratio.

The AE2 mRNA expression level was found increased in all PDAC cells studied with a particularly significant elevation in Panc-1 with a fold change of 4.62. Previously it was reported that compound DIDS induces apoptosis in HA22T hepatocellular carcinoma cells overexpressing AE2 (Liu et al 2008). Thus it is reasonable to postulate that this compound may confer the same antitumour properties in PDAC with elevated AE2 expression. AE3 was found only significantly up-regulated in MIA Paca-2 (Figure 1.3A). Other SLC4 gene products include electrogenic NBCe1 (SLC4A4) and NBCe2 (SLC4A5) and electroneutral Na\textsuperscript{+}-HCO\textsubscript{3}{−} co-transporters NBCn1 (SLC4A7). NBCe1 was found to be significantly up-regulated in only BxPC-3 (9.04 fold change) whereas NBCe2 is exceptionally up-regulated in MIA Paca-2 with a fold change of 33.2. All PDAC cells except AsPC-1 showed up-regulation of NBCn1 expression levels with MIA Paca-2 having the highest with a fold change of 6.78. Furthermore, all PDAC cell lines studied demonstrated up-regulation of Na\textsuperscript{+}-dependent Cl/HCO\textsubscript{3}{−} exchangers NDCBE (SLC4A8). Of these, Panc-1 showed a particularly high elevation with a fold change of 63.19 relative to HPDE expression level. Collectively, the differential expression profiles of bicarbonate transporters
among the cell lines studied point to an interesting direction of specific targeted therapy according to individual subtype of PDAC.

When compared to HPDE, only NHE2 was detected to be up-regulated in Panc-1 (Figure 1.3B); all NHEs studied were up-regulated in BxPC-3, with NHE1 (2.87 fold change) and NHE8 (2.31 fold change) being significantly increased; in AsPC-1, NHE2 and NHE8 demonstrated significant up-regulation of 3.39 and 2.56, respectively (Figure 1.3B). NHE2 and NHE8 are significantly up-regulated though with a slight NHE1 decrease was detected in AsPC-1. This might suggest a conceivably more important role of these two isoforms compared to that of NHE1 in these cells.

The mRNA expression levels of both MCT 1 and MCT 4 from the monocarboxylate transporters of SLC16 family showed a down-regulation in the PDAC cell lines tested when compared to those levels in the control HPDE cells (Figure 1.3C), with one exception where Panc-1 displayed an increase in the expression level relative to that in HPDE (a fold change of 1.32). Further information will be discussed in Chapter 2 Section 2.2 in this thesis.

No notable change of the mRNA levels of the selected SLC26 genes (SLC26A4 and SLC26A6; Figure 1.3D) has been detected among the PDAC cell lines when compared to the control HPDE. This might be attributable to the inconsistency in the data acquired from experimental repetitions. More experiments would be required to verify the data already collected.

While ATP6V0A1 of the V-ATPase family did not show any appreciable differences in the expression levels in all the PDAC cell lines relative to HPDE (Figure 1.3E), the ATP6V0A3 gene expression was found to be significantly elevated with a 2.48 fold change in MIA Paca-2, 3.52 fold change in Panc-1, 2.93 fold change in BxPC-3 and the highest change in AsPC-1 of nearly 7-fold of the up-regulation. On the other hand, overall mRNA expression of ATP6V1B2 were detected to have decreased in PDAC when compared to HPDE, with one significant down-regulation detected in Panc-1.

Differential mRNA expression profiles of pH regulatory transporters in different PDAC cell lines might indicate the specific roles of each transporter played in the respective cell lines are based on a key assumption that the mRNA expression is informative in predicting protein expression and hence the functions of each protein, though such an assumption is hardly established. Nonetheless, differential mRNA expression profiles could, if further patterns would be established, advocate
the possibility as first line tumour risk factors or biomarkers in predicting the development of the respective PDAC subtypes.

The current availability of specific antibodies allows further investigation of protein expression of NHE1 and NHE8, as well as NBCn1 in PDAC cell lines by western blotting and immunofluorescence analysis as described in Appendix A. Overall, the protein expression levels of these transporters correlated to the mRNA expression levels demonstrated, suggesting minor post-transcriptional modifications of mRNA into proteins of these transporters. Up-regulation of NHE1 and NHE8 protein level were observed in BxPC-3 (Figure 1.4A and 1.4B). Immunofluorescence-staining demonstrated that NHE1 is localised on the plasma membrane of HPDE and PDAC cell lines (Figure 1.4D). On the other hand, MIA PaCa-1 and Panc-1 demonstrated significant NBCn1 up-regulation at protein level (Figure 1.4C) as compared to the expression level of HPDE. Immunofluorescence analysis revealed mostly cell surface localisation of NBCn1 in all PDAC cell lines studied, whereas strong cytoplasmic staining of NBCn1 was observed in HPDE. Further experimental investigations are needed to explain this observation. This might suggest a distinct role of NBCn1 or the presence of dissimilar trafficking mechanisms of this protein in cancer cells versus healthy epithelial cells.
Figure 1.3: pH regulatory transporter mRNA were differentially expressed in HPDE and PDAC cell lines. Relative mRNA expression levels of AEs and NBCs (A), NHEs (B), MCTs (C), transporters of SLC26 Family (D), and V-type H⁺ ATPases (E) in HPDE cells and PDAC cell lines (MIA PaCa-2, Panc-1, BxPC-3, AsPC-1) were assessed by qRT-PCR. Quantitative data were acquired as mean ± SEM using β-actin as a housekeeping gene calculated as described in Appendix A-Materials and Methods. Statistical analysis was performed with one-way ANOVA in Tukey-Kramer post hoc test. Data are shown as mean with S.E.M. error bars, of three independent experiments per cell line.
Figure 1.4: Protein expression of NHE1, NHE8 and NBCn1 in HPDE and PDAC cell lines. Protein expression of NHE1 (A), NHE8 (B) and NBCn1 (C) was assayed by Western blotting. Upper panels showed representative blots and lower panels show densitometric quantification of normalized NHE1, NHE8 and NBCn1 protein levels in PDAC cell lines to HPDE. Densitometric data is shown as mean with S.E.M. error bars, of three independent experiments per cell line. *p < 0.05 and **p < 0.01: Significantly different from the level in HPDE cell by one-way ANOVA with post hoc Tukey-Kramer test. D, Representative immunofluorescence images of HPDE cells and PDAC cell lines. Cells were paraformaldehyde-fixed and stained with antibodies against NHE1 (green) or NBCn1 (red), respectively. Cells were counterstained with DAPI (blue) for nuclei staining. Data are representative of three independent experiments per cell line. Scalebars: 20 µm.
1.5 References


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Chapter 2
Monocarboxylate Transporters
Chapter 2  Monocarboxylate Transporters (MCTs)

The monocarboxylate transporters (MCTs) belong to a family of transporters encoded by the human solute carrier 16 (SLC16) gene. Members of this transporter family are predicated to consist of 12 transmembrane helices (TM) with C- and N-terminal located within the cytoplasm. The helices are arranged in two 6 helix bundles which are linked by a large intracellular loop between TMs 6 and 7 (Figure 2.1) (Halestrap 2012, Halestrap et al. 2014). To date, 14 putative members have been identified in this family on the basis of sequence homologies and protein structure. Each isoform is distinguished by kinetic properties, tissue distribution and respective substrate specificity. Of these, only isoforms 1-4 (MCT1-MCT4) are proton-coupled symporters that mediate the transmembrane transport of single-carboxylate metabolites in an electroneutral transport mode of 1 proton: 1 monocarboxylate with different substrate affinities (Halestrap & Price 1999a). These MCT isoforms function to mediate both the influx and the efflux of monocarboxylates such as L-lactate, pyruvate and the ketone bodies (acetoacetate and D-β-hydroxybutyrate). The net direction of this facilitated diffusion is dependent on the concentration gradients of protons and monocarboxylates across the plasma membrane and no energy input is required (Halestrap & Meredith 2004a, Poole & Halestrap 1993).

![Figure 2.1: Predicted membrane topology of the MCTs. Adapted from Halestrap (1999).](image)

**MCT1.** MCT1 is ubiquitously distributed in human tissues. It is prominently (though not exclusively) expressed in heart, brain, liver, kidney, intestine, muscles, white adipose tissue, red blood cells, testis and placenta (Halestrap & Meredith 2004b, Price et al. 1998). It is the most well-studied and functionally characterized isoform of the MCT family largely because of its exclusive expression in erythrocytes and indeed widest tissue distribution as compared to other MCT isoforms.
(Merezhinskaya & Fishbein 2009). Though tissue distribution of MCT1 is ubiquitous, cellular localisation of this protein within specific tissues varies (Halestrap 2013b). For example, the expression of MCT1 is restricted to the apical membrane in the retinal pigment epithelium (Halestrap & Price 1999b); whereas in the stomach and intestine, MCT1 is robustly expressed on the basolateral surface of gut epithelial cells (Garcia et al 1995, Juel & Halestrap 1999). Interestingly, MCT1 is noticeably absent is the β-cell of the pancreas under physiological conditions. It has been demonstrated that under strict repression and a mutation in MCT1 promoter, MCT1 expression in this cell type would lead to a specific disease in humans which causes improper insulin secretion triggered by blood lactate during exercise bouts (Carneiro & Pellerin 2015, Otonkoski et al 2007).

MCT1 with an affinity intermediate between MCT2 and MCT4, is suitable for both efflux as well as influx of monocarboxylates (Halestrap 2013a). While initial studies focused on the lactate transport by MCT1, successive studies discovered that the substrate specificity of MCT1 was much less specific than initially thought (Broer et al 1998, Halestrap & Meredith 2004b, Morris & Felmlee 2008). Lactate transport with MCT1 is stereoselective, with a ten-fold preference for L-lactate ($K_m$ value 3–5 mM) over D-lactate (Broer et al 1998). However for β-hydroxybutyrate, whose normal metabolite is in D-isomer, MCT1 demonstrates no stereospecificity (Halestrap 2013a). MCT1 was also found to transport branched oxo-acids with a greater affinity than for the transport of short-chain (C2-C5) monocarboxylic acids such as lactate (Broer et al 1999, Halestrap 2012).

As early as in the 1970s, Halestrap and Denton (1974) had discovered the compound α-cyano-4-hydroxycinnimate (4-CIN or CHC) with a $K_i$ value of 64 µM for MCT1. This inhibitor however was reported to be lack of specificity as it was found to also inhibit other transporter such as the mitochondrial pyruvate transporter. This widely used MCT inhibitor 4-CIN, has in fact been reported to be two orders of magnitude more potent at inhibiting pyruvate transport into mitochondria than it is at inhibiting lactate transport (Halestrap 1976). Later, other inhibitors such as stilbene-derived compounds including 4, 4-Odiisothiocyanostilbene-2,20-disulphonate (DIDS), which cause rapid reversible MCT1 inhibition, and 4,4-O-dibenzamidostilbene-2,2O-disulphonate (DBDS), were also discovered to inhibit MCT1 with $K_i$ values of 2-500 µM (Poole & Halestrap 1991). Nonetheless, the non-specificity of these agents has rendered these unsuitable for a specific inhibition of MCT1 (further discussion in Section 2.2).

**MCT2.** Unlike other MCTs, the sequence of MCT2 appears to be less conserved across species, and the tissue expression profile varies among species. Relatively low expression levels of MCT2 were detected in human tissues except for testis (Garcia et al 1995, Halestrap 2013a, Jackson et al
1997). This MCT isoform is generally categorized into the same cluster with MCT1 in phylogenetic analysis due to their range of substrate specificity and affinities detected in mammals. Characterization of MCT2 in Xenopus laevis oocytes by Broer and colleagues (Broer et al 1999) revealed that MCT2 has a 5-10 fold higher affinity for most substrates than MCT1 and is considered to be the highest among all MCTs. Its $K_m$ values for L-lactate and pyruvate were found to be about 0.1 and 0.74 mM, respectively, in comparison to the values of around 1 and 3.5 mM for MCT1. This feature can be particularly important in the brain where MCT2 is specifically expressed in the post-synaptic terminals of neurons to facilitate lactate uptake for oxidative metabolism (Bergersen 2007, Pierre et al 2002).

**MCT3.** This isoform was initially identified by Philp and colleagues (Philp et al 1998) as a developmentally expressed protein in the chick retinal pigment epithelium. It displays a tissue-specific expression pattern whereby its expression in human and mouse is restricted to the choroid plexus epithelia and retinal pigment epithelium with a proposed role in enabling the transport of glycolytically-derived lactic acid out of the retina (Bergersen et al 1999, Philp et al 1998).

**MCT4.** On contrary to MCT1, MCT4 is expressed particularly strongly in tissues with high glycolytic activities such as white skeletal muscle, astrocytes and white blood cell, suggesting that the major role of this transporter is lactate efflux (Dimmer et al 2000, Halestrap 2013b, Price et al 1998, Wilson et al 1998). This is supported further by a high expression in the placenta where it is implicated in the rapid transport of lactate from the foetal into the maternal circulation (Halestrap & Meredith 2004b). Although MCT4 demonstrates similarities to MCT1 with substrate and inhibitor specificity, it is known as a low affinity transporter for a range of monocarboxylates and inhibitors, with $K_m$ and $K_i$ values higher by a factor of between 5 and 10 (Dimmer et al 2000, Fox et al 2000). For example, the $K_m$ values for L-lactate and pyruvate were 28 and 150 mM, respectively (Halestrap 2013a). This relatively low affinity of MCT4 constitutes a good physiological rationale, proposed to retain pyruvate in a cell that relies on glycolysis in order to remove NADH produced in glycolysis by reduction of pyruvate to lactate, failing so, glycolysis would cease; also, a low affinity of MCT4 prevent further systemic lactic acidosis following an excessive lactate production during skeletal muscle fatigue (Halestrap 2013b, Juel & Halestrap 1999).

**Basigin/CD147.** MCTs require ancillary proteins to be correctly expressed at the plasma membrane. This was evidenced from that both MCT1 and MCT4 were retained in the Golgi apparatus or endoplasmic reticulum and both failed to reach the plasma membrane when these proteins were over-expressed in various cell lines. The proteins were however properly targeted to the plasma
membrane when co-expressed with basigin/CD147 (Kirk et al 2000, Wilson et al 2005). Also known by other names EMMPRIN/OX47/5A11, basigin is an integral plasma membrane glycoprotein of the immunoglobulin superfamily with metalloproteinase-inducing ability (Iacono et al 2007). In addition, by co-immunoprecipitation of MCT1 or MCT4 with basigin, as well as by demonstration of co-migration of both basigin and MCT1 into a cap at one end of a cell when treated with a cross-linking antibodies against basigin (Kirk et al 2000), MCTs were proven to be strongly associated with basigin. While MCT1, MCT3 and MCT4 were found to preferably associate with the mature, glycosylated form of basigin, in order for these MCTs to be expressed and be functional on either plasma or mitochondrial membranes (Deora et al 2005, Philp et al 2003); embigin (also known as gp-70), a cell-adhesion molecule of the immunoglobulin superfamily, is the preferred binding partner of MCT2 (Kirk et al 2000, Wilson et al 2005).

Basigins are found to be highly enriched on many human epithelial cancer cells including malignancies of the pancreas (Riethdorf et al 2006, Schneiderhan et al 2007). In our study, we demonstrated by western blotting and immunofluorescence analysis that basigin was expressed in HPDE and all PDAC cell lines applied and colocalised strongly in these cells with MCT1 and MCT4 at the plasma membrane (Paper II, Figure 2A and 2B; Appendix C). Our findings are consistent with the study performed by Zhao and colleagues (2001) where MCT1 was reported to localise exclusively to the plasma membrane in normal pancreas, colocalising with basigin. Schneiderhan and co-workers (2009) also showed silencing of basigin reduces the expression of MCT1 and MCT4 in PDAC cell lines and functionally, a reduction in lactate transport kinetics was observed following the basigin/CD147 silencing. The expression of basigin on the cell surface is also dependent on the co-expression with an MCT protein. It was shown that after MCT4 knockdown of MDA-MB-231 cells, abrogation of fully glycosylated basigin was detected and the core-glycosylated form was restricted to the endoplasmic reticulum (Gallagher et al 2007). In other experiments, knockdown of MCT1 in colorectal adenocarcinoma Caco-2 cells, the accumulation of the immature, core-glycosylated form and disappearance of the mature glycosylated form of basigin occurred with no difference in the mRNA expression (Deora et al 2005). Added to their role in enabling lactate transport activities by MCTs, several other roles of basigin have been implicated. Various studies have reported their interaction with integrins, calveolin-1 and cyclophilins, and further their implications in tumour cell migration and invasion in recent years due to these intriguing interactions (Bonuccelli et al 2009, Liao et al 2011a, Takahashi et al 2012, Yurchenko et al 2006, Zhao et al 2013). It is also well established that soluble basigin/CD147 (also known as extracellular matrix metalloproteinase inducer, EMMPRIN) acts in a paracrine fashion on stromal cells in tumour
microenvironment to stimulate the production of MMPs, which in turn contributes to tumour metastasis (Hanata et al 2007, Tang et al 2004, Toole 2003, Wu et al 2014).

**Carbonic Anhydrase (CA) and MCTs.** Activities of several pH regulatory transporters including AE1, NBCe1 and NHE1 have been reported previously to be improved through the interaction with carbonic anhydrase II (CAII), an enzyme that catalyzes reversible hydration of carbon dioxide (Becker & Deitmer 2007, Li et al 2002, Pushkin et al 2004, Vince & Reithmeier 1998). Later, Becker and colleagues also reported that the lactate transport activity of both MCT1 and MCT4 could also be augmented by the interaction with CAII. They also demonstrated that this augmentation is indeed not dependent on the catalytic aptitude of CAII on the reversible conversion of CO$_2$ and HCO$_3$-/H$^+$, since the augmentation persisted upon inhibition/inactivation of CAII catalytic activity as well as upon the removal of CO$_2$/HCO$_3$-. More recently, they found that extracellular CAIV increased transport activity of MCT1 and MCT4 when heterologously co-expressed in oocytes, and further the cooperation of intracellular CAII with extracellular CAIV was shown to enhance MCT activity even further (Klier et al 2014). Interestingly, studies performed by the same group also observed functional interaction between MCT1 and sodium bicarbonate co-transporter (NBC) stimulated the lactate transport activities, as seen when MCT1 was co-expressed with NBC the in *Xenopus* oocytes (Becker et al 2004).

### 2.1 Monocarboxylate Transporters in Cancer

As previously noted, tumour cells undergo a series of metabolic adaptations to develop the phenotypes essential for their survival within the tumour microenvironment. Among which, expression of membrane pH regulators including those discussed in Chapter 1 Section 1.4.1 are often seen up-regulated in these tumoural cells. Exploring the dys-regulated expression profiles of these transporters involving in tumour pH homeostasis might help in discovering essential biomarkers and further translate the knowledge in the clinic context. In highly glycolytic cells such as tumour cells, the efflux of lactate is mediated via MCTs, in particular MCT1 and MCT4 (Halestrap 2012). These transporters not only facilitate the efflux of lactate, which act as both a metabolic fuel and a signalling molecule beneficial for cancer progression; at the same time, they contribute to the preservation of the intracellular pH by co-transporting a proton along with lactate for malignant cells survival in acidic tumour microenvironment (Baltazar et al 2014).

The expression of MCTs in human tumours has been extensively reviewed by Pinheiro and coworkers (Pinheiro et al 2012). Briefly, increased levels of MCT1 have been discovered in breast
(Pinheiro et al 2010a), colorectal (Pinheiro et al 2008), gastric (de Oliveira et al 2012), as well as in cervical cancer (Pinheiro et al 2009b), though dissimilar findings had also been reported particularly in colon carcinoma and breast cancer. MCT4 expression is significantly increased in renal cell carcinoma (Kim et al 2015) and cervical cancer (Pinheiro et al 2009a); yet it was reported to be down-regulated in gastric cancer (Pinheiro et al 2009c). Expression of MCT2 and MCT3 are found as the major isoforms in glioblastoma with no MCT4 being detected in any tumour tissues (Mathupala et al 2004). Contradictory findings on MCT expression in tumours have been reported by other studies on the same tumour types. For instance, earlier studies reported the down-regulation of MCTs in colon carcinoma (Le Floch et al 2011, Ritzhaupt et al 1998) and breast cancer (Asada et al 2003), opposite to the latter reports stating that these transporters are in fact highly up-regulated in these tumours (Pinheiro et al 2010a, Pinheiro et al 2010b). No conclusions can be drawn from the inconsistency between these results, though the differential expression of MCTs among tumours might reflect the different metabolic profiles in different tumours (Baltazar et al 2014).

Current work focuses on MCT1 and MCT4 in PDAC. MCT2, although this has previously been implicated in gliomas (Colen et al 2011, Mathupala et al 2004), western blotting revealed the lack of this protein expression in PDAC cell lines applied (data not shown). MCT3 is excluded due to their restricted distribution as described above and thus irrelevant to current work in pancreatic cancer.

At present we applied bioinformatics analysis on publicly available cancer genome databases to elucidate the expression profiles of MCTs in PDAC (see Paper I). Previous comparative transcriptomic data analyses of six cancer types revealed transcriptional up-regulation of MCT1 and MCT4 in all the cancer types studied, except for prostate cancer in which no particular increase of MCT gene expression has been detected (Xu et al 2013). From our data-mining analyses, an increased mRNA level of MCT4 in PDAC has been reported from all three indicated databases, namely Oncomine, The Cancer Genome Atlas (TCGA) and the Pancreatic Expression Database (PED); also mRNA level of MCT1 has been reported to be up-regulated in PDAC from two of the three databases. However, both mRNA and the protein expression levels of MCT1 and MCT4 were detected to be generally lower in the PDAC cells lines studied when compared to the control cell line HPDE; except for MCT4, where a higher expression level of mRNA and protein was detected (Paper II, Figure 1). This result is in accordance with an individual study reported by Makawita and colleagues (2011) in PED, where the MCT4 expression level was particularly high in Panc-1, with a fold change of 11.05 as compared with the expression in HPDE cell lines. The lower expression of MCT1 and MCT4 in current PDAC cell lines studied relative to the expression in our control
HPDE is speculated to be due to the construction of this immortalized HPDE cell lines, which make the cell lines hardly to be considered as a good mimic as the normal epithelium in regard to many features including their metabolisms, and hence their MCT expression profile. Although, as previously mentioned, broad analyses on the expression of MCTs in cancer tissues by Pinheiro et al (2012) revealed contrasting observations in which overexpression of one or more MCT isoforms was detected in some tumours, while an opposing trend was reported for other cancer types.

Evidences in transcriptional regulation of MCT expression has been presented. Transcriptional regulation of the expression of MCT1 has been demonstrated in various tissues in response to various cues (reviewed in (Doherty & Cleveland 2013, Halestrap & Wilson 2012)). It has been corroborated that histone methylation at the promoter and miRNAs contribute to the silencing of MCT1 in pancreatic β cells (Pullen et al 2011, van Arensbergen et al 2010). Also, MCT1 promoter methylation of CpG islands and transcriptional repression have been reported in the invasive breast cancer cell line MDA-MB-231 (Asada et al 2003, Doherty & Cleveland 2013). The major transcriptional regulatory mechanism responsible for the up-regulation MCT4 expression in all tissues and cell lines is hypoxia inducible factor-1α (HIF-1α) mediated mechanism (Halestrap 2013a, Ord et al 2005). Under hypoxic conditions as seen in a solid tumour, HIF-1α binds to two hypoxia response elements (HREs) in the MCT4 just upstream of the transcription start site and stimulates the activity of MCT4 promoter. The expressions of MCT1 and MCT2 on the other hand are not affected by hypoxia due to the lack of the corresponding HREs in their promoters (Halestrap 2013b, Ullah et al 2006).

Elevated expression of MCTs in cancers provides a therapeutic repertoire whereby disabling these transporters functionally could represent promising strategies in anti-cancer treatment. Previous studies have adapted this approach to exploit the potential using several classical MCT inhibitors identified in both in vitro and in vivo studies. These inhibitors include the substituted aromatic monocarboxylates 4-CIN or CHC, DIDS and DBDS, as well as bioflavonoids like quercetin and phloretin (Halestrap & Price 1999b). The sensitivities of the different MCT isoforms towards these inhibitors vary according to their affinity for the substrates. These agents are likely promiscuous, being neither selective nor specific to MCTs, as they often also target other molecules. For instance, quercetin and phloretin also act as a potent inhibitor to the anion exchanger isoform 1 (AE1) and other membrane transport processes (Halestrap & Meredith 2004b). Thus, conclusion should be drawn with caution when applying these agents in search of the therapeutic significance in cancers.

In recent years, a new class of small molecule compound, AR-C155858, has been developed by Astrazenca. This compound has been reported to exhibit a cytosolic \( K_i \) value of 2.3 nM, specifically
targeting both MCT1 and MCT2, yet be inactive towards MCT4, when expressed in oocytes of the frog *Xenopus laevis* (Ovens et al 2010). Another similar compound targeting MCT1, named AZD3965, has also been developed by the same company and is currently under phase I/IIa clinical evaluation in the United Kingdom.

### 2.1.1 Lactate Transport via MCTs in Cancer

As the name suggests, physiologically MCTs function as the transporters facilitating transmembrane transport of monocarboxylates with L-lactate, an anionic dissociated form of lactic acid at a physiological pH, being quantitatively the most important (Halestrap 2013a). Two studies have provided evidences for MCTs-mediated lactate shuttling within some tumours, in which both oxidative tumour cells and endothelial cells lining tumour blood vessels can adopt lactate released by glycolytic tumour cells through MCTs. In this model, a form of metabolic symbiosis is created where the glycolysis-derived lactate produced in the hypoxic centre of the tumour is exported thru the low-affinity lactate transporter MCT4, and later this exogenous lactate is taken up by peripheral oxidative tumour cells occurs through the high-affinity lactate transporter MCT1 (Sonveaux et al 2008, Vegran et al 2011).

The effects of MCT inhibition either by pharmacological means or gene-targeting approaches have been reported in many individual studies to have a profound impact on lactate transport in various cancer types, consequently impeding their malignancy development. Studies include those demonstrated a decrease in lactate efflux with 4-CIN in glioma (Miranda-Goncalves et al 2013) and in breast cancer cells (Morais-Santos et al 2014). Recent report from Polanski and colleagues (2014) showed AZD3965 treatment increased intracellular lactate in small lung cancer cells. It has also been reported that lactate export was repressed in RAS-transformed fibroblasts when MCT1/2 is inhibited with AR-C155858 (Le Floch et al 2011).

Lonidamine, a small molecule inhibitor selective for MCT1, has also been applied and reported to promote a decrease in initial glucose uptake and lactate formation in breast cancer cells (Benhorin et al 1995). Furthermore, Morais-Santos and coworkers (2014) demonstrated that lonidamine and quercetin were able to decrease lactate production in breast cancer, in a manner similar to that of 4-CIN. An earlier study using the same drug reported that the drug action is indeed involved in the inhibition of lactate efflux and intracellular acidification in glioma cultured cells (Ben-Yoseph et al 1998).
4-CIN and AR-C155858 were applied in the current studies (Paper II). 4-CIN is a widely applied inhibitors in studies on MCTs which has also been demonstrated to harbour a high $K_i$ (0.5 mM) against lactate transporters (except MCT3) in tumours (Philp et al 2001, Spencer & Lehninger 1976). AR-C155858 was selected due to their specificity as mentioned earlier in Section 2.1.

In line with the abovementioned studies, we showed that lactate influx in PDAC cells is strongly inhibited by 4-CIN while a lesser effect was observed when using AR-C155858. For specificity of MCT inhibition using proposed drugs or compounds is often limited, the RNA interference (RNAi) approach has also been applied to further elucidate the MCT inhibition effects on the lactate transport in PDAC cells. In accordance with our results, the silencing of MCT1 has been reported to reduce lactate efflux in glycolytic glioma cells (Miranda-Gonçalves et al 2013). We demonstrated in this study that the silencing of either MCT1 or MCT4 reduced lactate uptake in AsPC-1 cells, whereas only MCT4 knockdown reduced lactate uptake in BxPC-3 cells, though the effect is not significant. Interestingly, we also found that our control non-tumourous cell line HPDE exhibited a low lactate flux capacity, consistent with the observation that a considerable fraction of MCT1 and MCT4 staining did not localise to the plasma membrane in these cells, suggesting that these transporters were not available for lactate uptake. We speculated also that other MCT isoforms may contribute to the lactate influx capacity of PDAC cells, since silencing of either MCT1 or MCT4 is not capable of abolishing the transportation of lactate into the cells. Further studies would be carried out taking into consideration of the action of other potential but yet explicated transporters which are involved in lactate transport, namely the SMCT1 (SLC5A8) and SMCT2 (SLC5A12) of the SLC5 family responsible for transportation of monocarboxylates in a sodium dependent manner (Gopal et al 2007, Gupta & Massague 2006, Gupta et al 2006, Martin et al 2006).

2.1.2 Roles of MCTs in Cancer Motility and Invasion

Metastasis is a dynamic, complex and multistage process principally accountable for the mortality and morbidity of cancer patients (Gupta & Massague 2006, Steeg 2006). These series of processes comprise the events of firstly, loss of cellular adhesion, then increased migration ability and invasiveness, intravasation and survival in the circulation, extravasation, and lastly colonization at a distant site (Chambers et al 2002, Fidler 2003). Through this chain of well-defined cascades, malignant cells are able to spread from the tumour of origin to proliferate and form secondary tumours in distant organs.
Gatenby and Gawlinski (1996) proposed that the invasion mode of tumour into normal tissue is primarily due to the acidic pH in the surrounding space of a tumour. Due to the altered metabolism of tumours and excess acid production and hence the decrease in the intercellular pH, several crucial biochemical processes of the surrounding normal cells could be disrupted, and eventually the death of these cells resulted. This model proposed that tumour cells are then able to grow and develop through cell division by filling the hypo-cellular gap created after the death of adjacent normal cells (Gatenby & Gawlinski 1996). Successively, several studies described further how tumour acidity could facilitate metastasis by providing a favourable acidic tumour microenvironment for extracellular matrix (ECM) degradation and remodeling. In brief, acidic extracellular pH (pH 6.5–6.9) in malignant tumours promotes the activity of various cathepsin proteinases, matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator as often these enzymes involving in the ECM degradation and remodeling have a low pH optima (Kato et al 2007, Koblinski et al 2000, Robey et al 2009, Rofstad et al 2006, Rozhin et al 1994). A low pH also contributes to MMP activation by promoting the proteolytic cascade which converts pro-MMPs to active MMPs (Chambers & Matrisian 1997).

MCTs contribute largely to the tumour extracellular acidification and therefore tumour cell invasion. It was found earlier that cellular expression levels of MCT1 and MCT4 correlated with invasion activity of 11 human lung cancer cells and that MCTs inhibition reduced both migration and invasiveness in these cells (Izumi et al 2011). Interestingly, this study also showed that knockdown of MCT 1 and MCT4 potently decreases tumour cell migration and invasion to a larger extent than basigin/CD147 knockdown, suggesting a MCTs-mediated mechanism which is not overlapping the previously suggested pro-metastatic actions of basigin/CD147 (Curtin et al 2005, Liao et al 2011b). Their results however also showed that the enzymatic activity and the expression of both MMP-2 and MMP-9 were not correlated to the invasion activity in lung cancer cell lines. Another study showed that silencing of MCT4 decreased in vitro migration of breast cancer MDA-MB-231 cells, suggested through mechanisms involving the interaction between MCT4 and β1-integrin (Gallagher et al 2009, Gallagher et al 2007). A more recent study also pointed that migration and invasion of breast cancer cells were reduced by the inhibitors quercetin and lonidamine (Morais-Santos et al 2014).

Pancreas cancer is a highly aggressive and invasive malignancy, with almost all patients presenting with metastasis at the time of diagnosis or with local invasion involving major blood vessels (Philip 2011). The metastatic event is likely to be responsible for the poor survival rate of this malignancy (Farrell et al 1997). To our knowledge, there are few studies into the roles of MCTs in PDAC
motility and invasiveness. Seeing MCT involvement in the acidosis of tumour microenvironment as discussed, it is thus rational to speculate that MCTs play a major role in this aggressive phenotype of pancreatic cancer. Metastasis fundamentally involves migration and invasion elicited by the movement of cells from one site to another. Here we described the contribution of MCT activity to motility and invasiveness in PDAC cells with a scratch-wound migration assay to monitor cell movement (Paper II, Figure 4), and with Boyden chamber invasion assay which monitors cell movement through a layer of matrigel (Paper II, Figure 5) (Deer et al 2010). Stahle and coworkers (2003) demonstrated that Panc-1 cells had a 5-fold greater motility than BxPC-3 cells, a result dissimilar to what we have observed, in which BxPC-3 was found to be the fastest moving cell line in closing the wound in the wound-healing migration assay (Paper II, Supplementary Figure 2).

Collectively, in current work we observed that MCT1 inhibition by AR-C155858 had no detectable effect on migration of PDAC cells in wound-healing assays (Paper II, Figure 4C); whereas 4-CIN tended to reduce the motility of all cell types, and BxPC-3 in particular, the effective magnitude is similar to that of MCT4 knockdown (Paper II, Figure 4D and 4F). In the Boyden chamber assay, we demonstrated that BxPC-3 is the most invasive cell line followed by Panc-1 amongst all the cell lines tested (Paper II, Figure 5); similar to that being observed by Menke and colleagues (2001) in which Panc-1 and BxPC-3 demonstrated great invasiveness, though the migration of Panc-1 cells was reported to be greater than BxPC-3 cells on transwell filters coated with collagen I. The dissimilar observations were probably due to the different adhesive ability and interaction of the cells with a different reconstituted extracellular matrix, i.e. matrigel (composed of a mixture of laminin, type IV collagen, entactin and heparin sulfate), as applied in present study.

4-CIN application inhibited invasiveness of the cell lines tested. While MCT1 inhibition with AR-C155858 and MCT1 silencing showed a nominal effect on the BxPC-3 cell migration, 4-CIN treatments exerted significant inhibition towards the invasiveness of Panc-1 and BxPC-3 cells. Substantial reduced invasiveness of these cells was also observed following the silencing of MCT4. Furthermore, we studied the invasiveness of BxPC-3 in a 3D spheroid cell model, a model mimicking the solid tumours in vivo. We found that inhibition of either MCT4 (and to a lesser extent MCT1) or MCT1 and MCT4 in combination, reduced the invasiveness in 3D outgrowth assays. Taking these data together, we speculated a more important role of MCT1 in PDAC cell invasiveness than that of 2D-migration seeing the modest effect of AR-C155858 treatment and MCT1 silencing on PDAC motility. Similar to our findings, Izumi and colleagues (2011) demonstrated in A110L lung tumour cells a reduced invasiveness of these cells without their migration capability being affected after knockdown of MCT1 and MCT4. Additionally, upon both 4-CIN treatment and siRNA
transfection, we observed that BxPC-3 lost their usual tightly packed sheet form and the cells appeared as dispersed single cells on the other surface of the filter in a Boyden chamber. However no obvious change was detected with AR-C155858 treatment. This might indicate that the membranes of these cells were disturbed upon treatment, which conceivably interferes with their migratory machineries. Further studies need to be performed to explicate whether this observation was indeed a MCTs-involved event or it was due to the cytotoxicity natures of the agents applied.

2.1.3 Roles of MCTs in Cancer Proliferation and Survival

One of the critical events underlying the idiopathy of all cancers is the unbridled proliferation and survival of tumoural cells. Targeting and impeding the key players associating with the proliferative capability of these cells underscore potent therapeutic effects in treating cancer.

Several in vitro studies indicated that cell proliferation and cell viability of various cancer cells could be repressed by applying established MCT inhibitors such as 4-CIN and lonidamine (Fang et al 2006, Hamdan et al 2013, Miranda-Goncalves et al 2013, Morais-Santos et al 2014, Wahl et al 2002). Importantly, 4-CIN has also been reported to impede the tumour growth and render tumour cell sensitization to radiation in in vivo models, namely mouse xenografts and chick chorioallantoic membrane (CAM) models (Colen et al 2011, Miranda-Goncalves et al 2013, Sonveaux et al 2008).

In current retrospective study, we aimed to assess the impacts of MCT1 and MCT4 on the proliferation of PDAC cells via specific silencing of these transporters. The cell proliferation fractions were evaluated by BrdU incorporation assay and IncuCyte ZOOM® live cell imaging proliferation assay as described in Appendix A. Overall, all 3 PDAC cell lines treated with siMCT4 have decreased cell proliferation as compared with control mock-transfected cells (Figure 2.2), among which a statistical significant decreased proliferation was detected for Panc-1 treated with siMCT4 (Figure 2.2A). siMCT1 treatment resulted in no significant proliferative differences when compared to mock siRNA-transfected cells among indicated cells. To further address the effects of MCT1 and MCT4 silencing on PDAC cells for a longer span of time, we conducted cell proliferation assay using Incucyte ZOOM™ system, a live cell imaging system which allows hourly monitoring of cell growth by using non-label cell monolayer confluence approach. We observed that Panc-1 demonstrates the highest proliferation rate among three cell lines. Both MCT1 and MCT4 silencing effects were sustained on indicated PDAC cells over a period of more than 120 h of recordings (Figure 2.3). Consistence with BrDU incorporation assay, we found that siMCT4 treatment resulted in a larger inhibitory effect on cell proliferation than siMCT1 treatment amongst all 3 cell lines. In particular, proliferative ability of AsPC-1 cells was almost diminished with siMCT4 treatment.
Cell proliferation of BxPC-3 was only slightly inhibited after siMCT1 and siMCT4 treatments (Figure 2.3B). Taken together, these results provide evidence that both MCT1 and MCT4 are involved in the proliferation of PDAC cells where MCT4 has a bigger impact. While the underlying mechanisms of current proliferation inhibition on PDAC cells after MCT1 and MCT4 silencing were yet studied, previously Zhu and colleagues (2014) demonstrated that siRNA-mediated silencing of MCT4 inhibited the proliferation of oral squamous cell carcinoma cells and the proliferation inhibition was associated with two main cell proliferation pathways, Akt and MEK-ERK. More recently, Gao and colleagues (2015) showed that MCT4 silencing decreased phosphorylation of Akt in hepatocellular carcinoma. We conjecture our current observation in PDAC cells may be elicited via similar signalling mechanisms. Further experiments should revise various signalling molecules associated with the cell proliferation mechanisms to endorse current speculation.

![Graphs showing cell proliferation inhibition](image)

**Figure 2.2:** Proliferation of PDAC cells is inhibited by silencing of MCT1 and MCT4 in BrdU incorporation assay. All indicated cell lines PanC-1 (A), BxPC-3 (B) and AsPC-1 (C) were transfected with siRNA against MCT1 or MCT4, or non-silencing mock control, respectively, and subsequently assayed for BrdU incorporation at 48 h post transfection as described in Appendix A. Data are shown as mean ± S.E.M. of 3-5 independent experiments per condition. Significant differences between non-silencing mock control and siMCT1 or siMCT4 treated cells are indicated with asterisks (*) by one-way ANOVA with post hoc Tukey-Kramer test.
Figure 2.3: Proliferation of PDAC cells is inhibited by silencing of MCT1 and MCT4 in Incucyte ZOOM® cell proliferation assay. PDAC cell lines were transfected with siRNA against MCT1 or MCT4, or non-silencing mock control, respectively and re-seeding into 96-well plate before registering into the Incucyte ZOOM® real-time video imaging system. Figure showed representative growth curves generated from confluence measurements of Panc-1 (A), BxPC-3 (B) and AsPC-1 (C). Independent experiments were repeated for 3-4 times. WT: non-treated cells.
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Chapter 3
Vacuolar-type H\(^+\)-ATPases
Chapter 3  Vacuolar-type H⁺-Adenosine Triphosphatases (V-ATPases)

The vacuolar-type H⁺-adenosine triphosphatases (V-ATPases) are ubiquitously expressed, ATP-dependent proton pumps which can be found in all eukaryotic cells and in numerous bacteria. Among eukaryotic cells, the structure of these proton pumps is greatly conserved from yeast to human, as a large enzymatic complex with a molecular mass exceeding 850 kDa (Nishi & Forgac 2002). V-ATPases transport protons across membranes and acidify intraorganelle compartments. In some cases, these transporters translocate the protons across the plasma membrane of cells and involve in the regulation of cell pH\textsubscript{i} and pH\textsubscript{e}.

3.1 Structure of V-ATPases

Structurally, V-ATPases are heteromultimeric complexes that work as molecular rotary motors. They are composed of a cytosolic catalytic $V_1$ domain responsible for ATP hydrolysis, and an integral, membrane bound $V_0$ domain. Each domain comprises different subunits with various copy numbers (Figure 3.1). The catalytic $V_1$ complex consists of eight subunits $A_3B_3CDEFGH$ (numbers designate the putative stoichiometry of the subunits) with the core assembling a hexameric arrangement of alternating A and B subunits, playing a role in ATP-binding and hydrolysis. Other subunits include two copies of E and G subunits which act as the stator of the complex, one copy each of regulatory C and H subunits, and one copy of D and F subunits that form a central rotor axle. The $V_0$ domain participate in proton translocation across the membranes consists of a ring with five subunits of $ac_{de}$ with the proteolipid subunit $c$ and its possible isoforms $c'$ and $c''$ (no $c'$ has been found in mammals) located next to subunits $a$ and $e$ (Wilkens & Forgac 2001). The C, E, G, H, and $a$ subunits have two to four isoforms each (Sun-Wada et al 2004). These subunits has been suggested to associate with the reversible

Figure 3.1: Schematic representation of V-Atpase structure and subunit composition. $V_1$ domain composed of 8 subunit (A-H) is responsible for ATP hydrolysis, and integral $V_0$ domain composed of at least 5 subunits ($ac_{de}$) is responsible for proton translocation. Adapted from Forgac (2007b).
disassembly/reassembly regulatory mechanism specific to the V-ATPase in yeast and tobacco hornworm (Forgac 2007a).

In the V₁ domain, subunits A present the ATPase hydrolytic activity, while subunits B are thought to be regulatory and interact with the actin cytoskeleton (Holllanday et al 2005, Holliday et al 2000). Briefly, the free energy released from ATP hydrolysis by subunit A is converted into a rotational movement of the central stalk formed by subunits D and F of V₁ and subunit d of V₀, causing the transmembrane cc’’ subunit ring as the torque to rotate around the DFd stalk, and actively translocates possibly up to two protons per ATP consumed in a single 360° clockwise rotation. (Nakanishi-Matsui et al 2010). The multiple peripheral stalks comprised of subunits C, E, G, H, the N-terminal domain of subunit a surface remain stationary and serve as a stator to prevent the rotation of the A₃B₃ hexamer during ATP hydrolysis (Lu 2012), a mechanism which is important during proton translocation via the interplay of two hydrophilic hemichannels residing in subunit a and an acidic amino acid residues residing in each of the c subunits (Holllanday et al 2000, Inoue & Forgac 2005). Potent plecomacrolide V-ATPase inhibitors bafilomycin A1 and concanamycin have been found to selectively bind to the subunit c of V₀ domain, thereby causing the inhibition of proton transportation by mechanically perturbing rotation of the b/c-ring (Bowman & Bowman 2002, Huss et al 2002). Bafilomycin A1 has also been revealed to bind to subunit a (Wang et al 2005, Zhang et al 1994). However, clinical usage of these inhibitors has been limited due to their lack of cell-type or tissue specificity. Inhibitors selective for specialized V-ATPases like those found in the plasma membrane of osteoclasts or intercalated cells of the kidney have not been identified (see Section 3.2). As their mechanism of action is based at the specific and highly conserved c subunit across species and since there are no isoforms in humans, specific inhibition targeting V-ATPases in different forms is unlikely (Hesselink et al 2008, Huss et al 2002, Xie et al 2004). Thus, the potential application of these inhibitors application in a clinical context such as cancer therapy has been hitherto hindered.

V-ATPases are constitutively present in various endomembranous systems including Golgi-derived vesicles, lysosomes, endosomes and phagosomes. There they responsible for the acidification of these intracellular organelles. This V-ATPases-mediated acidification has been implicated in several critical physiological processes in the organelles, including proton-coupled transport of small molecules, receptor recycling, proper protein trafficking via endosomal compartments, zymogen activation, pro-hormone synthesis, and intracellular microbial degradation (Stevens & Forgac 1997). In addition, V-ATPases have also been associated with functions such as membrane fusion and virus entry (Kielian et al 2010). Apart from these
implications in intracellular organelles, expressions of V-ATPases have also been detected in some cells at the plasma membrane, where they serve several functions.

3.2 Physiological Functions of V-ATPases in Plasma Membrane

Cellular plasma membrane V-ATPases principally carry out cell type-specific roles, including renal H⁺ secretion, cytoplasmic pH homeostasis, bone reabsorption, sperm maturation and tumour metastasis.

In the distal tubule and collecting duct of the mammalian kidney, V-ATPases are located at the apical membrane of type A intercalated cells and here, they function in proton secretion into the renal fluid; while type B intercalated cells responsible for bicarbonate secretion contain V-ATPases localised between the apical and basolateral membranes (Brown et al 1987, Schulz et al 2002, Wagner et al 2004). Together they maintain the acid-base balance in the kidney.

In macrophages, plasma membrane V-ATPases aid in sustaining a neutral cytoplasmic pH (pHₖ) to retain the cell functionality under conditions of substantial acid load, such as in the local environment of an infection or tumor; in neutrophils, plasma membrane V-ATPases eliminate protons generated during respiratory burst. (Nanda et al 1996, Stevens & Forgac 1997, Swallow et al 1990). V-ATPase expression in neutrophils has also been associated in apoptotic mechanisms (Gottlieb et al 1995).

Osteoclasts represent a non-epithelial type of V-ATPase–rich cell (Gluck 1992). The process of bone reabsorption required acidic pH in the enclosed extracellular space (Figure 3.2C, in red) created by osteoclasts, both for increasing the activity of secreted hydrolases as well as for the solubilization of bone matrix. This osteoclast-mediated extracellular acidification is achieved by V-ATPases which are targeted to the domain of the osteoclast plasma membrane surrounding the extracellular compartment (Blair et al 1989).

Plasma membrane V-ATPases has also been identified to control motility and maturation of sperm by stabilising the sperm medium (Breton et al 1996, Brown & Breton 2000). High density of V-ATPases was found in the apical membrane of cells which line the epididymis and vas deferens. Here they function in controlling the acid secretion into epididymal clear cells that helps to sustain an acidic pHₖ in the epididymis, a feature needed for sperm maturation (Brown & Breton 2000, Pietrement et al 2006).
In fitting with the important roles plasma membrane V-ATPases demonstrate in physiological processes, many tumour cells also appear to have dysregulated expression V-ATPases at the plasma membrane, teleologically to mediate proton extrusion in these malignant cells, which further contribute to the tumour aggressive phenotype. As V-ATPases are found in endo- and exocytotic vesicles, it has been proposed that the presence of V-ATPases in the plasma membrane of tumour is attributed to the enhanced membrane-recycling mechanisms (Martinez-Zaguañan et al 1999, Nishi & Forgac 2002, Raghunand et al 1999). Additional roles of plasma membrane V-ATPases in tumour will be discussed further.


3.3 V-ATPases in Cancer

The tumour microenvironment is acidic often due to the marked production of lactate as discussed earlier. However, an acidic environment can also be created with reduced lactate production via glycolysis, suggesting the aerobic metabolism is not the sole mechanism responsible for the development of this malignant acidic environment within solid tumours (Newell et al 1993, Yamagata et al 1998). In tumour cells, V-ATPases can directly transport protons into the tumour microenvironment; also, it could help maintaining a relatively neutral-alkaline intracellular pH of these cells by sequestering protons into lysosomal compartments which are subsequently to be released into extracellular space, together successively creating an acidic extracellular milieu favoured for tumour metastasis (Sennoune et al 2004b). In addition, overexpression of V-ATPases following chemotherapy treatments has been proposed to be involved in tumour defensive response and drug resistance mechanisms (Murakami et al 2001,
Torigoe et al 2002). Thus, targeting V-ATPases is desirable as yet another promising anti-cancer approach.

3.3.1 Expression of V-ATPases in Cancer

Increased level of expressions and specific spliced variants of V-ATPase subunits have been detected in various tumours, yet the regulation of their expression and localisation has not been extensively explored. In mammals, subunit $a$ exists in four isoforms, namely $a1$-$a4$ (Oka et al 2001, Smith et al 2000). The $a1$ and $a2$ isoforms localise primarily at intracellular compartments. $a1$ localises at synaptic vesicles (Morel et al 2003), while $a2$ is present in endosomal compartments and the Golgi (Toyomura et al 2003). Particularly, the $a3$ subunit has been reported to be expressed at high levels in pancreatic beta cells (Sun-Wada et al 2006). Only two isoforms of the B subunit are expressed in mammals, namely $B1$ and $B2$ (Vanhille et al 1994). $B1$ is found to demonstrate restraint expression in renal type A intercalated cells, in epithelial plasma membranes of the epididymis as well as in cochlea and the endolymphatic sac of the inner ear (Karet et al 1999, Miller et al 2005), whereas 57-kDa $B2$ is ubiquitously expressed. Together, these determined the selection of our candidate isoforms for further investigation.

In this work we performed bioinformatics analyses on V-ATPases mRNA expression profile in pancreatic cancer (Paper I). From The Cancer Genome Atlas (TCGA) database, only subunit ATP6V0A3 (also known as TCIRG1) showed up-regulation tendency among the 88 human tissue samples included; whereas V-ATPase subunits ATP6V0A1 and ATP6V1B2 did not. In the Oncomine database, the V-ATPase subunits ATP6V1B2 were found to be up-regulated in an individual study by Logsdon and colleagues (2003); other subunits ATP6V0A1, ATP6V0A2 and TCIRG1 were reported with no significant observation when comparing PDAC to normal cell lines/tissues. Data on the ATP6V1B2 expression reported in the Pancreatic Expression Database (PED) by two different studies were inconsistent. Friess and coworkers (2003) reported that the gene was up-regulated whereas Pogue-Geile and groups (2006) observed a down-regulation when PDAC tissues were compared with normal pancreas. ATP6V0A2 was found to be down-regulated. Data on the ATP6V0A1 expression were not available in PED.

Compiling previous experimental findings from different studies: an earlier study showed the expression level of subunit $c$ was up-regulated and related to the metastatic potential of pancreatic cancer. This study compared the expression level of subunit $c$ between normal and pancreatic carcinoma tissues as well as between invasive and non-invasive pancreatic cancers, and found
marked up-regulation in invasive cancers, yet neither non-invasive nor benign cystic neoplasm expressed detectable proteins (Lu 2012, Ohta et al 1996); also found in pancreatic cancer, the expression of the V1E subunit was found to be the highest in PDAC. V-ATPase expressions were found to localise at the plasma membrane of Panc-1 cells and the increased expression level correlates with the increasing cancer grade (Chung et al 2011); all the isoforms of subunit a were detected in highly metastatic MDA-MB-231 and MCF10CA1a breast cancer cells (Capecci & Forgac 2013, Sennoune et al 2004a); the a3 isoform was also observed in highly metastatic mouse melanoma B16-F10 cells and significant V-ATPase localisation was displayed at the plasma membrane (Nishisho et al 2011). von Schwarzenberg and colleagues (2013) also detected higher expression of the a3 isoform in breast cancer cells SKBR3, consistent with the data of Hinton et al. (2009). Further, subunit c1 was detected to be the most strongly overexpressed gene at the mRNA level when in comparison to other genes of V-ATPase complex in oral squamous cell carcinoma (Otero-Rey et al 2008).

In accordance with these data, we observed that a3 is highly up-regulated at the mRNA and protein level in all our tested PDAC cell lines relative to the control non-cancerous pancreatic epithelial cells, whereas the overall ATP6V1B2 mRNA and protein level are lower in PDAC cells (Paper III, Figure 1).

We observed that a substantial fraction of the V-ATPase subunit B2 was found staining at intracellular compartments in our present study without co-localisation of our membrane marker E-cadherin (Paper III Figure 2A), and their partial co-localisation of this with lysosome associated membrane protein 1 (LAMP-1) further revealed that this V-ATPase isoform was expressed in late endosomes and lysosomes (Paper III Figure 2B). This is in line with previous report that B2 staining is predominantly detected as vesicular or diffuse cytoplasmic (Paunescu et al 2004). Our immunofluorescence staining unfortunately did not produce specific subunit a3/TCIRG1 labeling in the PDAC cells studied, and further analysis is thus hindered. However, previous findings from Chung and colleagues (2011) reported that only very little plasma membrane staining of the TCIRG1 isoform was detected in PDAC cells. Nishisho and colleagues (2011) on the other hand, reported that the expression of a3 on plasma membrane or in cytoplasm correlates with the metastatic potential of low-metastatic and low-metastatic B16 melanoma cells, an observation possibly could be reflected in current PDAC cell lines studied according to their observed invasiveness in vitro.
3.3.2 V-ATPases in Cancer Invasion and Metastasis

Metastasis and invasion involve a series of actions by tumour cells of breaking through the basement membrane, ECM degradation, angiogenesis, vascular system invasion and redistribution in the distant host sites. During the process, a low pH of extracellular microenvironment is required for inducing the secretion, activation and cellular distribution of several proteases which are responsible for degradation and remodeling of ECM (Martinez-Zaguilan et al 1996, Rofstad et al 2006). These proteases, including bone morphogenetic protein-1–type metalloproteinases, caphepsin L, matrix metalloproteinases (MMP), gelatinase, adamalysin-related membrane proteases and tissue serine proteases, typically have an acid pH optima (Gocheva & Joyce 2007, Joyce & Hanahan 2004, Martinez-Zaguilan et al 1996). Plasma membrane V-ATPases in tumour cells are able to provide such aberrant pH gradient between the alkaline cytosol and the acidic extracellular milieu via their proton extrusion aptitude, thus leading to the invasive behaviour of these cells and supporting malignancy development. It has also been suggested that V-ATPase may influence the expression of proteases directly and independent of the whole enzyme V-ATPase function as reported by Kubota and colleagues (2000). Here, transfectants overexpressed with V-ATPase subunit c at mRNA level showed enhanced invasiveness in vitro with a concomitant increases in MMP-2 secretion (Kubota & Seyama 2000).

Indeed the invasive phenotype of tumours has been evidenced to be closely related with high V-ATPase activity. The initial direct evidence for the role of V-ATPases in tumour cell invasiveness was described by Martínez-Zaguilán and colleagues (1993) when they screened 19 normal and human tumour cell lines for the presence of plasmalemmal H⁺-ATPase activity using bafilomycin A1 to inhibit V-type H⁺-ATPases. They then demonstrated that highly invasive breast cancer MDA-MB-231 cells were found to exhibit significant plasma membrane V-ATPase activity as compared to the poorly metastatic MCF7 cells (Martinezzaguilan et al 1993). Furthermore, the invasiveness of MB231 cells, but not of MCF7 cells, was significantly inhibited by treatment with the specific V-ATPase inhibitors concanamycin and bafilomycin (Sennoune et al 2004a). In addition, they also demonstrated in a later study that MDA-MB-231 cells express higher levels of a3 and a4 isoforms of V-ATPases when compared to MCF7 cells. Subsequent knockdown of either a3 or a4 isoforms also appeared to inhibit invasion of these cells (Hinton et al 2009). Similar findings were reported by Capecci and coworkers (2013) where MCF10CA1a breast cancer cells showed significantly higher invasion with much higher expression levels of both a1 and a3 subunit isoforms relative to the non-invasive, immortalized MCF10a breast epithelial cells. In vitro migration and invasion of human lung tumour cells and further spontaneous metastases in nude
mice engrafted with human lung carcinoma were also shown to be inhibited by another V-ATPase inhibitor, NiK-12192 (Supino et al 2008).

In addition, other studies have also reported that improper activation of V-ATPases associates with an invasive phenotype of several other tumour types, including melanoma (Nishisho et al 2011), hepatocellular carcinoma (Lu et al 2005) and pancreatic cancer (Chung et al 2011). Pancreatic cancer in particular, Chung and colleagues (2011) reported that the expression of the V₁E subunit is highest in PDAC and the expression pattern correlates with cancer stage, an observation similar to a previous study in which the mRNA levels of V-ATPase V₀c subunit were reported to have increased in PDAC eightfold over normal pancreas (Ohta et al 1996). Furthermore, V-ATPase inhibition with concanamycin or short-hairpin RNA targeting the V₁E subunit has also been shown to reduce activity of MMP-9 in PDAC; yet stimulated that of MMP-2, probably due to the differential regulation and activation of MMP-2 and -9 precursors (Chung et al 2011).

In agreement with these earlier reports, silencing of subunit a3 has been shown to significantly inhibit the invasiveness of BxPC-3 cells by about 50% in our transwell Boyden chamber assay, and inhibit Panc-1 invasion to a lesser extent (Paper III, Figure 4). Conspicuously, AsPC-1 cells were detected to have the highest a3 expression level in our studies, yet the cells demonstrated minimal invasiveness in the Boyden chamber assay (Paper I, Figure 5), and were thus excluded from further investigations. This low invasiveness observed however is contradictory to a previous study where AsPC-1 cells were found to be indeed invasive and its invasiveness is comparable to that of Panc-1 cells (Tan et al 2005, Zang et al 2015). Further experiments are needed to elucidate the differences in our reports and the high a3 expression level is indeed intriguing as this might represent a typical malignant phenotype of these cells.

Interestingly in Panc-1 and BxPC-3 cells, silencing of a3 was found to significantly increase the migration velocity, directionality and the total distance on a 2D matrix simulating PDAC ECM (constituted of laminins [4.46%), fibronectin [4.46%], collagen IV [0.602%], collagen III [1.34%], collagen I [89.15%]). Our data were found to contradict previous studies where inhibition of V-ATPases with concanamycin A obstructs the migration of PDAC (Chung et al 2011) and breast cancer cells (Cotter et al 2015), and that NiK-12192 treatment on lung tumour cells demonstrates a retardation of migration in wound-healing assay (Supino et al 2008). Nevertheless, it was also indicated that invasiveness and migratory capability of cells could vary and be arranged according to different milieu given. Mouse B16V melanoma cells were found to behave inconsistently on two different matrices. They were found to be more adhesive and migrated quicker on a basement
membrane yet were hardly invasive on this matrix; on the other hand these cells were found to strongly invade a dermis-like matrix than to migrate on it (Vahle et al 2014). While the exact mechanisms leading to our results are currently not known, they might involve the activity of various MMPs and/or cathepsins as corroborated by previous works (Chung et al 2011, Kulshrestha et al 2015, Nishisho et al 2011, Rozhin et al 1994).

3.3.3 V-ATPases in Various Cancer-assciated Signalling Pathways

Human cancers essentially develop from an imbalance of cell growth and cell death. Several crucial cell cycle regulatory proteins, such as those of cyclin-dependent kinases (CDKs) and the inhibitors of CDKs, are responsible for upholding this balance via specific signalling pathways.

Upon activation, the serine-threonine-specific protein kinase Akt enhances survival of the cells by stimulating cell cycle progression through the inhibitory phosphorylation at Thr^{145} and Ser^{146} of the CDK inhibitors p21\(^{CIP1/WAF1}\) (p21), a known tumour suppressor (Abukhdeir & Park 2008, Li et al 2002). Our data showed that both concanamycin A treatment and silencing of the \(a3\) subunit reduced Akt activity with concomitant increase in p21 levels (Paper III, Figure 6 and 7). A significant decrease in the activity of Akt and an increase in p21 protein level were seen after \(a3\) silencing, the magnitude of which correlated with the magnitude of knockdown. Similar effects were observed with concanamycin A treatment where Akt phosphorylation was decreased and p21 protein level was increased in a dose-dependent manner. These data suggest that PDAC cell cycle progression are in part regulated by V-ATPase activity, with underlying mechanisms similar to those which have been investigated and proposed previously in other studies upon pharmacological V-ATPase inhibition (Lim et al 2006, Schempp et al 2014, von Schwarzenberg et al 2014). An earlier report by Lim and coworkers (2006) demonstrated that bafilomycin A1 induces HIF-1\(\alpha\), which in turn causing the cell cycle arrest in the G\(_1\) phase by inducing p21 in various cancer cells. In line with this observation, we further detected a dose-dependent increase in HIF-1\(\alpha\) protein level by concanamycin A treatment in PDAC cells, suggesting a similar signalling pathway is involved considering the analogous mode of actions of the two V-ATPase inhibitors.

Evidence indicates macroautophagy or autophagy is required during the early stages of oncogenesis, serving as an alternative energy source for the adaptation of tumour cells with high metabolic demands while in their microenvironment with poor nutrients and oxygen to thereby sustain the survival and growth of these cells (Degenhardt et al 2006, Lu et al 2008, White &
DiPaola 2009, Yang et al 2011). Guo and colleagues (2011) in their studies indicated that down-regulating essential autophagy proteins in human cancer cell lines bearing activating mutations in H-ras or K-ras impaired the growth of these cells, suggesting that inhibiting autophagy in autophagy-addicted Ras-driven cancers (as seen in PDAC with universal K-ras mutation) could be a potential treatment approach. p62, also known as sequestosome 1 (SQSTM1) in humans, is an ubiquitin-binding scaffold protein which co-localises with those ubiquitinated protein destined to be degraded by autophagy. For its degradation is dependent on autophagy, the level of p62 increases in response to inhibition of autophagy (Bjorkoy et al 2005). From our data, the protein level of p62 was not affected by subunit a3 silencing, indicating that autophagic degradation activity (flux) was not significantly affected after the knockdown; on the other hand, treatment with concanamycin A elicited an increase in p62, indicative of reduced autophagic flux. We speculated that the current observation may be attributed to a dosing effect between the knockdown and inhibition effects in the experiments. Future experiments should seek to achieve a complete silencing of subunit a3 in PDAC cells in order to verify present postulation.

We later used phosphorylated retinoblastoma protein (p-Rb) as a proliferation marker and found that upon concanamycin A treatment, proliferation of BxPC-3 cells was lowered in a dose-dependent manner; increased of PARP cleavage was also detected with the same treatment, indicating an increase in apoptotic cell death. These effects are in agreement with those being reported in colon cancer upon bafilomycin A1 treatment (Wu et al 2009). Current work had not explicated in depth the underlying signalling mechanisms that lead to this decreased proliferation and the cell death observed. Also reported by Wu and collegues (2009), the anti-proliferative and pro-apoptotic effects of V-ATPase inhibition are found to be mediated in part by the p38 mitogen-activated protein kinases (MAPK) signalling pathway. We however did not observe an effect on the activity of extracellular signal-regulated kinases 1 and 2 (ERK1/2) though there were some noticeable effects on STAT3 by a3 knockdown (Paper III, Supplementary Figure 2), suggesting that different cell cycle signalling modules and/or another V-ATPase isoforms were involved in the anti-proliferative and apoptotic mechanisms currently seen in PDAC cells. Since both bafilomycin A1 and concanamycin A bind to subunit c of V0 domain, it is reasonable to postulate that current observations correlate with the V-ATPase-coupled proton translocation machinery and therefore with the acidification events in PDAC cells, though this has to be verified by further experiments.
3.4 References


Chapter 4
Concluding Remarks and Perspectives
Chapter 4  Conclusion Remarks and Perspectives

In solid tumours, cells often undergo extensive metabolic changes coupled with increased acid production in cells. In order to circumvent this increased intracellular acidosis, tumoural cells up-regulate expressions and activities of various membrane acid-base transporters, thereby creating an aberrant pH profile favourable for tumour development. The study of this thesis was set out to address the expression profiles of various pH regulatory transporters in pancreatic ductal adenocarcinoma (PDAC) and the general roles played by these pH regulatory transporters, focusing on two transporter families, i.e. monocarboxylates transporters (MCTs) and vacuolar-type H\(^+\)-ATPases (V-ATPases), in the events leading to PDAC progression. We first show that the expression of many of the pH-regulating transporters are dys-regulated at mRNA levels in different PDAC cell lines. Exploring further the expression profiles of these transporters or their protein products might help in discovering essential biomarkers for PDAC prognosis and aid the development of targeted cancer therapy. The work later on focuses on MCT1 and MCT4 as well as V-ATPases to evaluate their functional roles in contributing to the aggressive behaviour of PDAC cells, such as migration and the invasion capacity of the cells.

In our *in vitro* studies, we show that all PDAC cell lines applied in the study display lactate uptake capacity and that this lactate uptake was greatly inhibited by MCT inhibitor 4-CIN though to a lesser extent by the MCT1 and -2 inhibitor AR-C155858. Subsequent silencing of MCT1 as well as MCT4 reduced lactate uptake in AsPC-1 cells, whereas only MCT4 knockdown reduced lactate uptake in BxPC-3 cells. PDAC cell migration was not detectably altered by AR-C155858 or after MCT1 silencing, but was inhibited by 4-CIN. Notably BxPC-3 cell migration was significantly impeded after MCT4 knockdown. Both pharmacological inhibition as well as MCT1 and MCT4 knockdown reduced the PDAC cell invasiveness. Further, MCT1 and/or MCT4 knockdown reduced BxPC-3 spheroid invasiveness in the spheroid outgrowth assays. We later also show that silencing of MCT1 and MCT4 inhibited PDAC cell proliferation, where MCT4 silencing demonstrated bigger inhibitory effects than MCT1 silencing. Collectively, MCT4 seems to play a greater role than does MCT1 in PDAC cells.

We studied two V-ATPase subunit \(a3\) (ATP6V0A3/TCIRG1) and subunit B2 (ATP6V1B2) and found that subunit \(a3\) but not subunit B2 was significantly up-regulated in all PDAC cell lines applied relative to the control HPDE cell lines. To our knowledge, this is the first evidence of up-regulation of subunit \(a3\) in PDAC. Subsequently our data demonstrate that silencing of subunit \(a3\)
impeded the invasiveness of PDAC cells while increased PDAC cell migration on a 2D matrix, an observation which could be related to various MMPs and caspase activities. We also observed an up-regulation of HIF-1α and p21 in PDAC cells upon subunit α3 silencing and V-ATPase inhibition by concanamycin A, suggesting a role of this transporter in PDAC cell cycle and cell survival. Moreover, we detected that concanamycin A treatment lowered the cell proliferation with increased apoptosis in BxPC-3 cells in a dose-dependent manner. Our data also indicate a role of V-ATPase in PDAC cell autophagy, a mechanism associated with oncogenesis.

All in all, we demonstrate in this work that pH regulatory transporter MCT1, MCT4 and V-ATPases play a role in PDAC development. Specific targeting of these transporters could be beneficial in PDAC anti-cancer therapy.

This PhD project endorses the involvement of pH regulatory transporters in tumour progression, particularly in the context of invasion and metastasis. While providing appreciated preliminary insights for PDAC clinical paradigm, new questions and issues have also emerged from this study.

While the current studies concentrate on MCTs and V-ATPases, contributions of other transporter families in pH-regulating and PDAC tumour progression cannot be overlooked as several transporters have also been found to be up-regulated in PDAC as described at the beginning of this thesis. Further characterization of the expression of these pH regulatory transporters would be of great value to decipher specific pH-regulating mechanisms carried out by tumoural cells in PDAC.

Considering the major roles of MCTs in cancer metabolic adaptations as aforementioned, inhibition of MCTs and subsequently inhibition of lactic acid efflux from cells warrants a promising therapeutic solution. However, problems remain with a lack of specific pharmacological inhibitors for individual MCT isoforms. 4-CIN has long been used as a classical non-specific MCT1 inhibitor and was applied as a broad spectrum MCT inhibitors in the current study, yet cautions should be taken for this approach since it has also been found to greatly inhibit mitochondrial pyruvate transport and later, glucose oxidation with a potency of at least two orders of magnitude greater than MCT1, in addition to AE1 inhibition (Halestrap 2013, Halestrap & Denton 1975). The possible cofounding effect of the mitochondrial pyruvate transport and AE1 inhibition in conjunction with MCTs inhibition should be recognized while interpreting the outcomes observed from applying these agents. The lack of isoform specific pharmacological inhibitors impedes the current research targeting various MCT isoforms vital in tumour
malignancies. Alternative approaches such as gene knockdown with siRNAs or shRNAs should be explored further to bring about specific silencing of these candidate transporters.

In current work, only a physical interaction between basigin and MCT1 or -4 has been demonstrated. Given the importance of these proteins as already mentioned, there is a compelling rationale to continue the work to investigate in PDAC the functional interplay among MCTs, basigin, MMPs and not least, β1-integrin as implicated in several previous studies (Gallagher et al 2009, Le Floch et al 2011, Sangboonruang et al 2014), to reveal their potential intertwined underlying molecular pathways that contribute to PDAC progression.

Present studies have demonstrated the overall effect of V-ATPase subunit a3 on single cell migration of Panc-1 and BxPC-3 cells. As differences between these cells have been observed from the real time recordings, it could be interesting to revisit the assays to elucidate further respective modes of translocation of different PDAC cell types, which could potentially uncover information to impede distance metastasis in various PDAC subtypes. Questions remain to be explicated- how would the morphological changes of PDAC cells on different ECM substrata contribute to the movement of cells? How do expression changes of individual transporters, followed by the change in the pH, relate to the migratory machineries of the cells? Many of these questions could be availed by an application of advanced fluorescent cell labeling and live cell imaging microscopy in future studies to elucidate different aspects of migration.

The current study is also evidently limited by the use of mainly tumour cell lines cultured in 2D-monolayers which are hardly considered as analogous to physiologically contexts. Comparable hypotheses and approaches in this study could be advanced by adapting better defined and more complex models simulating native solid tumors. There are fundamental differences in physical and chemical properties between 2D-monolayer and 3D-spheroid cell culture in terms of gradients for oxygen/hypoxia, nutrient infiltration, lactate transport, matrix structure and signaling, which potentially affect the tumourigenicity of a tumour mass. 3D-spheroid cell models allow the monitoring of tumour progression in vitro in a format which is more physiologically relevant as well as being a more time- and cost-effective approach. Although this model, complementary to the simple 2D-monolayers, was applied as described in Paper II, and the general impacts of MCTs in one PDAC cell model (BxPC-3) have been demonstrated, an in-depth understanding of the specific differences at molecular level is still lacking. Further experimental studies should exploit these models to answer the research questions addressed. Other novel models such as collagen
organotypic models are currently being explored and optimized. These models could also be adapted to advocate present work at a later date.
4.1 References


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Paper I
Acid-base transport in pancreatic cancer: Molecular mechanisms and clinical potential

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Abstract: Solid tumors are characterized by a microenvironment that is highly acidic, while intracellular pH (pHi) is normal or even elevated. This is the result of elevated metabolic rates in the highly proliferative cancer cells, in conjunction with often greatly increased rates of net cellular acid extrusion. Studies in various cancers have suggested that while the acid extrusion mechanisms employed are generally the same as those in healthy cells, the specific transporters upregulated vary with the cancer type. The main such transporters include Na+/H+ exchangers, various HCO3− transporters, H+ pumps, and lactate-H+ cotransporters. The mechanisms leading to their dysregulation in cancer are incompletely understood but include changes in transporter expression levels, trafficking and membrane localization, and posttranslational modifications. In turn, accumulating evidence has revealed that in addition to supporting their elevated metabolic rate, their increased acid efflux capacity endows the cancer cells with increased capacity for invasiveness, proliferation, and chemotherapy resistance. The pancreatic duct exhibits an enormous capacity for acid-base transport, rendering pH dysregulation a potentially very important topic in pancreatic ductal adenocarcinoma (PDAC). PDAC — accounting for about 90% of all pancreatic cancers — has one of the highest cancer mortality rates known, and new diagnostic and treatment options are highly needed. However, very little is known about whether pH regulation is altered in PDAC and, if so, the possible role of this in cancer development. Here, we review current models for pancreatic acid-base transport and pH homeostasis and summarize current views on acid-base dysregulation in cancer, focusing where possible on the few studies to date in PDAC. Finally, we present new data-mining analyses of acid-base transporter expression changes in PDAC and discuss essential directions for future work.

Key words: PDAC, NHE, MCT, H+/K+−ATPase, V-ATPase, NBCs, H+, proton, bicarbonate, metabolism.

Introduction

Recent years have seen a surge of interest in how dysregulation of intra- and extracellular pH (pHi, pHe) occurs early in cellular transformation and contributes, via multiple different mechanisms, to the development of cancer (for reviews, see Gatenby and Gillies 2008; Amith and Fliegel 2013; Webb et al. 2011; Parks et al. 2013; Boedtkjer et al. 2012; Andersen et al. 2014; Cardone et al. 2005b; Swietach et al. 2014). The high glycolytic activity in the proliferative cancer cells increases the need for acid extrusion. Consequently, upregulation of net acid extrusion early in transformation is evolutionarily favored and confers a clear advantage to the cancer cells by allowing a highly acid-generating metabolic
phenotype to persist without detrimental intracellular acid accumulation. Thus, the dysregulation of normal pH homeostasis is at least in part causally linked with the metabolic changes occurring in the cancer cells (Andersen et al. 2014). It can also be a direct result of oncogenic signaling, as shown decades ago for the Ras-mediated activation of a Na+/H+ exchanger (Doppler et al. 1987) and very recently for the ErbB2-mediated upregulation of the electroneutral Na+/HCO₃⁻ cotransporter NBCn1 (SLC4A7) (Gorbatenko et al. 2014b). Importantly, increased net acid extrusion is favorable for cancer development not only because it maintains pH₁ at or even above the normal level. The extensive acid extrusion, in conjunction with the inefficiently functioning vasculature in most solid tumors, also creates the highly acidic pH₈, often in the range of pH 6–6.5, that is characteristic for most solid tumors (Cardone et al. 2005b; Helmlinger et al. 1997; Carmeliet and Jain 2000; Gillies et al. 2002; Vaupel 2004; Zhang et al. 2010). As discussed in several recent reviews (Gatenby and Gillies 2004; Cardone et al. 2005b; Schwab et al. 2012; Webb et al. 2011; Parks et al. 2013; Boedtkjer et al. 2012; Amith and Fliegel 2013), this acidic microenvironment confers additional advantages to the cancer cells, including increased motility, decreased susceptibility to some chemotherapeutic treatments, and possibly also the elimination of non-transformed cells that do not possess the greatly upregulated acid extrusion capacity of the cancer cells. As will be discussed below, there is evidence that changes in expression levels and (or) activity occur across all families of pH-regulatory ion transport proteins and in a wide variety of cancers, although their importance for tumor growth and metastasis likely varies greatly with the type of tumor and the specific microenvironmental conditions. Before we embark on this topic, the experimental limitations of most existing studies of ion transport in cancer should be pointed out. The activity of most ion transport proteins is strongly regulated by posttranslational modifications and interaction partners, meaning that their expression pattern may say little about their activity. Yet, the understanding of the correlation between, for example, phosphorylation pattern and activity is still so limited for most transporters that it is not possible to predict transporter activity based on a fixed patient tissue sample. With a few exceptions, studies assessing transporter activity and roles in cancer have been carried out in cell culture, and in fact the great majority have been conducted in cells in monolayer growth, which have little in common with a patient tumor (for general insight into this, see for example Gadaleta et al. 2011). Hence, although there is strong evidence that altered pH-regulatory ion transport is an essential component in cancer development, more advanced models and a better understanding of, for example, the posttranslational regulation of acid-base transporters in cancer are urgently needed. Finally, the roles of altered acid extrusion in various aspects of cancer cell function have been studied most extensively in the context of breast cancer (e.g., Robey et al. 2009; You et al. 2009; Busco et al. 2010; Lauritzen et al. 2010, 2012; Lee et al. 2014; Boedtkjer et al. 2013) and melanomas (e.g., Stock et al. 2005, 2007; Krahling et al. 2009; De Milito et al. 2010), while only a few studies exist for most other cancers, including ovarian (Hulikova et al. 2011), prostate (Robey et al. 2009), and colorectal cancers (Le et al. 2011; gIomas (McLean et al. 2000), and the topic of this review, pancreatic cancers (Olszewski et al. 2010).

With an estimated global incidence of 250 000 individuals annually, which is expected to increase further in the future with increasing population age, pancreatic cancers represent a significant global burden on society (Yeo and Lowenfels 2012). In contrast to the major diagnostic and treatment advances made in past decades for many other cancers, pancreatic cancers remain absolutely devastating, with their mortality rate equal to their incidence rate, and a 5-year survival rate of around 5% (Koorstra et al. 2008; Jemal et al. 2009). They are of particular interest in the context of pH regulation because the pancreatic ducts (from which the great majority of pancreatic cancers, known as pancreatic ductal adenocarcinoma (PDAC), originate; see below) are endowed with an enormous and highly regulated acid-base transport capacity, which drives their central function of HCO₃⁻ secretion (Novak et al. 2013). Despite the diagnostic and therapeutical potential, very few studies to date have directly evaluated how the exceptional acid-base transport machinery of pancreatic duc
tal cells is altered during PDAC development and how this might in turn contribute to cancer progression.

The aim of the present review is to provide the reader with a platform for studying the still largely unexplored potential of acid-base transport proteins as diagnostic and therapeutic tools in PDAC. An up-to-date overview of the central aspects of acid-base dysregulation in cancer is followed by an introduction to the current model(s) of acid-base transport in the healthy pancreas and an overview of the involvement of specific transport proteins in cancer, including the limited literature available specifically on pH regulation in the PDAC setting. Finally, we provide, for the first time, extensive bioinformatics analyses of acid-base transporter expression changes in PDAC. The review closes with some brief recommendations for essential open questions to be explored in the immediate future.

**pH regulation in healthy pancreatic ducts — players and mechanisms**

The main function of pancreatic ducts is to secrete a bicarbonate-rich fluid. In humans, pancreatic juice can contain up to 140 mmol/L NaHCO₃; the precise concentration depends on secretory rates, the extent of stimulation, and modification along the ductal tree (Novak et al. 2013). It is clear, therefore, that the pancreatic ducts face a major acid-base homeostasis challenge in secreting HCO₃⁻ across the epithelium (from interstitium to lumen) while at the same time maintaining their pH₁.

In pursuit of unraveling the cellular and molecular mechanisms of acid-base transport in the pancreatic duct, researchers have faced several limitations. First, it is challenging to distinguish the acid-base transport across the epithelium as a whole from transport across the individual plasma membrane. In fact, many studies dealing with the physiological or secretory mechanism in duct cells are based on pH₁ measurements performed on single duct cells rather than the polarized epithelium. Second, functional studies on normal human duct epithelial cells are sparse, if available at all, and therefore many researchers resort to PDAC cell lines, such as PANC-1, CFPAC-1, and Capan-1 cells, which may have dysregulated acid-base transporters (see below). Nevertheless, by comparing data obtained on isolated ducts from mice, rats, guinea pigs, and pigs, it is possible to derive some common mechanisms, although this is complicated by the fact that HCO₃⁻ secretion (and thereby pH₁ regulation) may also depend on the origin of the isolated ducts within the heterogeneous ductal tree.

Below, we summarize what is known about the cellular and molecular mechanisms of pancreatic acid-base transport, as revealed mainly by pH₁ regulation studies, which are commonly considered cornerstones to understanding pancreatic HCO₃⁻ secretion (Fig. 1). More complete cellular models for pancreatic duct transport, including Cl⁻ and K⁺ channels, are given in recent reviews (Novak et al. 2013; Wilschanski and Novak 2013; Steward et al. 2005; Lee et al. 2012). Studies at the cellular level in isolated rat pancreatic ducts showed the presence of functional Na+/H⁺ and Cl⁻/HCO₃⁻ exchangers before the underlying molecular entities were identified (Novak and Greger 1988a, 1988b; Stuenkel et al. 1988; Zhao et al. 1994). Subsequent studies have added molecular understanding, although in many cases the mechanisms are incompletely understood.

In the secretory model, a Na+/H⁺ exchanger (NHE) would export H⁺ across the basolateral membrane, leaving intracellular HCO₃⁻ (generated by carbonic anhydrase; see below) for secretion to the...
lumen. In addition, or alternatively, NHE activity, particularly that of the ubiquitous NHE1 (SLC9A1), could serve as the major pHi regulator. NHE activity (assessed as pHi recovery sensitive to amiloride derivatives) has been revealed in pig, guinea pig, rat, and mice ducts and human duct cell lines (Veel et al. 1992; Szucs et al. 2006; de Ondarza and Hootman 1997; Ishiguro et al. 2000; Lee et al. 2000; Demeter et al. 2009; Novak and Christoffersen 2001; Rakonczay et al. 2006; Olszewski et al. 2010). There is some molecular evidence for NHE1 expression in normal ducts, where its localization appears to be on the basolateral membrane (Lee et al. 2000; Roussa et al. 2001), in agreement with its known localization in most other tissues (Boedtkjer et al. 2012). In addition, the NHE2 and NHE3 isoforms are expressed on the luminal membrane of main ducts and are proposed to interact with the cystic fibrosis transmembrane conductance regulator (CFTR) via PDZ domains and to conduct HCO$_3^-$ “salvage” (reabsorption) (Lee et al. 2000; Ahn et al. 2001).

The major coupled HCO$_3^-$ transporters in the pancreatic ducts are members of the SLC4 and SLC26 families. Cl$^-$/HCO$_3^-$ exchangers from both families have been identified in the pancreatic duct. It is generally accepted that in pancreatic ducts, Cl$^-$/HCO$_3^-$ exchangers would primarily serve a transport role in conjunction with CFTR, with the net effect being recirculation of Cl$^-$ and secretion of HCO$_3^-$ (Novak and Greger 1988b; Novak et al. 2013). However, Cl$^-$/HCO$_3^-$ exchangers can also regulate pHi, as deduced from a number of studies. The most important candidates for this function in the pancreatic duct are DRA/SLC26A3 and PAT1/SLC26A6, which are both expressed in PDAC cell lines (Greeley et al. 2001; Lohi et al. 2000). These exchangers have a transport stoichiometry for Cl$^-$:HCO$_3^-$ of 2:1 and 1:2, respectively. Therefore, HCO$_3^-$ secretion by the SLC26A6 isoform would be thermodynamically more favorable. Furthermore, in congruence with the existence of a functional complex, SLC26A6 interacts directly with CFTR via sulfate transporter and antisigma-factor antagonist (STAS) domains (Ko et al. 2004; Wang et al. 2006). However, the cistern distribution and physiological roles of SLC26 anion exchangers in pancreatic ducts remain controversial. For example, down-regulation of one exchanger can upregulate the other exchanger, thus affecting overall anion exchange stoichiometry (Song et al. 2012). An anion exchanger from the SLC4 family, SLC4A2 (AE2), which could regulate pHi after an alkaline load, has been identified in pancreatic ducts (Zhao et al. 1994; Rakonczay et al. 2006). However, immunohistochemical studies have not conclusively determined which membrane the transporter is localized to (Roussa et al. 2001; Kulaksiz and Getin 2002).

One group of HCO$_3^-$ transporters that have been particularly associated with HCO$_3^-$-transporting epithelia are the Na$^+$/HCO$_3^-$ transporters of the SLC4 family (also known as NBCs). In the pancreas, they are proposed to play a significant role in secretion, as they could transport HCO$_3^-$ from the interstitium to the duct cells. One NBC isoform, nNBC (NBCe1B), was cloned from the pancreas and found to be expressed on the basolateral membrane of ducts (Abuladze et al. 1998; Choi et al. 1999). Another isoform, the electroneutral NBCn1 (also known as NBC3, SLC4A7), is expressed on the luminal membrane and interacts with CFTR (Park et al. 2002), possibly regulating HCO$_3^-$ salvage. It is accepted that the role of these transporters is in secretory processes, however, most studies leading to these conclusions came from experiments monitoring pHi, and therefore it is expected that they would also have a role in pHi regulation.

H$^+$ pumps have also been associated with some secretory epithelia, including pancreatic ducts. First of all, the vacuum...
H^+ pump, which can also locate to plasma membranes under some conditions, has been proposed to be functional at the plasma membrane in the pig and guinea pig pancreatic ducts and in PDAC (Villanger et al. 1995; Zhao et al. 1994; Ishiguro et al. 1996; de Ondarza and Hootman 1997; Cheng et al. 1998). However, the molecular evidence is sparse and there are incongruent results, perhaps because of species differences or the use of different detection methods (pHi measurements, secretion assays). More recently, it has been shown that rat and human pancreatic ducts express gastric (ATP4A) and non-gastric (ATP2A2) H^+\textendash K^+ pumps (Novak et al. 2011; Wang et al., unpublished data). Again, both types of pumps have been functionally revealed during pHi measurements when other transporters have been suppressed, and some of the abovementioned studies show that they can also contribute to duct secretion.

Monocarboxylates such as lactate, pyruvate, and ketone bodies play major roles in metabolism and are transported across the plasma membranes of numerous cell types. Four monocarboxylate transporters that carry lactate, MCT1–4, have been well characterized (Halestrap 2013). Since monocarboxylate transport is linked to transport of H^+, these transporters will affect transmembrane pH gradients, although it should be noted that they are not strictly pH regulators, since their activity is not regulated by pH. Several MCT isoforms have been detected in pancreas, but apart from immunohistochemical identification of MCT1 in pancreatic acini, recent publications have focused on MCTs only in β-cells (Halestrap 2013; Bonen et al. 2006; Pullen and Rutter 2013; Zhao et al. 2001). It is not clear which isoforms are expressed in normal pancreatic ducts and what functions they have. In a normal pancreas stimulated with secretin, lactate concentration in the pancreatic juice is sub-millimolar (Kuroshima et al. 1986; Wang et al., unpublished data). Nevertheless, one may expect that in PDAC cells, the expression and function of MCTs would be crucial modulators of pHi and metabolite transport (see below).

Carbonic anhydrases (CAs), which catalyze the reaction H_2O + CO_2 ↔ H_2CO_3, thus allowing rapid interconversion between H_2O + CO_2 and H^+ + HCO_3^−, are some of the most important non-transport proteins regulating H^+ and HCO_3^− transport in acid-base transporting epithelia. CAs I through III and VII are cytosolic, whereas CAs IV, IX, and XII are membrane associated, CA V is mitochondrial, and CA VI is secreted. Several isoforms, mostly identified by immunohistochemistry, are found in human ducts and pancreatic tumors or PDAC cells (Nishimori et al. 1999; Nishimori and Onishi 2001; Kivelä et al. 2000; Alvarez et al. 2001). CA II and CA IV interact with H^+\textendash HCO_3^− transporters; however, localization of the CA isoforms does not always match the predicted localization of the transporters in pancreatic ducts. For example, CA II is found intracellularly and on the luminal membrane (Alvarez et al. 2001), and it seems to interact with NHE1 and NBC3 (Li et al. 2002; Loiselle et al. 2004). CA IV is also located on the luminal membrane (Mahieu et al. 1994), but expression studies have shown that it interacts with NBCe1 (Alvarez et al. 2003). CA IX and CA XII are expressed on the basolateral membranes of normal and pathological samples of pancreas (Kivelä et al. 2000; Juhasz et al. 2003). In one study of H^+ and HCO_3^− transport in CFPAC-1 cells, membrane-permeable CA inhibitors were found to have a stronger effect on pHi than a membrane-impermeable inhibitor, suggesting that at least in this context, the intracellular CAs were quantitatively the most important (Rakonczay et al. 2006). Carbonic anhydrases are key enzymes in pancreatic duct function, as their inhibition leads to marked effects on pancreatic secretion (Steward et al. 2005).

**Acid-base transporters implicated in cancer development, both generally and in PDAC**

**The Na^+/H^+ exchanger NHE1**

Of the acid-base transport proteins implicated in the development, growth, metastasis, and chemotherapy resistance of various cancers, by far the most studied are Na^+/H^+ exchangers (NHEs). NHE activity was shown in the eighties to be regulated by the Ras oncogene in NIH3T3 fibroblasts (Doppler et al. 1987), and around the same time, growth factor- and mutagen-induced NHE activity was identified in multiple contexts (Cassel et al. 1983; Sardet et al. 1990; Wakabayashi et al. 1994). Following the cloning in 1989 of the first human NHE, a ubiquitous basolateral isoform since denoted NHE1 (SLC9A1) (Sardet et al. 1989), it became clear that oncogene- or growth factor-induced NHE activity is generally mediated by this isoform.

Multiple lines of evidence link NHE1 to cancer development. First, although studies directly determining NHE1 protein expression in human cancer tissue are still scarce, available studies have indicated upregulation of NHE1 protein in human breast cancer (Boedtkjer et al. 2013; Lee et al. 2014), cervical cancer (Chiang et al. 2008), and hepatocellular carcinoma (Yang et al. 2011), compared with corresponding normal tissue. Second, studies of NHE1-mediated acid extrusion, performed in cultured cells, have also indicated increased NHE1 activity in transformed cells compared with normal cells (McLean et al. 2000) or upon oncone True expression (Lauritzen et al. 2010). In contrast, a recent study of organoids from human normal breast and breast carcinomas indicated that net NHE activity was unaffected by carcinogenesis, despite an increased NHE1 expression level in the carcinomas (Lee et al. 2014). Third, NHE1 has been shown to regulate cancer cell motility and invasiveness (Stock et al. 2005; Cardone et al. 2005a; Busco et al. 2010; Lauritzen et al. 2012), cell cycle progression (Putney and Barber 2003), and survival, the latter especially in conjunction with chemotherapy (Lauritzen et al. 2010; Rich et al. 2000). While the number of studies is limited, in vivo studies in mouse models have supported an important role of NHE1 in tumor growth, whether employing NHE1 knockdown (Chiche et al. 2012) or pharmacological inhibition (Comimaggio et al. 2013). Finally, in the in vivo setting, the roles of NHE1 in cancer might extend beyond the cancer cells themselves, as, for instance, NHE1 activity in human umbilical cord endothelial (HUVEC) cells has been shown to be important for angiogenesis (Gao et al. 2011; Mo et al. 2011). Whether or not this extends to the tumor endothelium remains to be explored.

With respect to the possible role of NHE1 in PDAC, interestingly, a recent study employed the NHE1 inhibitor ethylisopropylamiloride to inhibit macropinocytosis in PDAC tumors (Comimaggio et al. 2013), in line with a series of studies demonstrating the importance of NHE1 activity for this process (Ivanov 2008). Furthermore, several PDAC cell lines show NHE1 expression and function (Rakonczay et al. 2006; Olszewski et al. 2010). Olszewski and coworkers studied NHE1 activation by neurotransmitters, such as, for instance, NHE1 activation under these conditions was associated with direct evidence of a sodium-dependent accumulation of the exchanger, counteracted a neurotensin-induced intracellular alkalization, and eventually led to intracellular and extracellular acidification (Olszewski and Hamilton 2009; Olszewski et al. 2010). Furthermore, a study in CFPAC-1 cells showed functional and mRNA-level expression of NHE1 in these cells (Rakonczay et al. 2006).

**Bicarbonate transporters**

Several bicarbonate transport proteins have been implicated in cancer, as recently reviewed in Gorbatenko et al. (2014a). The most widely studied bicarbonate transporter in this regard is DRA, an SLC26 family Cl^−\textendash HCO_3^− exchanger (SLC26A3), which, as noted above, is an important transport protein in the normal pancreatic duct. A group of transporters that have only been implicated in cancer development more recently are the Na^+/HCO_3^− transporters of the SLC4 family, which, by importing HCO_3^−, mediate the net influx of acid equivalents. The SLC4 family member most studied in this context is NBCn1, which is upregulated in human breast cancer (Boedtkjer et al. 2013; Lee et al. 2014) and, as noted
above, specifically upregulated by the ErbB2 oncogene (Lauritzen et al. 2010; Gorbatenko et al. 2014b). There is also some, albeit limited, evidence pointing to the altered regulation of other members of both the SLC4 and SLC26 families in various cancers (Gorbatenko et al. 2014a).

Only a few studies have investigated the possible roles of Na\(^+\)-HCO\(_3\)^− cotransporters in PDAC, and only in the context of expression and pH regulation. One study showed functional Na\(^+\)-HCO\(_3\)^− cotransport in CFPAC-1 cells and identified by qPCR the presence of pNBC (SLC4A4) (Rakonczay et al. 2006). Similarly, Szucs and coworkers showed DIDS-sensitive Na\(^+\)-HCO\(_3\)^− cotransport and pNBC mRNA in Capan-1 cells (Szucs et al. 2006b).

H^+-ATPases

V-type H^+-ATPases (ATP6 family) have been quite widely implicated in cancer development. In most tissues, V-ATPases reside primarily in the endosomal and lysosomal membranes. However, in osteoclasts, renal intercalated cells, and epididymal clear cells, V-ATPase function at the plasma membrane is well characterized (Forgac 2007). Inhibition of V-type H^+-ATPases has been shown to induce the death of both melanoma (De Millogo et al. 2010) and breast cancer cells (You et al. 2009). Moreover, changes in the expression of H^+/K^+ATPases (ATP4A, ATP4B, ATP72A) have been noted in gastrointestinal cancers especially. In the case of ATP4A and ATP4B, the change has consistently been reported to be a downregulation involving intergenic hypermethylation (Raja et al. 2012). While the possible role of H^+/K^+ATPase downregulation in gastric cancers remains to be elucidated, loss of ATP4A is associated with gastric achlorhydria, mucosal hyperplasia, and increased growth factor levels (Judd et al. 2005). Conversely, for ATP12A (also known as ATP1AL1), overexpression compared with normal tissue has been reported in colorectal (Takahashi et al. 2009). The authors showed that both MCT1 and MCT4 were upregulated by hypoxia via HIF-1 \(^\alpha\) responsive elements in the MCT4 promoter (Ullah et al. 2006). The MCTs serve as essential extruders of lactic acid, cotransported with H^+, from highly glycolytic cancer cells. When the combined driving force for lactic acid and protein transport so dictates, the MCTs can also mediate the net uptake of lactic acid into cancer cells in well-oxygenated areas of the tumor, thereby reducing MCT1 and MCT4 expression, intracellular lactate accumulation, and reduced proliferation of PDAC cells (Schneiderhan et al. 2009).

Carbonic anhydrases

As noted above, CAs are essential regulators of H^+ and HCO\(_3\)^− transport. Accordingly, they are also important in cancer development and are in fact one of the most extensively studied groups of pH-regulatory proteins in cancer. This is particularly true for CA IX, which is upregulated in numerous cancers (Bartosova et al. 2002; Chen et al. 2005; Perez-Sayans et al. 2012; Supuran 2008; Sedlakova et al. 2014) and the knockdown of which reduces colon cancer xenograft growth in nude mice (Chiche et al. 2009). However, several other CA isoforms, including CA XII, have been implicated in cancer development in various tissues (Neri and Supuran 2011). Clinical trials are evaluating the potential applications of CAs in cancer treatment, as both diagnostic and therapeutic targets (Zatovicova et al. 2010; Neri and Supuran 2011).

As mentioned previously, pancreatic ducts express multiple CA isoforms. CA IX overexpression in at least a subset of human PDAC patients has been proposed (Juhasz et al. 2003; Kivela et al. 2000), whereas CA XII is expressed only at very weak levels (Kivela et al. 2000). The functional roles of CA dysregulation in PDAC are, to our knowledge, unexplored.

Bioinformatic analyses of pH-regulatory transporters in pancreatic cancers

To identify acid-base transporter of potential interest in PDAC, we analyzed three different databases: Oncomine (Rhodes et al. 2007); The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/), using the Cancer Genomics Browser (https://genomics-cancer.ucsc.edu); Goldman et al. 2013; Zhu et al. 2009); and the Pancreatic Expression Database (PED; Cutts et al. 2011; Dayem Ullah et al. 2014; Gadaleta et al. 2011). While the TCGA data are based on human tissue samples only, both Oncomine and PED include both human tissue studies and studies carried out on cell lines. More details on the data analyzed, including the sample types, and the number of samples in each study can be found in Supplementary Table 1.

Figure 2 provides an overview of the acid-base transporter genes reported in these databases to be upregulated (A) and downregulated (B) in pancreatic cancers at the mRNA level. In general, when interpreting this type of data, several precautions must be taken, and this is also the case for the pancreatic cancer data. First, as discussed in Gadaleta et al. (2011), the lack of standardization of histological procedures in PDAC may compromise the quality of “normal” control tissue, which generally stems from the area surrounding the tumor and may be contaminated by transformed tissue. Second, databases based on data deposited from individual studies may be confounded by poor-quality data or highly variable sample sizes. Finally, mRNA expression, while sometimes very informative, is frequently poorly correlated with protein expression, which again does not correlate with function. Hence, although the data presented show some very interesting trends, they should be interpreted with these caveats in mind.

As shown in Fig. 2, two genes, namely the NHE1 and MCT4 genes, are reported to be upregulated in all three databases and one gene, namely the ATP4 gene, is reported to be downregulated in all three. In addition, several genes, spanning all the families of transporters presented above, are reported as up- or downregulated in two databases. In the following discussion, we will briefly describe the results. Only genes showing altered expression in at least two databases will be discussed in detail.
TCGA database analyses

TCGA data on the expression of the abovementioned transporters and CAs are presented in Fig. 3. Since these data are based on only 88 samples, of which only 3 are normal controls, they are highly variable and should be interpreted with care. It should also be noted that the results are shown with respect to mean level normalization and not with respect to control tissues. However, the TCGA data do point to upregulation of several NHEs (NHE1–4), MCT1 and MCT4, and CA IX and (to a lesser extent) CA XII in the majority of PDAC patients. Furthermore, the data indicate the downregulation of two SLC4 family HCO$_3^-$ transporters, the anion exchanger AE3 and the electrogenic Na$^+$/HCO$_3^-$ cotransporter NBCe1 (SLC4A4). Finally, all three H$^+$/K$^+$-ATPases appear to be expressed at low levels in PDAC.

Pancreatic Expression Database analysis

PED analysis indicated that several pertinent acid-base transporters are upregulated in both PDAC and pancreatic intraepithelial neoplasia, a histologically well-defined precursor to invasive ductal adenocarcinoma of the pancreas (Hruban et al. 2008). These transporters include NHE1, NHE3, and NHE4, the anion transporters AE3 and DRA (SLC4A3 and SLC26A3), and MCT4. Among these, MCT4 was particularly highly expressed in the PDAC cell line Panc-1, with a fold change of 11.05 as compared with expression in the normal human pancreatic duct epithelial cell line (HPDE). Finally, the V-ATPase subunit ATP6V1B2 was found to be upregulated. Several transporter genes were found to be downregulated, primarily those encoding NHE8, NBCe1 and NBCe2, and the V-ATPase subunit ATP6V0A2. Data on expression of another V-ATPase subunit, ATP6V1B2, were contradictory, in that Friess et al. (2003) showed that the gene was upregulated while Pogue-Geile et al. (2006) observed downregulation of the gene when comparing PDAC tissue with normal pancreas. Finally, ATP4A was found to be downregulated. This was the case in both chronic pancreatitis and PDAC, as shown in Friess et al. (2003). In contrast, ATP12A was found to be upregulated in the study of Buchholz et al. (2005), which compared PDAC with normal pancreatic duct, yet downregulated in the study of Friess et al. (2003), which compared PDAC with normal pancreas. Results for ATP4B were not available in PED (Gadaleta et al. 2011).

Oncomine data analysis

Oncomine analysis showed upregulation of the SLC9 family members NHE1, NHE2, NHE4, and NHE8, the SLC4 family transporters AE3, NBCe1, NBCn1, NDCBE, and NBCn2, and the monocarboxylate transporters MCT1 and MCT4. Notably, MCT4 demonstrated a much greater upregulation than other upregulated transporters, which is similar to the data collected from the other two databases. In addition, the V-ATPase subunits ATP6V1B2 and TCIRG1 (ATP6V0A3) were found to be upregulated. In contrast, Oncomine analysis showed downregulation of ATP4A, ATP12A, and ATP4B in most studies, albeit with some variation between individual studies (Suppl. Table 1). Interestingly, the downregulation of these three genes is also visible in pancreatitis, a pancreatic inflammation that many studies show increases the risk of pancreatic cancer (Whitcomb 2004). As illustrated in Fig. 4, Oncomine indicates a downregulation at the mRNA level of ~2-fold for ATP4A (A), ~22-fold for ATP4B (B), and ~2-fold for ATP12A (C) during chronic pancreatitis compared with normal pancreas. The particularly marked downregulation of ATP4B may reflect that both $\alpha$ subunits (gastric and non-gastric) share this $\beta$ subunit to make a functional pump.

Summary and perspectives

In this review, we have outlined the importance of pH dysregulation in cancers and pointed out why we consider PDAC particularly interesting in this regard. We have described the mechanisms of acid-base transport in the healthy pancreas, and we have summarized the literature on the dysregulation and involvement of the various families of pH-regulatory transporters in cancer, highlighting, wherever possible, studies in PDAC. Finally, new data-mining analyses from three different databases have...
been presented, demonstrating the patterns of dysregulation of acid-base transport proteins in PDAC at the mRNA level. Collectively, these studies illustrate some major trends that we consider important areas for future research. First, all three databases point to increased mRNA levels of NHE1 and MCT4 in PDAC compared with normal pancreas. In conjunction with the widely recognized roles of these transporters in tumor development and metastasis in other cancers (Pinheiro et al. 2010, 2012; Stock et al. 2005; Cardone et al. 2005a; Busco et al. 2010; Lauritzen et al. 2010, 2012; Rich et al. 2000) and the fact that their functional expression in PDAC has already been established (Schneiderhan et al. 2009; Commisso et al. 2013; Rakonczay et al. 2006; Olszewski et al. 2010), this finding renders these transporters obvious targets for further studies in PDAC. Finally, although our analyses indicate that more acid-base transporters are upregulated than downregulated in PDAC, it is interesting that the mRNA level of the H+/K+-ATPase ATP4A is reported to be downregulated in all three databases, and its β-subunit ATP4B is reported to be downregulated in two databases. To substantiate this observation, we assessed the expression level of the H+/K+-ATPase genes in cancers derived from tissues with a very well-established physiology (albeit different from that of the pancreas), namely the gastrointestinal system and kidneys. We noted that gastric cancers are also associated with a marked downregulation of ATPA4 and ATP4B mRNA levels compared with those in normal tissue, whereas kidney carcinomas show marked downregulation of ATP12A and a less marked downregulation of the shared subunit ATP4B (data not shown). As noted above, the downregulation of ATP4A and ATP4B mRNA levels in gastric cancers has been found to reflect intergenic hypermethylation; however, whether the downregulation is causally involved in gastric cancer has not, to our knowledge, been elucidated.

In addition to these genes that stood out as dysregulated at the mRNA level in all three databases, a broad array of acid-base transporter genes were reported as dysregulated by two databases, and in many cases this concurred with their known dysregulation and (or) functional roles in other cancers. Thus, the present work points to several interesting directions from which to start
Fig. 4. Oncomine™ RNA array database (http://www.oncomine.com) analyses. The results are based on the study of Logsdon et al. (2003), which compared chronic pancreatitis (n = 5) versus normal pancreas (n = 5). (A) Downregulation of ATP4A. (B) Downregulation of ATP4B. (C) Downregulation of ATP12A.

References


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### Pancreatic Expression Database (PED)

#### Up-regulation/Down-regulation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tissue/Condition</th>
<th>Technique</th>
<th>Platform</th>
<th>Length</th>
<th>Normalization</th>
<th>Fold Change</th>
<th>P Value</th>
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#### References

## Oncomine

### Analysis Type: Cancer vs. Normal Analysis

#### Cancer Type: Pancreatic Carcinoma

### Up-regulation

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### Down-regulation

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### References


Paper II
Monocarboxylate transporters MCT1 and MCT4 regulate migration and invasion of Pancreatic Ductal Adenocarcinoma Cells

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**Running title:** MCT1 and MCT4 in PDAC cell migration and invasion

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**Disclosure:** The authors declare no conflicts of interest.

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Abstract

Objectives: Novel treatments for Pancreatic Ductal Adenocarcinoma (PDAC) are severely needed. The aim of this work was to explore the roles of H⁺-lactate transporters MCT1 and -4 in PDAC cell migration and invasiveness. Methods: MCT expression, localization, activity, and function were explored in human PDAC cells (MIAPaCa-2, Panc-1, BxPC-3, AsPC-1) and normal pancreatic ductal cells (HPDE), by qPCR, immunoblotting, immunocytochemistry, lactate-flux, migration and invasion assays. Results: MCT1 and -4 (mRNA, protein) were robustly expressed in all PDAC lines, localizing to the plasma membrane. Lactate influx capacity was highest in AsPC-1 cells and lowest in HPDE cells and was inhibited by MCT inhibitor α-cyano-4-hydroxycinnamate (4-CIN), MCT1/-2 inhibitor AR-C155858, or knockdown of MCT1 or -4. PDAC cell migration was largely unaffected by MCT1/-2 inhibition or MCT1 knockdown, but was reduced by 4-CIN, and by MCT4 knockdown (BxPC-3). Invasion measured in Boyden chamber (BxPC-3, Panc-1) and spheroid outgrowth (BxPC-3) assays was attenuated by 4-CIN and AR-C155858 and by MCT1 or -4 knockdown. Conclusion: Human PDAC cells exhibit robust MCT1 and -4 expression and partially MCT1 and -4-dependent lactate flux. PDAC cell migration is partially dependent on MCT4, and invasion on MCT1 and -4. Inhibition of MCT1 and -4 may have clinical relevance in PDAC.

Key words: PDAC, lactate, cell motility, , cancer, AR-C155858, acid-base regulation
Introduction

Pancreatic cancer, of which about 90% is pancreatic ductal adenocarcinoma (PDAC), is among the five most aggressive and deadly cancers globally, with a 5-year survival rate of less than 5%\(^1,^2\). At least 250,000 new cases are diagnosed globally each year, a number which can be expected to rise with the increasing age of the world population\(^3\). The abysmal survival rates for pancreatic cancer in large part reflect that 80% of patients are diagnosed at the time of already locally advanced or metastatic disease, rendering surgical resection unfeasible. Furthermore, response rates to the common chemotherapeutic regimens used (e.g. the FOLFIRINOX combination, or gemcitabine plus nab-paclitaxel) are low, and reliable prognostic and predictive biomarkers are lacking\(^4\). Thus, novel diagnostic and treatment options for PDAC patients are urgently needed.

In general, a hallmark of solid tumors, which has been increasingly appreciated within the last decade, is their profoundly altered intra- and extracellular pH (pH\(_i\), pH\(_e\)) and acid-base transport dynamics. These pH characteristics impact on multiple aspects of cancer development including metabolism, proliferation, chemotherapy resistance, and invasiveness\(^5^-^8\). Dysregulation of pH in cancers is causally linked to the often markedly altered metabolic profile and high proliferative activity of cancer cells, which greatly increase metabolic acid production. This in turn necessitates increased acid extrusion in order to maintain the neutral or slightly alkaline pH required for maintaining glycolytic flux\(^8^-^10\). Several acid-extruding transporters have been shown to exhibit increased activity and/or expression in various cancers. These include the Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1, SLC9A1), V-type H\(^+\)-ATPases, the Na\(^+\),HCO\(_3\)\(^-\) cotransporter NBCn1 (SLC4A7), and the H\(^+\)-linked monocarboxylate transporters MCT1 and -4 (SLC16A1 and –A3)\(^7,^8,^10,^11\). In addition to maintaining pH\(_i\) despite the increased metabolic acid production, some of these transporters, including NHE1 and V-ATPases, have been demonstrated to confer other advantages to the cancer cells, including increased motility and invasiveness\(^12^-^17\); for an excellent review, see\(^18\). A few studies have also implicated MCT1 and
MCT4 in the regulation of motility in various cancer cell lines. Indeed, application of α-cyano-4-hydroxycinnamate (4-CIN), a broad-spectrum inhibitor of both MCTs and of the mitochondrial pyruvate carrier, MPC 19 reduced migration and invasion of high-grade glioma cells, and knockdown of MCT1 reduced their migration 20. Further, 4-CIN inhibited and MCT1 overexpression stimulated the migration of SiHa cervical cancer cells 21; MCT1 knockdown inhibited migration and invasion of MNNG/HOS osteosarcoma cells 22; MCT4 knockdown inhibited migration and invasion in oral squamous cell carcinoma (ORCC) cell lines and in ORCC patients, MCT4 overexpression was associated with lymphatic and distant metastases and poor prognosis 23; and finally, in human lung cancer cell lines, MCT1 and MCT4 expression correlated with invasiveness and knockdown of either transporter inhibited invasiveness without affecting migration 24.

Despite the fact that the pancreatic duct exhibits an enormous capacity for acid-base transport under normal conditions 25, and that PDAC is associated with major metabolic changes 26,27, little is known about the roles of altered acid/base transport in PDAC (for a recent review, see 28). Roles for NHE1 as well as for bicarbonate-dependent mechanisms in PDAC pH regulation have been demonstrated 29-31. While membrane protein basigin/CD147, which is a chaperone and obligatory cofactor for both MCT1 and MCT4, has been shown to be important for PDAC growth and invasiveness 32,33, no studies to date have directly addressed the expression and activity of MCT1 and -4 in PDAC and their roles in PDAC cell motility.

The aim of this study was to determine whether MCT1 and MCT4 are differentially regulated in PDAC cell lines compared to normal pancreatic ductal epithelial cells, and to assess the contributions of these transporters to lactate flux, cell migration, and invasion in PDAC. We show that MCT1 and -4 are highly expressed at the mRNA and protein level in several PDAC cell lines, and colocalize with CD147 in the plasma membrane. All PDAC cell lines studied exhibited basal lactate influx to which both MCT1 and MCT4 contributed. PDAC migration and invasion were strongly inhibited by the broad MCT- and pyruvate transport inhibitor 4-CIN,
which, however, also elicited substantial cell death. Finally, knockdown of MCT4 reduced migration of BxPC-3 cells, and knockdown of either MCT1 or MCT4 reduced invasiveness of BxPC3 cells in Boyden chamber assays as well as in 3D spheroid assays. Collectively, our findings indicate that while both MCT1 and MCT4 are expressed at the plasma membrane of all cell lines studied, MCT4 generally plays a somewhat greater role than MCT1 in lactate flux, migration and invasion of PDAC cells.

Part of these data have previously been published in abstract form \(^\text{34}\).
Materials and Methods

Cell Culture

Human PDAC cell lines MIAPaCa-2, Panc-1, BxPC-3 and AsPC-1 were acquired from the American Type Culture Collection (ATCC) and maintained in culture with DMEM or RPMI 1640 media with stable glutamine supplemented with 10% (v/v) fetal bovine serum and 100 U/ml penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO2. MIAPaCa-2 was further supplemented with 2.5% (v/v) heat-inactivated horse serum. Immortalized normal human pancreatic ductal epithelial (HPDE H6c7) cells were kindly provided by Dr Ming-Sound Tsao at Ontario Cancer Institute, Toronto, Canada and cultured in keratinocyte basal medium supplemented with epidermal growth factor and bovine pituitary extract. All tissue culture media and supplements were obtained from Invitrogen, Carlsbad, CA, USA.

Antibodies and Reagents

Antibodies against MCT1 were purchased from Millipore, Massachusetts, USA and antibodies against MCT4 were purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Antibodies against Basigin/CD147 and p150Gluco were obtained R&D Systems Inc., Minneapolis, MN, USA and Cell Signaling Technology, Danvers, MA, USA, respectively. α-Cyano-4-hydroxycinnamate (4-CIN) was obtained from Sigma–Aldrich and was dissolved in DMSO (CHEMSOLUTE®, Renningen, Germany). MCT1 and -2 specific inhibitor AR-C155858 was acquired from AdooQ BioScience, Irvine, CA, USA. Giemsa stain modified solution was acquired from Sigma–Aldrich Chemie Gmbh, Munich, Germany. L-[14C(U)]-Lactic Acid, Sodium Salt and Ultima Gold™ liquid scintillation cocktail were purchased from Perkin Elmer, Massachusetts, USA.

qPCR analysis

Isolation of total RNA from cultured cells was performed using the NucleoSpin® RNA II (Macherey-Nagel, Germany) according to the manufacturer’s instructions. Total RNA isolated
was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and cDNA transcripts were amplified by qPCR using SYBR Green (Applied Biosystems, Cheshire, UK) and the qPCR machine ABI7900, the reaction was repeated for 40 cycles with denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute and extension at 72°C for 30 seconds. Primer sequences were: MCT1, sense 5'-CGTTGTTGCAAATGGAGTGTGTCATAT-3'; antisense 5’-AAGTCGATAATTGATGCCCATGCCAA-3’ and MCT4, sense 5’-CCATGCTCTACGGGACAGG-3’; antisense 5’-GCTTGCTGAAGTAGCGGTT-3’. Triplicate samples were used. Expression levels of each mRNA were evaluated using the comparative threshold cycle (Ct) method as normalized against house-keeping β-actin sense 5’-AGCGAGCATCCCCAAAGTT-3’, antisense 5’-GGGCACGAAGGCTCATCATT-3’. Data were expressed as relative expression to the control cell line HPDE.

Western Blotting analysis

To detect individual protein expression, HPDE and PDAC cells were seeded in 10 cm² culture plates and cells were harvested at 80% confluence. Cells were washed twice with 1x PBS and subsequently lysed in lysis buffer containing sodium orthovanadate (Sigma-Aldrich, Germany). After the removal of the cell debris by centrifugation (12,000g, 20 min), the concentration of the protein in the supernatant was measured using the Bradford Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard. 20 μg whole cell lysate from each sample were boiled for 10 min in sample buffer, fractionated by 10% SDS–PAGE and transferred to a PVDF membrane. Nonspecific reactivity was blocked in 5% nonfat dry milk in TBST [10 mmol/L Tris–HCl (pH7.5), 150 mmol/L NaCl, and 0.05% Tween20] for 1 h at 37°C. The membranes were blotted with respective primary antibodies specific for each protein: MCT1, MCT4 and p150Glued with dilutions made according to the manufacturer’s recommendation. After incubation, membranes were washed 3 times in TBST for 15 min and subsequently incubated with the appropriate secondary antibodies (Sigma-Aldrich, Germany), followed by
development with the BCIP/NBT Phosphatase Substrate System (Pierce Biotechnology, Rockford, IL). p150\textsuperscript{Glued} were used as loading control.

**Immunocytochemistry analysis**

Immunofluorescence analysis was performed on HPDE and PDAC cells grown on glass coverslips and fixed with 4% (w/v) paraformaldehyde in PBS for 10 min, followed by a Triton X-100 permeabilization step. After blocking with 5 % (w/v) BSA for 30 min, cells were incubated with primary antibodies specific for MCT1, MCT4 and Basigin/CD147 with recommended dilutions overnight at 4°C in a humidified container. After several washes in PBS, cells were incubated with fluorescently-conjugated secondary antibodies (Alexa Fluor 594 goat anti-rabbit IgG (H+L) and Alexa Fluor 488 goat anti-mouse IgG (H+L); Invitrogen) for 1 h at room temperature. Cells were counterstained with 4', 6-diamidino-2- phenylindole (DAPI) and mounted with antifade reagent. Samples were visualized and images were acquired using an Olympus Bx63 epifluorescence microscope (100x/1.4 NA objective), and image processing (intensity adjustment only, quantitatively equal for all samples) was performed in Adobe Photoshop (Adobe Systems).

**Lactate uptake assay**

L-[^14]C(U)]-Lactic Acid or ^14^C-lactate (Perkin Elmer, Massachusetts, USA) were used to estimate the uptake of lactate via MCT1 and MCT4. HPDE and PDAC cells were grown to approximately 80% confluency in 6-well culture plates in complete growth medium. Cells were pre-incubated with 10 mmol/L of 4-CIN for 15 min and 10 µmol/L of AR-C155858 for 1 hour in the experiments performed with pharmacological inhibitors. Medium was subsequently replaced with 1 mL of isotonic ringer solution containing respective inhibitor and cells were incubated for 5 min at room temperature; on the experiments involving MCTs-knocked down cells, cells were first seeded and transfected with respective siRNA and used for experiments after 48 h of culturing. Medium was then replaced with 1 mL of isotonic ringer solution followed by incubation for 5 min at room temperature.
The lactate uptake experiment was initiated by adding 50 µL ¹⁴C-lactate solution to each well at different time points (5, 4, 3, 2, 1 and 0.5 min) during a 5 min incubation period. The influx was terminated by rapidly removal of the ringer solution containing ¹⁴C-lactate and lysing the cells in 200 µl 96% EtOH after a brief washing of cells with PBS containing 10 mmol/L 4-CIN. Subsequent to the complete evaporation of the EtOH, each well were added 1 mL ddH₂O and incubated 1 h on an orbital shaker to dissolve the isotope. The dissolved isotope was transferred to scintillation vials and each well was washed three additional times in 1 mL ddH₂O, giving a total of 4 vials per well. The activity was determined by β-scintillation counting after the addition of 3.5 mL Ultima Gold™ per vial. Cellular amino acid activity (cpm-well⁻¹) was converted to nmol·g protein⁻¹, using the extracellular specific activity (cpm/nmol) and the protein content (mg protein per well), and finally plotted versus time. Lactate uptake (nmol·g protein⁻¹ min⁻¹) was determined by linear regression.

*siRNA knockdown of MCT1 and MCT4*

siRNA transfections were performed according to the manufacturer’s instructions. Briefly, the following RNA oligonucleotides were commercially generated and purchased from Sigma Aldrich, Chemie Gmbh, Munich, Germany: siMCT1, 5’ GAAACGAUCAGUCUUCCAA-3’ (fw) and 5’-UUGGAAGACUGAUCGUUUC-3’ (rv); siMCT4, 5’- CGACCCACGUCUACAUCAUGUUG-3’ (fw) and 5’-AACACGUACAGACGUGGGUCG-3’ (rv). Cells were seeded and grown to 60%-80% confluency prior to the siRNA transfection. 50 nM of siRNA were mixed with Lipofectamine®RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions and incubated for 20 min at room temperature. Oligomer–lipofectamine complexes were then gently added to cells. Cells were harvested after 48 h culture for Western blotting analysis, migration assays, invasion assays, and spheroid outgrowth assays.

*Analysis of cell migration by in vitro wound healing assay*
HPDE and PDAC cell lines were plated respectively on 96-well plates (Essen ImageLock, Essen Instruments, Ann Arbor, MI) and allowed to grow overnight. A scratch was introduced on the bottom of each well with the 96-pin WoundMaker device (Essen BioScience, Ann Arbor, MI). The medium was aspirated from each well, and wells were washed twice with PBS to prevent settling and reattachment of dislodged cells. The cells were then cultured in the complete medium with respective MCT inhibitors along with appropriate controls. 5 µM of Aphidicolin (Sigma-Aldrich, Germany) was added to inhibit cell proliferation. The wound confluence was acquired and analyzed by using the IncuCyte phase-contrast imaging and scratch wound assay system and software (Essen instruments, Ann Arbor, MI). Wound closure was observed every 2 h for 72 h by comparing the mean relative wound density of three biological replicates in each experiment.

*In vitro cell invasion assay*

Invasion assay was performed using 24-well transwell BioCoat Chambers (BD Biosciences) according to manufacturer’s recommendation. Briefly, HPDE and PDAC cell lines were first starved with low serum media supplemented with 0.1% BSA. 24 h post-starvation, cells were harvested, resuspended in serum-free medium with or without respective MCT inhibitors, and 5 x 10⁴ cells/mL aliquoted cells were seeded onto the matrigel present in the 8.0 μm microporous inserts of 24-well chamber. 10% fetal bovine serum was added to the lower chambers as a chemoattractant and chamber was incubated at 37°C and 5% CO₂ for 22-24 h. The non-invading cells and matrigel were next gently removed by scrubbing with a cotton swab. Invasive cells located on the lower side of the chamber were fixed with absolute methanol and stained with diluted Giemsa staining solution for 30 min. Membranes were destained in phosphate buffer solution, pH 6.8, air-dried, and placed where the side with migrated cells facing down onto a glass slide. Five random fields of view per membrane were imaged (40x objective, Olympus Bx63 microscope, Olympus D73 camera). Data are shown as the total number of invaded cell over these 5 fields of view.
**Spheroid invasion assays**

BxPC-3 cells (48 h previously transfected with mock or MCT1 and/or MCT4 siRNA as indicated) were trypsinized, brought into suspension, and counted. In order to form spheroids, 50,000 cells/ml (200 uL working volume, 10000 cells/well) were dispensed in each well of an ultra-low attachment round-bottom 96-well plate (Corning), followed by centrifugation (750 rcf, 15 min, 4°C) of the plate. After 3 days, 20 µl droplets of 70% Geltrex (7mg/ml, Gibco® #: A1413202) and 30% bovine collagen I (3mg/ml, Gibco® #: A10644-01) were placed in a Petri dish. The spheroids were gently transferred to the center of the droplet using a 20-200 µL pipette tip. The dish was incubated upside down at 37°C for 30 min to allow for consistent positioning of the spheroid. A 10 µl droplet of Geltrex/collagen I mix was placed on top of the existing droplet, followed by another 30 min incubation at 37°C, and addition of growth medium. Spheroids were incubated at 37°C/5% CO₂, and images were acquired at day 0, 2, 5, and 7 using a Leica MZ16 microscope with a Plan Apo 11.5x objective.

**Statistical Analysis**

All data are presented as mean ± S.E.M. of at least three independent experiments. Data analysis was performed by Graphpad Prism 6.0 (GraphPad Software, Inc., CA). Multiple groups were analyzed by one-way and two-way ANOVA with post-hoc Tukey test. Single group data was assessed using the Student’s t-test. *p*-values <0.05 were considered statistically significant.
Results

*MCT1 and MCT4 are expressed in PDAC cells and colocalize with basigin in the plasma membrane*

To determine the expression levels of MCT1 and MCT4 in PDAC cells relative to normal pancreatic epithelial cells, qPCR and Western Blotting was performed on lysates from MIAPaCa-2, Panc-1, BxPC-3, and AsPC-1 human PDAC cells and the immortalized human pancreatic ductal epithelial cell line, HPDE. Interestingly, mRNA levels of both MCT1 and MCT4 were generally reduced by 40-60% in PDAC cell lines compared to that in HPDE cells (Fig. 1A,C). The only exception to this pattern was Panc-1 cells, in which the MCT4 mRNA level was similar to that in HPDE cells. The protein level of MCT1 also tended to be reduced in PDAC cells compared to that in HPDE cells, although less pronounced than the mRNA level (Fig. 1B), and the MCT4 protein level tended to be increased in BxPC-3 cells compared to that in HPDE cells (Fig. 1D). The specificity of both antibodies was confirmed by siRNA knockdown (see below). Immunofluorescence analysis confirmed the relative protein expression levels of MCT1 (Fig. 2A) and MCT4 (Fig. 2B) observed by Western Blotting and showed that both transporters predominantly localized in or closely adjacent to the plasma membrane of all the PDAC cells tested and colocalized strongly with the MCT1/4 chaperone basigin. HPDE cells differed from the PDAC cell lines in that both MCT1 and -4 staining also exhibited substantial intracellular localization (Fig. 2A-B).

*PDAC cell lines exhibit lactate transport inhibitable by 4-CIN, and modestly by AR-C155858, and MCT1 and -4 knockdown*

To assess whether the observed differences in MCT1 and MCT4 expression in the HPDE and PDAC cell lines correlated with differences in lactate transport, we first determined basal [$^{14}$C]-lactate uptake in all cell lines, the absence and presence of AR-C155858, a selective inhibitor of MCT1 and -2, and $\alpha$-cyano-4-hydroxycinnamate (4-CIN), a broad-spectrum MCT inhibitor (see 19). All PDAC cell lines as well as HPDE cells exhibited detectable lactate uptake, with
magnitudes in the order AsPC-1 > MIAPaCa-2 ~ Panc-1 ~ BxPC-3 > HPDE. Lactate influx was inhibited 10-20% by AR-C155858 (10 μmol/L), and by 35-80% by 4-CIN, depending on the cell line (Fig. 3A-C).

To validate these findings, MCT1 and MCT4 were next knocked down by siRNA in BxPC-3 and AsPC-1 cells (which both exhibited robust lactate fluxes yet differ in their migratory/invasive capacity, see below). Knockdown efficiencies ranged between 55 and 70% (Suppl. Fig. 1). No compensatory upregulation of MCT4 expression was seen after MCT1 knockdown or vice versa (Suppl. Fig. 1). Knockdown of either MCT1 or MCT4 reduced lactate uptake in AsPC-1 cells, whereas only MCT4 knockdown reduced lactate uptake in BxPC-3 cells, and this was not quite statistically significant (Fig. 3D-E).

Collectively, these data show that lactate uptake capacity is greater in PDAC cell lines than in HPDE cells, and appears to be mediated primarily by MCT4, but with a smaller contribution also from MCT1.

Migration of some PDAC cell lines in wound healing assays is partially dependent on MCT4

We next asked whether PDAC cell motility was dependent on MCT activity. To this end, we employed automated high-throughput cell migration analysis, in the presence of aphidicolin to prevent confounding effects of cell proliferation. A wound was introduced in confluent cell cultures, images were acquired every 2 h, and relative wound closure –an estimate of migration into the wound - was calculated at time 8 h. Representative images are shown in Fig. 4A-B for BxPC-3 cells, and in Suppl. Fig. 2 for remaining cell lines. MCT1/-2 inhibition by AR-C155858 had no detectable effect on relative wound closure at any of the time points tested (Fig. 4C). In contrast to AR-C155858, 4-CIN tended to reduce relative wound closure in all cell types except for Panc-1 cells (Fig. 4D).

To validate these findings, migration experiments were repeated after siRNA mediated knockdown of MCT1 or MCT4 in BxPC-3 cells (Fig. 4E-F). Supplemental Video 1 and 2 show representative movies from migration experiments performed on BxPC3 cells after MCT4
knockdown (Supplemental Video 1 and Supplemental Legends) or in corresponding cells treated with mock siRNA (Supplemental Video 2 and Supplemental Legends). Significant inhibition of migration was seen after knockdown of MCT4 (Fig. 4F). Importantly the effects of 4-CIN treatment (Fig. 4D) and MCT4 knockdown (Fig. 4F) on BxPC-3 cells were comparable in magnitude, suggesting that the effect of 4-CIN predominantly reflected MCT4 inhibition. These data collectively indicate that MCT4, but not MCT1, plays a major role in 2D migration of BxPC3 cells, and that this seems to be similar for other PDAC cell lines tested, with the exception of Panc-1 cells, in which migration was not attenuated by MCT inhibition.

Invasion of PDAC cell lines in Boyden chamber assays is partially dependent on MCT1 and -4

To assess the invasive capacity of the PDAC cell lines, chemotactic Boyden chamber invasion assays were performed. Serum-starved cells were allowed to invade for 24 h through matrigel-coated inserts and 8 µm pores towards the serum-containing lower chamber, in the absence or presence of MCT inhibitors. As expected, HPDE cells were incapable of invading through the matrix. Similarly, MIAPaCa-2 and AsPC-1 cells exhibited essentially no invasive capacity. In marked contrast, Panc-1 and BxPC-3 cells showed migration rates that were ~20- and 50-fold higher, respectively, than that of HPDE cells (Fig. 5). In contrast to the other cell lines tested, yet in agreement with previous reports on this cell line 38, BxPC-3 cells appeared to move collectively, appearing as large clusters on the trans side of the filters. MCT1 inhibition by AR-C155858 significantly reduced invasion of BxPC-3 cells, but had no or only marginal effect on invasion in the other cell lines tested (Fig. 5A-B). In contrast, 4-CIN strongly attenuated invasion in Panc-1 and BxPC-3 cells, and markedly dispersed the BxPC-3 cells (Fig. 5C-D; arrows in panel C). Since long-term incubation with 4-CIN could exert unspecific effects on the mitochondrial pyruvate carrier (see 19), MCT1 and -4 were also knocked down in BxPC-3 cells to validate these findings. In agreement with the pharmacological data, knockdown of either MCT1 or MCT4 significantly reduced BxPC-3 cell invasion, to about 35% and 40% of that in mock-transfected controls (Fig. 6A).
Knockdown of MCT1 and -4 inhibits outgrowth from BxPC-3 spheroids

Similar to other cancer cells, the phenotype of PDAC cell lines more closely mimics their original patient phenotype in a 3-dimensional (3D) spheroid context, and hence 3D outgrowth assays mimic more closely than Boyden type assays the conditions within a solid tumor. Furthermore, the 3D setting is of particular importance in the context of acid/base transporters since 3D growth poses an additional challenge to pH-regulation in the form of diffusion limitations. To assess 3D invasiveness, MCT1 and/or MCT4 were knocked down in BxPC-3 cells (which readily grow as well-defined spheroids), which were subsequently grown as 3D spheroids and subsequently embedded in a matrigel/collagen-I mix to monitor invasive outgrowth (Fig. 6B). Mock-transfected spheroids showed progressive infiltration into the surrounding matrix, and this effect was strongly reduced after knockdown of either MCT4 or both transporters in combination, whereas knockdown of MCT1 alone had a detectable but less marked effect (Fig. 6B). These data indicate that siRNA treatment targeting MCT1 and/or -4 reduces invasive behavior in BxPC-3 cells grown in 3D.
Discussion

The importance of lactate transport for metabolic function is well recognized across a variety of cancers. PDAC is one of the most deadly cancer forms globally, and new treatment options are urgently needed. To our knowledge, the only studies of MCTs in PDAC to date are our recent bioinformatic analyses indicating that MCT1 and MCT4 mRNA levels are increased in human PDAC tissue, and a study showing that knockdown of the MCT chaperone basigin elicits reduced MCT1 and MCT4 expression and concomitant intracellular lactate accumulation in PDAC cell lines. Here, we provide the first analysis of MCT1 and MCT4 mRNA and protein expression across a panel of human PDAC cell lines of moderately/poorly (BxPC-3) to poorly (MIA PaCa-2, Panc-1, AsPC-1) differentiated phenotype, and assess their importance for lactate uptake capacity, motility and invasiveness.

**PDAC cell lines exhibit varying levels of MCT1 and MCT4 expression and tend to show increased lactate uptake capacity compared to HPDE cells**

In most cell types, MCT1 and MCT4 mediate the majority of L-lactate transport across the plasma membrane. MCT1 has high (Km ~3.5-10 mmol/L) and MCT4 low (Km ~22-28 mmol/L) affinity for L-lactate. Accordingly, although they are bidirectional, secondary active transporters driven by the combined driving forces for H+ and lactate, MCT1 will most commonly function in the inward direction in oxidative tumor cells, while MCT4 is particularly well adapted for export of lactate from glycolytic cells. Upregulation of MCT1 and/or MCT4 has been reported in several cancer types, although the pattern is not straightforward, and reduced expression of one or the other isoform has also been reported in several tumor types (for an excellent overview, see). In PDAC, human patient data from publically available databases and studies of mouse models of PDAC have suggested the upregulation of both MCT1 and MCT4.

All PDAC cell lines tested in the present study showed robust MCT1 and/or MCT4 mRNA and protein expression, however, not exceeding that in the “normal” pancreatic ductal epithelial cell
line, HPDE cells. MCT1, but not -4, was detected in the normal exocrine rat pancreas sections fixed in situ 43, suggesting that MCT4 may be particularly strongly upregulated in PDAC, consistent with its known upregulation in other cancers 42:44. The apparent reduction in MCT1 and -4 mRNA levels in most PDAC cell lines compared to HPDE cells is likely an artifact related to the creation of HPDE cells, which were immortalized using the HPV16 E6/E7 gene, and consequently lack p53. While HPDE cells retain many genetic properties of normal pancreatic epithelium 35:36, the known role of p53 in glycolysis suppression via TP53-induced glycolysis regulator (TIGAR, an inhibitor of the fructose-2,6-bisphosphate 45) suggests that glycolysis, and consequently lactate flux capacity, i.e. MCT1 and/or MCT4, are upregulated in HPDE cells. Furthermore, in contrast to normal pancreatic epithelial cells, HPDE cells are flat and fail to form tight monolayers (Fig. 2; our unpublished data), likely reflecting the known effect of HPV on PDZ-domain containing proteins 46, and we speculate that this underlies the present observation that these cells are quite motile, albeit not invasive. Thus, while the HPDE cells are, to our knowledge the only available immortalized “normal” human pancreatic epithelial cell line, they are unlikely to reliably mimic the normal epithelium with respect to metabolism, polarity, and motility.

Both MCT1 and MCT4 were predominantly expressed at or closely adjacent to the plasma membrane in all cell lines, colocalizing with their chaperone basigin. Consistent with our findings, MCT1 was found to localize exclusively to the plasma membrane in normal pancreas, colocalizing with basigin 43. Interestingly, there was no strong correlation between mRNA and protein levels of MCT1 and -4 in the PDAC cell lines, likely indicating posttranscriptional regulation of expression, which has previously been reported for several MCTs, including MCT1 19.

All cell lines exhibited a basal lactate uptake, which was greatest in AsPC-1 cells and lowest in HPDE cells. In all cell lines, lactate uptake was attenuated strongly by the broad MCT inhibitor 4-CIN and modestly by the MCT1/2 inhibitor AR-C155858. While 4-CIN also inhibits the MPC
this is unlikely to have an effect on the measurements of rapid (0-5 min) plasma membrane lactate flux measurements. Confirming the role of MCT4, knockdown of this transporter also reduced lactate uptake in BxPC-3 cells, while knockdown of MCT1 did not. It is interesting to note that the low flux capacity of HPDE cells is consistent with the finding that a substantial fraction of the MCT1 and -4 staining did not localize to the plasma membrane in these cells, suggesting that they were not available for lactate uptake under the conditions studied. However, also in the PDAC cell lines, in which both MCT1 and -4 localized strongly to the plasma membrane, net expression levels did not appear to correlate with the relative lactate flux capacities. Furthermore, knockdown of neither MCT1 nor MCT4 abolished lactate transport, despite lack of compensatory upregulation of either of these isoforms, suggesting that other MCT isoforms may contribute to the lactate uptake capacity.

**MCT activity contributes to migration and invasion in PDAC cell lines**

Having demonstrated MCT1 and MCT4 expression and activity in the PDAC cell lines, we asked what roles these transporters have in regulating cell motility. We and others have demonstrated that other acid/base transporters play major roles in regulation of cancer cell motility through mechanisms involving local changes in pHᵢ and pHₑ. The roles of MCTs in motility have been addressed in a few previous studies. Thus inhibitory effects of 4-CIN on migration and invasion of high-grade glioma cells and on migration of SiHa cervical cancer cells have been demonstrated. Confirming the specific involvement of these transporters, knockdown of MCT1 or MCT4 was shown to reduce migration and/or invasion of various cancer cell lines, but nothing was so far known about the roles of MCTs in PDAC motility. Indicative of a role of MCT4 in this process, 4-CIN treatment inhibited PDAC cell motility to an extent similar to that of MCT4 knockdown (as shown in BxPC-3 cells). 4-CIN also inhibited invasiveness in all cell lines tested, as did MCT4 knockdown in BxPC-3 cells. Importantly, while inhibition/knockdown of MCT1 activity had no detectable role in cell migration these treatments did inhibit invasiveness in Boyden chamber assays. Furthermore,
inhibition of either MCT4 (and to a lesser extent MCT1) or MCT1 and -4 in combination reduced invasiveness in 3D outgrowth assays of BxPC-3 cells. Thus, an important finding of this study is that in PDAC cell lines, MCT1 activity seems to be more important for invasiveness (to which both MCT1 and -4 contribute) than for 2D migration. Interestingly, knockdown of MCT1 or MCT4 in human lung cancer cell lines, in analogy with our findings, was found to inhibit invasiveness without affecting migration\(^4\).

Essentially no studies have addressed the mechanisms of how MCTs might regulate cell motility. However, in the non-cancer epithelial cell lines, ARPE-19 and MDCK cells, MCT4 colocalized with β1 integrin in leading edge lamellipodia, and knockdown of MCT4, but not of MCT1, inhibited migration and increased focal adhesion size\(^4\). Furthermore, in MCF-7 breast cancer cells, we find that MCT1 localizes to invadopodial rosettes (Andersen, A.P, and Pedersen, S.F., unpublished). While further work is required to delineate the molecular mechanisms involved, these findings point in the direction of MCT activity serving a role similar to that of other net acid extruding transporters in regulation of cell invasion possibly via invadopodia function.

*In conclusion,* we show here that a panel of human PDAC cells exhibit plasma membrane expression of MCT1 and MCT4 and a corresponding lactate flux, mediated predominantly by MCT4. Migration of PDAC cells in wound healing assays was partially dependent on MCT4, in a cell-line dependent manner. In contrast, invasion of most PDAC cell lines was dependent on MCT1, and knockdown of MCT1 or MCT4 reduced invasiveness in 3D spheroid outgrowth assays. These findings indicate that MCT activity contributes to the motility and invasiveness in PDAC, with MCT4 primarily involved in migration and MCT1 in invasion. These findings have potential therapeutic importance in PDAC.

**Acknowledgements**

The HPDE cell line was a kind gift of Dr. M-S. Tsao from University Health Network in Toronto. We are grateful to Katrine Franklin Mark and Pernille Roshof for excellent technical
assistance, to the BRIC High Throughput Facility, Copenhagen, and Prof. L. Pardo, Max-Planck-Institute of Experimental Medicine, Göttingen, Germany, for use of IncuCyte equipment, and to Dr. Ian H. Lambert, University of Copenhagen, for introduction to lactate flux measurements.
Reference List


**Figure legends**

*Figure 1: mRNA and protein expression of monocarboxylate transporter 1 (MCT1) and MCT4 in HPDE and PDAC cell lines*

A, C. Relative mRNA levels of MCT1 and MCT4 in HPDE cells and PDAC cell lines (MIAPaCa-2, Panc-1, BxPC-3, AsPC-1) were assessed by qRT-PCR. Quantitative data was acquired as mean ± SEM using β-actin as a housekeeping gene calculated as described in Materials and Methods. Statistical analysis was performed with one-way ANOVA in Tukey post hoc test. Data are shown as mean with S.E.M. error bars, of three independent experiments per cell line. B, D. Protein expression of MCT1 and MCT4 was assayed by Western blotting. Upper panels showed representative blots and lower panels show densitometric quantification of normalized MCT1 and MCT4 levels in PDAC cell lines to HPDE. p150Glued was used as a loading control. 20 mg of total protein were loaded per lane. Densitometric data is shown as mean with S.E.M. error bars, of three independent experiments per cell line. *) (p < 0.05) and **) (p < 0.01): Significantly different from the level in HPDE cells, one-way ANOVA in Tukey post hoc test.

*Figure 2: Localization of MCT1, MCT4 and basigin in HPDE and PDAC cells*

Representative immunofluorescence images of HPDE cells and PDAC cell lines. Cells were paraformaldehyde-fixed and stained with antibodies against MCT1 (A, red), or MCT4 (B, red) and Basigin/CD147 (green), respectively. Cells were counterstained with DAPI (Blue) for nuclei staining. Scalebars: 20 µm. Data are representative of three independent experiments per cell line.
Lactate uptake measurements were performed on HPDE and PDAC cell lines with or without treatments as described in Methods and Materials. A. Lactate uptake (nmol*g protein\(^{-1}\) min\(^{-1}\)) of HPDE and PDAC cell lines, determined by linear regression of time traces as described in Materials and Methods. B-C. Inhibitory effect of AR-C155858 (10 \(\mu\)M) or 4-CIN (10 mmol/L) on lactate influx. Data are presented as the inhibition in %, calculated from the differences between control cells and treated cells. *) indicates significant differences (*p<0.05, **<0.05) as compared to respective normalized control, using Student’s t-test. D-E. Relative lactate uptake (nmol*g protein\(^{-1}\) min\(^{-1}\)) in BxPC-3 and AsPC-1 cells after knockdown of siMCT1 or siMCT4. *) significantly different from mock-transfected controls, Student’s t-test (*p<0.05, **<0.005, ***<0.0005). Data are shown as mean ± S.E.M. of 3 independent experiments performed in triplicate.

Figure 4: Effects of AR-C155858, 4-CIN, and MCT1 and -4 knockdown on PDAC cell migration.

HPDE and PDAC cells were grown to confluence in complete medium in 96 well plates, a wound introduced, and images recorded by IncuCyte analysis as described in Materials and Methods. A, B. Representative images of BxPC3 cell migration at 0 and 8 h, in absence and presence of AR-C155858 or 4-CIN as shown. Scalebars: 300 \(\mu\)m. C, D. Relative wound density at 8 h in the absence or presence of 10 \(\mu\)mol/L AR-C155858 (C) or 10 mmol/L 4-CIN (D). Abbreviations: H: HPDE; M: MIA PaCa-2, P: Panc-1; B: BxPC-3, A: AsPC-1. Data are shown as mean ± S.E.M. and statistical analyses were performed with two-way ANOVA followed by Tukey post-test. n = 3-5. Significant differences from the control cell line HPDE (*), and between untreated (control) and treated samples (#) are indicated. It may be noted that absolute migration rates varied between two different batches of HPDE and BxPC-3 cells used in panel C.
and D, resulting in the difference observed for these cells between the panels. E, F. Relative wound density at the indicated time points, in BxPC-3 cells treated with siRNA against MCT1 (E) or MCT4 (F), or mock siRNA. Data are shown as mean ± S.E.M. *) significantly different (p <0.05) from control, Student’s t-test.

Figure 5: Inhibitory effects of AR-C155858 and 4-CIN on PDAC cell invasiveness

HPDE and PDAC cells were plated onto matrigel invasion chambers after being serum-starved for 24 h and treated with respective inhibitors or DMSO (Control) for 22-24 h as described in Materials and Methods. A, B. Representative images of cells adhering to the lower chamber stained with Giemsa solution after the invasive process. Scalebars: 20 µm. Relative invasion was determined by the number of cells invaded through matrigel-coated inserts in the absence (Ctrl) or presence of inhibitors. C. 10 µmol/L AR-C155858, D. 10 mmol/L 4-CIN. Data are shown as mean ± S.E.M. of 4-8 independent experiments per condition. Significant differences from the control cell line HPDE (*), and between untreated (control) and treated samples (#) were indicated, two-way ANOVA with Tukey post-test.

Figure 6: Invasion of BxPC-3 cells is inhibited by knockdown of MCT1 and/or MCT4

A. BxPC-3 were transfected with siRNA against MCT1 or MCT4, or mock siRNA, respectively before seeding onto matrigel invasion chamber. Cells were culture for further 22-24 hs as described in Materials and Methods. Upper panels show representative images of cells adhering to the lower chamber stained with Giemsa solution after the invasive process. Scalebars: 20 µm. Relative invasion was determined by the number of cells invaded through matrigel-coated inserts. Data are shown as mean ± S.E.M. of 3 independent experiments per condition. Significant differences between non-silencing mock control and siMCT1 or siMCT4 treated cells are indicated as *), one-way ANOVA with Tukey post-test. B. Images depicting time course of BxPC-3 spheroids treated with siRNA targeting MCT1, MCT4 or MCT1+4 embedded in three-
dimensional (3D) matrix for the indicated times. Data are representative of 3 independent assays for each condition.

Supplementary Video 1: Representative videos from wound healing assays of BxPC-3 cells after knock down of MCT4.

BxPC-3 cells were transfected with siMCT4 after cells reaching 60-80% confluency. After transfection, cells were further grown to confluence in complete medium. A wound was introduced using 96-well WoundMaker. Images were subsequently recorded with IncuCyte™ (Essen BioSciences, USA) every 2 h as described in Methods and Materials.

Supplementary Video 2: Representative videos from wound healing assays of BxPC-3 cells transfected with unspecific siRNA control.

BxPC-3 cells were treated with unspecific siRNA control after cells reaching 60-80% confluency. After transfection, cells were further grown to confluence in complete medium. A wound was introduced using 96-well WoundMaker. Images were subsequently recorded with IncuCyte™ (Essen BioSciences, USA) every 2 h as described in Methods and Materials.
Figure 2 (Kong et al)

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Figure 4 (Kong et al)

A. BxPC-3
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   t=0hr
   
   t=8hr

B. BxPC-3
   Ctrl.  |  4-CIN
   
   t=0hr
   
   t=8hr

C. Chart showing relative wound density (%)
   - Ctrl.
   - AR-C155858
   - Relative Wound Density (%)
   - H, M, P, B, A

D. Chart showing relative wound density (%)
   - Ctrl.
   - 4-CIN
   - Relative Wound Density (%)
   - H, M, P, B, A

E. Chart showing relative wound density (%) over time:
   - Mock
   - siMCAT1
   - Relative Wound Density (%)
   - Time point (h): 4, 8, 12, 24

F. Chart showing relative wound density (%) over time:
   - Mock
   - siMCAT4
   - Relative Wound Density (%)
   - Time point (h): 4, 8, 12, 24
Figure 6 (Kong et al)

A

Mock  siMCT1  siMCT4

B

Number of Invaded Cells

Mock  siMCT1  siMCT4

Day 1  Day 3  Day 6  Day 8  Day 8 zoom

Mock  siMCT1  siMCT4  2 X Mock  siMCT1+4
Supplementary Figure 1: Knockdown efficacy of MCT1 and -4 in AsPC-1 and BxPC-3 cells
(a) Knockdown of MCT1 and/or MCT4 in AsPC-1 cells with siRNA significantly decreases expression of MCT1 and/or MCT4 respectively. (b) Knockdown of MCT1 and/or MCT4 in BxPC-3 cells with siRNA significantly decreases expression of MCT1 and/or MCT4 respectively. *P ≤ 0.05, **P ≤ 0.01, n=3 (exception of AsPC, relative MCT1 expression, siMCT4, n=2); one-way ANOVA, Dunnet’s multiple comparisons test.
Supplementary Figure 2: Representative images from wound healing assays of HPDE and PDAC cells.
HPDE and PDAC cells were grown to confluence in complete medium in 96-well plates, a wound introduced, and images recorded by IncuCyte analysis every 2 hours as described in Materials and Methods. Representative images of HPDE and PDAC cell migration at 0, 8 and 36 h are shown. Scalebars: 300 µm.
Paper III
Upregulation of the V-ATPase α3 subunit (ATP6V0a3) in pancreatic ductal adenocarcinoma: Contributions to the cancer phenotype

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Running title: ATP6V0a3 in pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most deadly cancers globally and its impact is predicted to increase over the next decades. Altered acid-base homeostasis is a major hallmark of solid tumors, yet its impact in PDAC is poorly understood. Here we address the regulation and roles in PDAC of the vacuolar H\(^{+}\) ATPase (V-ATPase), focusing on the a3 subunit of the V\(_{0}\) domain, ATP6V0a3 (hereafter a3), which is upregulated in PDAC patient tissue. The a3 mRNA and protein levels were increased in all PDAC cell lines tested (Panc-1, AsPC-1, and BxPC-3), compared to those in a non-cancerous pancreatic epithelial cell line, HPDE, while expression of the ubiquitous V-ATPase B2 subunit was unaltered. The V-ATPase (B2 immunostaining) mainly localized to a LAMP-1 positive compartment, suggesting predominantly late endosomal/lysosomal localization. Knockdown of a3 increased both migration velocity and directionality of BxPC-3 and Panc-1 cells, yet decreased invasiveness of BxPC3 cells in Boyden chamber assays. Recovery of intracellular pH (pH\(_{i}\)) in BxPC-3 and Panc-1 cells after an acid load in absence of Na\(^{+}\) and HCO\(_{3}^{-}\) was unaffected by a3 knockdown. a3 knockdown, similarly to V-ATPase inhibition by concanamycin A, resulted in upregulation of HIF-1\(\alpha\) and p21 and decreased Akt phosphorylation, as well as increased p62 levels. We conclude that despite its lack of detectable impact on global pH\(_{i}\) regulation after acid loading in PDAC cells, V-ATPase activity, and specifically a3, is important for motility, invasion and growth/survival signaling in PDAC cells.

Key words: pancreatic ductal adenocarcinoma; PDAC; PKB; Akt, p21; cell death; invasion; migration; proliferation; pH-regulation; TCIRG1; ATP6V0a3; OC116
Introduction

Pancreatic cancer, of which pancreatic ductal adenocarcinoma (PDAC) comprises about 90%, is one of the deadliest cancers globally, with a 5-year survival rate of less than 5% (1, 2). Recent analyses predict that pancreatic cancer will be the second leading cause of cancer-related death in the United States by 2030, reflecting changing population demographics and improved treatment options for other cancers (3). The majority of pancreatic cancer patients present with locally advanced or metastatic disease at the time of diagnosis, frequently rendering surgical treatment unfeasible. Standard treatment options for PDAC include gemcitabine combination therapies and the FOLFIRINOX combination (leucovorin, 5-fluorouracil, irinotecan, and oxaliplatin)(4). However, response rates are low, reliable biomarkers are lacking, and novel diagnostic and treatment options are urgently needed (5).

We and others have shown that solid tumors exhibit metabolic changes and high proliferative rates causing increased intracellular acid generation (6-10). Since normal cell function cannot be maintained at even modestly acidic intracellular pH (pHi), this necessitates increased net acid efflux, generally resulting in a very acidic extracellular tumor pH (pHe), while pHi is maintained normal or alkaline (6-9). In line with the increased demand for net acid extrusion, several acid-extruding transporters are upregulated in cancer cells and patient tumor tissue from various cancers (6-9, 11). Notably, it has been shown that upregulation of acid extrusion confers advantages to the cancer cells not limited to maintaining global cytosolic pH, such as increased survival, motility and invasiveness (12-16). Recent studies have focused primarily on the Na+/H+ exchanger isoform 1 (NHE1, SLC9A1), Na+,HCO3− cotransporters (NBCs, primarily SLC4A7), and the H+-linked monocarboxylate transporters MCT1 and -4 (SLC16A1 and -A3). However, also V-type H+-ATPases (ATP6) have been implicated in cancer development.

V-ATPases consist of a peripheral V1 domain responsible for ATP hydrolysis and a membrane-integral V0 domain, responsible for H+ translocation. Each of these domains is a multi-subunit structure, V1 consisting of subunits A-H, and V0 of subunits a, d, e, c, c′′ and accessory
subunit Ac45 (17). The subunit of the V-ATPase (ATP6V0a), which exists in 4 isoforms, a1-4, is a transmembrane protein which determines its subcellular localization (17-19). V-ATPases are ubiquitously expressed in intracellular organelles such as endosomes and lysosomes and are important for their luminal acidification. In addition, they are expressed in the plasma membrane of specialized cells, such as kidney intercalated cells, epididymal clear cells, and osteoclasts (17). Reflecting their essential role in bone resorption, genetic defects in ATP6V0a3, the predominant a3 isoform in osteoclasts, are responsible for about 50% of inherited forms of osteopetrosis (20). In addition to their established roles in acidification of endosomes and lysosomes, V-ATPases are also important for maintaining lysosomal homeostasis (21), and have been assigned roles as endosomal acid sensors with roles in regulation of traffic along the protein degradation pathway (22) and in the regulation of vesicle fusion and autophagy. The roles of V-ATPases in control of lysosomal function and autophagy render them particularly interesting in the context of PDAC, given the essential roles of macropinocytosis, increased lysosomal catabolic function and autophagy in this cancer type (23-26).

Highly invasive human breast cancer cells show higher levels of V-ATPase expression and activity at the plasma membrane than less invasive breast cancer cells, and in vitro invasion by MDA-MB-231 cells but not of MCF7 cells is inhibited by the V-ATPase inhibitors bafilomycin and concanamycin (27), in a manner recently proposed to reflect inhibition of plasma membrane-located V-ATPases (28). Pointing to specific roles of a3 and a4, MDA-MB-231 cells express higher levels of these isoforms relative to MCF7 cells and knockdown of either a3 or a4 inhibits invasion of these cells (29). Similar findings were reported in highly invasive MCF10CA1a cells (30). In melanoma cells, a3 expression determined the V-ATPase plasma membrane localization and increased invasiveness in vitro and metastasis in vivo, in part via upregulation of MMP2 and MMP9 (31). On the other hand, in many cell types, including NIH3T3 and RAW 264.7 macrophages, V-ATPases with the a3 subunit localize to late endosomes and lysosomes (19), and
it has also been reported on phagosomes (32) and other lysosome-related organelles such as pancreatic insulin granules (33).

A contribution of V-ATPases in the plasma membrane of normal pancreatic ducts to pH\textsubscript{i} regulation and potentially pancreatic secretion has been proposed, although the contribution to pH\textsubscript{i} regulation was modest and only detected in the absence of CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} and in presence of amiloride to inhibit NHEs (34). However, little is known about the roles of V-ATPases in PDAC, yet it has been reported that V-ATPase expression (the expression of the V\textsubscript{1}E subunit was assessed) is upregulated in PDAC and correlates with cancer stage (35). Furthermore, it was proposed that a3-containing V-ATPases localized to the plasma membrane of PDAC cells in a manner correlating with their invasiveness, and that V-ATPase inhibition reduced expression/activity of matrix metalloprotease-9 (MMP9), but stimulated that of MMP2 (35). Finally, the V-ATPase inhibitor bafilomycin was shown to induce apoptosis in PDAC cells and reduce xenograft growth of PDAC tumors (36). This is in accordance with studies from other cancer cell types demonstrating that this compound, or other V-ATPase inhibitors such as concanamycin A (ConA), elicits the activation of multiple signaling pathways favoring cell death and cell cycle arrest (37-42).

The aim of the present study was to characterize the regulation and roles of the V-ATPase subunit a3 in PDAC. We show that a3 is robustly upregulated at the mRNA and protein level in PDAC cell lines compared to non-cancerous pancreatic epithelial cells. The a3 subunit localizes mainly to an endosomal/lysosomal compartment and its knockdown has no detectable effect on pH\textsubscript{i}. However, a3 knockdown decreases invasiveness yet increases 2D migration of PDAC cells. Furthermore, a3 knockdown induces HIF-1\textalpha upregulation, growth arrest and apoptosis signaling in a manner similar, but not identical, to that induced by V-ATPase inhibition by ConA.

A part of this work has previously been reported in abstract form (43).
Materials and Methods

Computational analyses

Genome wide data (ChIP-seq, histone modification and CAGE) was obtained and visualized using the UCSC Genome Browser (http://genome.ucsc.edu; (44)). Dataset of genes co-expressed with ATP6V0a3 was generated using the CORD database (http://cord-db.org/ (45). Detection of over-represented transcription factor binding sites in the set of co-expressed genes was performed using the oPOSSUM online tool (http://opossum.cisreg.ca/oPOSSUM3/ (46)).

Reagents

Unless otherwise mentioned reagents were from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest analytical grade. Antibodies against PARP, phospho-Ser780 Rb, STAT3, phospho-Tyr705 STAT3, Akt, phospho-Ser473 Akt, p44/42 Erk1/2, phospho-Thr202/Tyr204 p44/42 Erk1/2, were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against p21 and Lamp-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against TCIRG1 and β-actin were from Sigma-Aldrich. Antibody against p62 was from Abcam (Cambridge, UK), HIF-1α and p150Glued antibodies were from BD Biosciences (San Jose, CA). B2 subunit antibody was kind gift from Michael Forgac, Tufts University, MA, USA. Secondary antibodies for Western blotting (goat anti mouse and goat anti rabbit) were from DAKO (Glostrup, Denmark). Concanamycin A was a kind gift from Dr. J. Nylandsted, Danish Cancer Society Research Center.

Cell culture and treatments

BxPC-3 and AsPC-1 were cultured in RPMI-1640 medium and Panc-1 cells in Dulbecco’s Modified Eagle Medium, all supplemented with Glutamax (Life Technologies, Camarillo, CA, USA), 10% “Gold” Fetal Bovine Serum (PAA Laboratories GmbH, Germany), and 100 U/ml penicillin, 100 μg/mL streptomycin. Immortalized normal human pancreatic ductal epithelial
(HPDE H6c7) cells were kindly provided by Dr M.-S. Tsao, Ontario Cancer Institute, Toronto, Canada (47, 48) and cultured in keratinocyte basal medium supplemented with epidermal growth factor and bovine pituitary extract. All cell cultures were maintained at 37°C, 95% humidity and 5% CO₂.

siRNA and Primers

Three pre-designed siRNA and a negative control siRNA against a3 were purchased from Origene (Cat. No. SR306986, Rockville, MD, USA). Cells were transfected with the relevant siRNA (10 nM final concentration) using Lipofectamine 2000 (Life Technologies) 48 h before start of the experiment. Primers were designed using online tool NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov) and synthesized by Eurofins Genomics, Germany. ATP6V0A3, sense 5’-GTGAATGGCTGGAGCTCCGATGA-3’; antisense 5’-AGGCCTATGCGCATCACCATGG -3’ and ATP6V1B2, sense 5’-AGTCAGTCGGAACTACCTCTC-3’; antisense 5’-CATCCGGTAAGGTCAAATGGAC -3’.

SDS-PAGE and Western blotting

After treatments as indicated, cells were lysed in 90°C SDS lysis buffer (0.1 M TRIS pH 7.5, 10% SDS, 1 mM NaVO₃) supplemented with Complete™ protease inhibitor mix (Roche Diagnostics, Germany). Protein concentrations were determined using DC assay (BioRad, Hercules, CA). Samples were mixed with loading buffer (5 mM Tris-Cl pH 6.8, 10% SDS, 1% bromophenol blue, 10% glycerol; Life Technologies) and equal amounts of protein per lane were separated by SDS-PAGE, using BioRad Tris/Glycine/SDS running buffer, premade 10% Bis-Tris gels (BioRad, Hercules, CA, USA), and BenchMark protein ladder (Life Technologies). Proteins were transferred to PVDF membrane using the Trans-Blot Turbo transfer system (BioRad). Membranes were Ponceau S stained, blocked for 1 h at 37°C in 5% nonfat dry milk in TBST (0.01 M Tris/HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.4), incubated with primary and secondary antibodies, and
developed using either (alkaline phosphatase-conjugated secondary antibodies) BCIP/NBT (KPL, Maryland, USA) or (HRP-conjugated secondary antibodies) ECL and the Fusion Fx system (Vilber Lourmat, Marne-la-Vallé, France). Densitometric analyses were carried out using UNSCAN-IT 6.1 software (Silk Scientific, Orem, Utah).

Quantitative real-time PCR (qPCR)

Isolation of total RNA from cultured cells was performed using NucleoSpin® RNA II (Macherey-Nagel, Germany) according to the manufacturer's instructions. Total RNA isolated was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and cDNA transcripts were amplified by qPCR using SYBR Green (Applied Biosystems, Cheshire, UK) and an ABI7900 qPCR machine. The reaction was repeated for 40 cycles with denaturation at 95°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 30 s. Triplicate samples were used. Primers were designed using the online tool NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov) and synthesized by Eurofins Genomics, Germany. Primer sequences were: ATP6V0A3, sense 5’-GTGAATGGCTGGAGCTCCGATGA-3’; antisense 5’-AGGCCTATGCGCATCACCATGG-3’ and ATP6V1B2, sense 5’-AGTCAGTCGGAACTACCTCTC-3’; antisense 5’-CATCCGGTAAGGTCAATGGGAC-3’; β-actin sense 5’-AGCGAGCATCCCCAAGTT-3’, antisense 5’-GGGCACGAAGGCTCATCATT-3’. Expression levels of each mRNA were evaluated using the comparative threshold cycle (Ct) method, normalized to β-actin Data were expressed as expression relative to that in HPDE cells.

Immunofluorescence imaging
Cells grown on glass coverslips were washed in ice-cold PBS, fixed in 2% paraformaldehyde for 15 min at room temperature and 30 min on ice, washed in TBST (2×5 min), permeabilized for 5 min in 0.1% Triton x-100 in TBST, blocked for 30 min in 5% BSA in TBST, and incubated overnight at 4°C with primary antibodies diluted in TBST + 1% BSA. Next day, preparations were
washed in TBST + 1% BSA (3×5 min), and incubated for 1 h at room temperature with the relevant fluorophore-conjugated secondary antibodies diluted in TBST + 1% BSA. Finally, preparations were washed in TBST + 1% BSA for 5×5 min, of which the second wash contained 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen) for nuclear staining. Coverslips were mounted in N-propyl-gallate antifade mounting medium on glass slides and sealed. Cells were visualized using the 40X/1.0 NA objective of an Olympus Bx63 epifluorescence microscope. No or negligible labeling was seen in the absence of primary antibody. Overlays and brightness/contrast adjustment was carried out using Adobe Photoshop software. No other image adjustment was performed.

*Measurements of intracellular pH (pHᵢ)*

Cells seeded on 15 mm glass coverslips were transfected with 10 nM a3 siRNA or corresponding control siRNA for 48 h. For pHᵢ measurements, cells were loaded with 2 μM BCECF-AM (Life Technologies) in a physiological saline solution (NaCl 125 mM, KCl 5 mM, CaCl₂ 1 mM, MgCl₂ 0.5 mM, Na₂HPO₄ 1 mM, glucose 11 mM, HEPES 25 mM). After 30 min, cells were washed twice in this solution. Coverslips were mounted in the perfused chamber of an Imic2000 microscope equipped with a gravity flow perfusion system, and images were acquired using a 40x oil immersion objective (Olympus, Tokyo, Japan), and a PolychromeV monochromator as light source (Till Photonics, Gräfelfing, Germany), appropriate Chroma filter set (Chroma Technology, Bellows Falls, VT, USA) and digitized by an Ixon 885 camera (Andor, Belfast, N. Ireland). Signals between 470-550 nm following 20 ms excitation at 440 nm and 485 nm were measured in 2 s intervals. Microscope control, signal visualization (binning 1x1) and analysis were performed in Live Acquisition software (Till Photonics). All experiments were done at 37⁰C and in absence of CO₂/HCO₃⁻ in order to facilitate isolation of the contribution of V-ATPases to the recorded pHᵢ changes. After achieving a stable baseline pHᵢ, cells were exposed to 20 mM NH₄Cl in the experimental saline for 10 min, followed by an ~3 min exposure to Na⁺-free saline (Na⁺ replaced equimolarly by N-methyl-D-glucammonium (NMDG⁺)), and return to the standard experimental
saline. Calibration to pH values was performed using the high-K/nigericin technique and a 3-point linear calibration curve(49), essentially as in (50). Intrinsic intracellular buffering capacity (βi) was calculated from the change in pH upon washout of NH₄Cl under Na⁺-free conditions, using the equation βi = Δ[NH₄⁺]/ΔpH, with [NH₄⁺] calculated using the Henderson-Hasselbalch equation and assuming a pKa of 8.9. Finally, net Na⁺-and CO₂/HCO₃⁻-independent acid efflux (J) was calculated as the change in pH during the last minute of the Na⁺-free phase, multiplied by βi, as: J=dpH/dt • βi.

Single cell migration analysis

T-12.5 flasks were coated with a substratum composed of extracellular matrix-mimicking components including laminins (4.46%), fibronectin (4.46%), collagen IV (0.602%), collagen III (1.34%), collagen I (89.15%). The coating was let solidify overnight in an incubator at 37°C. Transfected cells were thinly seeded at a density of 2 · 10⁴ cells per flask and allowed to adhere for 3–4 h at 37°C, 5% CO₂ prior to recording. The cells were placed in a heated chamber (37 °C) on the stage of an inverted microscope (Axio Vert.A1; 20x objective; Zeiss, Oberkochen, Germany). A representative field displaying at least eight cells was chosen for evaluation. Migration was monitored for 10 h with a video camera (Model: ORCA C8484-05G02; Hamamatsu, Herrsching, Germany) controlled by HC-IMAGE-Live software (version 4.2.5; Hamamatsu). Images were taken at 10 min intervals and stored as stacks of TIF-files. Single cell migration was manually tracked and quantified data were obtained using ImageJ (http://rsb.info.nih.gov/ij/). Migration is then represented by the movement of the nucleus during a defined time interval.

Migration velocity (μm min⁻¹) was calculated as the mean of the velocities for each time (10 min) interval, and is a measure of the average speed of movement during the experiment. Translocation was calculated as the mean distance between the position of the nucleus at the beginning and at the end of the experiment. The total path length was determined as the sum of
the single distances covered within each time interval. A directionality index as measure of sustained, directed migration was calculated dividing the translocation (start-end distance) by the total path length. Thus, a directionality index of "1" would represent a straight linear forward movement.

**Cell invasion analyses**

Invasion assay was performed using 24-well transwell BioCoat Chambers (BD Biosciences) according to manufacturer's recommendation. Briefly, HPDE and PDAC cell lines were first starved with low serum media supplemented with 0.1% BSA. 24 h post-starvation, cells were harvested, and resuspended in serum-free medium. 5 x 10⁴ cells/mL aliquoted cells were seeded onto the matrigel present in the 8.0 μm microporous inserts of 24-well chamber. 10% fetal bovine serum was added to the lower chambers as a chemoattractant and chamber was incubated at 37°C and 5% CO₂ for maximum 24 h. The non-invading cells and matrigel were next gently removed by scrubbing with a cotton swab. Invasive cells located on the lower side of the chamber were fixed with absolute methanol and stained with diluted Giemsa staining solution for 30 min. The membranes were briefly destained in phosphate buffer solution, pH 6.8, and air-dried. Membranes were cut off from the inserts and placed with migrated cells facing down onto a glass slide. Five random fields of view per membrane were photographed using a 10x objective on an Olympus Bx63 microscope and pictures were obtained with Olympus D73 camera. Data were shown as the total number of invaded cell over these 5 fields of view.

**Statistics**

Unless otherwise specified, data are presented as mean ± S.E.M. of at least three independent experiments. Data analysis was performed using SPSS software and Graphpad Prism 6.0. Multiple groups were analyzed by one-way ANOVA with Dunnett, Tukey, or Games-Howell post-hoc test
as relevant; Student’s $t$-test was used for comparisons of datasets with only two groups. $p$-values of $<0.05$ were considered statistically significant.
Results

The a3 subunit is upregulated in PDAC cells and mainly localizes to intracellular vesicles

Our recent bioinformatics analyses of expression patterns of acid-base transporters in human PDAC tissue indicated that mRNA levels of ATP6V0a3 as well as the ubiquitous V-ATPase subunit ATP6V1B2 are upregulated in PDAC tissue compared to normal pancreatic tissue, although this was not seen in all databases studied (51). To determine whether altered expression of these subunits occurs in in vitro models of PDAC, we first evaluated the mRNA levels and protein levels of ATP6V1B2 and ATP6V0a3 (hereafter: B2 and a3) in “normal” human pancreatic epithelial cells (HPDE cells) and three PDAC cell lines: Panc-1, BxPC-3 and AsPC-1. As seen, relative mRNA expression of B2 was significantly reduced in Panc-1 cells compared to HPDE cells, and not different from that of HPDE cells in the two other PDAC cell lines (Fig. 1A). At the protein level, evaluated by Western blotting, there was a slight decrease in the protein expression level of B2 in Panc-1 and AsPC-1 cells, and no change in BxPC-3 cells, compared to HPDE cells (Fig. 1B). In contrast, the relative a3 mRNA expression was increased in all of the 3 PDAC cell lines, with increases of 2- to more than 6-fold compared to that in HPDE cells (Fig. 1C). Similarly, a3 protein expression was upregulated in all PDAC cell lines compared to HPDE cells, to 3-4 fold in Panc-1 and BxPC-3 cells and more than 11-fold in AsPC-1 cells (Fig. 1D).

The subunit a3 has been implicated in the plasma membrane localization of V-ATPases in breast cancer and melanoma cells (27, 29, 31) and localization to the plasma membrane has been proposed in PDAC cells based on a3 immunofluorescence (35). We therefore next asked whether the increased a3 expression in PDAC cell lines was associated with increased membrane localization of the V-ATPase compared to that in HPDE cells, as visualized by immunofluorescence analysis of the ubiquitous B2 subunit (Fig. 2A). The great majority of B2 labeling was intracellular and punctate, and overlapped substantially but not completely with staining for LAMP-1, in agreement with the localization of the V-ATPase to the endosomal and lysosomal compartments (Fig. 2B).
In specialized cell types in which V-ATPases have well-defined plasma membrane functions, they have been shown to shuttle in a regulated manner between the plasma membrane and intracellular organelles (19, 52, 53). Increases in cellular cAMP and alkaline extracellular pH (pHe) increase plasma membrane V-ATPase insertion in epididymal clear cells (52). As alkaline pHe is unlikely to be relevant in cancers, which generally exhibit acidic pHe, we therefore asked whether stimulation with forskolin (10 µM, 30 min) to increase cAMP increased membrane insertion of B2. Forskolin treatment had no effects on V-ATPase localization detectable by immunofluorescence analysis, including no detectable translocation to the plasma membrane (Supplementary Fig. 1).

Collectively, these experiments show that the mRNA and protein expression of the a3 subunit of the V-ATPase are increased in PDAC cells compared to normal pancreatic epithelial cells, yet this is not associated with detectable changes in the localization of the V-ATPase to the plasma membrane, and the great majority of V-ATPase staining in PDAC cells is intracellular, predominantly lysosomal.

Knockdown of a3 does not alter recovery of global pH\textsubscript{i} after acid loading in PDAC cells

Next, we asked whether V-ATPase activity contributes to regulation of pH\textsubscript{i} in PDAC cells, and specifically whether a3 knockdown alters pH\textsubscript{i} regulation in these cells. All experiments were conducted in the nominal absence of HCO\textsubscript{3}\textsuperscript{-} to prevent contributions from HCO\textsubscript{3}\textsuperscript{-}-dependent transporters. BxPC-3 cells were loaded with the pH-sensitive fluorescent probe BCECF and exposed to an NH\textsubscript{4}Cl prepulse (20 mM NH\textsubscript{4}Cl, 5 min) to impose an intracellular acid load, and recovery of pH\textsubscript{i} was monitored over time. Despite the lack of visible recruitment of V-ATPases to the membrane by forskolin, in some experiments, cells were treated with forskolin (10 µM, 30 min) to maximize the chance of observing V-ATPase activity (Fig. 3B-E). In the absence of Na\textsuperscript{+}, intracellular acidification continued and no recovery was observed, and this was unaffected by a3 knockdown and forskolin treatment (Fig. 3A-D). Fig. 3E summarizes these data taking into
account the cellular buffering capacity, i.e. as the net H\(^+\) efflux (J\(_{H^+}\)) in the absence of Na\(^+\), and as a function of the pH\(_i\) at which the data were obtained. Comparable data were obtained in Panc-1 cells, using a3 siRNAs 1 and 2 (n=2 per condition, data not shown). When Na\(^+\) was re-introduced, the BxPC-3 cells in all cases recovered their pH\(_i\) efficiently after the acid load (Fig. 3), consistent with the very NHE1 expression in these cells (unpublished data). While not further studied here, it is interesting to note that while forskolin had no effect on recovery in the absence of Na\(^+\) (Fig. 3E), it clearly increased the rate of recovery in the presence of Na\(^+\) (compare Fig. 3A-B), suggesting that NHE activity in the BxPC3 cells may be stimulated by cAMP/PKA signaling.

Collectively, these results indicate that V-ATPase activity does not contribute detectably to regulation of global pH\(_i\) after an acid load in BxPC-3 cells. Notably, local effects on pH\(_i\) may have gone undetected in our approach. In fact, the pH\(_i\) values reached in BxPC3 cells after a3 knockdown were generally lower than those of mock-treated cells (Fig. 3E), suggesting that V-ATPase activity modestly impacts steady state pH\(_i\) maintenance in these cells and/or that the cellular energy stress created by V-ATPase inhibition increases net cellular acid production (see Discussion).

**Roles of a3 in invasion and migration of PDAC cells**

The a3 subunit has been assigned a specific role in invasion of breast cancer cells and melanoma cells (29-31). In PDAC cells, a3-containing V-ATPases were previously proposed to localize partially to the plasma membrane and ConA treatment to inhibit invasion (35). We therefore next asked whether knockdown of a3 impacted on the invasive and migratory capacity of PDAC cells, using Panc-1 and BxPC-3 cells as representative moderately and highly invasive PDAC cells, respectively (54). In Boyden chamber invasion assays, knockdown of a3 inhibited invasion of BxPC3 cells through matrigel by about 50% (Fig. 4A), whereas invasion of Panc-1 cells was slightly but not significantly attenuated (Fig. 4B).
Whereas effects of V-ATPase activity on invasion have been widely assessed, its impact on cell migration is much less investigated, and to our knowledge no data on the effect of a3 knockdown on migration of PDAC cells exist. We therefore next assessed the migratory capacity of Panc-1 and BxPC-3 cells on a 2D matrix simulating the desmoplastic microenvironment in PDAC tumors (in order of magnitude: collagen I, laminins (4.46%), fibronectin (4.46%), collagen III and collagen IV, reflecting the composition of the PDAC ECM (55-57)). Mock- and a3 siRNA-transfected cells were seeded sparsely on the matrix and single cell migration analyzed as total distance migrated, directionality index, migration velocity, and translocation (see Materials and Methods). Both directionality and velocity of migration was increased by knockdown of a3 (Fig. 5). Fig. 5A and 5B shows trajectories of single migrating cells, normalized to a common starting point. Fig. 5C-F show the quantified summary data obtained based on such trajectories. Notably, a3 knockdown increased 2D migration of both cell lines by 25-30% compared to that of mock-transfected controls, regardless of the migration parameter assessed.

**Signaling pathways elicited by a3 knockdown and V-ATPase inhibition in PDAC cells**

We next evaluated the impact of a3 knockdown on growth- and proliferation signaling in PDAC cells. BxPC-3 and Panc-1 cells were first subjected to a3 knockdown using three different siRNAs, and 48 h after transfection, cells were lysed and pathways relevant to cell cycle arrest, apoptosis and autophagy analyzed by Western blotting. Knockdown of a3 (Fig. 6A) resulted in a significant decrease in expression and phosphorylation of Akt (Fig. 6B), concomitant with an increase in p21 protein level (Fig. 6C). Notably, the protein level of HIF-1α was about doubled by a3 knockdown in BxPC-3 cells, while there was no consistent effect of a3 knockdown on the HIF-1α level in Panc-1 cells (Fig. 6D). The activity of ERK1/2 and STAT3, central signaling pathways in PDAC (58, 59) were not affected by a3 knockdown (Supplemental Fig. 2), and the p53 protein level was not significantly altered (n=4, data not shown). As V-ATPase activity has been implicated in the regulation of autophagy, we analyzed the protein levels of the polyubiquitin-binding protein
p62/SQSTM1 (p62), which interacts with LC3 and is itself a substrate for degradation in the autophagosome, the accumulation of which indicative of reduced autophagic flux (60). The knockdown of a3 tended to increase p62 levels in both cell types, although modestly so and not significantly across all 3 siRNAs (Fig. 6E).

To determine whether the effect of a3 knockdown was similar to that of V-ATPase inhibition, BxPC-3 cells were treated with ConA (2 and 10 nM) for 48 h, followed by Western blotting for signaling pathways previously implicated in V-ATPase-inhibition-mediated cell death (37-42). Similar to a3 knockdown, ConA treatment elicited a dose-dependent decrease in Akt phosphorylation (Fig. 7A) and p21 protein level (Fig. 7B), a strong, dose-dependent increase in HIF-1α protein level (Fig. 7C), and in this case a marked increase in p62 (Fig. 7D), indicative of reduced autophagic flux. Consistent with the notion that interfering with V-ATPase activity elicited general growth arrest, ConA treatment was also associated with a decrease in phosphorylated Retinoblastoma protein (p-Rb) (Fig. 7E), and increased PARP cleavage (Fig. 7F), indicative of decreased proliferation and increased apoptosis, respectively.

Possible mechanisms of regulation of the ATP6V0a3 promoter

In order to address possible mechanisms of a3 upregulation in PDAC cells, we used publicly available genome-wide data and used bioinformatical prediction tools, to possibly predict factors responsible for a3 upregulation. Cap analysis of gene expression (CAGE) data, publicly available in the Human Genome Browser, indicates that a3 gene (TCIRG1) has a single promoter, transcription initiation of which appears to be dispersed (Supplemental Fig. 3B). This is supported by the presence of a CpG island, a promoter element associated with initiation of transcription in a broad area of the promoter (61). The putative promoter region contains all features required for an active promoter: histone acetylation (H3K27Ac) 3-methylation (H3K4Me3 marks), DNAseI hypersensitivity clusters, as well as a plethora of transcription factors binding to the region, validated by chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq)
(Supplemental Fig. 3A). To investigate possible transcription activators or repressors involved in upregulation of a3 transcription in PDAC, we performed CORD-co-expression analysis (45). Using human gene expression microarray datasets from Gene Expression Omnibus (GEO), CORD algorithm predicted a set of genes, which expression correlated positively (Concordant, Supplemental Table 1) or negatively (Discordant, Supplemental Table 1) with expression of a3 subunit. The set of most significantly co-expressed genes (target genes names given in Ensemble format - Supplemental Table 2) were further subjected to Single Site Analysis (SSA) using the oPOSSUM online tool (46) (see Supplemental Table 2 “oPOSSUM results” for details used for analysis). Interestingly, the hits with the highest z-score (21.509 and 19.570 respectively), reflecting the occurrence of the TFBS in the target set of genes/sequences compared to background, were the transcription factors specificity protein 1 (SP1) and Krüppel-like Factor 4 (KLF4) (Supplemental Table 2), which we have previously demonstrated to be involved in the regulation of another cancer-associated acid-base transport protein, the Na⁺,HCO₃⁻ cotransporter NBCn1. Finally, although the very short 3’UTR of the TCIRG1 gene has no putative miRNA binding sites as predicted by TargetScan or microRNA.org (63-65), TCIRG1 mRNA was enriched in pulldowns of miR-34a, a tumor suppressor miRNA, often downregulated in pancreatic cancer (66).
Discussion

The understanding of how acid-base transporters contribute to PDAC is limited. A number of acid-base transporters have been shown to exhibit aberrant expression in both PDAC patients and cell lines, and/or have been implicated in PDAC in cell-line based studies (54, 67-69). Disturbed V-ATPases function has been implicated in a broad spectrum of diseases including, in addition to bone disorders, lysosomal storage diseases, neurodegeneration, myopathies, and cancer (70). In agreement with this notion, a role of V-ATPases in PDAC has been suggested (35, 36). However previous functional evidence was based on the use of bafilomycin, which also targets the SERCA Ca\(^{2+}\)-ATPase (71), a major concern given the central role of altered Ca\(^{2+}\) homeostasis in cancer development (72).

V-ATPase function is of particular interest in PDAC, given the reliance of this exceptionally aggressive cancer on macropinocytosis, increased lysosomal catabolic activity, and autophagy (23-26). Here, we focused on the a3 subunit of the V-ATPase, which our bioinformatics analyses indicated to be upregulated in PDAC patient tissue (43), and which has been proposed to target the V-ATPase to the plasma membrane in invasive breast cancer and melanoma cells (29-31) and to localize to the plasma membrane in PDAC cells (35). Indeed, in contrast to the ubiquitously expressed B2 subunit, we found a3 to be upregulated at the mRNA and protein level in all PDAC cell lines studied, compared to the non-cancerous HPDE cell line. Although V-ATPases per se have been proposed to be upregulated in some cancers including PDAC (73), the lack of B2 upregulation suggests that V-ATPase expression as such is not upregulated in cancer cells compared to non-cancerous cells, in line with recent work in breast cancer cells (41). Our analyses of publically available databases provided some clues to the mechanisms of a3 upregulation in PDAC. The TCIRG gene encodes, by alternative transcription start sites, two proteins: T-cell immune response cDNA 7 (TIRC7) protein, an ~75 kDa plasma membrane protein transiently expressed in some lymphocytes upon antigen stimulation and associated with inflammation (74) and T- and B- cell responses (75), and a3, also known as OC116 (as the name implies, 116 kDa) (76). The a3 ORF
appears to exhibit only a single functional promoter, of the dispersed type. Notably, the transcription factors SP1 and KLF4, which link to many forms of cancer and are involved in the regulation of the Na⁺,HCO₃⁻ cotransporter NBCn1 by the p95 HER2 oncogene (62), were identified as putative candidates for ATP6V0a3 transcription regulation. Most V-ATPase subunits appear to be strongly regulated by mRNA stabilization; specifically, a3 appears to be regulated both at the promoter level and by mRNA stabilization (77, 78). Consistent with the latter, TCIRG mRNA was enriched in pulldowns of miR-34a, a tumor suppressor miRNA, often downregulated in pancreatic cancer (66), hence, regulation of a3 at the miRNA level in PDAC is conceivable, and should be addressed in future work. Finally, it is interesting in the context of the acidic cancer microenvironment that acidic extracellular conditions (pH 5.5) was shown to increase the expression of V-ATPase a1, a2, a3, c, c" and d1 isoforms (31).

In our hands, the great majority of V-ATPase staining in PDAC cells localized to LAMP-1 positive intracellular vesicles, i.e. it was predominantly late endosomal/lysosomal, although we clearly cannot exclude the presence of a small fraction of cellular V-ATPases in the plasma membrane. In our hands, this distribution was not detectably altered by treatment with forskolin to increase cAMP, which is known to activate V-ATPase plasma membrane insertion in some cell types (52) and suggested to increase V-ATPase mediated H⁺ extrusion in the normal pancreatic duct (34). This result may not be surprising, as the PDAC cells have lost their polarization and thus are likely to exhibit altered responses to the signals regulating their function under normal, polarized conditions. Although previous work proposed a3 localization to the plasma membrane in PDAC (35), this claim was based on a very small fraction of total cellular V-ATPase staining, and the overall pattern appears similar to that found in the present study (35). In agreement with our findings, Sun-Wada and colleagues reported that although V-ATPase activity is important for melanoma cell motility (31), little if any V-ATPase expression was detectable in the plasma membrane of melanoma cells (18, 79). Rather, a3 was found to be involved in the localization of the V-ATPase to late endosomes, lysosomes, phagosomes, and, in specialized cells, to
melanosomes and insulin-containing granules (19, 32, 33, 79). In accordance with the lack of major V-ATPase localization to the plasma membrane in PDAC cells, knockdown of a3 had no detectable effect on cytosolic pH\textsubscript{i} regulation after an acid load. While substantial contributions of V-ATPase activity to cytosolic pH\textsubscript{i} regulation are clearly seen in some cell types (80) and a minor contribution has also been reported in intact pancreatic ducts (34), our data are in good agreement with the notion that in most cell types, including cancer cells, recovery after an acid load is generally largely or fully accounted for by the activity of Na\textsuperscript{+}/H\textsuperscript{+} exchangers and Na\textsuperscript{+}-HCO\textsubscript{3}\textsuperscript{-} cotransporters (16, 81). We did, however, observe an apparent reduction of pH\textsubscript{i} after a3 knockdown which might, as proposed in breast cancer cells (29) reflect reduced V-ATPase-mediated H\textsuperscript{+} pumping from the cytosol and into the endosomal/lysosomal compartment, whereas during an acid load, the fractional contribution of V-ATPases to recovery is too low to be detectable. Finally, in light of the marked impact of a3 knockdown on cell death/proliferation pathways demonstrated here, cellular pH homeostasis may be disturbed by a3 knockdown for reasons independent of a role in cytosolic acid extrusion.

We next determined the involvement of a3 in PDAC cell invasion and migration. In agreement with reports in melanoma and breast cancer cells on the importance of this subunit (29-31), and the inhibitory effect of ConA on invasion in PDAC cells (35), a3 knockdown inhibited invasiveness of BxPC-3 cells, although it had only a non-significant effect on invasiveness of Panc-1 cells. In agreement with our work, knockdown of a3 (and to an equal extent, a4), inhibited invasiveness in MDA-MB-231 breast cancer cells (29). In these cells, V-ATPases localize partially to the plasma membrane, however, this localization is mainly mediated by a4, with little if any contribution from a3 (29). Thus, our work, as well as studies in breast cancer (29), and in melanocytes (31) indicate that the role of a3 in invasiveness predominantly reflects roles of a3-containing V-ATPases residing in late endosomal/lysosomal compartments. A specific role of V-ATPases in the plasma membrane in breast cancer cell motility was nonetheless recently
demonstrated, suggesting that V-ATPases in one or both compartments may contribute, depending on the cell type (28).

In contrast to its inhibitory effect on invasiveness, a3 knockdown caused significant increases in migration speed and distance. While counter to at least one earlier report proposing reduced migration of PDAC cells by ConA (35) this is in line with recent work indicating that a given context can differentially affect invasiveness and migration. For instance, murine melanoma cells prefer to invade a dermis-like matrix to migrating on it, whereas they migrate on a basement membrane which they hardly invade (82), and for the PDAC cell lines MiaPaCa-2, Panc-1 and BxPC-3 it has been shown that the most invasive cell line is the least motile on a desmoplastic matrix (69). In conjunction with the previous work demonstrating that V-ATPases impact MMP expression/activity (31, 35, 83), we speculate that the V-ATPase mediated stimulation of invasion reduces 2D migration speed because 2D movement is arrested as the cells digest the matrix and begin to invade into it.

V-ATPases are emerging as important potential targets in cancer therapy (37-42, 84), yet given their central roles in normal physiology (17), their inhibition is likely to be associated with widespread toxicity. Specific targeting of a3 in cancers in which this subunit is overexpressed is therefore of therapeutic interest. Testifying to the validity of this concept, we demonstrated here that knockdown of a3 decreased Akt phosphorylation and increased p21, Hif-1α and p62 levels, qualitatively similar to the effect of inhibition of V-ATPase activity by ConA, and in agreement with the proposed role of V-ATPases in autophagy (32, 85). On the other hand, recent work challenged the proposed essential role of V-ATPases/a3 in autophagy (17, 71), by showing that cells lacking a3 or essentially lacking V₀ exhibited normal endo(lyso)osomal acidification and phagosome maturation, and that the latter was unaffected by neutralization of lysosomal pH (86) (the lack of dependence of autophagy on H⁺ transport by the V-ATPase is however, in agreement with previous reports (17, 71)).
The mechanisms through which a3 knockdown increases p21 levels and reduces Akt phosphorylation in PDAC cells appear to be similar to pathways previously reported in various cell types upon pharmacological V-ATPase inhibition, although the specific signaling effects of V-ATPase inhibition differ between studies, likely reflecting both cell-type-, inhibitor- and dosage-dependent effects (37-42). In our hands, both a3 knockdown and ConA treatment induced a robust increase in HIF-1α protein level, in accordance with previous work in several cancer cell types (37, 41). HIF-1α upregulation by bafilomycin A in PC3 prostate cancer cells was pH$_i$ independent (37), in congruence with the lack of effect of a3 knockdown on pH$_i$ in our study. It has been shown that Hif-1α undergoes not only proteasomal but also lysosomal degradation (specifically chaperone-mediated autophagy, CMA), and Hif-1α levels were consequently shown to be increased by inhibitors of lysosomal function in a range of cell types (87). Thus, we propose that the HIF-1α upregulation observed in the present study at least in part reflects reduced lysosomal degradation, possibly in conjunction with a stress response-mediated increase in HIF-1α levels as proposed in (41). With respect to the causal role of V-ATPase inhibition-mediated HIF-1α upregulation in downstream death/growth arrest, it has been proposed to be either protective (41) or, conversely, to be the mechanism of p21 upregulation and necessary for cell cycle arrest induction upon V-ATPase inhibition (37). Similar to our findings, p21 was upregulated in a HIF-1α-dependent, but p53-increase-independent manner by bafilomycin A in SiHa cervical cancer cells (37). Whether the role of a3/V-ATPase in cell death/proliferation signaling are dependent on altered H$^+$ pumping by the V-ATPase remains unresolved. Clearly, however, not all a3 functions are dependent on V-ATPase mediated endosomal/lysosomal acidification, including, e.g. insulin secretion in pancreatic β cells (33), and autophagic flux in Drosophila (71), in accordance with proposed pH-independent roles of the V$_0$ domain in membrane fusion events (for a review, see (17).

In conclusion, V-ATPase subunit a3 is robustly upregulated at the mRNA and protein level in PDAC cell lines compared to non-cancerous pancreatic epithelial cells. V-ATPases localizes
mainly to an endosomal/lysosomal compartment in PDAC cells, and does not contribute detectably to recovery of pHᵢ after an acid load. Knockdown of α3 decreases invasiveness yet increases migration of PDAC cells, and its knockdown has no detectable effect on pHᵢ, yet induces HIF-1α upregulation and growth arrest and apoptosis signaling in a manner similar to that induced by V-ATPase inhibition by ConA.

**Acknowledgements**

This work was supported by the Marie Curie Initial Training Network *IonTraC* (Grant Agreement No. 289648), and by the Hartmann Foundation (SFP), and by the Department of Biology, University of Copenhagen. The HPDE cell line was a kind gift of Dr. M-S. Tsao from University Health Network in Toronto. We are grateful to Katrine F. Mark and Pernille Roshof for expert technical assistance, and to Daniel Sauter for help in setting up the live imaging experiments.
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Figure legends

**Figure 1. ATP6V0a3, but not ATP6V1B2, is upregulated in all investigated PDAC cell lines.**
A, C. Relative mRNA levels of ATPV1B2 and ATPV0a3 in HPDE and PDAC cells were assessed by qRT-PCR. Data are shown as mean with S.E.M. error bars, of three independent experiments per cell line. B, D. Protein expression of ATP6V1B2 and ATP6V0a3 was assayed by Western blotting. Upper panels showed representative blots and lower panels show densitometric quantification of normalized to the level in HPDE cells. β-actin was used as a loading control. Data are shown as mean with S.E.M. error bars, of three independent experiments per cell line. * (p < 0.05), ** (p < 0.01) and *** (p < 0.005): Significantly different from the level in HPDE cells, using one-way ANOVA and Dunnett post hoc test.

**Figure 2. Immunofluorescence analysis of ATP6V1B2 localization in PDAC cells**
(A) ATP6V1B2 localization in PDAC cell staining (red). HPDE, PDAC, BxPC-3 and AsPC-1 cells were fixed using paraformaldehyde and subjected to immunofluorescence analysis using polyclonal B2 antibody and AlexaFluor488-conjugated secondary antibodies, and visualized by epifluorescence microscopy. Nuclei are stained with DAPI. (B) Co-localization of B2 with LAMP-1 in Panc-1 cells. Scale bars: Left row, 20 μm, right row, 5 μm.

**Figure 3. Recovery after acid loading in BxPC-3 cells.**
BxPC-3 cells were transfected with mock siRNA (A-B) or siRNA1 or -2 against a3 (C-D) as indicated, and 48 h later, were subjected to live imaging of pHᵢ using BCECF-AM under CO₂/HCO₃⁻ free conditions. Where indicated, forskolin (10 µM) was present during the 30 min loading of cells with BCECF. The horizontal bar indicates the switch from standard HEPES-buffered Na⁺-containing Ringer with 20 mM NH₄Cl to induce the acid load, to NMDG-Cl Ringer to observe Na⁺-independent pHᵢ recovery. E. The Na⁺-and CO₂/HCO₃⁻ independent net acid efflux
over time was calculated as the change in pH during the last minute of the Na\(^+-\)free phase, multiplied by \(\beta_i\) to obtain the flux, \(J_{H^+}\), and plotted against the mean pH during the minute of the measurement. BCECF ratios were calibrated to pH using the high K\(^+\)/nigericin technique. Data shown are representative of (A-D) or summarized from (E) 3 independent experiments. Error bars show SD of measurements on multiple cells in the same experiment (A-D) or S.E.M. of 3 independent experiments.

Figure 4. Knockdown of a3 decreases invasion of BxPC3 cells
A. PDAC cells were transfected with siRNA against ATP6V0a3 or mock siRNA, respectively before seeding onto matrigel invasion chambers. Experiments were terminated after another 22-24 h as described in Materials and Methods. Upper panels show representative images of cells adhering to the lower chamber stained with Giemsa solution after the invasive process. Scalebars: 20 µm. Relative invasion was determined by the number of cells invaded through matrigel-coated inserts. Data are shown as mean ± S.E.M. of 3-4 independent experiments per condition. Significant differences between non-silencing mock control and siTCIRG1 treated cells are indicated as *), one-way ANOVA with Dunnett post hoc test.

Figure 5. Migration of Panc-1 and BxPC-3 cell on a reconstituted extracellular matrix.
A-B. Trajectories of migrating mock- and a3 siRNA-transfected Panc-1 (A) and BxPC-3 (B) cells. The trajectories obtained were standardized to the same starting post represented by the center of a circle. The radii of the circles represent the mean distances (=translocation) covered within 10 h. C.-F. Total distance covered, translocation, velocity, and directionality index, calculated based on all data as in A-B. Data are representative of 4 independent experiments (44 mock and 34 a3 siRNA cells) for Panc-1 cells and 3 experiments (38 mock and 34 a3 siRNA cells) for BxPC-3 cells. Data given as means with SEM error bars. *) P<0.05, **) P<0.01, ***) P<0.001. Student’s t-test was used for comparison.
**Figure 6. Knockdown of a3 activates cell cycle arrest and apoptosis pathways and upregulates HIF-1α in Panc-1 and BxPC3 cells.**

BxPC3 and Panc-1 cells were transfected with 3 different a3 siRNAs for 48h, lysed and subjected to Western blotting for a3 (A), phospho-Ser473 Akt (B), p21 (C), HIF-1α (D) and p62 (E), followed by densitometric quantification. Data was normalized to β-actin and are shown as mean +/- S.E.M. of 2-6 n pr condition and siRNA. *, **: P < 0.05 and P < 0.01, respectively, indicate the comparison to control condition (mock siRNA) using Dunnetts post-hoc test. Representative blots of BxPC-3 are shown.

**Figure 7. Concanamycin A dose-dependently activates multiple cell cycle arrest and cell death pathways and upregulates HIF-1α in BxPC-3 cells**

BxPC3 cells were treated with 2 or 10 nM ConA for 48 h. (A) p21 protein level, representative blot and quantification. (B) phospho-Ser473 Akt protein level, representative blot and quantification. (C) HIF-1α protein level, representative blot and quantification. (D) p62 protein level, representative blot and quantification. (E) phospho-Ser795 Rb protein level, representative blot (n=4); (F) PARP cleavage, representative blot (n=2). One-way ANOVA with Tukey post-test. Quantification data are shown as mean with SEM error bars of 4 independent experiments per condition. *) P<0.05, **) P<0.01 compared to control. □) p<0.05 compared between treatments.

**Supplementary Figure 1. Effect of forskolin on V-ATPase localization in Panc-1 and BxPC3 cells.**

BxPC-3 (A) and Panc-1 (B) cells were stimulated with forskolin (10 µM) or vehicle control (DMSO) for 30 min and subjected to immunofluorescence analysis for B2 (red) and E-cadherin green. B2 and Lamp-1 antibodies in red, nuclei stained with DAPI. Scale bar: 10 µm.
Supplementary Figure 2. Knockdown of a3 has no effect on ERK and STAT3 activity in Panc-1 and BxPC3 cells.

Cells were treated essentially as in Fig. 5. Representative blots of, phospho-Thr202/Tyr204 ERK1/2, phospho-Tyr705 STAT3 and α-actin and their quantification. Data at least from 3 biological replicates. *: P < 0.05, indicate the comparison to control condition (mock siRNA) using Tukey post-hoc test.

Supplementary Figure 3. The putative promoter of ATP6C0a3.

(A) The figure shows a screen-shot of the putative ATP6V0a3 promoter from the human genome browser. CAGE data from at least 3 cell lines indicates a broader initiation site. Light blue lines indicate gene isoforms for TCIRG1 based on data from the USCF Human Genome Browser. Black lines indicate alignment of human mRNAs from GenBank. Two different histone modifications (H3K27Ac, H3K4Me3) from the ENCODE project are presented below. The predicted CpG-island is shown in green. Chip-seq results are shown as grayscale bars for a number of cell lines.

(B) Zoom of the CpG island from A section screenshot, to illustrate CAGE data and transcription initiation.

Supplementary Table 1. Cord analysis.

Cord online tool (http://cord-db.org/) uses publicly available microarray datasets from Gene Expression Omnibus to determine set of co-expressed genes, additionally it performs KEGG-pathway analysis and gene ontology. Following settings were using for analysis: Target Gene: “TCIRG1”, Method Type: “By Factors”, Factors: “Include Tissue Comparisons”, Species included in Experiments: “Human”, Fold Change Threshold: >2, p-value threshold: p<0.01, Maximum number of experiments: 3 or more.
Supplementary Table 2. oPOSSUM Single Site (SSA) analysis.

Detected over-represented conserved transcription factor binding sites in a set of genes co-expressed with a3. Settings given in detail in the Supplementary table 2 “Opposum results”.

Figure 1

A

ATP6V1B2

β-actin

B

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<td>β-actin</td>
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C

ATP6V0a3

β-actin

D

ATP6V0a3 (TCIRG1)

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Figure 4

A

**Panc-1**

Mock  siTCIRG1

B

**BxPC-3**

Mock  siTCIRG1

**Number of Invaded Cells**
Figure 5

A

Panc-1

B

BxPC-3

mock

siT

100 µm

C

D

E

F

**

**

***

n.s.

**

*
Figure 7

A

p21

β-actin

B

pAkt

Akt

β-actin

C

Hif1α

β-actin

D

p62

p150

E

p-pRb

p150

F

PARP

Cleaved PARP

β-actin
Suppl. Figure 1

A (BxPC-3)

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B (Panc-1)

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Suppl. Figure 3B

Scale

GM12878 whole cell polyA+ CAGE Plus start sites Rep 1 from ENCODE/RIKEN

Monocytes-CD14+ whole cell polyA+ CAGE Plus start sites Rep 1 from ENCODE/RIKEN

CD14 cell polyA+:

A549 whole cell polyA+ CAGE Plus start sites Rep 1 from ENCODE/RIKEN

CpG: 36

OpC Islands (Islands < 1000 Bases are Light Green)
Appendices
Appendix A – Materials and Methods

**Primers Design**
Primers used in the study were designed using Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) as described in Ye et al (2012). All primers were synthesized by Integrated DNA Technologies (Leuven, Belgium).

**Quantitative RT-PCR analysis**
Isolation of total RNA from cultured cells HPDE, MIA PaCa-2, Panc-1, BxPC-3 and AsPC-1 was performed using the NucleoSpin® RNA II (Macherey-Nagel, Germany) according to the manufacturer’s instructions. Total RNA isolated was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and cDNA transcripts were amplified by qPCR using SYBR Green (Applied Biosystems, Cheshire, UK) and the qPCR machine ABI7900, the reaction was repeated for 40 cycles with denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute and extension at 72°C for 30 seconds. Primer sequences applied as shown in Appendix B. Triplicate samples were used. Expression levels of each mRNA were evaluated using the comparative threshold cycle (Ct) method as normalized against house-keeping β-actin sense 5’-AGCGAGCATCCCCCAAAGTT-3’, antisense 5’-GGGCACGAAGGCTCATCATT-3’. Data were expressed as relative expression to the control cell line HPDE.

**Western Blot Analysis**
To detect individual protein expression, HPDE and PDAC cells were seeded in 10 cm² culture plates and cells were harvested at 80% confluence. Cells were washed twice with 1x PBS and subsequently lysed in lysis buffer containing sodium orthovanadate (Sigma-Aldrich, Germany). After the removal of the cell debris by centrifugation (12,000g, 20 min), the concentration of the protein in the supernatant was measured using the Bradford Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard. 20 μg whole cell lysate from each sample were boiled for 10 min in sample buffer, fractionated by 10% SDS-PAGE and transferred to a PVDF membrane. Nonspecific reactivity was blocked in 5% nonfat dry milk in TBST [10 mmol/L Tris–HCl (pH7.5), 150 mmol/L NaCl, and 0.05% Tween20] for 1 h at 37°C. The membranes were blotted with respective primary antibodies specific for each protein: NHE1, NHE8 (Santa Cruz Biotechnology, Dallas, TX, USA) and NBCn1 (a kind gift from Professor Jeppe Prætorius in Aarhus University, Denmark) with dilutions made according to the manufacturer’s recommendation. After incubation, membranes were washed 3 times in TBST for 15 min and subsequently incubated with the appropriate secondary antibodies (Sigma-
Aldrich, Germany), followed by development with the BCIP/NBT Phosphatase Substrate System (Pierce Biotechnology, Rockford, IL).

**Immunofluorescence Analysis**

Immunofluorescence analysis was performed on HPDE and PDAC cells grown on glass coverslips and fixed with 4% (w/v) paraformaldehyde in PBS for 10 min, followed by a Triton X-100 permeabilization step. After blocking with 5% (w/v) BSA for 30 min, cells were incubated with primary antibodies specific for NHE1, NHE8 (Santa Cruz Biotechnology, Dallas, TX, USA) and NBCn1 (a kind gift from Professor Jeppe Prætorius in Aarhus University, Denmark) with recommended dilutions overnight at 4°C in a humidified container. After several washes in PBS, cells were incubated with fluorescently-conjugated secondary antibodies (Alexa Fluor 594 goat anti-rabbit IgG (H+L) and Alexa Fluor 488 goat anti-mouse IgG (H+L); Invitrogen) for 1 h at room temperature. Cells were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) and mounted with antifade reagent. Samples were visualized and images were acquired using an Olympus Bx63 epifluorescence microscope (100x/1.4 NA objective), and image processing (intensity adjustment only, quantitatively equal for all samples) was performed in Adobe Photoshop (Adobe Systems).

**siRNA silencing of MCT1 and MCT4**

siRNA transfections were performed according to the manufacturer’s instructions. Briefly, the following RNA oligonucleotides were commercially generated and purchased from Sigma Aldrich, Chemie Gmbh, Munich, Germany: siMCT1, 5′ GAAACGAUCAGUCCUUCAA-3′ (fw) and 5′-UUGGAAGACUGAUUUC-3′ (rv); siMCT4, 5′-CGACCCAGUCUACAUGUACGUGUU-3′ (fw) and 5′-AACACGUACAUGUAGCGUGGGUCG-3′ (rv). Cells were seeded and grown to 60%-80% confluency prior to the siRNA transfection with mock control and siMCT1 or siMCT4. 50 nM of siRNAs were mixed with Lipofectamine®RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions and incubated for 20 min at room temperature. Oligomer–lipofectamine complexes were then gently added to cells. Cells were harvested after 48 h culture for subsequence assays and analyses.

**BrdU incorporation assay**

BrdU incorporation was performed using BrdU Cell Proliferation Assay Kit (#6813; Cell Signaling Technology, Massachusetts, USA), according to the manufacturer’s protocol. The assay measures the incorporation of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) into newly synthesised DNA of replicating cells by ELISA using an anti-BrdU monoclonal antibody. Briefly, transfected cells were seeded at a density of 5 × 10³ cells into a 96-well plate and allowed to attach overnight.
100 μL of fresh growth media was added to each well followed by 20 μL of BrdU labeling solution (final concentration 1x). Cells were incubated for 4 h. Labeling solution was discarded and 100 μL of the fixing/denaturing solution was added for 30 min at room temperature. 100 μL of 1x anti-BrdU antibody solution was added to each well and incubated for 1 h at room temperature. Cells were subsequently washed three times with wash buffer. 100 μL of peroxidase Antimouse IgG, HRP-linked secondary antibody solution was added and incubated for 30 min at room temperature. Cells were washed again three times with wash buffer. 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added and incubated for 30 min at room temperature prior of adding of 100 μL of stop solution. Absorbance was read at 450 nm within 30 min after the adding of stop solution.

**IncuCyte ZOOM® cell proliferation assay**

Cells were transfected with mock control and siMCT1 and siMCT4 for 48 h prior to the seeding of cells. Cells were seeded in a 96-well plate at a density of 6 x 10^3 cells/well. The growth curves were built from confluence measurements automatically acquired and registered during round-the-clock kinetic imaging by the IncuCyte ZOOM™ software system (Essen BioScience, Ann Arbor, MI, USA). Images were acquired every h of three biological replicates in each experiment.

**Statistical Analysis**

All data are presented as mean ± S.E.M. of three to four independent experiments. Data analysis was performed by Graphpad Prism 6.0 (GraphPad Software, Inc., CA). Multiple groups were analyzed by one-way ANOVA with post hoc Tukey-Kramer test.

**Reference**

# Appendix B – List of Primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'→3')</th>
<th>Tm</th>
<th>Product Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Na⁺/H⁺ Exchangers (NHEs)</strong></td>
<td></td>
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</tr>
<tr>
<td>SLC9A1 (NHE1)</td>
<td>Fw: CACACCACCATCAAATAACCCTTC</td>
<td>57.30</td>
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<tr>
<td></td>
<td>Rw: GAACCTTGGTTAGAAGAGAGGTCC</td>
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<td>SLC9A2 (NHE2)</td>
<td>Fw: TTGGACAGCTCCCTGCGTATGATG</td>
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<tr>
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<td>Rw: TCAGCTGCTGATGAGAAGAMATAACTG</td>
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<tr>
<td>SLC9A8 (NHE8)</td>
<td>Fw: CATGTGTGTGCTATTCTGCTGCT</td>
<td>63.20</td>
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<tr>
<td></td>
<td>Rw: GAAGGGGTTTCGATCGACTGGCAT</td>
<td>82.47</td>
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</tr>
<tr>
<td><strong>Anion Cl⁻/HCO₃⁻ Exchangers (AEs)</strong></td>
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<tr>
<td>SLC4A2</td>
<td>Fw: GAAGATCTCCTAGAGATGAGCG</td>
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<tr>
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<td>Rw: GTCCATGTGTCGACTTCCTG</td>
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<tr>
<td>SLC4A3</td>
<td>Fw: GTCTCATCCTGATCTCATG</td>
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<tr>
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<td>Rw: GGACACGCTGATCAGAC</td>
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<tr>
<td><strong>Na⁺ - HCO₃⁻ Cotransporters of the SLC4 Family (NBCs)</strong></td>
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<tr>
<td>SLC4A4 (NBCe1)</td>
<td>Fw: GGTGTGCTGTTACATGAGCTGTC</td>
<td>61.82</td>
<td>336</td>
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<tr>
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<td>Rw: GTCACTGTCAGACTTCCTG</td>
<td>61.29</td>
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<tr>
<td>SLC4A5 (NBCe2)</td>
<td>Fw: ATCTCTGAGACAGAGATGAC</td>
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<td></td>
<td>Rw: TGGTTGGCTGGAGTCAAGAG</td>
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<td>SLC4A7 (NBCn1)</td>
<td>Fw: ATCTTCGGAACCTCTGCTACT</td>
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<td>Rw: CGACTCTTTTCTACAAAAGGGAAC</td>
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<td>SLC4A8 (NBCe8)</td>
<td>Fw: GCTCTAAGAGCTGCCTTGCTAC</td>
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<td>Rw: CATGAAGACCTGAGACCCATC</td>
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<td><strong>Na⁺ - HCO₃⁻ Cotransporters of the SLC2 Family (SLC28)</strong></td>
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<tr>
<td>SLC28A4</td>
<td>Fw: GTTTACTAGCTGCTGCTTATGAAAGCTG</td>
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<td>Rw: CCTATGGGATTGGCGAGCTG</td>
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<td>SLC28A6</td>
<td>Fw: AAGCCACTCTGCTCGACACACAG</td>
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<td>Rw: ATGAAAGACAGGGTGAAGGAGCTGATAG</td>
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<td><strong>Monocarboxylate Transporters (MCTs)</strong></td>
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<td>SLC16A1 (MCT1)</td>
<td>Fw: CGGTGGTTGAAATTGAGTGTCTCATAT</td>
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<td>Rw: AAGTCGATAAATTGAGTGTCTGCCCAGGCAA</td>
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<tr>
<td>SLC16A3 (MCT4)</td>
<td>Fw: CCATGCTGCTGCTGAGGAGGAG</td>
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<td>225</td>
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<tr>
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<td>Rw: GCTTGCTGAAAGCTGCGGT</td>
<td>58.46</td>
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<td><strong>Vacuolar-type H⁺ ATPases</strong></td>
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<tr>
<td>ATP8V1B2</td>
<td>Fw: AGTCAGTCGGGAACACTACCGCTTC</td>
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<tr>
<td></td>
<td>Rw: CATCGGGTGAGGCATCGAGAC</td>
<td>59.06</td>
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<tr>
<td>ATP8V0A1</td>
<td>Fw: GTATTTAGGGAGAAACATTTGGGGA</td>
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<td>Rw: CATCGTGGCAATACGTCCAGACAG</td>
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<td>ATP8V0A3 (TCIRG1)</td>
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<td></td>
<td>Rw: AGGCGCTATGCTGGGATCAGAG</td>
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<td><strong>β-actin</strong></td>
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<tr>
<td>β-actin</td>
<td>Fw: AGCGGACATCCCAGAAAAGTT</td>
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<tr>
<td></td>
<td>Rw: GGGCAACGAGGCGCTCATATT</td>
<td>80.76</td>
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</table>
Appendix C – Protein Expression Level of Basigin

Protein expression of Basigin was assayed by Western blotting. Upper panels showed representative blots and lower panels show densitometric quantification of normalized basigin levels in PDAC cell lines to HPDE. p150\textsubscript{Glued} was used as a loading control. 20 mg of total protein were loaded per lane. Data is shown as mean ± S.E.M. n=3.

Appendix C: Relative expression levels of basigin in HPDE and PDAC cell lines

Protein expression of Basigin was assayed by Western blotting. Upper panels showed representative blots and lower panels show densitometric quantification of normalized basigin levels in PDAC cell lines to HPDE. p150\textsubscript{Glued} was used as a loading control. 20 mg of total protein were loaded per lane. Data is shown as mean ± S.E.M. n=3.