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Na^+/H^+ exchange in the tumour microenvironment: does NHE1 drive breast cancer carcinogenesis?

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ABSTRACT Ionic messengers signal several critical events in carcinogenesis, including metastasis, the leading cause of patient mortality. The aberrant metabolic, proliferative and anti-apoptotic nature of neoplastic cells can be traced to the abnormal expression of their ion transporters and related signalling networks. In this manuscript, we discuss Na^+/H^+ flux, as mediated by the sodium-hydrogen exchanger isoform 1 (NHE1), a major ion transporter involved in tumourigenesis. Allosteric activation of NHE1 by external stimuli is controlled by phosphorylation of key amino acids on its cytosolic C-terminal tail, which also acts as a signal scaffold for its regulation by intracellular protein and lipid binding partners. In breast cancer cells, pH homeostasis and proton dynamics are disrupted early in transformation. This constitutively activates NHE1, causing a reversal of the plasma membrane pH gradient, resulting in a more alkaline intracellular pH and a more acidic extracellular pH. NHE1-mediated cellular alkalinization potentiates cytoskeletal remodelling, mobilizing cells for directed migration. Concomitant redistribution of NHE1 to invadopodia, where increased proton extrusion promotes proteolytic digestion of the extracellular matrix, primes cells for invasion into the bloodstream. NHE1 hyperactivity therefore heralds an important stage in cancer cell development, critically facilitating the acquisition of the invasive phenotype necessary for metastasis to occur. The potential for targeting NHE1 in the development of novel chemotherapeutic applications is explored.

KEY WORDS: *NHE1, breast cancer, metastasis, carcinogenesis, chemotherapy*

Introduction

Ionic messengers are key participants in the most fundamental pathways of cell communication, both within the cytoplasm and at the intracellular-extracellular interface. Gap junctions and diffusion across the plasma membrane lipid bilayer allow for the free passage of ions between cells, while membrane-bound ion channels, transporters, and exchangers selectively control ion traffic at the organellar and cellular level. The constant, asymmetrical flux of ions (e.g. H^+, Na^+, K^+, Ca^{2+}, Cl^-, HCO_3^-) across the cell membrane results in an electrochemical gradient that is chiefly controlled by active transport. Therefore, ionic messengers can ubiquitously aid in metabolism as well as regulate cell volume, growth, proliferation and death in normal cells. In neoplastic cells, however, ion channels and their downstream signalling pathways can become dysregulated, thus contributing to transformation.

Research over the last decade has confirmed that a variety of ion transport proteins are expressed in cancer cells. Often, expression levels of these proteins and their perturbed functionality in tumour cells can be quite cancer-specific and play a significant role in both tumourigenesis and metastasis (Pedersen and Stock, 2013). Changes in ion channel expression can occur at genomic, transcriptional, post-translational or epigenetic levels and are associated with mechanistic and signalling events that are specific

Abbreviations used in this paper: CaM, calmodulin; CHP, calcineurin B homologous protein; EBP50, ERM-binding phosphoprotein 50 (also known as NHERF1); ECM, extracellular matrix; ER, estrogen receptor; ERM, ezrin-radixin-moesin proteins; HER2, human epithelial growth factor receptor 2 (also known as HER2/Neu or ErbB2); MMP, matrix metalloproteinase, $Na_V1.5$: voltage-gated sodium channels (*SCN5A*); NHE1, Na^+/H^+ exchanger isoform 1 (*SLC9A1*); NHERF1, Na^+/H^+ exchanger regulatory factor 1 (also known as EBP50); NBCn1, Na^+/HCO_3^- cotransporter 1 (*SLC4A7*); pH_i, intracellular pH, pH_e, extracellular pH; PR, progesterone receptor; TNBC, triple-negative breast cancer.

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for the stage and type of cancer being studied. For example, the expression of voltage-gated Na^+ channels is up-regulated in many types of carcinomas, while voltage-gated K^+ channel expression may become down-regulated as cancer progresses. The subsequent increase in intracellular Na^+ is thought to promote invasion of cancer cells (Fraser *et al.*, 2014), whereas altered K^+ flux can affect cell proliferation and migration, which aids in tumour progression (Fraser *et al.*, 2014, Pardo and Stuhmer, 2014). Transformed cells and tumour cell lines also lose their dependency on the Ca^{2+} signalling that drives proliferation. Indeed, the entire regulatory network of proteins and adaptor molecules that maintain Ca^{2+} signalling can become drastically remodelled to sustain the transformed phenotype (Roderick and Cook, 2008). Furthermore, in recent years, it has become abundantly evident that pH regulation has a dramatic impact on cancer progression. Therefore, the plethora of transporters that maintain pH homeostasis in cells have come under substantial scrutiny for the role they may play in cancer, from transformation to metastasis. On the plasma membrane, these include: Na^+/H^+ exchangers (NHEs), monocarboxylate transporters (MCTs), vacuolar-type proton pump ATPases (V-ATPases), anion exchangers (AEs), and bicarbonate-coupled transporters, particularly the Na^+/HCO_3^- transporter (Neri and Supuran, 2011). The preceding review articles provide a representation of the extensive body of research, both *in vivo* and *in vitro*, that reiterate the importance of ionic messengers, their transporters, regulatory partners and signalling networks in cancer. However, as the complex interaction between tumour cells and their immediate microenvironment becomes clearer, traditional roles for ion transporters are being redefined, sparking renewed interest in exploiting these proteins as potential targets in the development of novel chemotherapies. In the present review, we focus on one such ion transporter, the ubiquitously expressed Na^+/H^+ exchanger isoform 1 (NHE1), and its role and regulation in breast cancer cell development. Our aim is to summarize current knowledge and characterize the mechanisms underlying the dysregulation of NHE1 activity, a phenomenon that appears to drive carcinogenesis and metastasis within the tumour microenvironment. Finally, the potential for NHE1 as a target for chemotherapy is discussed.

pH homeostasis and breast cancer: the disruption of proton dynamics

Sodium-hydrogen exchangers (NHEs) belong to the SLC9A solute carrier family of ion transporters, of which there are nine known human isoforms (NHE1-9; *SLC9A1-9*). While much is known about the structural dynamics of NHEs (Hendus-Altenburger *et al.*, 2014), no precise crystal structure has been determined for any of the human isoforms. Human NHE1 (*SLC9A1*) is 815 amino acids in length. A topology model (Wakabayashi *et al.*, 2000) based on cysteine accessibility studies, suggests that the first 500 residues are the hydrophobic N-terminal transmembrane domain. This domain is responsible for ion transport. The 315-amino acid hydrophilic C-terminal cytosolic domain regulates activity of the membrane domain and ionic homeostasis through post-translational modifications (chiefly via phosphorylation) or through interactions with intracellular lipid or protein binding partners (Lee *et al.*, 2013). NHE1 is highly sensitive to amiloride and its lipophilic derivatives, as well as the benzolyguanidine class of inhibitors that include cariporide (Donowitz *et al.*, 2013). NHE1 does not require energetic input from ATP hydrolysis, instead it uses the energy of the inwardly directed sodium gradient to extrude protons. However, NHE1 is an ATP-binding transporter dependent on cellular ATP for its activation (Shimada-Shimizu *et al.*, 2013). NHE1 extrudes intracellular H^+, a by-product of metabolism or electrochemical transport, in an electroneutral 1:1 ratio in exchange for extracellular Na^+. The directionality of Na^+/H^+ exchange is reversible and dependent on the transmembrane concentration gradients for both ions, as well as intracellular pH (pH_i). Increased intracellular H^+ ions rapidly stimulate exchanger activity (Wakabayashi *et al.*, 1997b). Na^+/H^+ exchange results in cellular alkalinizition that simultaneously maintains both pH_i and cell volume. Hence, dysregulation of Na^+/H^+ exchange can have significant deleterious effects on cells. Indeed, many NHE isoforms can contribute to the pathophysiology of various human diseases including cancer (Fuster and Alexander, 2014).

It has long been known that the solid tumor cores acidify their cores (Hasuda *et al.*, 1994). Poor vascularization of tumours may account for some of the extracellular acidification. However, this phenomenon can be distinguished from the elevation of pH_i in various types of tumour cells that has, at least in some cell types, been shown to be due to an increase in Na^+/H^+ exchanger activity, even in cultures of monolayers of cells (Karki *et al.*, 2011). Elevated pH_i and acidification of extracellular space is reliant on the regulation of the Na^+/H^+ exchanger (Amith and Fliegel, 2013).

For breast cancer cells, when tested *in vitro*, highly invasive MDA-MB-231 cells were found to actively acidify their non-buffered balanced salt solution media in culture. This did not occur with estrogen receptor (ER)-positive, lowly invasive MCF-7 breast cancer cells, or normal mammary epithelial cells. This finding was particularly significant since MDA-MB-231 cells are representative of the metastatic triple-negative form of breast cancer (negative for the expression of estrogen (ER) and progesterone receptors (PR), and human epidermal growth factor receptor 2, HER2). The same study also found that metastatic breast tumour cells from pleural effusions were 200-fold more active in acidifying their extracellular culture media compared to non-malignant mammary cells, suggesting that extracellular acidification also occurs in tumours *in vivo* (Montcourrier *et al.*, 1997). In E7 oncogene-induced transformation of NIH3T3 murine fibroblasts and human keratinocytes, early cytoplasmic alkalinization was a direct result of an increase in NHE1 activity. This increase was driven by an underlying increase in the exchanger's affinity for allosteric proton binding. Interestingly, the reprogramming of cellular metabolism to aerobic glycolysis was found to be a result of, and dependent upon, E7 oncogene-mediated alkalinization. Furthermore, in the presence of the NHE1 inhibitor 5-(N,N-dimethyl) amiloride, or when pH_i was clamped to non-tumorigenic levels, cells did not exhibit the transformed phenotype, and no subsequent increased growth rate, serum- and anchorage-independent growth, glycolytic metabolism or *in vivo* tumour development in nude mice was observed (Reshkin *et al.*, 2000b). The continued rise in intracellular pH as the tumour progresses is associated with elevated aerobic glycolysis, the so-called "Warburg effect" (Vander Heiden *et al.*, 2009), metabolic reprogramming of pyruvate oxidation to lactic acid conversion, and extracellular oxygen and nutrient depletion as a result of poor perfusion (Parks *et al.*, 2013, Webb *et al.*, 2011).

The activation of NHE1 leads to a reversal of the pH gradient of tumour cells early in oncogenic transformation. The H^+ ions extruded chiefly by NHE1 build up in the interstitial spaces between cells of

the developing tumour; this acidifies the tumour microenvironment and facilitates metastasis. The resultant low pH is necessary for the optimal activity of matrix metalloproteinases and other proteolytic enzymes that degrade the extracellular matrix, and aid in the detachment and dissemination of tumour cells. Coupled with poor vasculature, nutrient deprivation, and hypoxic conditions in and around growing tumours, the acidic microenvironment promotes cancer cell survival, proliferation, migration, invasion and eventual metastasis to sites distant from the primary tumour (Cardone *et al.*, 2005b, Donowitz *et al.*, 2013).

The C-terminal domain of NHE1: a tail of complex regulation

The functional regulation of NHE1 activity in normal cells is both complex and myriad. Apart from the allosteric activation of NHE1 by intracellular protons, several external factors are also known to drive exchanger activity, including: growth factors and hormones in serum, and several classes of receptors on the plasma membrane (receptor tyrosine kinases, G-protein coupled receptors, and integrin receptors). Modulation of NHE1 activity by external factors is mediated through its cytosolic C-terminal tail, either via phosphorylation of specific amino acids, binding of interacting adaptor proteins or lipids, or conformational changes, all of which potentially alter the affinity of the exchanger's allosteric binding site for H^+ (Putney *et al.*, 2002). Early studies showed that, in physiological conditions, the activation of NHE1 by growth factors (like α-thrombin and insulin) is due to an increase in the allosteric affinity of the exchanger for internal H^+ (Paris and Pouysségur, 1984). While the transmembrane domain of NHEs is highly conserved amongst the isoforms, the C-terminal domain is not. In NHE1, deleting the C-terminal tail does not impact ion exchange but does affect H^+ "sensing", a measure of activity at a particular pH_i. In support of this proton-sensing model, one study examined four subdomains of the cytosolic tail of NHE1 in the unstimulated state: I, amino acids (aa) 516-590/595; II, aa 596-635; III, aa 636-659; and IV, aa 660-815. Truncations of these amino acid sequences suggested that subdomain I plays an important role in the maintenance of high pH_i sensitivity of the exchanger, while subdomains II and IV were shown to have no discernible role in pH sensing. Subdomain III is involved in autoinhibition of NHE1, which is facilitated by calmodulin binding and, potentially, the spatial arrangement of subdomain II, a proposed "flexible loop" domain that aids this interaction (Ikeda *et al.*, 1997) (Fig. 1).

Several major regulatory phosphorylation events on the NHE1 tail have been mapped to the region between aa 636 to 815 (Wakabayashi *et al.*, 1994). These include target residues for phosphorylation by: extracellular signal-regulated kinase (ERK1/2) (Liu *et al.*, 2004), mitogen-activated protein kinase (MAPK) (Malo *et al.*, 2007)], and B-Raf kinase (Karki *et al.*, 2011); see Hendus-Altenburger *et al.,* (2014) for a comprehensive listing. However, not all the protein kinases and phosphatases that phosphorylate NHE1 have been identified. Similarly, several kinases are known to mediate NHE1 activity, but their exact amino acid targets are yet to be determined. Based on the relative positions of these known and predicted phosphorylation sites on the NHE1 C-terminal tail, it has been intriguingly suggested that phosphorylation at these sites may be interdependent, forming "clusters" of phosphorylation events that could be involved in the temporal regulation of NHE1 (Hendus-Altenburger *et al.*, 2014). Recently, it was demonstrated that phosphorylation can induce structural changes in the C-terminal of NHE1 that mediate phosphorylation-induced regulation (Li *et al.*, 2013a). To date, however, it is unclear if these same regulatory phosphorylation sites and mechanisms are retained in breast cancer cells (Amith and Fliegel, 2013).

Of particular interest is Ser703, where activation of the exchanger is attributed to phosphorylation by p90 ribosomal S6 kinase ($p90^{RSK}$), a downstream kinase of ERK1/2. Site-specific mutation of Ser703 to a non-phosphorylatable alanine residue, did not affect acid-stimulated Na^+/H^+ exchange but completely prevented the increase in H^+ affinity of NHE1 usually associated with growth-factor stimulation (Takahashi *et al.*, 1999). Later, a novel

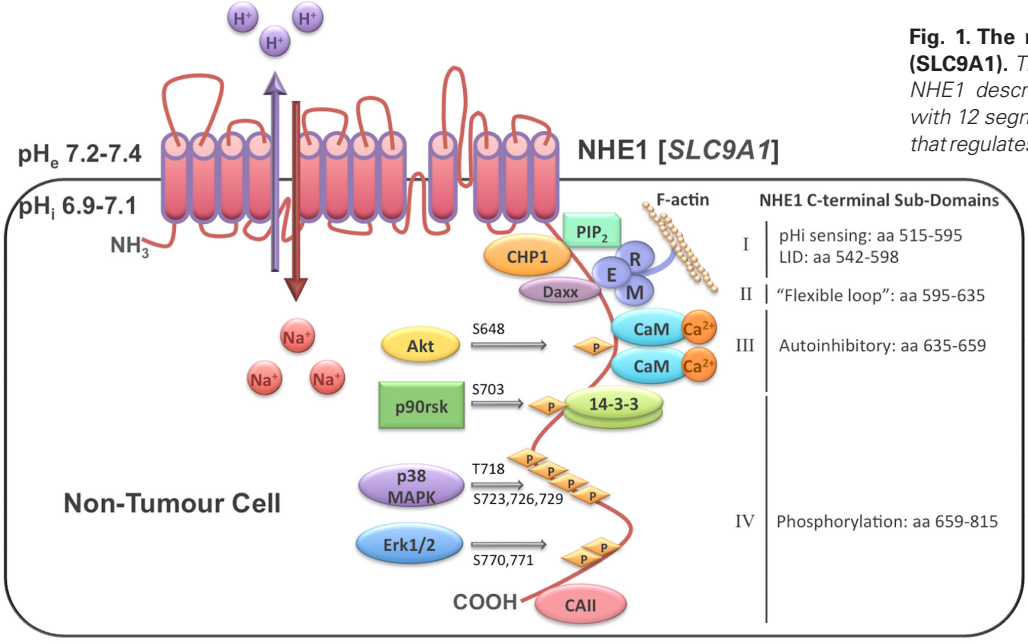

Fig. 1. The mammalian Na^+/H^+ exchanger, NHE1 (SLC9A1). *The widely accepted structural model of NHE1 describes a plasma transmembrane domain with 12 segments, and a cytosolic C-terminal domain that regulates Na^+/H^+ exchange. The C-terminal domain is further divided into four sub-domains characterized according to their interactions with intracellular protein and lipid binding partners and sites of phosphorylation. Basal NHE1 activity in normal (non-tumour) cells maintains intracellular pH between 6.9-7.1 and extracellular pH between 7.2-7.4. Akt, protein kinase B; CaM, calmodulin; CAII, carbonic anhydrase II; CHP, calcineurin homologous protein; E, ezrin; Erk1/2, Extracellular signal-regulated protein kinases 1 and 2; R, radixin; M, moesin; p38MAPK, p38 mitogen-activated protein kinases; P, protein phosphorylation site; PIP2, phosphatidylinositol 4,5-bisphosphate; p90rsk, ribosomal s6 kinase or MAPK-activated protein kinase-1.*

association of 14-3-3β with NHE1 at Ser703 in response to serum stimulation was demonstrated (Lehoux *et al.*, 2001). 14-3-3 binding to Ser703 in its phosphorylated state was necessary for serum-induced exchanger activity, and prevented its dephosphorylation. Mitogen-activated protein kinase kinase (MEK) inhibitors prevent 14-3-3 binding to NHE1 in serum-stimulated conditions, which has broader implications on MEK-ERK-RSK-mediated regulation of exchanger activity (Lehoux *et al.*, 2001) and, conversely, on the NHE1-mediated activation of MAPK signalling pathways (Pedersen *et al.*, 2007). In cancer cells *in vivo*, low serum is the norm in the tumour microenvironment, yet exchanger activity is unaffected. This is particularly evident in breast cancer cells where NHE1 becomes overactive with serum depletion. Exactly how the regulatory mechanisms underlying NHE1 activity is altered in low serum is not fully understood (see also below) but the activation of NHE1 in the low serum environment suggests that Ser703 is not the only regulator of NHE1. Two phosphorylatable residues upstream of Ser703 are Ser726 and Ser729. Substitution of Ser726 and Ser729 on NHE1 with alanine in non-tumour cells protected against cell death induced by serum deprivation, and prevented cellular alkalinization associated with apoptosis in a p38MAPK-dependent manner. Conversely, substituting these serines for phospho-mimetic glutamic acids resulted in a more alkaline basal pH_i and increased susceptibility to cell death signals (Grenier *et al.*, 2008). These studies allude to how tight the regulation over phosphorylation events on the NHE1 C-terminal can be, even at a single amino acid residue. Testing these mutations in a breast cancer cell model would therefore further elucidate the regulation of NHE1 in low serum conditions, and define exactly how much cell survival depends on Na^+/H^+ exchange.

Independent of phosphorylation by protein kinases and phosphatases, NHE1 interaction with its cofactors and binding partners is also important in its regulation, particularly in response to cell stressors and external stimuli. NHE1 is regulated by a multitude of binding proteins (reviewed in (Fliegel, 2009)). Several aspects of this regulation are of particular interest in breast cancer and in relation to the tumour microenvironment. NHE1 is proposed to be a scaffolding protein, an idea that stemmed from the discovery that it directly associates with the actin binding proteins ezrin, radixin and moesin (ERM) in the lamellipodia of fibroblasts, where NHE1 is predominantly localized (Baumgartner *et al.*, 2004). Mutations in the exchanger that disrupt binding of ERM proteins resulted in irregular cell shape with disorganization of focal adhesion assembly and actin stress fibers. While these mutations disrupted the cortical cytoskeleton of fibroblasts, no impairment of Na^+/H^+ exchange activity was observed (Denker *et al.*, 2000). NHE1-ERM interaction is also involved in cell survival signaling (Wu *et al.*, 2004). Death domain-associated protein Daxx stimulates exchanger activity under ischemic stress, and competes with ezrin in binding to NHE1 (Jung *et al.*, 2008).

Additional hormone-induced regulation of NHE1 is mediated through the lipid-interacting domain of the C-terminal tail proximal to the transmembrane domain. This region spans aa 542 to 598 and associates with acidic phospholipids (e.g. phosphatidylinositol-4,5-biphosphate, PIP_2) and other hydrophobic residues, as well as the ERM proteins and Daxx, in a competitive manner (Hendus-Altenburger *et al.*, 2014). Diacylglycerol, a by-product of PIP_2 hydrolysis mediated by hormone-induced G protein-coupled receptor activation, and tumour-promoting phorbol esters, are potent activators of protein kinase C (PKC). PKC is thought to be involved in the hormonal activation of NHE1, but is not known to phosphorylate the exchanger. Instead, it is proposed that diacylglycerol and phorbol esters bind directly to the lipid interacting domain, inducing conformational changes in this region by increasing its association with membrane lipids (Shimada-Shimizu *et al.*, 2014, Wakabayashi *et al.*, 2010).

As previously mentioned, calmodulin (CaM), a Ca^{2+}-binding protein that regulates NHE1-mediated Ca^{2+} signalling, is an important protein interacting with NHE1. CaM binds to two neighbouring sites on the C-terminal tail of NHE1: one high affinity site at aa 636-656, and a second intermediate affinity at aa 657-700 (Bertrand *et al.*, 1994). Deletion of this region induces a constitutively active NHE1, a shift in sensitivity towards alkaline pH_i, and a loss of exchanger responsiveness to increases in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (Wakabayashi *et al.*, 1997a). In quiescent cells, with low $[Ca^{2+}]_i$ and reduced CaM binding, this portion of the tail has an autoinhibitory effect on exchanger activity. Increases in $[Ca^{2+}]_i$ elicit CaM binding to the high affinity site on the NHE1 C-terminal tail. CaM binding is thought to weaken the association of the autoinhibitory domain with or near the H^+-modifier site on the transmembrane domain, thereby allowing H^+ to bind to and up-regulate the activity of the exchanger (Koster *et al.*, 2011) and CaM binding to NHE1 has recently been demonstrated *in vivo* (Li *et al.*, 2013b). However, it is unclear if CaM binding to NHE1 in breast cancer cells results in similar elevation of NHE1 activity under physiological conditions.

NHE1 regulation by second messenger Ca^{2+} signalling also occurs through its interaction with calcineurin B homologous proteins 1 and 2 (CHP1, CHP2) and tescalcin (also known as CHP3), which bind to the juxtamembrane region of the C-terminal tail. CHP1 binding to NHE1 increases its localization to the plasma membrane, and aids in antiporter activity by stabilizing the exchanger at the cell surface. Mutations to the CHP1 binding site of NHE1 do not prevent it from anchoring at the cell membrane, but do reduce the half-life of NHE1. This may be due to an unstable conformation that also negatively impacts NHE1 activity (Matsushita *et al.*, 2011). Similarly, CHP3 (tescalcin) binding to NHE1 also increases exchanger expression and half-life at the plasma membrane, likely by enhancing its biosynthetic maturation (Zaun *et al.*, 2008). Conversely, CHP2, which has ~60% amino acid sequence homology to CHP1, interacts more strongly with NHE1, but is found in much lower levels in the cytosol than CHP1. However, tumour cells express elevated CHP2 levels. In non-tumour cells co-expressing either CHP1 or CHP2 bound to NHE1, cytosolic pH was high (7.4-7.5) independent of serum in CHP2-NHE1 cells. In the CHP1-NHE1 cells, cellular alkalinization was only observed cells in the presence of serum. This indicated that CHP2 constitutively activated NHE1. In fact, CHP2-NHE1 cells were still viable after 7 days of serum deprivation while CHP1-NHE1 cells were not, suggesting that CHP2 aids in the maintenance of an alkaline pH_i. In this respect, CHP2-NHE1 cells were similar to malignantly transformed cells, indicating that NHE1-mediated cellular alkalinization under the regulation of CHP2 may be protective in nutrient-depleted conditions that mimic those of the tumour microenvironment (Pang *et al.*, 2002).

At the distal end of the C-terminal, NHE1 associates with cytosolic carbonic anhydrase isoform II (CAII) (Li *et al.*, 2006). Carbonic anhydrases are a class of enzymes that catalyze the hydration of CO_2 into HCO_3^- and H^+, and are involved in respiration, acid-base

regulation, and electrolyte secretion (Neri and Supuran, 2011). CAII is required for the efficient exchange of Cl^-/HCO_3^- by the red blood cell anion exchanger AE1 (Motais *et al.*, 1975, Sly and Hu, 1995). CAII binds directly to both AE1 (Vince and Reithmeier, 1998) and NHE1 (Li *et al.*, 2002). In NHE1, site-specific mutagenesis revealed that the critical amino acids involved in CAII binding are Ser796 and Asp797 (Li *et al.*, 2006). This association is thought to enhance the efficacy of proton extrusion. In many tumour cells, however, it is the overexpression of the transmembrane isozymes of carbonic anhydrase, CAIX and CAVII, which is related to poor prognosis and response to therapy (Neri and Supuran, 2011). CAIX and CAVII are also involved in the regulation of cellular pH homeostasis, and the pH of the tumour microenvironment, primarily via bicarbonate ion exchange (Meijer *et al.*, 2014). Could there be an association between the IX and VII isozymes of carbonic anhydrase and NHE1 at the plasma membrane of breast cancer cells, where pH regulation is so important to cancer progression? The prospect is intriguing given that CAIX overexpression is detected in the immunohistochemical analysis over 40% of ER-PR-positive/HER2-negative and over 80% ER-PR-negative/HER2-positive primary human breast tumours (Kaya *et al.*, 2012). Tissue microarray data from patients with various clinical subtypes of triple-negative breast cancer also showed a similar high expression of CAIX (Jeon *et al.*, 2013). Despite these observations, however, a direct association between NHE1 and CAIX has not been reported. As illustrated in Fig. 1, it is evident that piecing together the complex puzzle of signalling events and cofactor associations regulating NHE1 activity and ion exchange is hugely pertinent to both transformation and the latter stages of cancer cell development, as well as the establishment of the tumour microenvironment that mediates metastasis.

Na^+/H^+ exchange in the tumour microenvironment: triggering metastasis

Growth factor and nutrient-enriched serum activates NHE1 in normal cells while, paradoxically, serum deprivation activates NHE1 activity in cancer cells. This phenomenon becomes more pronounced in the latter stages of tumourigenesis. MCF-10A cells are a highly differentiated, non-tumorigenic human mammary epithelial cell line, while MCF-7 are minimally tumorigenic, lowly differentiated breast cancer cells. When comparing MCF-10A to MCF-7 cells, it was found that serum deprivation inhibited NHE1 activity in MCF-10A cells but increased exchanger activity in MCF-7 cells. This increase in NHE1 activity was due to an increase in the affinity of the internal H^+ regulatory site of the exchanger, and not due to changes in Na^+ kinetics or NHE1 protein expression (Reshkin *et al.*, 2000a). This effect also occurred in highly invasive and metastatic MDA-MB-435 cells; however, it was later determined that this is a melanoma cell line rather than a breast cancer cell line (Ellison *et al.*, 2002). We recently confirmed these results in MCF-7 cells, and further demonstrated that serum deprivation increases NHE1 activity in metastatic triple-negative, moderately invasive MDA-MB-468 cells, and in aggressively invasive MDA-MB-231 breast cancer cells (Amith *et al.*, 2015). Given that cancer cells survive and thrive in the low-serum conditions of the tumour microenvironment, these findings are unsurprising. In fact, despite the aberrant expression of growth factors, their receptors, and signalling pathways associated with the transformed phenotype, cell effector functions such as motility and invasiveness are neither negated nor limited by nutrient depletion, low oxygen and low pH. Instead, these conditions actually confer increased migratory and invasive capacity to breast cancer cells as they metastasize, characteristics thought to be potentiated by constitutively high NHE1 activity.

The mechanisms by which NHE1 activity is up-regulated in serum-deprived conditions are slowly coming to light. As noted above, CHP2 bound to NHE1 notably increased intracellular alkalinization and conferred a malignantly transformed phenotype to non-tumour cells (Pang *et al.*, 2002). Another possibly critical pathway is the RhoA/p160ROCK/p38MAPK signalling cascade (Cardone *et al.*, 2005a). In MCF-7 and MDA-MB-231 breast cancer cells, low serum activates NHE1 through a sequential RhoA/p160ROCK/p38MAPK signalling cascade gated by direct protein kinase A (PKA)-mediated phosphorylation of RhoA at Ser188, a key residue that blocks RhoA activation.

Serum deprivation also initiates a dynamic remodelling of the tumour cell cytoskeleton, priming it for directed migration. In migrating MCF-7 and MDA-MB-231 cells, there is a redistribution of NHE1, RhoA, and phospho-RhoA to the leading-edge compartments of pseudopodia (Cardone *et al.*, 2005a). Invadopodia are discrete extensions of invasive and metastatic tumour cells. In malignant MDA-MB-231 cells, NHE1 was also present in invadopodia. In these structures, NHE1 expression was associated with: well-defined areas of acidification in peri-invadopodial spaces; and focal regions of proteolytic extracellular matrix (ECM) degradation, as shown by co-localization of NHE1 with the invadopodial marker, cortactin (Busco *et al.*, 2010). Cortactin phosphorylation, an important precursor of invadopodia formation, was necessary for the recruitment of NHE1 to primary invadopodial structures (Magalhaes *et al.*, 2011).

As established above, the high rate of H^+ extrusion at the invadopodia of tumour cells creates areas of acidic pH that facilitates proteolytic digestion of the ECM. The serine proteases and matrix metalloproteinases (MMPs) required for ECM degradation at the invadopodia of breast cancer cells need the acid conditions established by NHE1 for optimal enzymatic activity (Fig. 2) (Greco *et al.*, 2014). In addition to facilitating the dissociation of cells from the ECM, extruded protons also promote cell-matrix interaction and adhesion at the cell front, mediated by ECM integrins, selectins and cadherins. Together, these processes culminate in migration of the tumour cell along the ECM (focal adhesion), and extravasation and invasion of cells through the ECM (focal digestion) into blood vessels where they are able to metastasize elsewhere in the body (Stock *et al.*, 2008).

Because of its critical role in proton extrusion, NHE1 must be tightly controlled by cell surface interacting partners, underlying signalling events, and associations with intracellular adaptor molecules. These all work in concert to form the NHE1 interactome that predicates cancer cell development. The invadopodial-ECM interface is therefore a hub of intricate physiological processes, mediated either directly or indirectly by NHE1 activity, that critically determine whether or not breast cancer cells acquire the invasive phenotype necessary for metastasis. Notably, these morphological changes are typical of epithelial-mesenchymal transition (EMT), an evolutionarily conserved developmental process by which tumour cells become invasive. In EMT, tumour cells undergo simultaneous molecular changes, typically: loss of epithelial marker, E-cadherin; and gain of mesenchymal markers, N-cadherin and vimentin

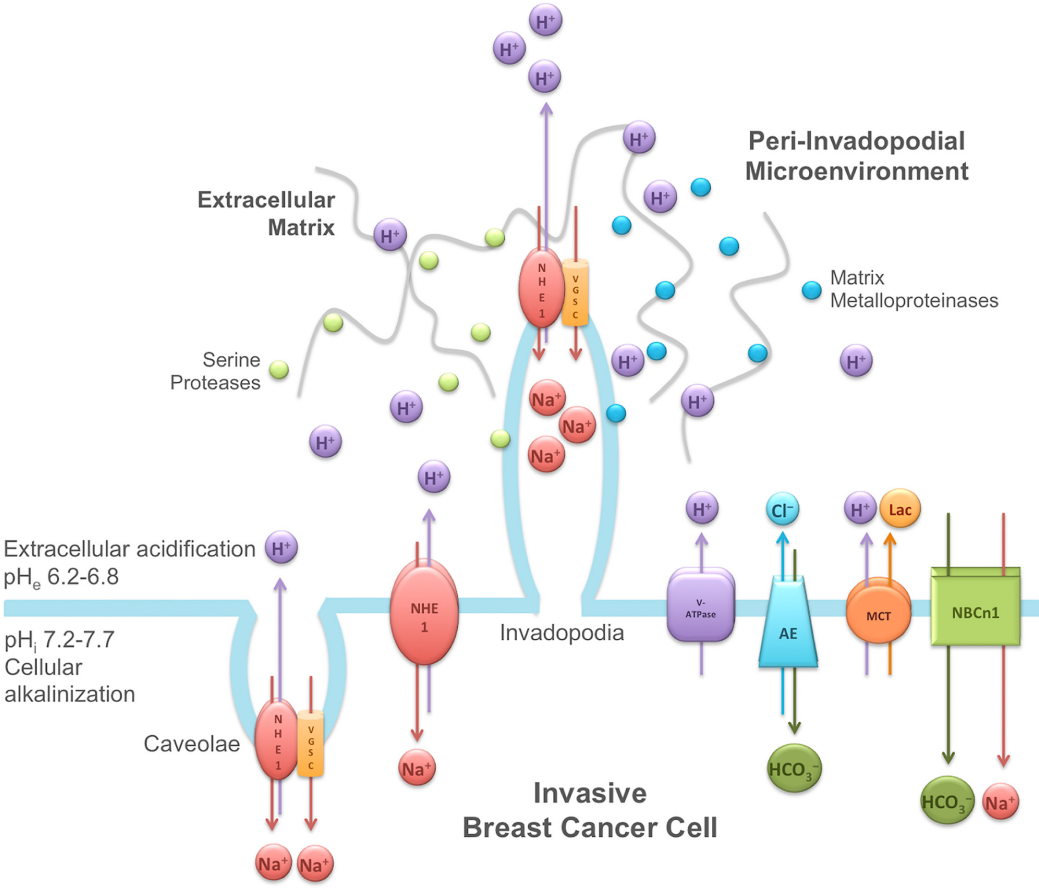

Fig. 2. Proton extrusion in the tumour microenvironment. *On the plasma membrane of tumour cells, NHE1 works in concert with other ion transporters (monocarboxylate transporters, vacuolar-type proton pump ATPases, anion exchangers, and bicarbonate-coupled transporters, including the Na^+/HCO_3^- transporter [NBCn1]) to facilitate directed migration and extracellular matrix degradation by serine proteases and matrix metalloproteinases. In caveolae and invadopodia, NHE1 co-localizes with voltage-gated sodium channels ($Na_V1.5$) to prime cells for invasion and metastasis. However, NHE1 is the major promoter of proton extrusion in the peri-invadopodial tumour microenvironment at the leading edges of invasive cells. AE, anion exchangers; Lac, lactate; MCT, monocarboxylate transporters; V-ATPases, vacuolar-type proton pump ATPases; VGSC, voltage-gated sodium channels.*

(Cheung and Ewald, 2014). Currently, there is no evidence linking NHE1 to the molecular mechanisms controlling EMT; research delving into this area could therefore be both provocative and promising.

Na^+/H^+ exchanger interactions with other transporters

Further mechanistic insights into the underlying network of pH regulation in tumour cells and their immediate microenvironment can also be gained by examining the interactions between NHE1 and other ion transporters. Here we examine four other ion transporters, voltage-gated sodium channels, V-ATPases, anion exchangers and bicarbonate transporters. Voltage-gated sodium channels are integral membrane proteins. Each channel consists of a large α-subunit and one or two auxiliary β-subunit. The α-subunit forms the sodium selective transporting pore. Nine isoforms of the sodium transporting α-subunit have been identified in higher vertebrates: $Na_V1.1$ - 1.9, reviewed in (Goldin, 2001). Interestingly, it is mainly the neonatal splice variant of $Na_V1.5$ ($nNa_V1.5$) that human breast cancer cells express both *in vitro* and *in vivo* (Brackenbury *et al.*, 2007, Fraser *et al.*, 2005, Fraser *et al.*, 2003, Onkal and Djamgoz, 2009), and $nNa_V1.5$ expression is suggested to be an integral part of the overall cancer process (Onkal and Djamgoz, 2009). $Na_V1.5$ is highly expressed in MDA-MB-231 breast cancer cells, less so in minimally tumourigenic MCF-7 cells, and not at all in non-tumour MCF-10A mammary epithelial cells. Specific inhibitors of $Na_V1.5$ (tetradotoxin, TTX) and NHE1 (5-(N-ethyl-N-isopropyl)-amiloride, EIPA) both reduce H^+ efflux in triple-negative, highly invasive MDA-MB-231 cells, TTX by ~25% and EIPA by ~90%. TTX and EIPA also inhibited cell invasiveness and extracellular gelatinolysis (a measure of ECM digestive capability) in MDA-MB-231 cells. However, neither inhibitor affects H^+ efflux in hormone receptor-positive, lowly invasive MCF-7 cells. $Na_V1.5$ also co-localizes with NHE1 in caveolae (Fig. 2), likely forming a complex that enhances NHE1-mediated acid extrusion and facilitates ECM degradation (Brisson *et al.*, 2011). Additionally, $Na_V1.5$ is expressed in a complex with NHE1 in the focal areas of ECM digestion around invadopodia (Fig. 2). $Na_V1.5$ activity increased Src kinase activity and cortactin phosphorylation, modifying the F-actin polymerization that ultimately results in cell polarization and cytoskeletal rearrangement towards a more invasive morphology (Brisson *et al.*, 2013). These studies suggest that $Na_V1.5$ is important in tumourigenesis in breast cancer cells and is in association with NHE1.

V-ATPases are multi-subunit membrane proteins localized in several intracellular compartments and on the plasma membrane. They translocate protons across cells and are overexpressed in tumour cells. HCO_3^--coupled transporters include Na^+/HCO_3^- co-transporters (NBCn1) and Cl^-/HCO_3^- exchangers reviewed in (Gorbatenko *et al.*, 2014)). Specific inhibitors of V-ATPases (bafilomycin) and anion exchangers (4,4(-diisothiocyanato-stilbene-2,2--disulphonic acid, which also negates the involvement of bicarbonate transport via AEs), did not affect H^+ flux in MDA-MB-231 cells, indicating that proton extrusion is largely due to NHE1 and $Na_V1.5$, albeit to varying degrees (Brisson *et al.*, 2011). MCF-7 cells are ERα-positive, but are negative for the expression of HER2 (also known as ErbB2), a receptor tyrosine kinase expressed in about 30% of metastatic breast tumours. N-terminal truncation of ErbB2 (NErbB2), renders

it constitutively active. In MCF-7 cells, expression of ErbB2 causes an up-regulation of NBCn1 at both the mRNA and protein levels that is not seen with NHE1. However, knockdown and chemical inhibition assays demonstrated that both transporters are involved in H^+ extrusion. Interestingly, NHE1 was more localized to lamellipodia in NErbB2-MCF7 cells, while NBCn1 localized predominantly at cell-cell contacts on the plasma membrane, shifting cell shape from an epithelial to a more fibroblast-like morphology (Lauritzen *et al.*, 2010). Na^+/HCO_3^- transport and its role in enhancing acid extrusion are also thought to be important in primary human breast carcinomas.

Ion transporter expression levels and cancer

ONCOMINE™ (www.oncomine.org) is a freely accessible cancer microarray database and web-based data-mining platform used to evaluate genome-wide expression analyses (Rhodes *et al.*, 2004). We used the *ONCOMINE™* database to compare changes in mRNA expression of NHE1 (*SLC9A1*), $Na_V1.5$ (*SCN5A*), and NBCn1 (*SLC4A7*) in cancerous versus normal tissue. As seen in Fig. 3, there is at least a two-fold increase in NHE1 mRNA expression in cancers in the breast, cervix, lungs, ovaries, pancreas, prostate and blood, while a three-fold or more increase in observed in bladder, esophageal, or renal cancer tissues (Fig. 3A, $P<0.05$). NBCn1 shows a 1.5-fold increase or greater in mRNA expression in brain, colorectal, gastric, liver, leukemic and pancreatic carcinomas, as well as in lymphomas (Fig. 3B, $P<0.05$). $Na_V1.5$ mRNA expression increases two-fold in lung carcinoma, whereas in breast cancers, the increase in expression is only one-fold (Fig. 3C, $P<0.05$). When we specifically examined the various clinical subtypes of breast carcinomas, a clearer picture of the expression of these transporters emerged. NHE1 expression increases at least 1.5-fold in invasive breast cancers, particularly those of lobular origin (Fig. 4A, $P<0.05$, Supplementary Table 1). NBCn1 expression appears to increase in invasive breast cancers, but due to the small sample size (either of normal or cancer tissue) used in these studies, this result will require further validation (Fig. 4B, Supplementary Table 2). Drawing conclusions from the data for changes in $Na_V1.5$ expression in breast carcinomas (Fig. 4C, Supplementary Table 3) are similarly inconclusive. It should be mentioned that the data obtained from the *ONCOMINE™* analysis are not from microdissected tissues, so adipocytes and other cell types are also present within the tissue. Nonetheless, these data are supportive of and reiterate the importance of NHE1 expression in various primary human carcinomas, and potentially in cancers of the breast.

Inhibiting NHE1: implications for breast cancer chemotherapy

Carcinogenesis is a dynamic process, so breast cancer manifests as a heterogeneous disease that clinically presents as a complexity of different cellular subtypes, based on the expression of: estrogen receptors (ERs), progesterone receptors (PRs), and human epidermal growth factor receptor-2 (HER2). In the broadest sense, breast cancer can be classed as: ER-positive, HER2-positive, and triple-negative (lacking ER, PR and HER2 expression). The majority of breast cancers are either ER-positive (~75%) or PR-positive (~55%), while HER2 expression is up-regulated in the tumour cells of ~20-30% of patients with the disease. Several targeted therapies for ER/PR-positive and HER2-positive cancers are known. How-

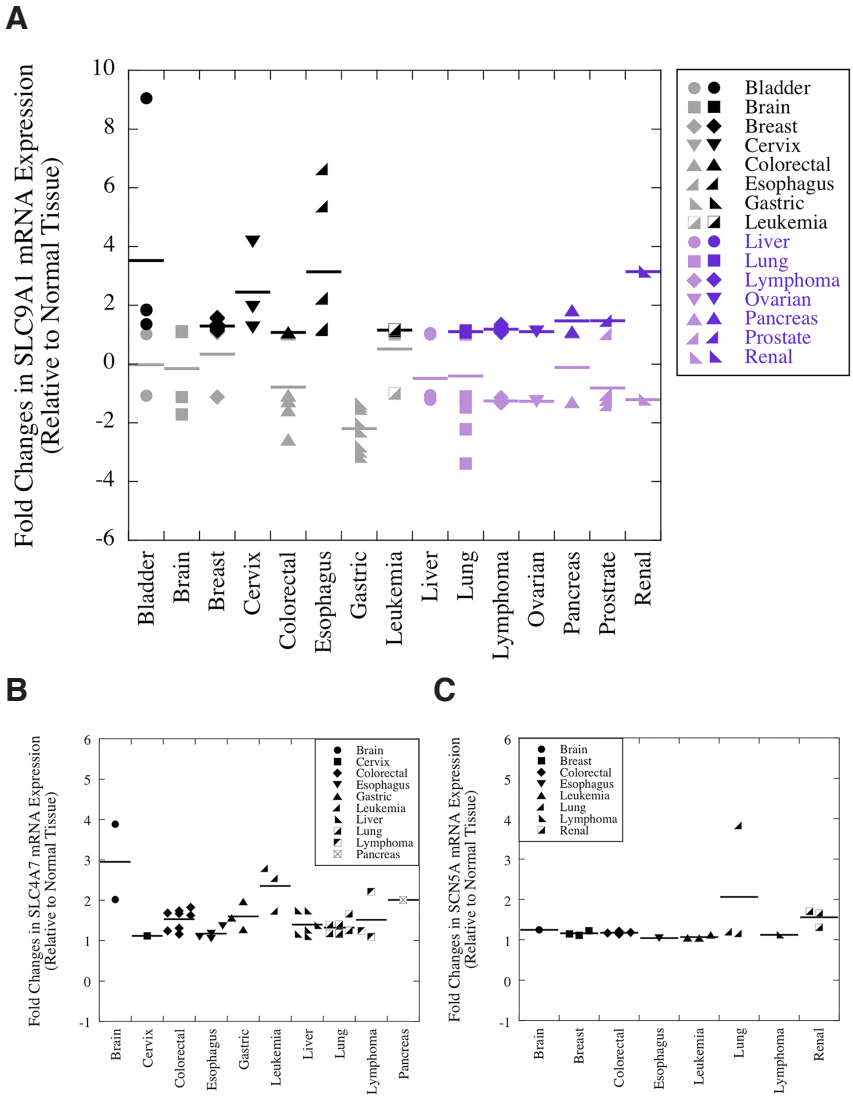

Fig. 3. Fold changes in mRNA expression of NHE1 *[SLC9A1]*, NBCn1 *[SLC4A7]* and $Na_V1.5$ *[SCN5A]* in various cancers relative to normal tissue. ONCOMINE *(www.oncomine.org) (Rhodes* et al.*, 2004) database analysis of various primary human cancers showing:* **(A)** *NHE1;* **(B)** *NBCn1; and* **(C)** *$Na_V1.5$ mRNA expression compared to normal tissues ($P<0.05$). Trial sizes and study references are detailed in Supplementary Tables 1-3.* **(A)** *Dark symbols indicate studies that were statistically significant at ($P<0.05$). Lighter symbols are studies that were not significant. Dark lines indicate mean of statistically significant studies. Lighter line indicates mean of non-significant studies.*

ever, hormone and HER2-targeted therapy is ineffective against triple-negative breast cancer (TNBC), which accounts for ~15% of breast cancers and often presents as highly aggressive with poor prognoses for patient survival (Cadoo *et al.*, 2013). Cytotoxic chemotherapy is standard in combatting TNBC, both at early and latter stages of the disease. Several classes of chemotherapy agents have been used in TNBC treatment to varying degrees of success. These include: platinum agents (e.g. cisplatin); taxanes (e.g. paclitaxel); and anthracyclines (e.g. doxorubicin) (Isakoff, 2010). Unfortunately, the development of a multidrug resistant phenotype often occurs during the course of chemotherapy, rendering it less effective (Martin *et al.*, 2014).

Evidence suggests that the acidic extracellular tumour microenvironment that is established by the constitutive activation of NHE1, actively promotes cancer progression. Also, the manipulation of intracellular or, in particular, the extracellular pH of tumour cells may aid cancer treatment strategies, possibly in combinatorial approaches to therapy (Harguindey *et al.*, 2013, McCarty and Whitaker, 2010). This may occur because chemotherapeutic agents are mildly basic and the acidic tumour microenvironment may partially neutralize their efficacy and actually contribute to chemotherapy resistance. Additionally, weakly basic agents may become protonated in low pH, whereby they are unable to permeate into the cell to reach their molecular targets (Webb *et al.*, 2011). Several studies have supported this suggestion. Xenograft tumours of MCF-7 cells, representative of ER-positive tumours, were more susceptible to doxorubicin *in vivo* at an extracellular pH of 7.4 versus 6.8 (Raghunand *et al.*, 1999). Lower extracellular pH also induced greater migration and higher resistance to paclitaxel and doxorubicin in MCF-7 cells. However, when bioenergetics modulators (e.g. glycolysis inhibitors), were used to pretreat cells, an increased sensitivity to paclitaxel and doxorubicin was observed (Tavares-Valente *et al.*, 2013). In a recent study, we knocked out NHE1 from aggressively tumorigenic triple-negative MDA-MB-231 cells. This dramatically decreased the ability of these cells to form subcutaneous xenograft tumours in athymic nude mice. *In vitro*, NHE1-knockout MDA-MB-231 cells were also markedly more sensitive to paclitaxel (Amith *et al.*, 2015). To date, however, despite the significant body of evidence supporting the role of NHE1 in breast cancer carcinogenesis from neoplastic transformation to metastasis, there is little direct evidence to confirm that pharmacological inhibition of NHE1 would be an effective chemotherapy strategy in patients with metastatic disease (Donowitz *et al.*, 2013). Clinical trials involving drugs that can inhibit NHE1 activity such as cariporide (Avkiran *et al.*, 2008), have been in the area of treatment for ischemic-reperfusion injury. Unfortunately, adverse effects associated with the use of cariporide outweighed its cardioprotective benefits. These off-target effects may have been due to the dose used and method of administration (Karmazyn, 2013). Future clinical trials in cancer patients need to address issues of dosage and cytotoxicity to minimize adverse effects and maximize drug benefits. A first step could be to target NHE1 in combination with adjuvant (administered post-surgery) or neoadjuvant (administered prior to surgery) chemotherapies, which could potentially be beneficial no matter what type of cancer is being treated, since NHE1 may be up-regulated in a multitude of carcinomas (Fig. 3A). Drug penetration of tumours is also an important aspect of chemotherapy (Minchinton and Tannock, 2006). At there present time however, it is not known whether NHE1 inhibitors themselves penetrate acidic core tumor environments, so further studies are needed in this area. Regrettably, there is also a dearth of studies exploring the use of NHE1 inhibitors themselves in treating metastasis and in combination

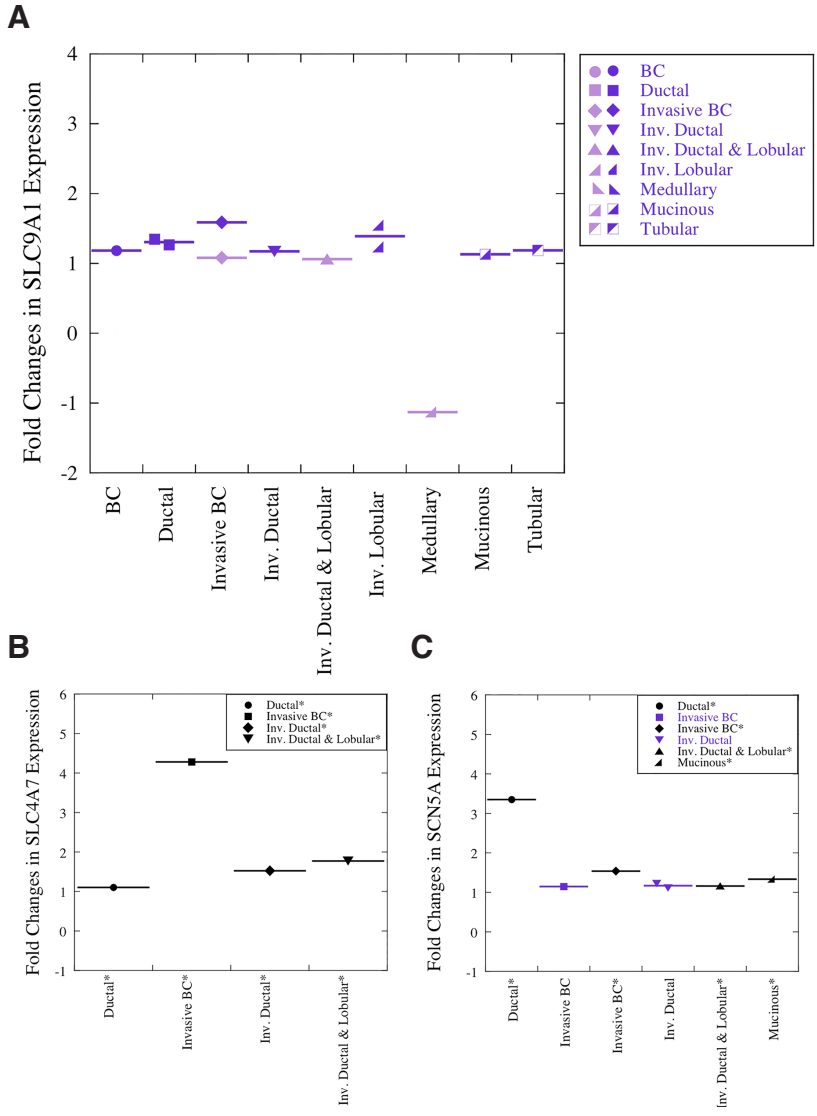

Fig. 4. Fold changes in mRNA expression of NHE1 *[SLC9A1]*, NBCn1 *[SLC4A7]* and $Na_V1.5$ *[SCN5A]* in breast cancer subtypes relative to normal breast tissue. ONCOMINE *database analysis of various primary human breast cancers showing:* **(A)** *NHE1;* **(B)** *NBCn1; and* **(C)** *$Na_V1.5$ mRNA expression compared to normal breast tissues (P<0.05). Trial sizes and study references are detailed in Supplementary Tables 1-3.* **(A)** *Dark symbols indicate studies that were statistically significant at (P<0.05). Lighter symbols are studies that were not significant. Dark lines indicate mean of statistically significant studies. Lighter line indicates mean of non-significant studies. Asterisk (*) indicates studies with small sample sizes of normal or cancer tissues (n<10).*

with chemotherapies. We recently showed that pretreating MDA-MB-231 and MDA-MB-468 breast cancer cells (representative of two different clinical subtypes of TNBCs) with NHE1 inhibitors increases their susceptibility to low-dose paclitaxel-mediated cell death, and decreases their metastatic potential *in vitro*. This was especially true in serum-depleted conditions that mimic those of the tumour microenvironment (Amith *et al.*, 2015). These findings indicate that there is merit to the hypothesis that NHE1 inhibitors could be used as co-adjuvants to chemotherapy; further testing in other breast cancer subtypes is worth pursuing.

Summary

The pivotal role that ionic messengers play in carcinogenesis is evident, but it is disrupted proton dynamics and the subsequent dysregulation of pH homeostasis that is associated with the evolution of cancer from neoplasia to metastasis. Oncogenic transformation initiates the NHE1-driven cellular alkalinization that facilitates glycolytic metabolism and promotes cancer cell survival in the hypoxic and nutrient-depleted tumour microenvironment. NHE1 relocalizes to the leading edges of migratory cells and invadopodia of invading cells, where its activity facilitates the onset of metastasis. Recently, this has sparked considerable interest in the potential modulation of pH in and around tumour cells as a novel means of broad-spectrum anti-cancer therapy that may be effective regardless of the type of tumour and where it originates. Nevertheless, we can conclude that proton flux, largely mediated by NHE1 and its interacting partners, promotes carcinogenesis and allows cancer cells to thrive in the harsh tumour microenvironment. The role of NHE1 has thus been redefined from a general "housekeeping" protein in charge of pH regulation and cell volume control, to an essential coordinator of cancer cell development. Likewise, its vast potential as a viable chemotherapeutic target, whether directly or indirectly in concert with cytotoxic drugs, must be reconsidered in our search for a cure.

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References

AMITH, S.R. and FLIEGEL, L. (2013). Regulation of the Na^+/H^+ Exchanger (NHE1) in breast cancer metastasis. *Cancer Res* 73: 1259-1264.

AMITH, S.R., WILKINSON, J.M., BAKSH, S. and FLIEGEL, L. (2015). Na+/H+ exchanger (NHE1) as a novel co-adjuvant target in paclitaxel therapy of triple-negative breast cancer cells. *Oncotarget* 6: 1262-1275.

AVKIRAN, M., COOK, A.R. and CUELLO, F. (2008). Targeting Na^+/H^+ exchanger regulation for cardiac protection: a RSKy approach? *Curr. Opin. Pharmacol.* 8: 133-140.

BAUMGARTNER, M., PATEL, H. and BARBER, D.L. (2004). Na(+)/H(+) exchanger NHE1 as plasma membrane scaffold in the assembly of signaling complexes. *Am J Physiol Cell Physiol* 287: C844-C850.

BERTRAND, B., WAKABAYASHI, S., IKEDA, T., POUYSSEGUR, J. and SHIGEKAWA, M. (1994). The Na^+/H^+ exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding proteins. Identification and characterization of calmodulin-binding sites. *J Biol Chem* 269: 13703-13709.

BRACKENBURY, W.J., CHIONI, A.M., DISS, J.K. and DJAMGOZ, M.B. (2007). The neonatal splice variant of Nav1.5 potentiates *in vitro* invasive behaviour of MDA-MB-231 human breast cancer cells. *Breast Cancer Res Treat* 101: 149-160.

BRISSON, L., DRIFFORT, V., BENOIST, L., POET, M., COUNILLON, L., ANTELMI, E., RUBINO, R., BESSON, P., LABBAL, F., CHEVALIER, S. *et al.,* (2013). NaV1.5 Na(+) channels allosterically regulate the NHE-1 exchanger and promote the activity of breast cancer cell invadopodia. *J Cell Sci* 126: 4835-4842.

BRISSON, L., GILLET, L., CALAGHAN, S., BESSON, P., LE GUENNEC, J.Y., ROGER, S. and GORE, J. (2011). Na(V)1.5 enhances breast cancer cell invasiveness by increasing NHE1-dependent H(+) efflux in caveolae. *Oncogene* 30: 2070-2076.

BUSCO, G., CARDONE, R.A., GRECO, M.R., BELLIZZI, A., COLELLA, M., ANTELMI, E., MANCINI, M.T., DELL'AQUILA, M.E., CASAVOLA, V., PARADISO, A. *et al.,* (2010). NHE1 promotes invadopodial ECM proteolysis through acidification of the peri-invadopodial space. *FASEB J* 24: 3903-3915.

CADOO, K.A., FORNIER, M.N. and MORRIS, P.G. (2013). Biological subtypes of breast cancer: current concepts and implications for recurrence patterns. *Q J Nucl Med Mol Imaging* 57: 312-321.

CARDONE, R.A., BAGORDA, A., BELLIZZI, A., BUSCO, G., GUERRA, L., PARADISO, A., CASAVOLA, V., ZACCOLO, M. and RESHKIN, S.J. (2005a). Protein kinase A gating of a pseudopodial-located RhoA/ROCK/p38/NHE1 signal module regulates invasion in breast cancer cell lines. *Mol Biol Cell* 16: 3117-3127.

CARDONE, R.A., CASAVOLA, V. and RESHKIN, S.J. (2005b). The role of disturbed pH dynamics and the Na^+/H^+ exchanger in metastasis. *Nature reviews. Cancer* 5: 786-795.

CHEUNG, K.J. and EWALD, A.J. (2014). Illuminating breast cancer invasion: diverse roles for cell-cell interactions. *Curr Opin Cell Biol* 30C: 99-111.

DENKER, S.P., HUANG, D.C., ORLOWSKI, J., FURTHMAYR, H. and BARBER, D.L. (2000). Direct binding of the Na-H exchanger NHE1 to ERM proteins regulates the cortical cytoskeleton and cell shape independently of H(+) translocation. *Mol Cell* 6: 1425-1436.

DONOWITZ, M., MING TSE, C. and FUSTER, D. (2013). SLC9/NHE gene family, a plasma membrane and organellar family of Na(+)/H(+) exchangers. *Mol Aspects Med* 34: 236-251.

ELLISON, G., KLINOWSKA, T., WESTWOOD, R.F., DOCTER, E., FRENCH, T. and FOX, J.C. (2002). Further evidence to support the melanocytic origin of MDA-MB-435. *Mol Pathol* 55: 294-299.

FLIEGEL, L. (2009). Regulation of the Na(+)/H(+) exchanger in the healthy and diseased myocardium. *Expert Opin Ther Targets* 13: 55-68.

FRASER, S.P., DISS, J.K., CHIONI, A.M., MYCIELSKA, M.E., PAN, H., YAMACI, R.F., PANI, F., SIWY, Z., KRASOWSKA, M., GRZYWNA, Z. *et al.,* (2005). Voltage-gated sodium channel expression and potentiation of human breast cancer metastasis. *Clin Cancer Res* 11: 5381-5389.

FRASER, S.P., OZERLAT-GUNDUZ, I., BRACKENBURY, W.J., FITZGERALD, E.M., CAMPBELL, T.M., COOMBES, R.C. and DJAMGOZ, M.B. (2014). Regulation of voltage-gated sodium channel expression in cancer: hormones, growth factors and auto-regulation. *Philos Trans R Soc Lond B Biol Sci* 369: 20130105.

FRASER, S.P., SALVADOR, V., MANNING, E.A., MIZAL, J., ALTUN, S., RAZA, M., BERRIDGE, R.J. and DJAMGOZ, M.B. (2003). Contribution of functional voltage-gated Na+ channel expression to cell behaviors involved in the metastatic cascade in rat prostate cancer: I. Lateral motility. *J. Cell Physiol.* 195: 479-487.

FUSTER, D.G. and ALEXANDER, R.T. (2014). Traditional and emerging roles for the SLC9 Na^+/H^+ exchangers. *Pflugers Arch* 466: 61-76.

GOLDIN, A.L. (2001). Resurgence of sodium channel research. *Annu Rev Physiol* 63: 871-894.

GORBATENKO, A., OLESEN, C.W., BOEDTKJER, E. and PEDERSEN, S.F. (2014). Regulation and roles of bicarbonate transporters in cancer. *Front. Physiol.* 5: 130.

GRECO, M.R., ANTELMI, E., BUSCO, G., GUERRA, L., RUBINO, R., CASAVOLA, V., RESHKIN, S.J. and CARDONE, R.A. (2014). Protease activity at invadopodial focal digestive areas is dependent on NHE1-driven acidic pHe. *Oncol Rep* 31: 940-946.

GRENIER, A.L., ABU-IHWEIJ, K., ZHANG, G., RUPPERT, S.M., BOOHAKER, R., SLEPKOV, E.R., PRIDEMORE, K., REN, J.J., FLIEGEL, L. and KHALED, A.R. (2008). Apoptosis-induced alkalinization by the Na^+/H^+ exchanger isoform 1 is mediated through phosphorylation of amino acids Ser726 and Ser729. *Am J Physiol Cell Physiol* 295: C883-C896.

HARGUINDEY, S., ARRANZ, J.L., POLO OROZCO, J.D., RAUCH, C., FAIS, S., CARDONE, R.A. and RESHKIN, S.J. (2013). Cariporide and other new and powerful NHE1 inhibitors as potentially selective anticancer drugs--an integral

molecular/biochemical/metabolic/clinical approach after one hundred years of cancer research. *J Transl Med* 11: 282.

HASUDA, K., LEE, C. and TANNOCK, I.F. (1994). Antitumor activity of nigericin and 5-(N-ethyl-N-isopropyl)amiloride: an approach to therapy based on cellular acidification and the inhibition of regulation of intracellular pH. *Oncology Res.* 6: 259-268.

HENDUS-ALTENBURGER, R., KRAGELUND, B.B. and PEDERSEN, S.F. (2014). Structural dynamics and regulation of the mammalian SLC9A family of Na(+)/H(+) exchangers. *Curr Top Membr* 73: 69-148.

IKEDA, T., SCHMITT, B., POUYSSEGUR, J., WAKABAYASHI, S. and SHIGEKAWA, M. (1997). Identification of cytoplasmic subdomains that control pH-sensing of the Na^+/H^+ exchanger (NHE1): pH-maintenance, ATP-sensitive, and flexible loop domains. *J Biochem* 121: 295-303.

ISAKOFF, S.J. (2010). Triple-negative breast cancer: role of specific chemotherapy agents. *Cancer J* 16: 53-61.

JEON, H.M., KIM DO, H., JUNG, W.H. and KOO, J.S. (2013). Expression of cell metabolism-related genes in different molecular subtypes of triple-negative breast cancer. *Tumori* 99: 555-564.

JUNG, Y.S., KIM, H.Y., KIM, J., LEE, M.G., POUYSSEGUR, J. and KIM, E. (2008). Physical interactions and functional coupling between Daxx and sodium hydrogen exchanger 1 in ischemic cell death. *J Biol Chem* 283: 1018-1025.

KARKI, P., LI, X., SCHRAMA, D. and FLIEGEL, L. (2011). B-RAF is associated with and activates the NHE1 isoform of the Na+/H+ exchanger. *J. Biol. Chem.* 286: 13096-13105.

KARMAZYN, M. (2013). NHE-1: still a viable therapeutic target. *J. Molec. Cell. Cardio.* 61: 77-82.

KAYA, A.O., GUNEL, N., BENEKLI, M., AKYUREK, N., BUYUKBERBER, S., TATLI, H., COSKUN, U., YILDIZ, R., YAMAN, E. and OZTURK, B. (2012). Hypoxia inducible factor-1 alpha and carbonic anhydrase IX overexpression are associated with poor survival in breast cancer patients. *J Buon* 17: 663-668.

KOSTER, S., PAVKOV-KELLER, T., KUHLBRANDT, W. and YILDIZ, O. (2011). Structure of human Na^+/H^+ exchanger NHE1 regulatory region in complex with calmodulin and Ca^{2+}. *J Biol Chem* 286: 40954-40961.

LAURITZEN, G., JENSEN, M.B., BOEDTKJER, E., DYBBOE, R., AALKJAER, C., NYLANDSTED, J. and PEDERSEN, S.F. (2010). NBCn1 and NHE1 expression and activity in DeltaNErbB2 receptor-expressing MCF-7 breast cancer cells: contributions to pHi regulation and chemotherapy resistance. *Exp Cell Res* 316: 2538-2553.

LEE, B.L., SYKES, B.D. and FLIEGEL, L. (2013). Structural and functional insights into the cardiac Na(+)/H(+) exchanger. *J Mol Cell Cardiol* 61: 60-67.

LEHOUX, S., ABE, J., FLORIAN, J.A. and BERK, B.C. (2001). 14-3-3 Binding to Na^+/H^+ exchanger isoform-1 is associated with serum-dependent activation of Na^+/H^+ exchange. *J Biol Chem* 276: 15794-15800.

LI, X., ALVAREZ, B., CASEY, J.R., REITHMEIER, R.A. and FLIEGEL, L. (2002). Carbonic anhydrase II binds to and enhances activity of the Na^+/H^+ exchanger. *J Biol Chem* 277: 36085-36091.

LI, X., KHAN, M.F., SCHRIEMER, D.C. and FLIEGEL, L. (2013a). Structural changes in the C-terminal regulatory region of the Na(+)/H(+) exchanger mediate phosphorylation induced regulation. *J Mol Cell Cardiol* 61: 153-163.

LI, X., LIU, Y., ALVAREZ, B.V., CASEY, J.R. and FLIEGEL, L. (2006). A novel carbonic anhydrase II binding site regulates NHE1 activity. *Biochemistry* 45: 2414-2424.

LI, X., PRINS, D., MICHALAK, M. and FLIEGEL, L. (2013b). Calmodulin dependent binding to the NHE1 Cytosolic Tail Mediates Activation of the Na^+/H^+ Exchanger by Ca+2 and Endothelin. *Am J Physiol Cell Physiol.* C1161-1169.

LIU, H., STUPAK, J., ZHENG, J., KELLER, B.O., BRIX, B.J., FLIEGEL, L. and LI, L. (2004). Open tubular immobilized metal–ion affinity chromatography combined with MALDI MS and MS/MS for identification of protein phosphorylation sites. *Anal. Chem.* 76: 4223-4232.

MAGALHAES, M.A., LARSON, D.R., MADER, C.C., BRAVO-CORDERO, J.J., GIL-HENN, H., OSER, M., CHEN, X., KOLESKE, A.J. and CONDEELIS, J. (2011). Cortactin phosphorylation regulates cell invasion through a pH-dependent pathway. *J Cell Biol* 195: 903-920.

MALO, M.E., LI, L. and FLIEGEL, L. (2007). Mitogen-activated protein kinase-dependent activation of the Na^+/H^+ exchanger is mediated through phosphorylation of amino acids Ser^{770} and Ser^{771}. *J. Biol. Chem.* 282: 6292-6299.

MARTIN, H.L., SMITH, L. and TOMLINSON, D.C. (2014). Multidrug-resistant breast cancer: current perspectives. *Breast Cancer (Dove Med Press)* 6: 1-13.

MATSUSHITA, M., TANAKA, H., MITSUI, K. and KANAZAWA, H. (2011). Dual functional significance of calcineurin homologous protein 1 binding to Na(+)/H(+) exchanger isoform 1. *Am J Physiol Cell Physiol* 301: C280-C288.

MCCARTY, M.F. and WHITAKER, J. (2010). Manipulating tumor acidification as a cancer treatment strategy. *Altern Med Rev* 15: 264-272.

MEIJER, T.W., BUSSINK, J., ZATOVICOVA, M., SPAN, P.N., LOK, J., SUPURAN, C.T. and KAANDERS, J.H. (2014). Tumor microenvironmental changes induced by the sulfamate carbonic anhydrase IX inhibitor S4 in a laryngeal tumor model. *PLoS One* 9: e108068.

MINCHINTON, A.I. and TANNOCK, I.F. (2006). Drug penetration in solid tumours. *Nature Rev. Cancer* 6: 583-592.

MONTCOURRIER, P., SILVER, I., FARNOUD, R., BIRD, I. and ROCHEFORT, H. (1997). Breast cancer cells have a high capacity to acidify extracellular milieu by a dual mechanism. *Clin Exp Metastasis* 15: 382-392.

MOTAIS, R., COUSIN, J.L. and SOLA, F. (1975). [Transmembrane exchange of C1- and HCO3- in erythrocytes: direct action of carbonic anhydrase inhibitors on the transport mechanism]. *C R Acad Sci Hebd Seances Acad Sci D* 280: 1119-1122.

NERI, D. and SUPURAN, C.T. (2011). Interfering with pH regulation in tumours as a therapeutic strategy. *Nat Rev Drug Discov* 10: 767-777.

ONKAL, R. and DJAMGOZ, M.B. (2009). Molecular pharmacology of voltage-gated sodium channel expression in metastatic disease: clinical potential of neonatal Nav1.5 in breast cancer. *Eur. J. Pharmacol.* 625: 206-219.

PANG, T., WAKABAYASHI, S. and SHIGEKAWA, M. (2002). Expression of calcineurin B homologous protein 2 protects serum deprivation-induced cell death by serum-independent activation of Na+/H+ exchanger. *J Biol Chem* 277: 43771-43777.

PARDO, L.A. and STUHMER, W. (2014). The roles of K(+) channels in cancer. *Nat Rev Cancer* 14: 39-48.

PARIS, S. and POUYSSÉGUR, J. (1984). Growth factor activate the Na+/H+ antiporter in quiescent fibroblasts by increasing its affinity for intracellular H+. *J. Biol. Chem.* 259: 10989-10994.

PARKS, S.K., CHICHE, J. and POUYSSEGUR, J. (2013). Disrupting proton dynamics and energy metabolism for cancer therapy. *Nat Rev Cancer* 13: 611-623.

PEDERSEN, S.F., DARBORG, B.V., RENTSCH, M.L. and RASMUSSEN, M. (2007). Regulation of mitogen-activated protein kinase pathways by the plasma membrane Na+/H+ exchanger, NHE1. *Arch Biochem Biophys* 462: 195-201.

PEDERSEN, S.F. and STOCK, C. (2013). Ion channels and transporters in cancer: pathophysiology, regulation, and clinical potential. *Cancer Res* 73: 1658-1661.

PUTNEY, L.K., DENKER, S.P. and BARBER, D.L. (2002). The changing face of the Na^+/H^+ exchanger, NHE1: structure, regulation, and cellular actions. *Annu Rev Pharmacol Toxicol* 42: 527-552.

RAGHUNAND, N., HE, X., VAN SLUIS, R., MAHONEY, B., BAGGETT, B., TAYLOR, C.W., PAINE-MURRIETA, G., ROE, D., BHUJWALLA, Z.M. and GILLIES, R.J. (1999). Enhancement of chemotherapy by manipulation of tumour pH. *Br J Cancer* 80: 1005-1011.

RESHKIN, S.J., BELLIZZI, A., ALBARANI, V., GUERRA, L., TOMMASINO, M., PARADISO, A. and CASAVOLA, V. (2000a). Phosphoinositide 3-kinase is involved in the tumor-specific activation of human breast cancer cell Na(+)/H(+) exchange, motility, and invasion induced by serum deprivation. *J Biol Chem* 275: 5361-5369.

RESHKIN, S.J., BELLIZZI, A., CALDEIRA, S., ALBARANI, V., MALANCHI, I., POIGNEE, M., ALUNNI-FABBRONI, M., CASAVOLA, V. and TOMMASINO, M. (2000b). Na^+/H^+ exchanger-dependent intracellular alkalinization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes. *FASEB J* 14: 2185-2197.

RHODES, D.R., YU, J., SHANKER, K., DESHPANDE, N., VARAMBALLY, R., GHOSH, D., BARRETTE, T., PANDEY, A. and CHINNAIYAN, A.M. (2004). ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* 6: 1-6.

RODERICK, H.L. and COOK, S.J. (2008). Ca^{2+} signalling checkpoints in cancer: remodelling Ca^{2+} for cancer cell proliferation and survival. *Nature Rev. Cancer* 8: 361-375.

SHIMADA-SHIMIZU, N., HISAMITSU, T., NAKAMURA, T.Y., HIRAYAMA, N. and WAKABAYASHI, S. (2014). Na^+/H^+ exchanger 1 is regulated via its lipid-interacting domain, which functions as a molecular switch: a pharmacological approach using indolocarbazole compounds. *Mol Pharmacol* 85: 18-28.

SHIMADA-SHIMIZU, N., HISAMITSU, T., NAKAMURA, T.Y. and WAKABAYASHI, S. (2013). Evidence that Na^+/H^+ exchanger 1 is an ATP-binding protein. *FEBS J* 280: 1430-1442.

SLY, W.S. and HU, P.Y. (1995). Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annu Rev Biochem* 64: 375-401.

STOCK, C., CARDONE, R.A., BUSCO, G., KRAHLING, H., SCHWAB, A. and RESHKIN, S.J. (2008). Protons extruded by NHE1: digestive or glue? *Eur J Cell Biol* 87: 591-599.

TAKAHASHI, E., ABE, J., GALLIS, B., AEBERSOLD, R., SPRING, D.J., KREBS, E.G. and BERK, B.C. (1999). p90(RSK) is a serum-stimulated Na^+/H^+ exchanger isoform-1 kinase. Regulatory phosphorylation of serine 703 of Na^+/H^+ exchanger isoform-1. *J Biol Chem* 274: 20206-20214.

TAVARES-VALENTE, D., BALTAZAR, F., MOREIRA, R. and QUEIROS, O. (2013). Cancer cell bioenergetics and pH regulation influence breast cancer cell resistance to paclitaxel and doxorubicin. *J Bioenerg Biomembr* 45: 467-475.

VANDER HEIDEN, M.G., CANTLEY, L.C. and THOMPSON, C.B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324: 1029-1033.

VINCE, J.W. and REITHMEIER, R.A. (1998). Carbonic anhydrase II binds to the carboxyl terminus of human band 3, the erythrocyte C1-/HCO3- exchanger. *J Biol Chem* 273: 28430-28437.

WAKABAYASHI, S., BERTRAND, B., SHIGEKAWA, M., FAFOURNOUX, P. and POUYSSEGUR, J. (1994). Growth factor activation and "H(+)-sensing" of the Na^+/H^+ exchanger isoform 1 (NHE1). Evidence for an additional mechanism not requiring direct phosphorylation. *J Biol Chem* 269: 5583-5588.

WAKABAYASHI, S., IKEDA, T., IWAMOTO, T., POUYSSEGUR, J. and SHIGEKAWA, M. (1997a). Calmodulin-binding autoinhibitory domain controls "pH-sensing" in the Na^+/H^+ exchanger NHE1 through sequence-specific interaction. *Biochemistry* 36: 12854-12861.

WAKABAYASHI, S., NAKAMURA, T.Y., KOBAYASHI, S. and HISAMITSU, T. (2010). Novel phorbol ester-binding motif mediates hormonal activation of Na+/H+ exchanger. *J Biol Chem* 285: 26652-26661.

WAKABAYASHI, S., PANG, T., SU, X. and SHIGEKAWA, M. (2000). A novel topology model of the human Na(+)/H(+) exchanger isoform 1. *J Biol Chem* 275: 7942-7949.

WAKABAYASHI, S., SHIGEKAWA, M. and POUYSSEGUR, J. (1997b). Molecular physiology of vertebrate Na^+/H^+ exchangers. *Physiol Rev* 77: 51-74.

WEBB, B.A., CHIMENTI, M., JACOBSON, M.P. and BARBER, D.L. (2011). Dysregulated pH: a perfect storm for cancer progression. *Nature reviews. Cancer* 11: 671-677.

WU, K.L., KHAN, S., LAKHE-REDDY, S., JARAD, G., MUKHERJEE, A., OBEJERO-PAZ, C.A., KONIECZKOWSKI, M., SEDOR, J.R. and SCHELLING, J.R. (2004). The NHE1 Na^+/H^+ exchanger recruits ezrin/radixin/moesin proteins to regulate Akt-dependent cell survival. *J. Biol. Chem.* 279: 26280-26286.

ZAUN, H.C., SHRIER, A. and ORLOWSKI, J. (2008). Calcineurin B homologous protein 3 promotes the biosynthetic maturation, cell surface stability, and optimal transport of the Na+/H+ exchanger NHE1 isoform. *J Biol Chem* 283: 12456-12467.

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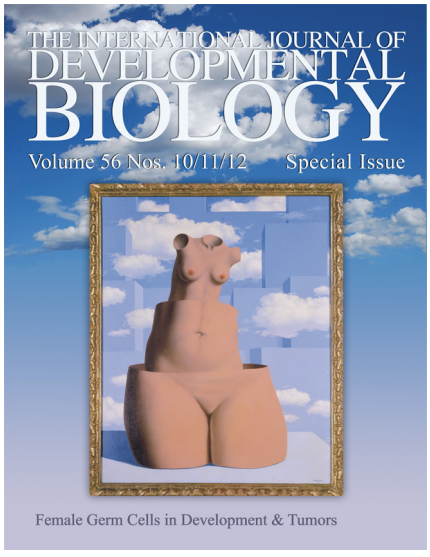

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