THE ROLE OF DISTURBED PH DYNAMICS AND THE NA$^+$/H$^+$ EXCHANGER IN METASTASIS

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Abstract | Recent research has highlighted the fundamental role of the tumour's extracellular metabolic microenvironment in malignant invasion. This microenvironment is acidified primarily by the tumour-cell Na$^+$/H$^+$ exchanger NHE1 and the H$^+$/lactate cotransporter, which are activated in cancer cells. NHE1 also regulates formation of invadopodia — cell structures that mediate tumour cell migration and invasion. How do these alterations of the metabolic microenvironment and cell invasiveness contribute to tumour formation and progression?

SET POINT
The intracellular pH value below which the Na$^+$/H$^+$ exchanger NHE1 becomes activated.

Both *in vitro* cell-culture studies and *in situ* tumour spectroscopic studies utilizing the ^{31}P isotope have reported that tumour cells have alkaline intracellular pH (pHi) values (7.12–7.65 compared with 6.99–7.20 in normal tissues) and acidic interstitial extracellular pH (pHe) values (6.2–6.9 compared with 7.3–7.4)[1]. So, the tumour extracellular environment is more acidic than the intracellular environment, creating a reversed pH gradient across the cell membrane that increases as the tumour progresses. The development and maintenance of this gradient is directly due to the ability of the tumour cells to secrete protons (H$^+$) and acidify their extracellular environment. Moreover, this ability increases with tumour aggressiveness[2,3]. This proton secretion depends on the buffering capacity of the cell and on membrane-based ion exchangers such as the Na$^+$/H$^+$ exchanger NHE1, the Na$^+$-independent and Na$^+$-dependent HCO_3^-/Cl$^-$ exchangers and the H$^+$/lactate cotransporter (known as the monocarboxylate transporter, MCT)[4].

Direct microinjection of the activated oncogene v-*Ha-ras*[5], long-term expression of various oncogenes by transfection[6–9], controlled expression of v-*Ha-ras* oncogene homologue (*HRAS*)[10] and transformation of human primary keratinocytes that are infected with human papilloma virus type 16 (HPV16)[9] have all been used to study the generation of the tumour pH gradient. These studies indicated that the reversed pH gradient is already apparent during the earliest step of neoplastic progression — transformation — and is caused by activation of NHE1.

Although these findings demonstrated that increased cytoplasmic pHi was associated with transformation, it was not clear whether the rise in pHi has a key role in the induction of cellular events that lead to transformation, or is itself a consequence of transformation (reviewed in REFS 11,12). Furthermore, the causal relationship between the shift to glycolytic metabolism and the increases in pHi and proliferation were unclear. However, a study that used an inducible expression system for the *E7* oncogene of HPV16 to determine the sequence and causality of early events that occur during transformation showed that oncogene expression results in a rapid increase in cytoplasmic pHi (FIG. 1a). Moreover, the pH change was shown to be driven primarily by the stimulation of NHE1 activity, as the increase in pHi was completely and reversibly inhibited by incubation with the NHE1 inhibitor, 5-*N*,*N*-dimethylamiloride (DMA)[9].

The activation of NHE1 is due to an increase in the affinity of its intracellular allosteric proton-binding site for its substrate[9]. This increases the resting pHi of the cell and raises the SET POINT for protons to a value that is greater than the resting pHi (FIG. 1b). Therefore, NHE1 becomes constitutively active, which results in an initial acidification of the

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Summary

- The gradient from the extracellular pH (pHe) to the intracellular pH (pHi) is reversed in tumours. That is, tumours become more acidic extracellularly and more alkaline intracellularly.
- This reversed pH gradient arises early in transformation and is driven primarily by oncogene-dependent stimulation of the Na$^+$/H$^+$ exchanger NHE1, which results in cellular alkalinization and subsequent aerobic glycolysis and H$^+$/lactate symport.
- Cellular alkalinization induces cell proliferation that is independent of serum and substrate anchorage, which results in dense disorganized cell masses that are poorly vascularized.
- Poor vascularization, together with the increased proton-extrusion ability of the tumour cell, produces the tumour-specific metabolic microenvironment. Owing to positive-feedback interactions between the tumour cell and this microenvironment, an ever higher reversed pH gradient is achieved as the disease progresses.
- Both the acidic pHe and the constitutive activity of NHE1 have roles in driving protease-mediated digestion and remodelling of the extracellular matrix. They also stimulate the invasive phenotypes of the cell — actin remodelling for increased motility and the formation of invasive structures such as leading-edge pseudopodia and invadopodia.
- Little is known about the signal-transduction systems that regulate NHE1 activity and that are associated with invasion.
- The formation of a tumour-microenvironment model of invasion and metastasis that integrates the interaction of cell structure with the biochemistry, physiology and regulation of NHE1 will lead to a better understanding of the dynamics of the invasive response of the tumour cell to the microenvironment.

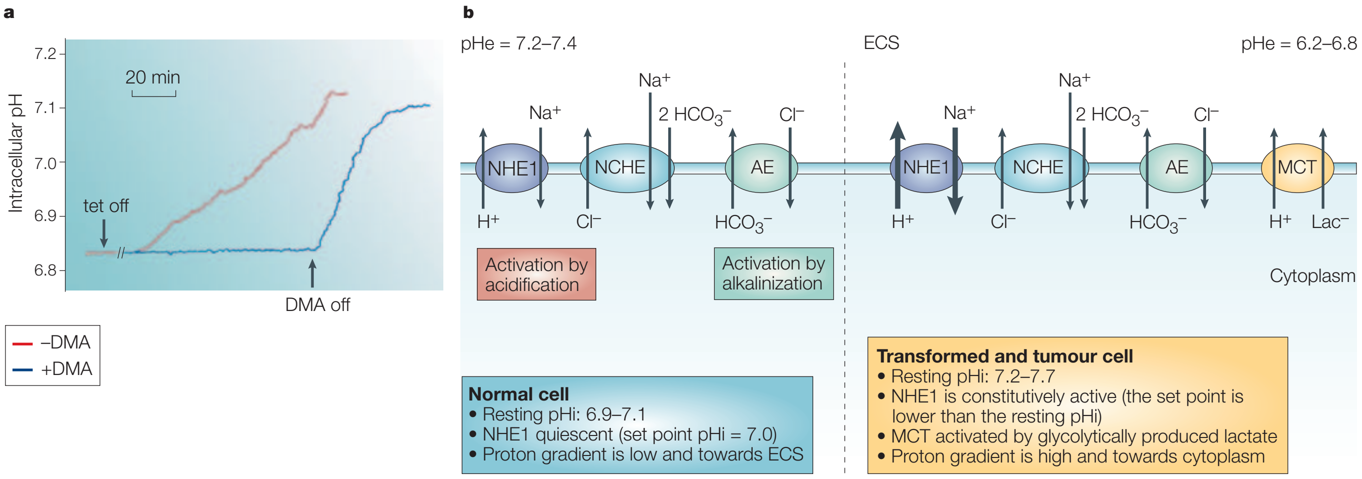


Figure 1 | **NHE1 in transformation and pHi homeostasis. a** | Experiments proving the role of NHE1 in regulating intracellular pH (pHi) in cancer cells involved the use of NIH-3T3 cells transformed with the E7 oncoprotein of the malignant human papilloma virus type 16 under the control of a tetracycaline (tet) inducible promoter [9]. The use of this inducible expression system permitted a finely controlled study of the sequence of early events during transformation and the effects of transformation on pHi. The expression of the E7 oncogene (after removal of tet, 'tet off') causes a rapid increase in cytoplasmic pHi (red line). Incubation with the inhibitor of the Na$^+$/H$^+$ exchanger NHE1, 5-*N,N*-dimethylamiloride (DMA) reversibly blocked the pHi increase (blue line), showing that the pH change that occurs during transformation is driven primarily by NHE1. Once DMA is removed (DMA off), the pHi increased again. **b** | The activity of the major pH-regulating transporters and the pHi values of normal and tumour cells. The relative activity of the ion transporters in the different cells is represented by the size of the arrows. In normal cells, NHE1 is quiescent because its set point for proton-dependent activation (BOX 1) is at the physiological, resting pHi of the cell (6.9–7.1). Resting pHi is primarily maintained by the Na$^+$-driven Cl$^-$/HCO$_3^-$ exchanger (NCHE), which is responsible for keeping pHi higher than its predicted equilibrium, and the Na$^+$-independent Cl$^-$/HCO$_3^-$ exchanger (AE), which, while regulating intracellular Cl$^-$ concentrations, also counteracts the activity of the NCHE to return pHi to physiological, resting values. The NHE1 and the AE are pHi 'house-keepers' in that they control pHi homeostasis by becoming activated only during intracellular acidification or alkalinization, respectively. If the pHi drops below the set point of NHE1, NHE1 becomes activated and returns the pHi to the resting value. In transformed and tumour cells, an increase in the affinity of the allosteric intracellular proton-binding site hyperactivates NHE1, resulting in an increase in pHi and acidification of the extracellular space (ECS). The increased pHi stimulates aerobic glycolysis, which increases the amount of cellular lactate that is transported out of the cell via the H$^+$/lactate cotransporter (MCT). The activity of the MCT exacerbates the alkalinization of the cell and acidification of the ECS, and further increases the reversed proton gradient. See main text and BOX 1 for further details. Part **a** is modified, with permission, from REF. 9 © (2000) The Federation of American Societies for Experimental Biology.

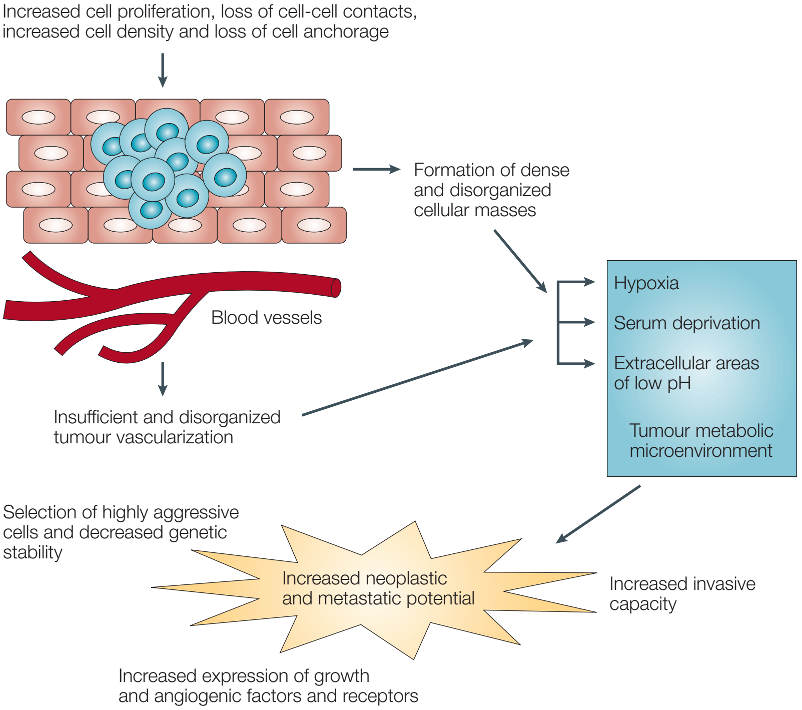


Figure 2 | **The development of the tumour metabolic microenvironment.** A general scheme that shows how the increased proliferation and density of tumour cells, along with the loss of cell–cell contacts and cell adhesion, affects the vasculature to produce the tumour metabolic microenvironment. This microenvironment is characterized by low serum levels, hypoxia and an acidic extracellular pH. This microenvironment drives progression of tumours to a metastatic phenotype.

extracellular space (that is, a decrease in pHe). The increased pHi directly drives the activation of aerobic glycolytic metabolism[9] and the resulting excess hydrogen ions are excreted from the cell through the MCT[13]. This further increases the pHi (REF. 14) and exacerbates the initial development of the acidic interstitial microenvironment and the reversed transmembrane pH gradient. Stable transfection of engineered ion-translocation-defective NHE1 into fibroblasts resulted in a decrease in gene expression of six of the enzymes of the glycolytic pathway, including lactate dehydrogenase. This evidence supports the fundamental role of NHE1 in regulating glycolysis[15]. Interestingly, this increase in aerobic glycolytic metabolism can protect hyper-proliferating cells against oxidative stress that arises from the reactive oxygen species that are produced during the increased proliferation[16].

Indeed, the increased pHi that is driven by the activated NHE1 of the transformed cell is concomitant with increased DNA synthesis[5,8,9], cell-cycle progression[5,6,9,17], both substrate- and serum-independent growth[9], and *in vivo* tumour growth[9,18], all of which result in a pathological and disorganized increase in cell number and density. A consequence of an increase in tumour cell density is a corresponding decrease in access to the circulatory system[19], which causes an inefficient washout of metabolic products (such as carbonic acid). This, together with the increase in aerobic glycolysis[9], gives rise to tumour METABOLIC MICROENVIRONMENTS — extracellular areas within tumours that are characterized by dynamic, interacting areas of hypoxia, low levels of serum nutrients and acidic pHe (REF. 20) (FIG. 2).

Acidification of the metabolic microenvironment

The conditions of the tumour metabolic microenvironment that are described above are not discretely distributed within the tumour and can all occur simultaneously (FIG. 2). The mechanisms by which malignant cells acidify their intratumoural microenvironment and in turn, the regulation of these processes by the other components of the microenvironment are still being clarified. Glycolytic lactate production/release is commonly considered to be the primary acidification mechanism of the microenvironment[1,21], and the hypoxic component of the metabolic microenvironment further stimulates glycolysis, providing additional pHe acidification[21,22]. However, tumours in nude mice that were derived from cells lacking lactate dehydrogenase are fully able to acidify their microenvironment[23], and the restriction of blood flow to murine tumours by vascular clamping stopped lactate production/release but the microenvironment continued to acidify[3]. This indicates that other mechanisms of cellular pH regulation contribute to extracellular acidification. In tumour cells, NHE1 activation by low serum[24–26], acidic pHe (REF. 22) and hypoxia (R.A.C., V.C. and S.J.R., unpublished observations) is another mechanism that contributes to the acidification of the microenvironment. A recent study has also demonstrated that within the STROMAL MICROENVIRONMENT of breast tumours, HYALURONAN interaction with its receptor, CD44, on breast cancer cells activates NHE1, resulting in acidification of the extracellular medium (REF. 27). These findings led to the recognition of a synergistic, positive-feedback interaction between the tumour cell and both the metabolic and stromal microenvironments that can lead to transient changes in the biochemistry and physiology of the tumour cells. These can elicit further changes in these microenvironments that support tumour progression.

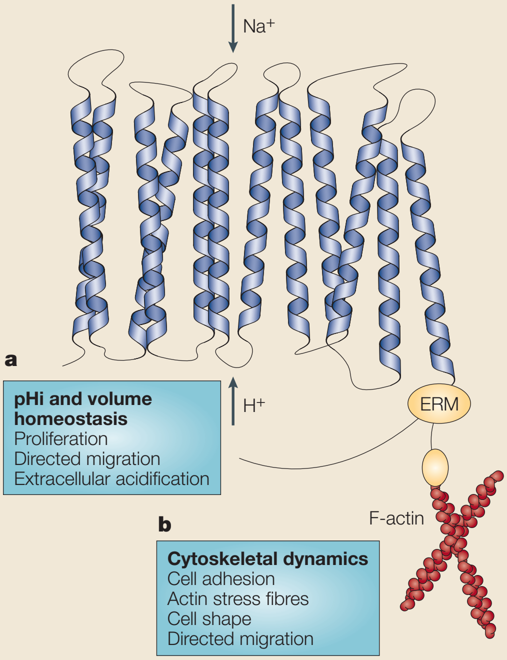
NHE1 in tumour metastasis and invasion

Metastasis involves alterations in the coordination and regulation of various cellular processes. These include neoangiogenesis, cell adhesion (required for cancer cell escape from the primary tumour), degradation and remodelling of the extracellular matrix (ECM) barrier (represented by basal laminae and connective tissue), directed motility, resistance to anoikis while in the circulation and sustained ectopic growth in the invaded organ[28]. Specific adaptation of tumour cells to their metabolic microenvironment makes this microenvironment a fundamental driving force in tumour progression to a more aggressive metastatic phenotype, which is independent of genomic changes in the tumour population. This provides a rationale to formulate a

METABOLIC MICROENVIRONMENT
The *in vivo* microenvironment within tumours is significantly different from that within normal tissues. It is characterized by the presence of closely linked, dynamic extracellular areas of low pH, low serum levels and hypoxia, and is known as the tumour-specific physiological or metabolic microenvironment. It arises as a consequence of the dysregulation of proliferation, apoptosis and growth-factor signalling and causes a pathological and disorganized increase in cell number and density and a decrease in access to circulation.

Box 1 | The Na^+/H^+ exchanger NHE1

The Na^+/H^+ exchanger NHE1 is a member of a family of secondary active acid extruders that mediates the 1:1 exchange of extracelluar sodium for intracellular protons across the cell membrane. Through NHE1 action, the inwardly directed sodium gradient can drive the uphill extrusion of protons, alkalinize intracellular pH (pHi) and acidify the extracellular pH (pHe). NHE1 is expressed ubiquitously in non-epithelial cells and on the basolateral membrane of epithelial cells. It is composed of 12 transmembrane segments and a long carboxy-terminal cytoplasmic tail that has a role in both its regulation and function (see figure). The first functions of NHE1 to be identified were its role in regulating pHi homeostasis, cell volume and proliferation in response to growth factor stimulation. One of the important features of NHE1 is its exquisite sensitivity to pHi (FIG. 1b), — when pHi drops below a threshold level NHE1 is activated through an internal allosteric proton-binding regulatory site. This pHi sensitivity determines the activity set point of NHE1 — that is, the pHi at which NHE1 is quiescent — and, in normal cells, this set point is at the physiological, resting pHi. Neoplastic transformation increases the affinity of the proton-regulatory site, thereby increasing the sensitivity of NHE1 to the normal pHi, constitutively activating it and raising pHi. NHE1 in tumour cells is always active and these cells can have pHi values as high as 7.8. Through binding to the actin-binding protein VIL2 (villin 2, a member of the ezrin, radixin and moesin (ERM) family), NHE1 can directly regulate cytoskeletal dynamics independently of its ion-transporting capabilities, which permits the separation of the downstream functions of NHE1 into two groups (see figure). The first consists of those regulated by its ion translocation (a). The second consists of those regulated by its ability to directly bind the ERM family and regulate cortical cytoskeleton dynamics by anchoring the cortical cytoskeleton to the plasma membrane (b)[63]. Very recently, NHE1 was also proposed to be able to act as a scaffolding protein through its direct association with a number of signalling proteins[64]. These three different modes of regulation make NHE1 an important membrane-bound integrator for many signalling networks and cellular processes.



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model of microenvironment-driven tumour metastasis. Indeed, both progression and patient mortality are correlated with microvascular density, which is a measure of the development of the metabolic microenvironment[29].

In particular, the acidic component of the intratumoural metabolic microenvironment increases metastatic potential by promoting neoangiogenesis[30], anchorage-independent growth[1], genetic instability[1] and invasion. Invasion occurs through a complex series of interactions with the host tissue in which the infiltration and penetration of the normal tissue by cancer cells takes place by four biochemical and physiological steps[31,32]. First, there is a loss of tumour-cell attachment to either basement membranes or the ECM. Second, direct local degradation of these structures dependent on tumour-cell acid extrusion occurs. The third step is the secretion of acid-dependent proteases and their activation by acid extrusion from the tumour cell. And the fourth is increased tumour-cell locomotion into the modified region. The second, third and fourth processes are regulated by both pHe and pHi.

Invasiveness is dependent on the pericellular proteolysis of ECM barriers. These barriers would otherwise prevent tumour-cell escape from the normal tissue architecture and eventual INTRAVASATION of blood-vessel walls. The use of REACTION-DIFFUSION MODELLING of tumour metabolism has demonstrated the important direct role of this extracellular acidification in the digestion and remodelling of the ECM and in the interactions of the tumour with normal tissue[33]. In severe combined immunodeficient (SCID) mice, *in situ* human metastatic tumours that were stably expressing the anti-metastatic gene *NM23* had a lower pHi, a higher pHe and a lower metastatic load (that is, number and size of metastases) than tumours derived from wild-type cells, supporting a role for disturbed pH dynamics in metastasis[34]. The ECM remodelling process is further accelerated by the low pH, which facilitates the action of acidic proteases that are secreted by the tumour cell. The action of such proteases is not limited to protein cleavage but also involves glycosaminoglycan degradation[35]. Most of these proteases belong to the urokinase-type plasminogen activator system[36] or the protease family of cysteine[37] and aspartyl cathepsins[38]. Although the

STROMAL MICROENVIRONMENT
Tumours have an extensive adjacent stromal compartment that provides the framework for the tumour. It is composed of connective tissue that contains fibroblasts, immune and inflammatory cells, and cells that are derived from blood vessels, nerves and the extracellular matrices. Cancer cells communicate with and alter the adjacent stroma to form a permissive and supportive environment for tumour progression — the 'reactive' tumour stroma microenvironment.

HYALURONAN
A large, negatively charged specialized glycoprotein with polysaccharide side chains that participates in defining the properties of pericellular matrices and in transducing signals in proliferating and migrating cells. Hyaluronan is the main ligand for CD44, a cell-surface glycoprotein receptor, and is overproduced in many types of human tumour.

INTRAVASATION
The passage of tumour cells from the tumour and its surrounding tissue into blood vessels (haematogenous dissemination) or lymph vessels (lymphatic dissemination).

REACTION-DIFFUSION MODELLING
A model that combines mathematical analyses, experimental data and clinical observations to describe the interaction of tumour and normal (host) tissue in determining the spatiotemporal diffusive distribution of proton concentration and its role in directing invasion of the host tissue by the tumour.

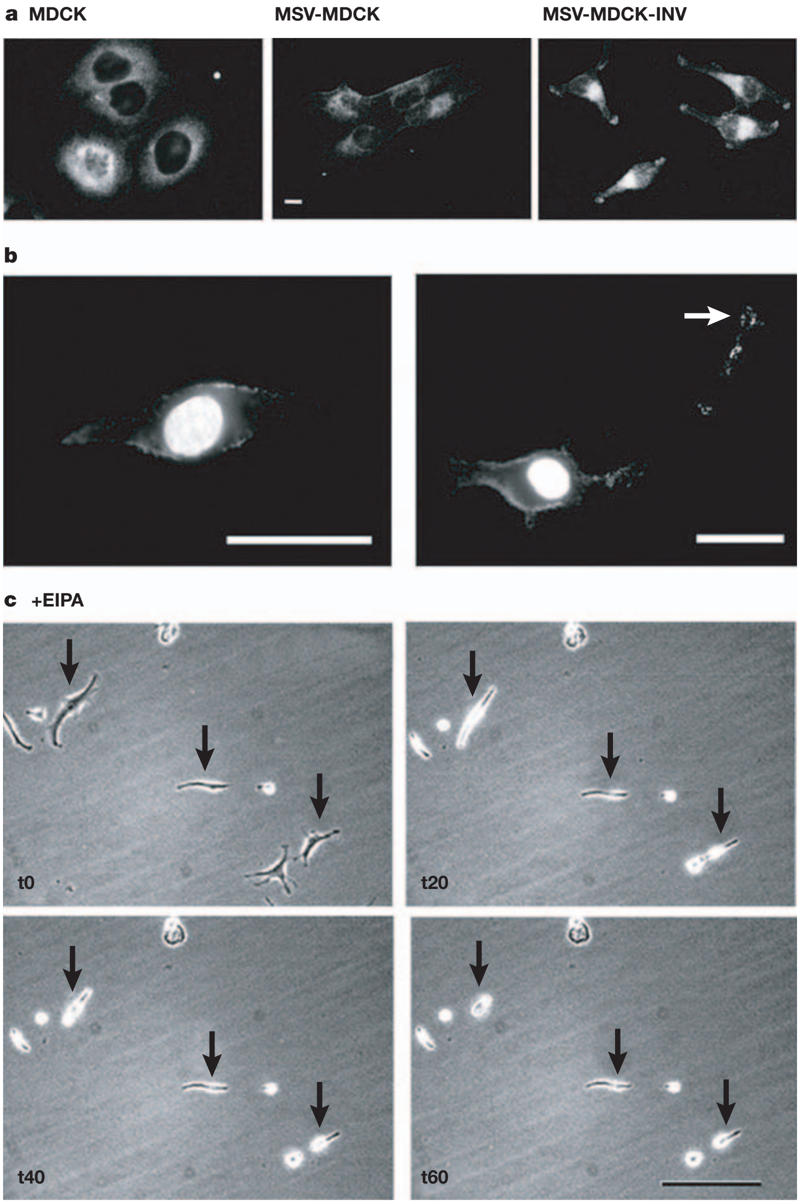

Figure 3 | **The location of NHE1 in invasive cells and role of NHE1 in pseudopod maintenance. a** | Immunofluorescence analysis reveals the distribution of NHE1 in three types of Madin–Darby canine kidney (MDCK) epithelial cells: wild-type cells (MDCK), left panel; Moloney sarcoma virus (MSV)-transformed cells (MSV-MDCK), centre panel; and invasive MSV-transformed cells (MSV-MDCK-INV), right panel. The wild-type MDCK cells express the Na^+/H^+ exchanger NHE1 in a diffuse manner. Upon transformation, MSV cells become elongated and NHE1 redistributes to the plasma membrane of the cell. The invasive variant of the transformed cells (MSV-MDCK-INV) have long pseudopodia with NHE1 preferentially expressed at the tip. **b** | Serum deprivation of metastatic breast cancer cells. The left panel shows a cell in normal culture conditions (10% fetal bovine serum) in which it has a fusiform, fibroblast-like shape and NHE1 is found in the plasma membrane of the main cell body (the scale bar represents 10 µm). The right panel shows a cell after 24 hours of serum deprivation. It has become elongated with a long leading-edge pseudopodia and a redistribution of NHE1 along the body of the pseudopodia and to the tip (arrowhead) (scale bar, 10 µm). **c** | A time course of the effect of specific inhibition of NHE1 by the amiloride derivative, 5-(*N*-ethyl-*N*-isopropyl)-amiloride on the leading-edge pseudopodia (scale bar, 50 µm). Arrows indicate cells in which treatment with ethylisopropylamiloride produces an initial retraction of the pseudopodia followed by the cell adopting a more round shape. For another example, see online Supplementary information S1 (movie). t, time in minutes. Parts **a** and **c** are reproduced, with permission, from REF. 47 © (2000) The Company of Biologists Ltd. Part **b** is reproduced, with permission, from REF. 26 © (2005) The American Society for Cell Biology.

metalloproteases[39,40] (MMPs) are not optimally active at the low pHe of the tumour microenvironment, the conversion of the latent MMP pro-form to the active form is dependent on the proteolytic activities of the other protease families, which do have their optimal activity at low pHe (REF. 41). Therefore, the low pHe of the microenvironment might provide a proteolytically active environment that surrounds the tumour. This optimizes not only the activity of the urokinase-type plasminogen activator and cathepsin systems, but also that of the interacting proteolytic cascades that, in turn, convert pro-MMPs to active MMPs. In a positive-feedback loop, low pHe has been shown to stimulate the release of cathepsin B[27,42] and MMP9 (REF. 43), presumably through the direct increase in cellular lysosomal volume and the stimulation of lysosomal migration towards the cell membrane, both of which are seen in invasive cancer cells[44]. Regulation of cathepsin B activity by the hyaluronan activation of CD44 promotes the interaction of CD44 with NHE1, which results in acidification of the tumour-cell ECM and increased invasiveness, thereby supporting a role for NHE1 in this loop[27]. Two lines of evidence are consistent with this loop. First, there is a reduction in tumour-cell invasive capacity that is caused by increasing the pHe, by incubating the cells with a cocktail of inhibitors for various secreted proteases[45] or by inhibiting NHE1 (REF. 27). Second, the stable transfection of an ion-translocation-defective NHE1 causes a decrease in both the activity of MMP9 and the expression of the gene that encodes it (REF. 15).

Acquisition of a motile and invasive phenotype is one requirement for a cell to become metastatically competent, and the ability of an epithelial cell to migrate individually is a hallmark of tumorigenic cells. The low pHe of the tumour microenvironment has been shown to increase tumour-cell motility by promoting formation of pseudopodia, which indicate cell migration, in less invasive cells. Low pHe also induces an increase in the number and length of pseudopodia in metastatic cells[44]. Both pesudopodial protrusion and migratory behaviour are reduced by increasing the pHe, which inhibits the activity of secreted proteases[45] and blocks NHE1 activity[25]. Furthermore, recent evidence indicates that NHE1 has an important role in directly coordinating tumour-cell motility[24,25,46–48] and invasion[24,25,27] independently of changes in pHe.

Altogether, the results of these studies are consistent with the hypothesis that increased tumour-cell invasiveness is the result of two complementary mechanisms: the disruption of cell–matrix interactions that arise from increased acid secretion and protease activity, and increased cell motility. NHE1 has a prominent role in coordinating both these mechanisms.

NHE1, pH and invasive structures

The increased chemotactic and invasive abilities of tumour cells depend on their ability to form pseudopodia and invadopodia at the cell's leading edge. Invadopodia are small, β1-integrin- and protease-rich actin structures that are often found

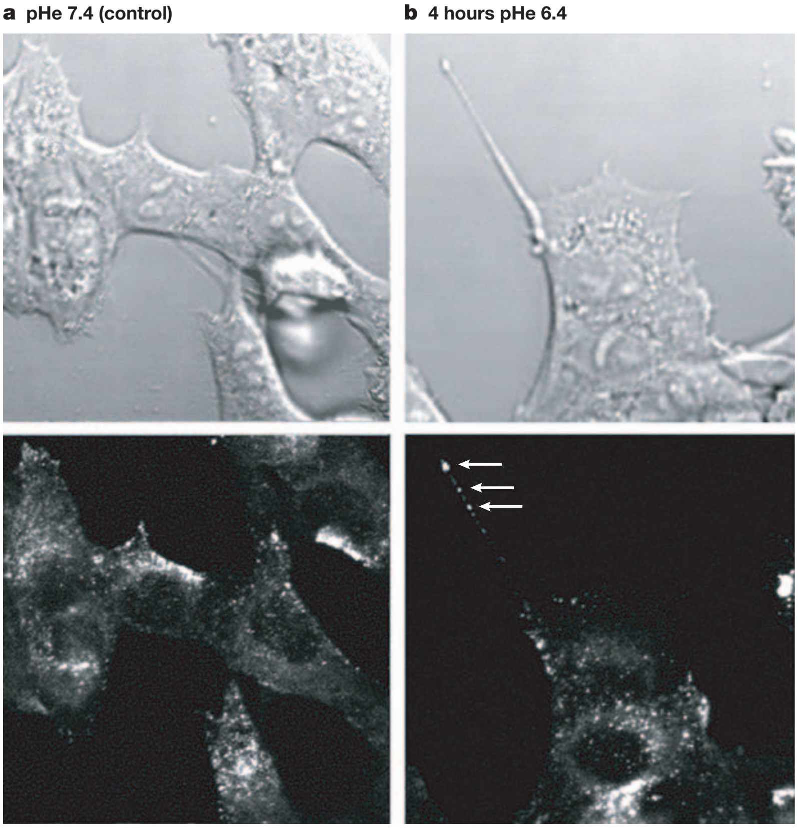

Figure 4 | **The effect of low pHe on lysosome transport in MDA-MB-435 breast cancer cells.** The upper panels are images aquired by transmitted-light differential interference contrast microscopy. The lower panels show the same continuously perfused living cells in which the position of lysosomes is imaged by laser scanning confocal fluorescence microscopy of 6-*O*-dansyl-$GlcNH_2$-labelled glycosylated lysosomal proteins (white, fluorescent areas). **a** | At the normal extracellular pH (pHe) of 7.4, lysosomes are preferentially perinuclear. **b** | Four hours exposure of cells to a pHe of 6.4 produces elongated pseudopodia and increases lysosome transport towards the plasma membrane in the pseudopodia. Arrows show that some of the lysosomes are found near and at the pseudopodial tip. Reproduced, with permission, from *Neoplasia* REF. 44 © (2003) Macmillan Magazines Ltd.

at the tip of the pseudopod. The creation of these specific cellular domains is one of the most intriguing properties of tumour cells and we still know little about the biochemical processes that mediate formation of these structures in tumour cells.

Leading-edge pseudopodia. Conditions that increase invasive capacity often provoke a dramatic shift in cell morphology from AMOEBOID MORPHOTYPE to MESENCHYMAL MORPHOTYPE, which corresponds with the increase in invasive capacity[45,49–51]. In many tumour cells mesenchymal morphology includes the projection of long 'leading-edge pseudopodia', which are distinct from lamellipodia and filopodia, are commonly observed in invasive tumour cells[49,52], and have been shown to be involved in tumour-cell invasion[25,47,53,54]. Intravital multiphoton microscopy of tumours has shown that these pseudopodia project in the direction of tumour-cell movement towards circulatory capillaries[55]. Pseudopod formation can be stimulated by oncogene expression[47], HGFR (hepatocyte growth-factor receptor) activation[56] and CD44-dependent adhesion[57], as well as by exposure to low pHe (REFS 44,58), hypoxia (R.A.C., V.C. and S.J.R., unpublished observations) or serum deprivation[25,26] — conditions that are characteristic of the tumour microenvironment.

This establishment of cell polarity and directed movement in an invasive cell requires dynamic remodelling of the cytoskeleton[25,47] and a redistribution of activated NHE1 to the the tip of the pseudopodial compartment[25,27,47] (FIG. 3a,b). Pharmacological inhibition of NHE1 blocks both the formation of this invasive structure (FIG. 3c, supplementary information S1 (movie)) and the ability to invade[24,27]. A tight interdependence between pseudopod formation, the processes of NHE1 activation, and protease secretion is demonstrated by the preferential localization of lysosome production along the pseudopodia and at its tip, which is stimulated by low pHe (FIG. 4). The reversion from mesenchymal morphotype to amoeboid morphotype, along with the reduction in motility that is observed when cells are incubated with a protease-inhibitor cocktail, further supports this interdependence[45].

Recent studies from the laboratories of Albrecht Schwab and Christian Stock used human melanoma cells to show that the protons extruded by NHE1 at the focal-adhesion sites might stabilize the bond between adhesion molecules, such as between plasma-membrane $\alpha_2\beta_1$-integrin receptors and the ECM[58]. When the local pHe is optimal for binding between membrane proteins and the ECM components, cells remain attached to the matrix and start to form long processes in all directions. In normal mammalian cells[59] and in *Dictyostelium discoideum*[60] NHE1 has been shown to localize to the leading edge of polarized cells and is necessary for the formation of lamellipodia and efficient chemotaxis. This indicates evolutionarily conserved roles for NHE1 in the processes of cytoskeletal assembly and for maintenance of polarity in motile cells.

AMOEBOID MORPHOTYPE
Amoeboid invasion is characterized by an amorphous cell morphology and rapid changes in direction that are made possible by rapid remodelling of the cell-cortical actin cytoskeleton. It is thought that amoeboid movement involves very weak cell–extracellular matrix (ECM) interactions and is protease-independent, because cells move through gaps that already exist in the ECM.

Invadopodia. Invadopodia are highly organized and specialized plasma-membrane structures that extend into the ECM, relaying signals from matrix components and mediating ECM degradation during tumour cell invasion[61]. The protein interactions that are involved in the regulation of invadopodia composition and formation, and the organization of protease docking and secretion are starting to be defined[61]. Little is known, however, about how the proteases that are secreted by the invadopodia become activated. Acidification of the tumour microenvironment is one factor known to activate these proteases. A vital clue to understanding the interplay of molecular, physiological and structural mechanisms that cause this acidification can be found by comparing the process of tumour cell invasion to that of bone degradation by the podosomes (structures that are similar to invadopodia) of OSTEOCLASTS[61].

Osteoclasts contain highly organized actin-rich membrane extensions that mediate adhesion to the bone, creating a tightly sealed subosteoclastic

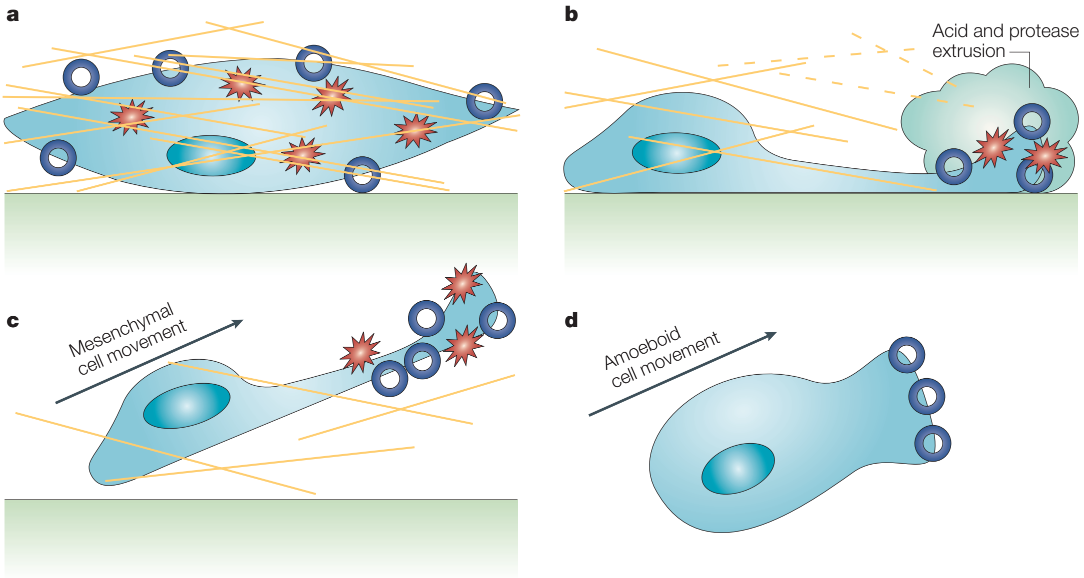

Figure 5 | **A proposed multistep invasion scenario. a** | Normally the extracellular matrix (ECM) (yellow lines) is tight and highly organized and cells localize the Na^+/H^+ exchanger NHE1 (hollow blue circles) generally along the plasma membrane, and proteases (red stars) to intracellular lysosomes. **b** | Interaction with elements of the stromal and/or metabolic microenvironment activates a coordinated set of mesenchymal morphotype behaviours. NHE1 is activated, which results in cytoskeletal reorganization and the formation of pseudopodia at the leading edge of the cell. NHE1 and lysosomes become redistributed to the tip of the pseudopod, resulting in focalized extrusion of acid and protease. This causes focalized proteolysis of the ECM and compromises cell attachments, which results in increased cellular motility and invasion into the digested areas of the ECM. **c** | A deteriorating metabolic microenvironment and digestion of the ECM cause the exposure of cryptic adhesion sites and the release of ECM-associated latent factors. These signals work in a positive-feedback loop to further activate NHE1 and stimulate protease secretion, thereby further increasing the invasive capacity of the tumour cell. **d** | When the ECM has been digested and the supplies of uncovered adhesion sites and released growth factors are exhausted, the tumour cell is able to make a mesenchymal to amoeboid transition. In doing so, the cell responds rapidly to growth and motility factors that are released from the nearby microcirculation to move through the gaps in the ECM towards the capillaries by chemotaxis. This transition can be reversed when the tumour cell returns to its original microenvironmental conditions. NHE1 is localized to the leading edge of cells that move by chemotaxis.

environment where proteases that are secreted by these membrane extensions can reach very high concentrations[35,61]. A highly regulated acidification of the podosome–bone compartment by VACUOLAR H^+-TRANSPORTING ATPASES (V-ATPases), lactate secretion and NHE1 is an essential component of the bone digestion and reabsorption process[62]. Analogously, proton extrusion by the invadopodia might mediate the localized degradation of the ECM, resulting in the directed movement of the leading-edge of the invading tumour cell through the digested tissue. Circumstantial evidence for the involvement of NHE1 in this process comes from a study that demonstrates the co-localization of the CD44 with MMP9 in the invadopodia of invasive breast cancer cells[57]. Furthermore, a recent study shows that the invasive activity of breast cancer cells is due, in part, to CD44 interaction with and activation of NHE1, a process which causes the subsequent extracellular acidification[27].

A multistep invasion scenario (FIG. 5) can be constructed in which the tumour cell is highly adaptable to the rapidly changing cues of the extracellular environment, and in which those cells that are genetically predisposed to a higher invasive capacity can transiently shift from one optimal invasive cell shape and physiological/biochemical phenotype to another. In this scenario, the tumour metabolic microenvironment activates a coordinated set of mesenchymal morphotype behaviours[44,50,51], including activation and redistribution of NHE1 (REFS 24–26). These behaviours allow the tumour cell to break down the ECM and infiltrate the surrounding stromal compartment. During this process, the tumour cell can increase its interaction with the stromal microenvironment through receptors, such as CD44 and integrins, that sense various components of the ECM. This activates another set of responses, again including activation of NHE1 (REFS 27,58), that further reduce the structure and density of the ECM and increase tumour-cell infiltration. The loosening of the matrix structure reduces the requirement for protease activity, which could be accompanied by a shift in morphology from a mesenchymal morphotype to an amoeboid morphotype. This shift would allow tumour cells to undergo rapid chemotaxis along growth-factor gradients towards blood vessels, guided, in part, by NHE1 redistribution to the leading edge of the pseudopodia[60], resulting in intravasation[55]. Is the NHE1 relocalization to the tip of the pseudopodia primarily to the invadopodia, and is the relocalization of NHE1 coordinated with the relocalization of lysosomes[44], and with protease secretion from invadopodia[61]?

MESENCHYMAL MORPHOTYPE
Mesenchymal invasion is characterized by an elongated polarized cell shape and is dependent on secreted proteases to digest the extracellular matrix. The initiating event for mesenchymal invasion is usually activation of tyrosine-kinase receptors or adhesion receptors, or exposure to the metabolic microenvironment. These events lead to the formation of a complex, tubulin- and actin-rich pseudopodial protrusion, the tip of which is rich in small integrin-dependent focal contacts where the secretion of proteases occurs.

OSTEOCLASTS
Specialized multinucleated bone-resorbing cells that are responsible for the degradation of bone.

VACUOLAR H^+-TRANSPORTING ATPASE
A complex enzyme composed of numerous subunits that is ubiquitously expressed in eukaryotic cells, where it is located in intracellular acidic organelles. In some specialized acid-extruding cells, such as osteoclasts, an increase in V-ATPase-dependent proton secretion correlates with an increase in V-ATPase plasma-membrane expression owing to a rapid recycling between intracellular vesicles and the plasma membrane.

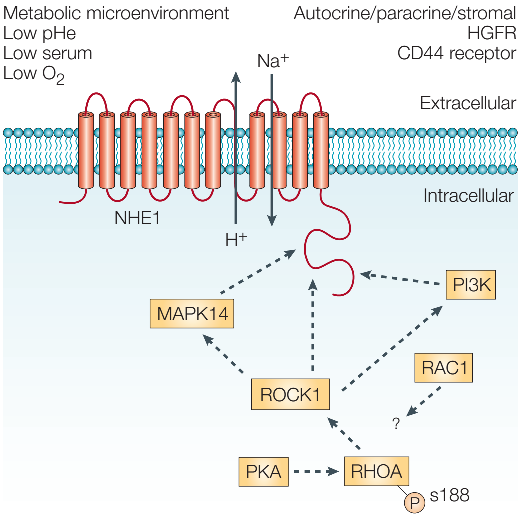

Figure 6 | **Signal transduction mediating NHE1-dependent invasion.** This diagram illustrates the signalling pathways that might regulate the activity and function of the Na^+/H^+ exchanger NHE1during tumour cell invasion. NHE1-activating signals from both the stromal compartment (mediated through CD44, the hyaluronic-acid-binding receptor) and the metabolic microenvironment (serum deprivation) probably involve phosphatidylinositol-3-kinase(PI3K)[68] and the small G-proteins RHOA (*ras*-homologue protein family, member A)[25,26,68] and RAC1 (*ras*-related C3 botulinum-toxin substrate 1)[25,67]. Signalling from RHOA through ROCK1 (rho-associated coiled-coil containing kinase 1) has been implicated in regulating PI3K and NHE1 — either directly[24,27,68] or through regulation of MAPK14 (mitogen-activated protein kinase 14)[26]. The downstream components that are involved in RAC1 regulation of NHE1 activity and invasion have not yet been defined (represented by ? in the figure). The role of these signalling components in pseudopodia formation and cell invasion has been indicated in recent work that shows serum-deprivation-mediated activation of a pseudopodia-located protein kinase A (PKA), which phosphorylates (p) RHOA at serine188 (s188)[24]. PKA activates this same RHOA–ROCK1–MAPK14 signalling pathway in breast cancer cells and during HGFR (hepatocyte growth-factor receptor) activation of the invasive variant of Moloney-sarcoma-virus-transformed Madin–Darby canine kidney cells[24].

Signal transduction

Determining the sequence and spatial organization of signalling events that direct the reorganization of the actin cytoskeleton and NHE1 during invasion is important for understanding the invasive process. The primary extracellular signals and the subsequent signal-transduction systems that regulate NHE1 activity and downstream function are well understood in normal cells[63–65]. But little is known about the extrinsic invasion-promoting signals that activate NHE1 in cancer cells, or about the signal-transduction systems that drive and regulate this process.

Studies in breast, colon and kidney cancer cells have elucidated some of the signalling systems that regulate the NHE1-dependent motility and invasion that is stimulated by the metabolic microenvironment and by interaction with the ECM (FIG. 6). Serum deprivation increases motility and invasion through a dynamic NHE1-dependent remodelling of the actin cytoskeleton of the cell. The formation of pseudopodia[25,26] is accompanied by a sorting of NHE1 (FIG. 3b) and the regulation of NHE1 activity at the pseudopodia's distal tips by the PKA–RHOA–ROCK1–MAPK14 signal cascade[26] (where PKA is protein kinase A; RHOA is *ras*-homologue protein family, member A; ROCK1 is rho-associated coiled-coil-containing protein kinase 1, also known as p160ROCK; and MAPK14 is mitogen-activated protein kinase 14, also known as p38MAPK). This process is also mediated by PI3K (phosphoinositide-3-kinase)[24] and by an antagonistic reciprocity between RAC1 (*ras*-related C3 botulinum-toxin substrate 1) and RHOA, in which RHOA is inhibited and RAC1 is stimulated[25]. This reciprocal pattern is identical to that reported for the invasion of colon cancer cells, which is stimulated by paracrine HGFR and TNC (tenascin C) activation[66].

Interestingly, in invasive variants of Moloney sarcoma virus (MSV)-transformed epithelial kidney cells, autocrine stimulation of the HGFR induces the formation of pseudopodia that localize an activated form of NHE1 at their tips[56]. This expression occurs through the activation of the RHOA–ROCK1–MAPK14 signalling cascade within the pseudopodia (J. Noël and I. R. Nabi, personal communication). Furthermore, data from the Bourguignon laboratory show that invasive breast tumour cells localize CD44 (the hyaluronan receptor) to the invadopodia that form at the tip of leading-edge pseudopodia[57]. CD44 activation stimulates NHE1 activity and invasion through activation of the RHOA effector, ROCK1 (REF. 27). The same authors have also shown that a RAC1 (REF. 67) and a RHOA–ROCK1–PI3K pathway[68] are involved in CD44-induced cell invasion as well. Altogether, these data indicate that PI3K, PKA, RHOA, ROCK1 and MAPK14 are all components of signal-transduction systems that are activated in tumour cells to drive NHE1-dependent establishment of the directed cell polarity that is involved in invasion (FIG. 6). This polarity is the result of the formation of leading-edge pseudopodia. Both MAPK14 (REF. 69) and ROCK1 (REF. 70) have been shown to directly phosphorylate NHE1 in normal cells, which indicates that a similar mechanism could occur in tumour cells.

The signalling pathways that regulate NHE1 activity are also among the principal systems that are thought to be involved in driving metastasis[32]. This indicates that NHE1 could have a crucial role in driving invasion, possibly by acting as an integrator protein that links the cytoskeleton and various metastasis-specific signalling complexes to mediate invasive activity. This could be facilitated by the common feature of NHE1, CD44 and HGFR — they are all binding partners of VIL2 (villin 2, a member of the ezrin, radixin and moesin family)[71]. As extensive cross-talk between components of signalling systems is an important aspect of cellular regulation, an important future direction will be to determine the specificity and possible interactions of these signal-transduction pathways in the NHE1-dependent regulation of invasion. It will also be important to identify the molecular architecture — orchestrated by scaffolding, anchoring and adaptor proteins[72] — that forms the basis for the coordination of these metastasis signal-transduction systems. Lastly, it will be useful to formulate a tumour-microenvironment invasion and metastasis model that integrates the interaction of NHE1-induced cell structure, biochemistry and physiology with signal transduction in the invasive response of the tumour cell to the stromal and metabolic microenvironments.

Future directions

Tumour cell invasive activity is an important component of metastasis, and many research efforts have focused on developing new strategies that target this process. There are two different pH-dependent systems that are involved in regulating cell invasion, which are not mutually exclusive and are potential therapeutic targets. One approach is to interfere with the disturbed pH dynamics that are linked to the reversed pHe–pHi gradient using agents that either disperse the gradient or inhibit the essential mechanisms that are responsible for forming it[14,73–76]. The other approach is to improve our understanding of the mechanisms by which NHE1 controls pseudopod formation and invadopod function, so we might produce reagents that target either NHE1 itself or critical components of its regulatory pathway that function in invasion.

NHE1 inhibitors have been developed and have been tested in Phase II and Phase III clinical trials for myocardial protection during ischaemia and reperfusion[77]. They have been shown to have very low levels of toxicity, indicating that they might also be useful as anti-cancer agents. The high basal activity of NHE1 in tumours and the low basal activity in normal tissues should facilitate the transition of these drugs into trials for their anti-tumour and anti-metastatic capabilities.

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Competing interests statement

The authors declare no competing financial interests.

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