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Na^+,HCO_3^- cotransporter NBCn1 accelerates breast carcinogenesis

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Abstract

Cell metabolism increases during carcinogenesis. Yet, intracellular pH in solid cancer tissue is typically maintained equal to or above that of normal tissue. This is achieved through accelerated cellular acid extrusion that compensates for the enhanced metabolic acid production. Upregulated Na^+,HCO_3^- cotransport is the predominant mechanism of net acid extrusion in human and murine breast cancer tissue, and in congruence, the protein expression of the electroneutral Na^+,HCO_3^- cotransporter NBCn1 is increased in primary breast carcinomas and lymph node metastases compared to matched normal breast tissue. The capacity for net acid extrusion and level of steady-state intracellular pH are lower in carcinogen- and ErbB2-induced breast cancer tissue from NBCn1 knockout mice compared to wild-type mice. Consistent with importance of intracellular pH control for breast cancer development, tumor-free survival is prolonged and tumor growth rate decelerated in NBCn1 knockout mice compared to wild-type mice. Glycolytic activity increases as function of tumor size and in areas of poor oxygenation. Because cell proliferation in NBCn1 knockout mice is particularly reduced in larger-sized breast carcinomas and central tumor regions with expected hypoxia, current evidence supports that NBCn1 facilitates cancer progression by eliminating intracellular acidic waste products derived from cancer cell metabolism. The present review explores the mechanisms and consequences of acid-base regulation in breast cancer tissue. Emphasis is on the Na^+,HCO_3^- cotransporter NBCn1 that accelerates net acid extrusion from breast cancer tissue and thereby maintains intracellular pH in a range permissive for cell proliferation and development of breast cancer.

Keywords Acid-base homeostasis · Breast carcinogenesis · Cancer metabolism · Cell proliferation · Sodium-bicarbonate cotransport · Tumor perfusion

Cancer cells differ from normal cells in their capacity for sustained proliferation and ability to evade growth suppression, resist cell death, attain replicative immortality, and enable invasion and metastasis [1]. Key among the neoplastic characteristics is enhanced metabolism that provides the energy and chemical components necessary for cell growth and division. In cells with fluctuating energy needs—such as skeletal muscle cells alternating between rest and exercise—net ATP hydrolysis under high cellular activity results in considerable net H^+ release and acidification [2]. In cancer cells with a raised but relatively constant energy demand, H^+ release from ATP hydrolysis is at steady state balanced by H^+ consumption during ATP synthesis and therefore does not represent a net acid load on the cells. Instead, the increased generation of acidic waste products in cancer cells compared to normal cells results from glycolysis coupled to lactic acid fermentation and from oxidative phosphorylation with accompanying hydration of CO_2 and dissociation of the carbonic acid [3]. In addition to a change towards anaerobic glycolysis because of tissue hypoxia, biochemical changes known as the Warburg effect favor fermentative glycolysis in cancer cells even under aerobic conditions [1, 4–6].

The increased metabolism in solid cancer tissue, in combination with insufficient blood supply, results in local interstitial accumulation of acidic waste products that causes extracellular pH in poorly perfused tumor regions to fall as much as one unit below that observed in corresponding normal tissues [7]. The composition of the tumor microenvironment—including the extracellular pH pattern—is spatially and temporally heterogeneous and reflects, at least in part, the variation in tissue perfusion [8]. Whereas the intratumoral blood vessels are structurally dysmorphic [9]—which interferes with normal blood flow regulation and tissue homeostasis—the extratumoral feed arteries maintain regular structural features and are specialized towards reduced resistance [10, 11], which is expected to maximize nutrient and oxygen delivery. The

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interaction between metabolism and intracellular pH in cancer tissue is bidirectional: the metabolic pathways are not only the primary source of intracellular acid loading, they are also themselves modulated by intracellular pH. Thus, the high rate of metabolic acid production puts cancer cells at risk of developing intracellular acidification that can retard glycolytic activity [12, 13], cell growth, and proliferation [14] and putatively inhibit tumor development and progression.

1 Transporters of acid-base equivalents maintain intracellular pH

In addition to contribution from net metabolic acid production, cells are typically prone to intracellular acidification because of their inside-negative membrane potential. At a membrane potential of −50 mV and an extracellular pH of 7.4, equilibration of H^+ across the plasma membrane according to the Nernst equation would result in an intracellular pH of 6.6. Typically, however, the resting intracellular pH is actively kept above equilibrium [15]: in normal breast epithelial cells at extracellular pH 7.4, intracellular pH is usually around 7.2, whereas intracellular pH of breast cancer cells is further elevated and can be as high as 7.6 [16–20].

Net acid extrusion from cells takes place through primary and secondary active transport mechanisms. In normal and malignantly transformed breast epithelium, acid extrusion is largely Na^+-dependent and mediated *via* Na^+,HCO_3^- cotransport and Na^+/H^+ exchange [16–18]. For some sources of cancer tissue, H^+-ATPases [21] and monocarboxylate transporters [22] may also contribute to cellular acid extrusion.

The Na^+-coupled HCO_3^- transporters of the SLC4 family constitute a clustering of related membrane proteins with diverse transport functions. The Na^+,HCO_3^- cotransporters (NBCs) mediate symport of HCO_3^- and Na^+ across cell membranes [23]. In addition to the ionic gradients and the membrane potential, the transport stoichiometries determine the directionality of the transport processes. At typical resting membrane potentials and in the presence of normal ionic gradients, cotransport of one or two HCO_3^- with each Na^+ is directed into cells, whereas cotransport of three HCO_3^- with each Na^+ is directed out of cells [23]. Notably, the transmembrane ionic gradients and the membrane potential can be markedly altered during cellular activation and in disease states (e.g., cancer and ischemia), which may influence the direction of transport and the activity of the acid-base transporters [24]. In the cytosol and interstitial space adjacent to the site of transport, the transfer of HCO_3^- across the membrane shifts the chemical reaction $CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$, which is catalyzed by carbonic anhydrases. Under equilibrated conditions, transport of HCO_3^- is equivalent to transport of H^+ in the opposite direction. As shown in Fig. 1, NBCn1 (SLC4A7) is an electroneutral Na^+,HCO_3^- cotransporter with a 1:1 stoichiometry [25] whereas NBCe1 (SLC4A4) and NBCe2 (SLC4A5) can transport Na^+ and HCO_3^- with either 1:2 or 1:3 stoichiometry [26–28] depending on cell type and phosphorylation state [29, 30]. At least one SLC4 family member (NDCBE, SLC4A8) mediates electroneutral Na^+-dependent Cl^-/HCO_3^- exchange [31, 32]. As illustrated in this figure, it is still debated whether the Na^+-dependent HCO_3^- transport mediated by NCBE/NBCn2 (SLC4A10) is coupled to net Cl^- transport [33, 34] or if it is only associated with Cl^- self-exchange [35]. AE4 (SLC4A9) is also a candidate Na^+,HCO_3^- cotransporter in some cell types [23].

It has been questioned if the SLC4 family members transport HCO_3^- or if at least some of the members translocate another HCO_3^--related species (e.g., CO_3^{2-} or the $NaCO_3^-$ ion pair) [36]. Due to the rapid interconversion between HCO_3^- and CO_3^{2-}, this is unlikely to have dramatic consequences for the integrated role of the transporters. Still, it may affect the local buffering mechanisms and the potential pH transients generated in membrane-near regions at the intracellular and extracellular surface of the acid-base transporters [37].

The Na^+/H^+ exchangers (NHEs) of the SLC9 family mediate electroneutral extrusion of H^+ in exchange for Na^+ under normal physiological conditions [38]. However, at least experimentally, substantial changes in the extracellular concentration of Na^+ can reverse the direction of the transport [39, 40].

2 NBCn1-mediated Na^+,HCO_3^- cotransport regulates intracellular pH in breast cancer tissue

The first indication that NBCn1 is linked to breast cancer came from a phosphoproteomic study showing differential expression of NBCn1 in xenografts based on a series of increasingly transformed MCF10AT breast cancer cell lines modeled after normal epithelium and various grades of breast cancer lesions [41]. Subsequently, multiple genome-wide association studies demonstrated that the single-nucleotide polymorphism rs4973768 located in the 3′ untranslated region of NBCn1 is associated with breast cancer in women of multiple ethnicities [42–52]. In some cohorts, rs4973768 associated only with estrogen receptor–positive breast cancer [52, 53] and with mammographic density only in pre-menopausal women [54]. The genetic association between NBCn1 and breast cancer does not apparently depend on menstrual or reproductive history or on body mass index [45, 48, 51]. Whereas rs4973768 is associated with breast cancer susceptibility, it is not apparently associated with overall or breast cancer–specific survival [55]. The possible consequences of the breast cancer–associated single-nucleotide polymorphism rs4973768 for expression and function of NBCn1 requires further documentation, but recent work shows that different hypertension-related single-nucleotide polymorphisms

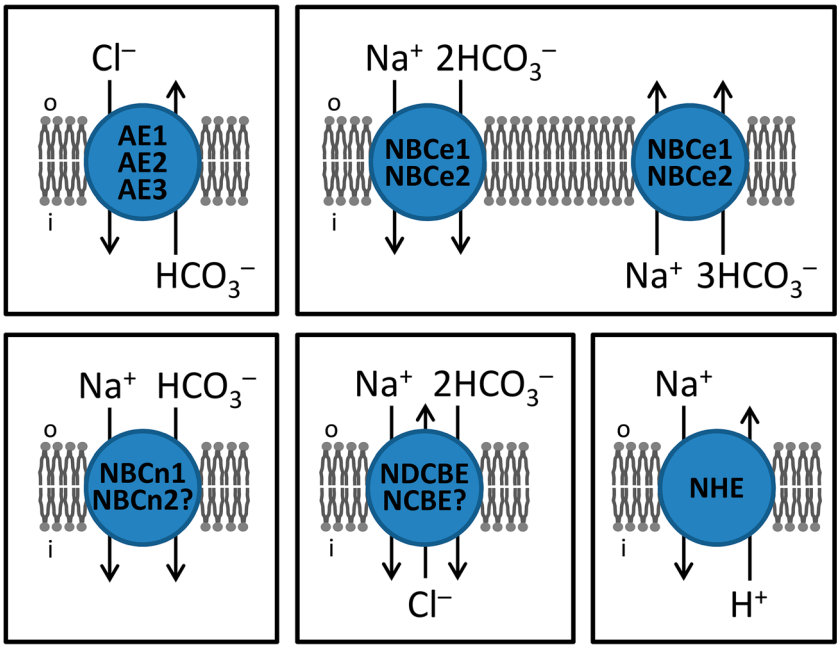

Fig. 1 Transport stoichiometries for the acid-base transporters of the SLC4 and SLC9 families at typical intracellular (i) and extracellular (o) ion concentrations and membrane potentials. Note that NBCn2 and NCBE are two alternative names suggested for SLC4A10 depending on whether the transporter mediates net Cl^- transport or only Cl^- self-exchange

rs820430 [56] and rs13082711 [57] near SLC4A7 are associated with increased NBCn1 expression and function in vascular smooth muscle cells.

Subsequent studies substantiated the role of NBCn1 in breast malignancy as they demonstrated NBCn1-mediated Na^+,HCO_3^- cotransport in the MCF-7 human breast cancer cell line [58]. Furthermore, NBCn1 protein expression in MCF-7 cells was found to increase twofold in response to heterologous overexpression of a constitutively active N-terminally truncated ErbB2 receptor [58], which is linked to increased breast malignancy and poor prognosis [59, 60]. Positive correlation between ErbB2 and NBCn1 expression in breast carcinomas was later confirmed in human tissue microarrays [61].

In order to identify mechanisms of acid-base control in an experimental setting that more realistically resembles the cellular composition and configuration of human breast cancer tissue, we next studied the role of NBCn1 in human biopsy material [16]. We demonstrated that NBCn1 expression is upregulated in biopsies of human primary breast carcinomas and metastases compared to normal breast tissue and established that Na^+,HCO_3^- cotransport is the principal mechanism of net acid extrusion in slices of human primary breast carcinomas [16]. Modest sensitivity of the identified Na^+,HCO_3^- cotransport to the nonspecific anion transport inhibitor 4,4-diisothiocyanatostilbenedisulphonic acid (DIDS) supports predominant contribution from NBCn1 [16]. In order to explore effects of breast carcinogenesis on protein expression and acid-base regulatory function, we next studied breast epithelial organoids—multicellular conglomerates isolated by partial collagenase digestion—that are dominated by epithelial cells and structurally comparable when produced from either normal breast tissue or breast cancer tissue [17, 18]. Based on freshly isolated breast epithelial organoids, we found that NBCn1 protein expression is twofold increased and Na^+,HCO_3^- cotransport robustly upregulated in human primary breast carcinomas compared to normal breast tissue [17].

The studies listed above provide compelling support—but not direct evidence—that NBCn1 mediates the Na^+,HCO_3^- cotransport in breast cancer tissue. Since no selective pharmacological inhibitors are currently available for NBCn1 [38, 62, 63], we instead used knockout mice and murine breast cancer models—induced by carcinogens (dimethylbenz(α)anthracene and medroxyprogesterone acetate) or ErbB2 overexpression—in order to directly evaluate the importance of NBCn1 in breast cancer tissue [18, 64]. We demonstrate that steady-state intracellular pH is elevated in carcinogen-induced breast cancer tissue from wild-type mice both when compared to normal breast tissue and relative to breast cancer tissue from NBCn1 knockout mice [18]. Additionally, the Na^+-dependent net acid extrusion in carcinogen-induced breast cancer tissue from wild-type mice showed a very substantial CO_2/HCO_3^- dependency that was completely abolished in breast cancer tissue from NBCn1 knockout mice [18]. Likewise, the upregulation of the CO_2/HCO_3^--dependent net acid extrusion and the steady-state alkalinization developing during ErbB2-induced breast carcinogenesis in wild-type mice were absent in NBCn1 knockout mice [64]. The protein expression of NBCn1 increased twofold to threefold during breast carcinogenesis irrespective of whether it was driven by carcinogens or ErbB2 overexpression [18, 64]. Based on the very similar patterns of pH regulation in human and murine breast cancer tissue and the comparable upregulation of NBCn1 protein expression during human and murine breast carcinogenesis, it is reasonable to propose that NBCn1 is

responsible for the Na^+,HCO_3^- cotransport activity in human as well as murine breast cancer tissue. Nonetheless, definitive functional evidence for the involvement of NBCn1 in human breast cancer tissue still needs to be provided.

It is notable that other Na^+,HCO_3^- cotransporters do not markedly compensate for the absence of NBCn1 in breast tissue even in mice with global constitutive knockout of NBCn1 [18, 64]. Despite functional redundancy within the families of acid-base transporters, the molecular mechanisms of transporter regulation apparently differ sufficiently that other transporters do not make up for the NBCn1 deficiency. This obviously strengthens the argument for NBCn1 as therapeutic oncology target.

3 Other mechanisms of intracellular pH regulation in breast cancer tissue

In addition to Na^+,HCO_3^- cotransport, cellular acid extrusion in malignant tissue is supported by a number of other acid-base transport mechanisms; see Fig. 2. A recent study of breast xenografts based on the MDA-MB-231 human cell line of triple-negative breast cancer showed that NBCn1 knockdown causes the most prominent extension of tumor latency and inhibition of tumor growth, but knockdown of the Na^+/H^+ exchanger NHE1 or monocarboxylate transporter MCT4 also reduced the growth rate of xenograft tumors, albeit to a smaller extent [61].

Multiple studies indicate that cancer cells lacking NHE1 or exposed to Na^+/H^+ exchange inhibitors show decelerated proliferation and reduced invasive abilities [65–67]. This is particularly true under CO_2/HCO_3^--free conditions and when extracellular pH is low [65]. In the presence of CO_2/HCO_3^-, we find prominent contribution from Na^+/H^+ exchange to elimination of intracellular acid in human and murine breast cancer tissue only at very low intracellular pH [16–18, 64]. Whereas the Na^+/H^+ exchangers have high capacity for acid extrusion during severe intracellular acidification, the Na^+/H^+ exchangers do not contribute substantially to establishing the steady-state intracellular pH in human breast cancer cells with a normal complement of Na^+,HCO_3^- cotransporters [16]. In congruence with the limited involvement of Na^+/H^+ exchangers in intracellular pH control in breast cancer tissue, we observe very similar protein expression levels for NHE1 in normal breast tissue and breast cancer tissue from mice [18] and only a smaller upregulation of NHE1 during human breast carcinogenesis [17]. Consistent with previous studies showing a role of NHE1 for regulation of pH in diffusion-restricted compartments [67, 68], it is very possible that NHE1 contributes to pH regulation in subcellular domains also in breast cancer tissue, but this needs to be further investigated.

Monocarboxylate transporters and H^+-ATPases—that can mediate symport of H^+ and lactate or directly extrude H^+ during ATP hydrolysis, respectively—have been proposed as important regulators of acid-base homeostasis in cancer cells [69, 70]; see Fig. 2. However, despite almost fivefold upregulation of MCT1 and MCT4 during breast carcinogenesis [18], only

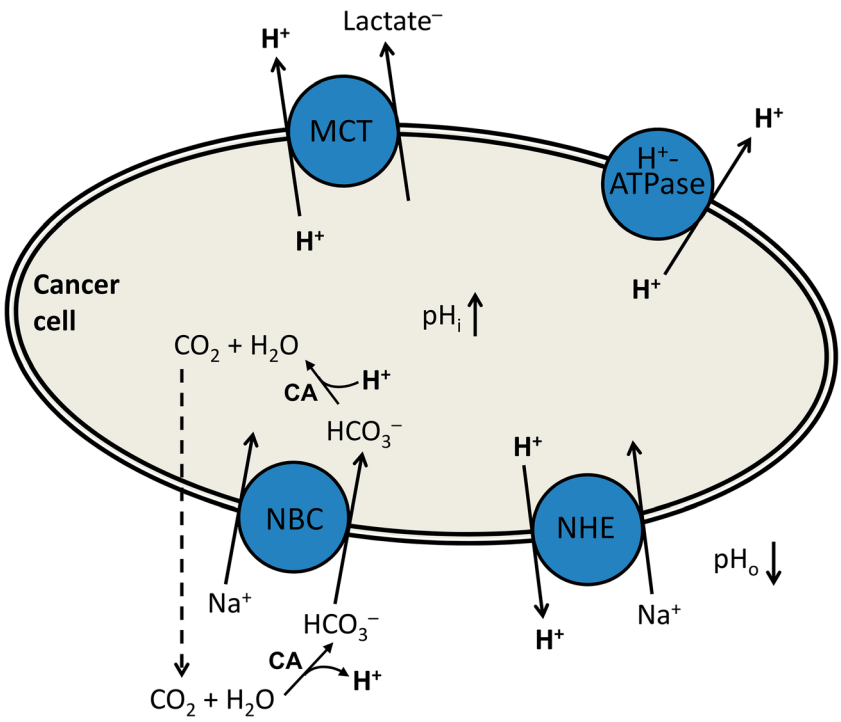

Fig. 2 Key acid-base transport mechanisms that mediate net acid extrusion from cancer cells and thereby contribute to establishing the relatively high intracellular and low extracellular pH. CA, carbonic anhydrase; MCT, monocarboxylate transporter; NBC, Na^+,HCO_3^- cotransporter; NHE, Na^+/H^+ exchanger; pH_i, intracellular pH; pH_o, extracellular pH

around 10% of overall net acid extrusion is Na^+-independent in human and murine breast cancer tissue even in experiments where the outward gradient is maximized by omission of extracellular lactate [16–18, 64]. This finding suggests that the monocarboxylate transporters have insufficient capacity for acid extrusion or that the metabolic source of lactate, under the experimental conditions, is inadequate to extrude H^+ at quantitatively prominent rates. A prime function of monocarboxylate transporters in cancer cells appears to be elimination of intracellular lactate, which could otherwise accumulate and block metabolism *via* product inhibition. Indeed, knockdown of MCT1 and MCT4 in human breast cancer cell lines inhibits lactate transport, glycolytic metabolism, as well as formation and growth of xenograft tumors [71].

Cellular elimination of acid in cancer tissue has been proposed to occur not only *via* direct extrusion across the plasma membrane of acid loaded cells but also through intercellular transfer. This may occur by transmission of lactate or HCO_3^- between neighboring epithelial cells coupled by gap junctions or through syncytia of stromal cells that can absorb extracellular acid *via* membrane transporters such as Cl^-/HCO_3^- exchangers [72–74] (see Swietach, this volume). The extent to which acid-base equivalents are eliminated through intercellular *versus* extracellular pathways determines among others the degree of extracellular acidification. Partial elimination of intracellular acid *via* intercellular pathways may thus contribute to setting a pH distribution within the cancer tissue that provides the cancer cells with a growth and survival advantage compared to normal cells without being toxic for the cancer cells themselves. Because extracellular acidity inhibits net acid extrusion *via* NHE1 and NBCn1 [75, 76], this alternative intercellular pathway for elimination of cytosolic acid may be particularly important for cells in very acidic tumor regions where direct elimination across the cell membrane is impaired.

Although the growing body of literature on acid-base regulation in cancer cells and tissues supports that acid extrusion is a general requirement for cancer cells, it also demonstrates that the relative importance of different transport systems varies between cancer models. Currently, it remains unclear whether the differences observed between cell lines are explained, for instance, by their origin from different cell types, dominance of different growth signals (e.g., because of mutations in specific proto-oncogenes), changes occurring during the culture procedure, or biological variation between the patients from whom these cells derive. If interindividual variation turns out to be prominent even for specific cancer types *in vivo*, it is possible that anti-neoplastic therapies will need to be selected based on expression profiles or functional assays that can be used to predict the dependency on specific druggable transport systems.

4 Carbonic anhydrases

The carbonic anhydrases—that catalyze equilibration of the CO_2/HCO_3^- buffer system, see Fig. 2—contribute to acid-base regulation in cancer tissue. Upregulation of membrane-bound extracellular carbonic anhydrase IX and XII is important for acid-base homeostasis in the extracellular space of simulated tumors [77, 78] and is of prognostic relevance for patients with solid cancer [79–82] (see Pastoreková, this volume).

Membrane acid-base transporters work in concert with intracellular and extrafacial carbonic anhydrases that catalyze CO_2/HCO_3^- equilibration in the cytosolic and interstitial space, respectively. The carbonic anhydrases are suggested to bind directly to intracellular and extracellular aspects of acid-base transporters and augment their transport activity [83]. The proposed transport metabolons encompass several acid-base transporters with relevance for solid cancer tissue including NBCn1 [84], NBCe1 [85], NHE1 [86], MCT1, and MCT4 [87]. The existence of acid-base transport metabolons and their functional consequences are still controversial [36], among others, because the applied methodology for identifying binding between the transporters and carbonic anhydrases has been disputed [88]. It is in some cases argued that the transporter-bound carbonic anhydrases are ideally located for catalyzing CO_2 hydration and directing HCO_3^- to or from the transporters [89]. In other cases, carbonic anhydrases are suggested to act as "H^+ collecting antenna" that nonenzymatically facilitate shuttling of H^+ to the relevant binding site for ion translocation [90]. Based on mathematical modeling of very simplistic cell systems, opposing arguments have been put forward that uniform spatial distribution of carbonic anhydrase activity throughout the cytosol accelerates transmembrane fluxes of acid-base equivalents more than if carbonic anhydrase activity is up-concentrated preferentially at the cell membrane [91]. This view may be challenged, however, if unstirred layers and areas of poor accessibility for larger buffers exist near the plasma membrane. In this instance, carbonic anhydrase activity at the cell membrane may be required for shuttling acid-base equivalents on the CO_2/HCO_3^- buffer between the acid-base transporters and the mobile buffers that are excluded from direct access to the transport proteins in the membrane [92].

5 Extracellular pH in cancer tissue

The high production rate of acidic metabolic waste and upregulated cellular net acid extrusion from cancer cells lead to enhanced acid loading of the extracellular tumor microenvironment. The degree of local extracellular acidification is determined by the balance between the rate at which acid is added to the extracellular space from cell metabolism and how efficiently the

acid equivalents are transferred from the interstitial space to the blood stream. Because the fraction of H^+ that is free in the aqueous phase is low compared to that bound to buffers, the apparent H^+ mobility in solutions is determined by the relative presence of protonatable sites on the available spatially mobile and fixed buffers [93]. The CO_2/HCO_3^- buffer is prominent among the mobile buffers but requires carbonic anhydrase activity for fast equilibration and efficient shuttling of H^+ equivalents [76, 77, 94]. Acidification of the extracellular tumor microenvironment varies between cancer types; but in general, extracellular pH in solid tumors—including breast cancer tissue—can fall as much as one unit below corresponding normal tissue levels [95].

For cancers to develop and progress, they need to escape the immune control mechanisms that would otherwise be triggered by the changes in cellular surface antigens expressed by transformed cells [96]. Although the mechanisms of immune escape are still under intense investigation, the acidic extracellular tumor microenvironment has potential to contribute substantially by modulating the function of immune cells [97, 98]. Growing evidence also supports that the acidic tumor microenvironment promotes cancer progression by selecting cancer cells with more malignant phenotypes [99]. Supplying large amounts of HCO_3^- or other small molecule buffers can modify the selection pressure exerted by the acidic extracellular tumor conditions. This approach can reduce metastatic disease development [100, 101] likely because the buffer therapy neutralizes the acidic tumor microenvironment and thereby inhibits extracellular matrix degradation [102] and reduces the selection pressure that usually favors highly malignant subpopulations of cancer cells [103] (see Ibrahim-Hashim, this volume).

The tumor microenvironment can modify the local architecture of cancer tissue. As chemoresistance can evolve as a consequence of desmoplasia—e.g., fibrosis related to development of primary or secondary tumors [104]—it is an important challenge to determine the microenvironmental factors contributing to tissue remodeling and how they may be targeted therapeutically [105, 106]. In addition to effects of pH on extracellular matrix components and stromal cells, acidosis can also interfere with tissue integrity by modifying cell-cell and cell-stroma adhesion, and the increasingly disjointed tissue structure likely facilitates cancer cell invasion. We have previously found that human normal breast epithelium structurally disintegrates in response to low extracellular pH whereas breast cancer epithelial organoids remain remarkably intact even at an extracellular pH of 6.8 [17]. The ability of cancer cells to withstand the acidic and hypoxic tumor microenvironment, which is incompatible with the function of most normal cells, allows them to displace the normal tissue and expand. The upregulated capacity for acid extrusion may contribute to the acid resistance of breast cancer cells, but the adaptive processes facilitating acid resistance during breast carcinogenesis need further investigation.

6 Tumor perfusion

In order to eliminate the exported acidic waste products from the interstitial tumor space, the acid equivalents must be transferred to the blood supply. A major focus has been placed on the chaotic architecture and unusual permeability of intratumoral blood vessels [9]. These dysmorphic blood vessels contribute to the heterogeneous perfusion pattern and acid-base profile within many tumors. In contrast, the architecture of cancer feed arteries—that provide blood supply to tumors but are not directly imbedded in cancer tissue—resemble the structure of other resistance arteries with maintained tunica intima, media, and adventitia [10, 11]. The cancer feed arteries are interesting from a pharmacological point of view because they maintain contractile ability and therefore can be acutely modified functionally in order to increase the hypoxic stress (by vasoconstriction) or enhance drug delivery or oxygen tension in the tumor tissue (by vasorelaxation) during chemotherapy or radiotherapy. Our recent findings show that cancer feed arteries are specialized towards reduced resistance [10, 11]. The mechanism of reduced vascular resistance in murine breast cancer feed arteries involves attenuated vasocontraction due to a thinner tunica media and lower α_{1A}-adrenoceptor expression on vascular smooth muscle cells [10]. In human colon cancer feed arteries, we show that the reduced vascular resistance can be explained by increased endothelial NO production that favors smooth muscle relaxation [11]. Other investigators have found that larger feed arteries isolated at greater distance from human colon cancer tissue respond with increased sensitivity to the vasoconstrictor endothelin-1 and have normal endothelial function [107]. Intriguingly, the resistance-sized feed arteries supplying human colon cancer tissue also show reduced vasomotion [11] that are rhythmic contractions superimposed on the tonic increase in baseline tone during stimulation with vasoactive agonists. This oscillatory contractile pattern has been suggested to enhance tissue dialysis and oxygen delivery [108]. In the context of the acidic tumor microenvironment, it is interesting that vasomotion is reduced in arteries where the vascular smooth muscle cells are chronically exposed to intracellular acidification due to knockout of NBCn1 [109].

7 Consequences of intracellular pH disturbances in breast cancer tissue

The upregulated cellular net acid extrusion in breast cancer tissue depends, as described above, on NBCn1-mediated HCO_3^- uptake [17, 18, 64]. Therefore, experimental studies based on NBCn1 knockout mice allow us to study the influence of pH on breast carcinogenesis. Knockout of NBCn1—that causes steady-state intracellular pH in breast cancer tissue to decrease by 0.2—delays breast cancer development after

carcinogen induction by around 50% (from a median tumor-free survival of 83 to 125 days) and decelerates tumor growth by 65% compared to wild-type mice [18]. Furthermore, the carcinogen-induced breast tumors of NBCn1 knockout mice are of lower aggressiveness and more benign histopathology [18]. Disrupted expression of NBCn1 also delays ErbB2-induced breast cancer development from a median latency of 9.5 months in wild-type mice to 12 months in NBCn1 knockout mice and decelerates the rate of tumor growth by approximately 35% [64].

In order to explore the mechanistic role of NBCn1 in breast carcinogenesis, we evaluated glycolytic metabolism by introducing microdialysis probes into breast cancer tissue and matched normal breast tissue [18, 64]. Phosphofructokinase-1 catalyzes the committing step of glycolysis and is particularly pH-sensitive as even pH changes of 0.1–0.3 can dramatically influence its catalytic activity *in vitro* [12, 13], and in congruence, glycolysis is strongly attenuated by intracellular acidification in skeletal muscle cells *in vivo* [12]. Although we find that glycolytic activity was lower in carcinogen-induced breast carcinomas of NBCn1 knockout mice compared to wild-type mice, it remains unclear whether the difference in metabolic profile is a direct consequence of the lower intracellular pH in the breast cancer tissue or a secondary consequence of the smaller tumor sizes [18]. Our data [18] as well as data from previous reports [110] show that glycolytic activity is generally lower in smaller compared to larger tumors. In ErbB2-induced murine breast cancer tissue, we find no evidence that NBCn1 knockout alters glycolytic activity [64]. This may imply that intracellular acidification is not acting through inhibition of metabolism. However, glycolytic metabolism is much lower in ErbB2- compared to carcinogen-induced breast carcinomas as signified by a tenfold lower ratio of interstitial [lactate] to [glucose] [18, 64]. The greater dependency on fermentative glycolysis in carcinogen-induced breast carcinomas will elevate the cellular acid load and consequently the requirement for NBCn1-mediated HCO_3^- uptake. It is possible that acid-induced inhibition of glycolysis becomes rate limiting only in cells that rely to a great extent on fermentative glycolysis. The higher metabolic activity of carcinogen-induced tumors is also reflected in the aggressiveness of tumor development, which appeared with a shorter latency and faster growth rate compared to ErbB2-induced tumors [18, 64].

Consistent with the slower tumor growth in NBCn1 knockout mice, the rate of cell proliferation is lower in medium- and large-sized carcinogen-induced breast carcinomas of NBCn1 knockout mice compared to wild-type mice [18]. Accordingly, cell proliferation is reduced at the core of ErbB2-induced murine breast carcinomas lacking NBCn1 expression [64]. Although previous studies support that low intracellular pH inhibits cell proliferation because DNA synthesis and cell cycle progression require a sufficiently elevated intracellular pH [111, 112], the exact cellular mechanisms limiting cell division during intracellular acidification are still unsettled. Cyclical changes in intracellular pH during the cell cycle have been demonstrated in MCF-7 breast cancer cells and are associated with variation in protein expression levels for NBCn1 and NHE1 [113]. Based on our studies in NBCn1 knockout mice [18, 64], we propose that NBCn1 in breast carcinomas is particularly important for establishing a level of intracellular pH that is permissive for cell division under conditions of increased acid loading brought about by enhanced glycolysis coupled to lactic acid fermentation.

Because most proteins show some degree of pH dependency, it is oftentimes difficult to pinpoint the exact molecular mechanism(s) responsible for specific acid-base-induced changes in cell behavior. However, some key cellular proteins—such as receptors, enzymes, and ion channels—are particularly sensitive to pH changes that modify the function by altering the protonation state of individual amino acids, in particular histidines (see Barber, this volume). In this way, protonation serves as an important reversible post-translational modification. Changes in charge distribution within proteins can affect their tertiary structure, their ability to interact with other molecules and ions, and ultimately their functional activity. Acid-base disturbances influence, in particular, a family of G protein–coupled receptors (e.g., OGR1, GPR4, TDAG8) with very prominent pH sensitivities that have been proposed as extracellular pH sensors [114] and are putatively linked to malignancy [115]. Although altered intracellular and extracellular pH mediate many of the cellular responses to acid-base disturbances, concomitant changes in the local buffer composition also likely contribute under *in vivo* circumstances. Changes in the concentrations of CO_2 and HCO_3^- are candidate signals for initiating cellular adaptations to acid-base disorders. Particularly, the initial identification of putative intracellular and extracellular sensing mechanisms for HCO_3^- [116, 117] suggests that HCO_3^- serves dual roles, providing substrate for membrane transporters that regulate intracellular pH and mediating cellular responses to acid-base disturbances [118].

The rate of transporter-mediated extrusion of acid-base equivalents can vary spatially between different regions on cancer cell membranes [119]. As described earlier for vascular smooth muscle cells, spatially constrained acid extrusion *via* NBCn1 can lead to intracellular pH gradients along the length of filopodia and contribute to directional migration [120]. The spatial variation in acid-base transporter expression and activity likely generates corresponding local extracellular pH gradients in the tortuous extracellular tumor space [121, 122]. Increased acid extrusion at the leading edge of cancer cells and at invadopodia facilitates degradation of the extracellular matrix through activation of matrix metalloproteinases and cathepsins [67, 119, 123]. The pH difference between the front and rear end of cells can enhance directional migration, for

instance, through modulation of integrin function: the lower local extracellular pH increases binding at the leading end of cells whereas the higher local extracellular pH promotes detachment at the rear end of cells [124]. For breast cancer cells, the relationship between acid-base transporters and directional migration is not yet clear, and in MCF-7 human breast cancer cells, inhibition of acid extrusion *via* Na^+/H^+ exchange has even been found to accelerate cell migration [125]. Because cellular pH gradients were not investigated in these studies, the underlying mechanism is still unclear. Effects of acid-base transporters on local pH dynamics occur through an interplay with metabolism, buffers, and the associated chemical reactions. Carbonic anhydrases typically enhance buffer mobility [94], and in vascular smooth muscle cells, endogenous carbonic anhydrase activity dissipates intracellular pH gradients along filopodia [120]. In congruence, carbonic anhydrase inhibitors increase the number and length of filopodia and the rate of vascular smooth muscle cell migration [120]. On the other hand, interaction of carbonic anhydrase IX with NBCe1 and AE2 appears to facilitate acid-base transport in lamellipodia of the renal MDCK cell line and thereby enhance cell migration [126]. This duality, where carbonic anhydrases depending on conditions can either accelerate migration (by amplifying acid-base transporter activity) or decelerate migration (by increasing buffer mobility), could relate to the compartmentalized expression of different carbonic anhydrase isoforms in the cytosol, mitochondria, or extracellular space. Irrespectively, further investigations are required in order to determine the anti-cancer therapeutic potential of carbonic anhydrase inhibitors.

While the primary focus of this review is on the role of NBCn1 in breast cancer, cell culture experiments *in vitro* and xenograft studies *in vivo* have also suggested a role for NBCe1 (SLC4A4) and AE4 (SLC4A9) in MDA-MB-231 breast cancer cells [127, 128]. The consequences of NBCe1 and AE4 for intracellular pH control and the mechanisms by which they modify malignant cell functions need further investigation. The observation that knockdown of NBCe1 reduces cancer cell proliferation and migration despite limited impact on global intracellular pH regulation particularly at steady state [127] could suggest that NBCe1 plays pH-independent roles or contribute to intracellular pH regulation in subcellular compartments.

8 Adaptation of NBCn1 expression during breast carcinogenesis

The vast majority of evidence supports that NBCn1 protein expression is twofold to threefold higher in human and murine breast cancer tissue compared to normal breast tissue [16–18, 64]. A previous study of xenografts based on MCF10AT cells modeled after various grades of breast carcinomas suggests that NBCn1 expression increases during development from normal epithelium to low-grade malignancy but then decreases as the condition progresses [41]. We find that upregulation of NBCn1 in breast tissue occurs early during carcinogenesis and observe no difference in NBCn1 expression levels between small and large breast carcinomas [18].

Hypoxia leads to upregulation of several acid-base-related proteins (e.g., carbonic anhydrase IX, NBCe1, and AE4) in cancer tissue [128–130]. However, in a screen of ten cancer cell lines—including three derived from breast cancer—NBCn1 mRNA levels were insensitive to hypoxia [128]. Notably, however, NBCn1 mRNA levels do not always reflect the protein level [38]: in murine breast cancer tissue induced by genetic overexpression of ErbB2, NBCn1 expression increases twofold whereas mRNA levels are reduced by 75% [64]. Also, during metabolic acidosis induced by NH_4^+ loading of rats, NBCn1 protein expression in the renal inner stripe of outer medulla increases threefold to fivefold whereas NBCn1 mRNA levels are essentially unchanged [131]. The increase in NBCn1 protein expression in breast cancer tissue compared to normal breast tissue despite lower mRNA levels [64] suggests that the half-life of the NBCn1 protein may lengthen during breast carcinogenesis. In the MCF-7 human breast cancer cell line, NBCn1 mRNA and protein expression both increase in response to heterologous overexpression of an N-terminally truncated, constitutively active ErbB2 receptor through a mechanism that involves the Akt, Erk, and Src kinases and the transcription factor Kruppel-like factor 4 [132].

Clearly, more evidence is required before we understand the mechanisms that regulate NBCn1 expression during breast carcinogenesis. For instance, studies are required to investigate how cancer-associated genetic polymorphisms and oncogenic signaling modulate the expression and function of NBCn1. Interactions between the acidic and hypoxic tumor microenvironment and the expression and function of NBCn1 could depend on one or more of the identified sensing mechanisms for H^+ [114] and/or HCO_3^- [116, 117] but are currently largely unknown.

9 Conclusions and future challenges

As detailed in this review, elimination or neutralization of intracellular acidic waste products in breast cancer tissue occurs predominantly *via* Na^+,HCO_3^- cotransport and Na^+/H^+ exchange [16–18]. Particularly the Na^+,HCO_3^- cotransport is upregulated in human and murine breast cancer tissue compared to normal breast tissue [17, 18]. During breast carcinogenesis, protein expression of the electroneutral Na^+,HCO_3^- cotransporter NBCn1 increases approximately twofold [17, 18], and in accordance, NBCn1 is responsible for the Na^+,HCO_3^- cotransport activity in primary carcinomas from mice with carcinogen- or ErbB2-induced breast cancer [18,

64]. When the capacity for neutralizing intracellular acidic waste products is lowered due to genetic disruption of NBCn1 expression, breast cancer development is delayed, tumor growth decelerated, and proliferative activity in the breast cancer tissue reduced [18, 64]. Further investigations are required in order to determine whether lower glycolytic metabolism or other mechanisms of acid-induced inhibition of cell proliferation explain the delayed breast cancer development and slower tumor growth.

The molecular mechanisms of cellular acid-base regulation have been studied intensively over the last few decades [23, 133]. Based primarily on findings from heterologous expression systems such as *Xenopus laevis* oocytes, we now have substantial knowledge regarding the molecular physiology of these transporters, their possible molecular mechanisms of regulation, and their structure-function relationships [134]. Still, we are only beginning to understand the integrated physiological roles of the acid-base transporters and their possible involvement in disease development. NBCn1 conducts Na^+, mediates Na^+,HCO_3^- cotransport, and interacts with other proteins [25], yet it is not currently clear to what extent each of these molecular functions promote malignancy.

Although most pH regulatory mechanisms support narrow control of intracellular pH, spatially restricted transport of acid-base equivalents can change pH locally and serve signaling purposes, for instance, by coordinating the function of multiple pH-sensitive proteins along gradients of intracellular or extracellular pH [120]. It is an important future challenge to identify the putative pH-sensitive mechanisms that are modified by the local pH and determine whether H^+ and HCO_3^- serve as dynamic cellular signals in diffusion-restricted spaces acting to coordinate complex cellular functions such as directional migration. Current evidence supports that local pH in cancer tissue promotes the invasive phenotype of cancer cells [67], but additional studies are needed to establish the contribution of Na^+,HCO_3^- cotransporters for cancer cell invasion and metastasis.

Based on the marked differences in cell metabolism and handling of acidic waste products between cancer and normal cells, improved understanding of the carcinogenesis-related changes in cell function and the adaptations to the hostile tumor microenvironment are crucial for rational design of new treatment options targeted specifically at cancer cells without major side effects in normal tissue. Studies are ongoing with regard to inhibitors of Na^+/H^+ exchangers, H^+-ATPases, and monocarboxylate transporters, whereas lack of selective Na^+,HCO_3^- cotransport inhibitors with appropriate pharmacokinetic profiles has been a major limitation [38, 62, 63]. Our recent results based on genetic disruption of NBCn1 [18, 64] are very promising and support the therapeutic potential of acid-base transport inhibition in breast cancer tissue.

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