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Tumor pH and metastasis: a malignant process beyond hypoxia

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Abstract
Tumors often show, compared to normal tissues, a markedly decreased extracellular pH resulting from anaerobic or aerobic glycolysis in combination with a reduced removal of acidic metabolites. Several studies indicate that acidosis induces (independently from hypoxia) hematogenous and lymphatic spread of tumor cells worsening the long-term prognosis of tumor patients. This review gives an overview on the impact of low pH on different steps of metastasis including (a) local tumor cell invasion and angiogenesis, (b) intravasation of tumor cells and detachment into the circulation, and (c) adherence of circulating tumor cells, transmigration and invasion in the new host tissue. The review describes pH-dependent cellular mechanisms fostering these steps such as endothelial-to-mesenchymal transition (EMT), activation of cell migration, degradation of the extracellular matrix, or angiogenesis. The review discusses mechanisms of tumor cells for proton sensing including acid-sensitive ion channels (ASICs, TRPs) or ion transporters (NHE1) and G protein coupled H^+-sensors. Finally, the review describes several intracellular signaling cascades activated by H^+ sensing mechanisms leading to transcriptional, post-transcriptional, or functional changes in the cell relevant for the metastatic spread. From these studies, different therapeutical approaches are described to overcome tumor acidosis or to interfere with the signaling cascades to reduce the metastatic potential of tumors.

Keywords Tumor acidosis · Mechanisms of metastasis · Endothelial-to-mesenchymal transition · Proton (H^+) sensing mechanisms · Intracellular signaling

Abbreviations

CREB	cAMP response element-binding protein
ASIC	Acid-sensing ion channel
EMT	Endothelial-to-mesenchymal transition
GPR	G protein-coupled receptor
HIF	Hypoxia-inducible factor
MAPK	Mitogen-activated protein kinase
MMP	Matrix-metalloproteinase
TRP	Transient receptor potential channel

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1 Introduction

1.1 The metabolic microenvironment of tumors

Tumors are rapidly growing tissues not limited by natural tissue boundaries. The dividing cells require nutrients (glucose, amino acids), oxygen, and reducing equivalents (e.g., NADPH) for the synthesis of energy and biomass [1]. This cellular metabolism produces numerous end-products which have to be removed from the tissue. In the beginning of tumor growth, the supply with nutrients and the disposal of waste products is carried out by diffusion from the surrounding normal tissue. However, due to the limited diffusion radius of these substances, the growth of the malignant tissue is limited until the tumor develops its own vascular network. In order to initiate this process, tumor cells and/or associated fibroblasts (and other stromal cells) secrete vascular growth factors leading to sprouting of newly formed vessels [2, 3]. With this improved supply and waste removal, tumors are able to rapidly grow to macroscopic size. During the last 40 years, numerous studies clearly indicated that the histological structure of the newly formed tumor vessels fundamentally differs from that of normal tissues. The regular hierarchical structure of vascular trees is replaced by a chaotically oriented mess of

macro- and microvessels. The vascular wall often lacks smooth muscle cells and the endothelial lining can be replaced by tumor cells [4]. Functionally the vascular network exhibit numerous distinct features, for instance, tumor blood flow is not homogenous, it shows pronounced spatial and temporal fluctuations [5].

Taking these facts together (high demand of nutrients and waste removal on the one hand and a structural and functional insufficient vascular network on the other hand) it becomes obvious that in many cases tumor perfusion is incapable to adequately supply the tissue. The best studied characteristic of the tumor, which is of high therapeutic interest, is the presence of a pronounced oxygen deficiency (hypoxia) with pO_2 values in vital tissue even below 1 mmHg [6, 7] that shows pronounced temporal fluctuations [5]. One consequence of hypoxia is a metabolic switch to anerobic glycolysis to ensure the necessary energy demand and which leads to an increased production of lactic acid. But for almost 100 years, it is also known that tumors switch to glycolytic metabolism even if sufficient oxygen is available, the so-called Warburg effect (aerobic glycolysis) [8]. This process provides the dividing tumor cell with reducing equivalents for the synthesis of complex organic compounds needed for cell growth (e.g., fatty acids) [1].

Both anaerobic and aerobic glycolysis lead to an accumulation of H^+ in the extracellular space of tumors causing a marked acidification of the tissue. Due to the insufficient vascular network, H^+ will not be removed adequately resulting in pH_e values even below 6.0 [6]. Comparable to tissue perfusion and oxygenation, the local tumor pH shows marked spatial and temporal heterogeneities [9].

1.2 Steps of metastatic dissemination

Dissemination of tumor cells from the primary tumor to distant locations *via* blood or lymph is a multi-step process [10, 11]. At the site of primary tumor growth, the proliferating malignant cells have to invade the surrounding normal tissue either as a collective tissue structure or as detached single cells or small cell clusters [12, 13]. Invasion of cell clusters or single cells is facilitated if the tumor cells loosen their tight tissue structure by reducing cell-cell-adhesion, which is a characteristic feature of the process of epithelial-to-mesenchymal transition (EMT). During EMT, tumor cells lose their epithelial properties (e.g., decrease of E-cadherin expression) gaining mesenchymal features, resulting in a reduced cell adherence and apico-basal polarity, a change in the cytoskeletal structure and an increase of cell motility [13–16]. Therefore EMT is an essential process of tumor progression associated with increased metastatic potential [12, 13]. During invasion, the tumor has not only to replace normal cells but also to reorganize the extracellular matrix (ECM) by degrading the ECM with secreted proteases (e.g., matrix-metalloproteinases MMPs) [13, 17].

After invading the normal tissue and the formation of a new vascular network by secreting angiogenic factors, the tumor cells will get in contact with small blood vessels (venules or capillaries) or lymphatics. By this, single tumor cells or cell clusters can enter the circulation. The process of intravasation and detachment is facilitated by EMT during which the tumor cells lose their cell-cell contacts [12]. Local invasion of tumor cells and infiltration of the vascular system is enhanced by various cytokines (e.g., TGF-ß, CSF-1) which are not only secreted by tumor cells but also from tumor-associated fibroblasts (TAF) or macrophages (TAM) [13, 18]. Circulating tumor cells (or tumor cell clusters) have to protect themselves from hostile conditions of the blood such as mechanical shear stress or attacks of NK cells, for instance by binding platelets to their cell surface [12, 13]. These platelet-covered circulating tumor cells can interact with circulating neutrophils which then facilitate the next step of metastatic dissemination, namely the interaction with the endothelial lining in a secondary host tissue and extravasation. Activation of platelets by bound tumor cells can signal, e.g., by secreted ATP or expression of selectins, to intensify contacts between endothelial and tumor cells [12]. Bound neutrophils may also foster extravasation of tumor cells by secretion of MMPs [19]. This process of forming a so-called pre-metastatic niche can even be facilitated by other cells, e.g., hematopoietic cells rerouted to the new host tissue. In this model, the primary tumor releases soluble factors which induce hematopoietic progenitor cells to home to the future tumor site and modify the tissue niche by secreting MMPs [20]. In the end, the tumor cells can leave the blood stream and invade the secondary tissue. In this process once again active migration of the tumor cell (as the result of EMT) and the secretion of proteases are supporting factors enhancing invasion and colonization [10, 12].

1.3 Impact of tumor acidosis on metastatic spread

Quite early, numerous observations revealed that the adverse metabolic tumor environment affects malignant progression of cancers and prognosis of patients. More than 60 years ago, it was shown that low oxygen concentration in a tissue limits the efficacy of sparsely ionizing radiation [7]. After systematically analyzing the tissue oxygenation status of human tumors, it was shown for many tumor entities that the presence of hypoxia reduces long-term prognosis (overall survival, disease-free survival, progression-free survival, local control) after primary radiotherapy [21, 22]. But even after treatment modalities that are not oxygen-dependent (e.g., primary surgery), tumor hypoxia predicted for disease recurrence and progression [21, 23]. Tumor oxygenation has been identified to modulate angiogenesis and the local invasion of tumor cells into the surrounding normal tissue [24–26]. Several

studies also clearly indicated that oxygen deficiency leads to a higher rate of far distant metastases [27–29]. Various mechanisms have been discussed which may be involved in the processes hypoxia-induced metastasis. Most of these processes are related to the activation of hypoxia-inducible factor (HIF) which regulates the expression of numerous relevant proteins and, by this, modulates important signaling cascades [30]. It was shown that hypoxia causes a remodeling of extracellular matrix in a HIF-dependent manner, for instance by a reduction of the activity of collagen modifying enzymes (e.g., prolyl-4-hydroxylase) [26] or by an induction matrix-degrading enzymes [31–33]. In addition, hypoxia induces EMT through different cascades, all resulting in a reduced intercellular adherence and increased migration [31–34]. HIF has also been shown to foster the formation of the pre-metastatic niche, for instance by inducing the production of several members of the lysyl oxidase (LOX) family [24, 31, 32, 34]. Hypoxia is therefore a metabolic factor which induces metastatic spread of tumors independently from therapeutic interventions.

Since tumor hypoxia intensifies glycolytic metabolism, the production of lactic acid is increased. By measuring the intratumoral concentration of lactate using bioluminescence [35], it was shown that primary tumors with high lactate levels show a significantly higher rate of metastases [35–37]. Some authors claim that the lactate anion *per se* accounts for the malignant behavior of these tumors [38]. Lactate can be taken up by monocarboxylate transporter-1 (MCT1) and is then metabolized to pyruvate which can inhibit prolylhydroxlyase 2 interfering with the HIF-1 signaling pathway. However, it was also shown that MCT1 expression affects tumor cell migration and metastasis formation independently from its function in transporting lactic acid [39].

Glycolytic metabolism enhances proton production leading to an acidification of the tumor tissue. In early studies, a combination of hyperthermia (43 °C) together with hyperglycemia led to a tumor pH of 6.0–6.5 and induced lung metastases in 90% and kidney metastases in 35% of the animals [40]. In the following years, these results were analyzed systematically. Schlappack et al. [41] incubated tumor cells in an acidic medium (pH 6.5) and injected them intravenously. They found a significant increase in the number of lung metastases; however, this was only seen if the tumor cells were re-cultivated at normal pH for at least 24 h. In similar experiments using different tumor and animal models the increased metastatic rate of acidotically primed tumor cells was also seen if the tumor cells were injected immediately after the acidic incubation [42–44]. Kalliomäki and Hill [45] performed another experimental setting to show the impact of the tumor pH on metastasis. Instead of injecting acidically primed tumor cells, they used a treatment protocol combining high glucose concentrations together with meta-iodobenzylguanidine (MIBG), an inhibitor of the respiratory chain, to intensify glycolytic metabolism *in situ* so that the implanted tumor became more acidic (reduction of the pH by ≈ 0.5). By this, the number of lung micrometastases increased moderately and these were further increased by simultaneous inspiratory hypoxia, which led to a more pronounced tumor acidosis. In a therapeutic study in dogs Lora-Michiels et al. [46] showed that after thermoradiotherapy, the extracellular pH was predictive of metastasis formation and of therapeutic outcome. In a study using a near-infrared fluorescent probe, it was shown that the pH distribution within the heterogenous tumor tissue was correlated with the metastatic potential. In primary tumors with high metastatic potential, low pH values were found in the tumor periphery whereas in low-metastatic tumors the pH distribution was more homogenous [47]. But not only has the formation of far distant metastases been shown to be pH-dependent. Also, the local invasiveness of tumors is correlated with the local pH, with the highest invasive potential at regions of lowest pH [48]. This could be the result of extracellular matrix destruction in the surrounding normal tissue by degrading enzymes (e.g., cathepsin B) by the tumor cells, fibroblasts, or macrophages at low extracellular pH [49, 50].

2 Mechanisms

Several mechanisms have been discussed by which the metabolic microenvironment may affect different steps of metastasis. Metabolic factors can reduce tumor cell adherence, may induce angiogenesis, loosen the structure of the extracellular matrix, decrease the anti-cancer immune response or induce intensified tumor cell migration [51–56] (Fig. 1). In particular, the process of endothelial-to-mesenchymal transition (EMT) and its reverse, mesenchymal-to-epithelial transition (MET), seem to play an important role for the detachment of tumor cells from their primary site and adherence and colonization in a new tissue [57, 58]. In the following sections, results from studies analyzing different mechanisms will be discussed.

2.1 Endothelial-to-mesenchymal transition (EMT)

EMT of cells is characterized by loosening their epithelial properties and gaining mesenchymal features. These changes lead to a loss of tight cell-cell and cell-matrix junctions and of cellular apico-basal polarity, a change of cytoskeletal structure and an increase in motility, all resulting in an invasive phenotype [14–16]. EMT is characterized by a reduced expression of epithelial markers (e.g., downregulation of E-cadherin) in combination with a higher expression of mesenchymal related proteins such as N-cadherin and/or vimentin [16, 57, 59]. Tumor cells undergoing EMT can leave the cancer cell complex and migrate into the surrounding normal tissue leading to local tumor progression [16, 57]. But also the invasion of blood circulation, as well as the transmigration through the

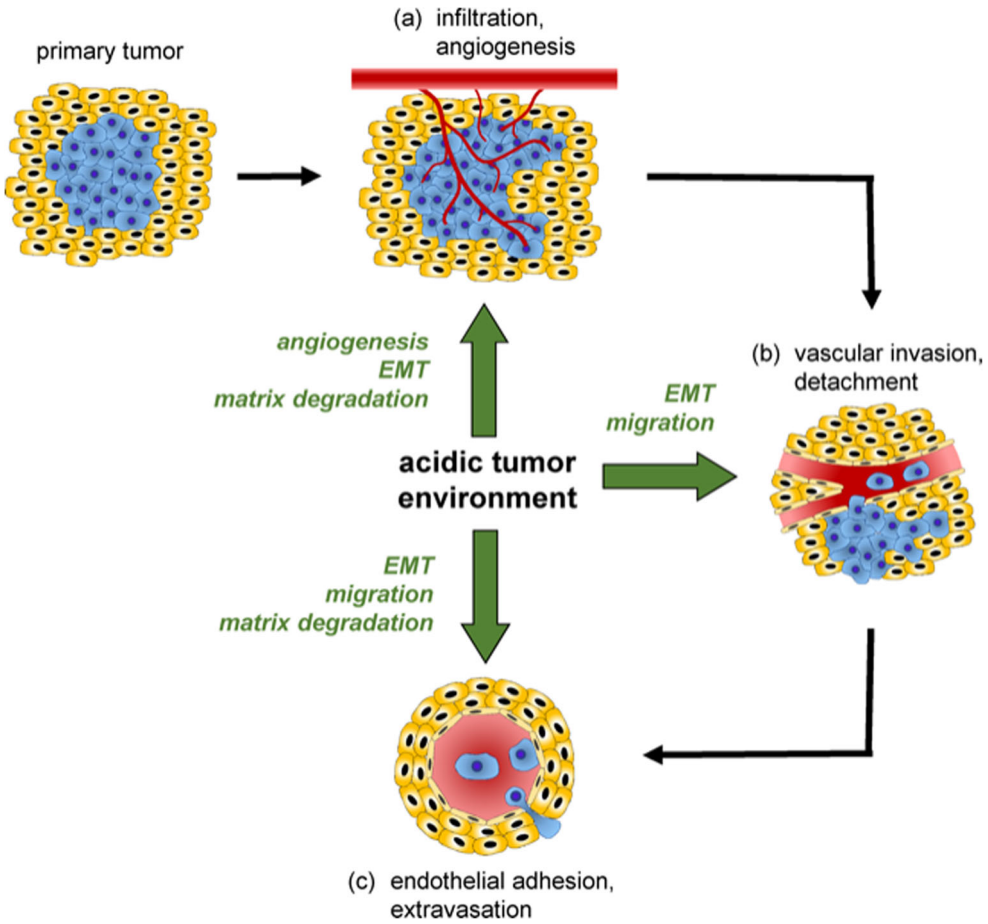

Fig. 1 Impact of the acidic tumor environment on different steps during metastasis formation: **a** local tumor cell invasion and angiogenesis, **b** intravasation of tumor cells and detachment into the circulation, **c** adherence of circulating tumor cells, transmigration, and invasion in the new host tissue

epithelial lining in a new tissue, increases by EMT. This leads to a higher rate of far distant metastases and a worse prognosis of tumor patients [14, 16].

EMT can be triggered by cytokines (e.g., transforming growth factor ß TGF-ß, platelet-derived growth factor PDGF) [60] but is also induced by metabolic parameters. Hypoxia has clearly been shown to induce EMT, either by a direct impact of HIF-1 which regulates EMT-related signaling pathways (e.g., SNAIL, NOTCH, ZEB) [31, 61, 62], or by hypoxia/HIF-dependent growth factors (e.g., VEGF) which foster EMT [31, 63]. Also, extracellular acidosis is able to induce changes in EMT markers. Peppicelli et al. [44] described a doubling of N-cadherin and vimentin expression in a human melanoma cell line, but only a slight decrease in E-cadherin expression after 24 h at pH 6.7. An inhibition of cell migration was only seen if simultaneously to lowering pH, cells were treated with an inhibitor of matrix metalloproteinases. In a recent study by Riemann at al. [64], it was shown in three different tumor cell lines that moderate extracellular acidosis (pH 6.6) changed the expression of EMT-related cell markers *in vitro* and *in vivo*. However, analyzing mRNA and protein expression revealed that N-cadherin showed disparate regulation with a significant increase in mRNA but a significant decrease in N-cadherin protein. In addition, the changes of EMT markers were cell-line specific. N-cadherin and vimentin were upregulated in two cancer cell lines, whereas in a prostate carcinoma a decrease of the expression was seen. A pH-induced loss of E-cadherin was also observed in several normal cell lines [64, 65] and seems therefore not to be a tumor-specific answer to low pH.

The pH-dependent mechanisms that may induce EMT are still not fully understood. It has been discussed that EMT-promoting factors (e.g., TGF-ß) are secreted by the tumor cells in a pH-dependent manner [66]. Another indirect mechanism could be the cellular metabolism which can be modulated by extracellular acidity [67]. Possibly, low pH activates EMT *via* factors in glucose or lipid metabolic pathways, for instance, downregulation of fumarate hydratase (Fh) or upregulation of acyl-CoA synthetase long-chain family member 1 (Acsl1) [68], which are known to activate EMT [59]. In addition to the cadherins, membrane-bound catenins, important for the cell-cell-interaction at adherens junctions, have also been shown to be pH-dependent [69]. Additionally, the cell-matrix-adherence *via* $\alpha 2ß1$-integrins was increased at low pH, an effect that was even more pronounced if the local pH regulation at the cell surface was altered by inhibiting Na^+/H^+-exchangers, NHE1, which are located closely adjacent at focal adherens contacts [70]. For $\alpha_5ß_1$- as well as for $\alpha_v ß_3$-integrins,

an activation by reduced pH_e has been reported, which promoted focal adhesion and cell protrusion [71, 72].

Functional analysis of tumor cell adherence at different pH revealed that acidic incubation of already adherent cells reduced significantly the stability of the contact [64]. This is in accordance with other studies, in which a loss of E-cadherin led to a significant reduction of cell adherence [73]. However, if tumor cells were primed at an acidic pH prior to the adherence measurements (which reflects the situation of circulating tumor cells released from an acidic tumor) a cell-line specific effect was observed as reduced or increased cell adherence, respectively [64]. It was described that extracellular protons lower the strength of cell-cell adherence independently from the expression of adhesion molecules [74]. Some studies postulate that low pH affects the structure of the cytoskeleton [71, 75] which then induces changes in cell morphology or modulate the dynamics of the actin cytoskeleton affecting cell migration [75].

2.2 Migration

Active movement of tumor cells is an essential process of malignant dissemination [13]. Tumor cells migrate in the primary tumor to enter the vasculature (invasion and transmigration) but also active shaping is necessary to leave the intravascular space in a new host tissue (extravasation). Therefore cells form protrusions at the front in the direction of migration (adhering to the surroundings) and retract the trailing end [76] by which a target-oriented movement is possible. Several studies indicate that a directed or randomly oriented movement of tumor (and normal tissue) cells is modulated by the extracellular pH (see also Bødkjaer, this volume).

Numerous studies described an increase in tumor cell migration if the cells were incubated at a moderately acid pH (≈ 6.7). This effect was seen either by the fraction of actively migrating cells measured for instance in transwell migration or scratch assays [44, 69, 76–79] or by the speed of cell migration assessed by time-lapse microscopy [43, 70, 76, 80, 81]. Enhanced migration was also seen if cells were adapted to low pH during a longer period and then re-transferred to medium at control pH [80] indicating long-lasting adaptation of tumor cells to low pH. Peppicelli et al. [44] described a strong increase of migrating cells in Matrigel at pH 6.7 for 24 h. The migration was inhibited if the cells were simultaneously treated with an inhibitor of matrix metalloproteinase (ilomastat).

Indirect evidence that H^+ may induce tumor cell migration comes from studies in which the expression of carbonic anhydrase IX (CAIX) was found to be correlated with the ability to migrate [82]. Since CAIX exports H^+, the local pH at the cell surface is reduced which leads to more prominent focal contacts which play an important role for active migration [83, 84]. Zhou et al. [85] found an increase in migrating tumor when cells were transfected with an acid-sensitive ion channel (ASIC2). This effect was even more pronounced if the cells were kept at low pH.

An increased fraction of migrating cells was also described for normal cells (osteoclasts) [86] and therefore seems not to be a tumor-specific property. Srivastava et al. [87] indicated that cell migration was depending on intracellular pH (pH_i) in normal fibroblasts. At low pH_i migratory speed was significantly reduced probably due to a pH-dependent increase in focal adhesion lifetime. The important role of the pH dependency of the actin cytoskeleton was underlined by the fact that cofilin, a regulator of actin stability, which is known to be pH-dependent [88], determines the migratory behavior of metastatic cancer cells [89].

In contrast, a few studies have described a reduced migratory ability of tumor cells at low pH [75], which was attributed to a pH-dependent phosphorylation of an actin-binding protein, HLJ1. Huang et al. [90] described reduced migration in normal bone marrow-derived endothelial progenitor cells, which was accompanied with reduced cell-matrix adhesion.

2.3 Degradation of the extracellular matrix

One essential process for invasion into normal tissue but also for transmigrating the vascular wall is the destruction of the extracellular matrix [13, 17]. Important proteases for the reorganization of extracellular matrix are matrix-metalloproteinases (MMPs) and cathepsins (e.g., cathepsins B or L). An increased proteolytic activity of these enzymes will foster the invasiveness of tumor cells [49, 83]. Several studies have shown that either the expression or enzymatic activity of these proteases is pH-dependent.

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are secreted proteinases which are known to be directly related to metastatic spread [91, 92]. The proteases are not only produced by tumor cells but also by macrophages, mast cells or fibroblasts [91] which are part of the tumor mass and may also been affected by the acidic microenvironment. In several studies, the expression of MMP-9 either at the mRNA or protein level increased when extracellular pH was reduced to values of 5.9 to 6.8 [42, 44, 93–97]. The number of studies in which MMP-2 was analyzed is smaller; however, a clear trend to an upregulation at low extracellular pH has been described [42, 96, 98]. For studies in which the expression at the mRNA level was measured, it has to be taken into account that the proteins are produced in an inactive pro-form which is activated by intracellular proteases [91]. For this reason, studies in which the functional MMP activity is correlated with the extracellular pH are even more significant. Subsequent studies indicated that, at low pH, the proteolytic activity of MMP-9 [42, 93, 98] and MMP-2 [42] is markedly increased.

Rofstad et al. [42] analyzed the role of MMPs for metastatic spread *in vivo*. They discovered that the number of lung

metastases after i.v. injection of tumor cells was significantly increased if the cells were primed at pH 6.8 for 48 h. If these cells were additionally treated with MMP inhibitors the number of metastases was reduced almost to the control level of cells at pH 7.4. These results indicate that the MMP activity of tumor cells play an important role for the process of transmigration and invasion in the new host tissue.

Besides MMPs, cathepsins (especially cathepsin B) are known to affect the metastatic spread of tumor cells [99]. Cathepsins are produced by various tumor cells, but also by normal cells (e.g., hepatocytes, chondrocytes). Analyzing the cathepsin B expression in tumor cells at low pH showed a significantly higher secretion [42, 50, 100]. Rozin et al. [50] could show that in an acidic environment, cathepsin containing intracellular vesicles were translocated to the cell periphery close to the cell membrane. It was also shown that the increased expression goes along with a higher proteolytic activity [50, 96]. Some studies have also indicated a correlation of the expression of cathepsin L with an acidic extracellular pH [42, 78, 100].

Taking all these results together, it becomes apparent that an extracellular acidosis induces an enzymatic degradation of the extracellular matrix in the primary tumor site as well as in the new host tissue which fosters the metastatic spread and invasion of tumor cells.

2.4 Angiogenesis

Hematogenous or lymphatic metastatic dissemination needs the presence of a vascular system within the tumor mass. For this reason, angiogenesis of blood vessels or lymphatics is an important step for the access of tumor cells to the intravascular space [10]. Hypoxia is known to strongly induce tumor angiogenesis by HIF-dependent angiogenic growth factors such as VEGF. But acidosis *per se*, independent from O_2 deficiency, also seems to modulate vascular growth [57].

Several studies analyzed the expression of VEGF (VEGF-A) on mRNA or protein level when reducing extracellular pH. Mostly an inverse correlation was found with increasing VEGF levels at low pH [97, 101–103]. Jang et al. [100] described a strong increase in VEGF expression after pH 6.5 incubation but only if the cells were kept at normal pH for 48 h after acidic priming. Promotor studies revealed an activation of VEGF transcription at low pH at least at moderate acidosis [101, 102]. Shi et al. [102] also showed that the stability of VEGF mRNA increased at low pH. Low pH appears to differentially regulate the expression of various VEGF isoforms. Acidosis promotes the expression of angiogenic VEGF-121, but not VEGF-165 or 189 [104]. Acidosis-induced VEGF protein was able to induce vascular growth [97].

In brain tumors, *in vivo* the activation of VEGF transcription was only weakly correlated with low local tumor pH [101]. However, if the whole tumors were divided in hypoxic and well-oxygenated regions, the author could show that in normoxic regions the effects of low pH were much stronger, whereas in hypoxic regions VEGF induction is predominately regulated by pO_2 and pH did not play a role [101].

Some indirect indications on the role of acidosis for angiogenesis came from studies of a H^+-sensitive G protein-coupled membrane sensor, GPR4. Knocking-out GPR4 reduced the formation of new vessels [105] whereas GPR4 overexpression promoted VEGF secretion and angiogenesis *in vitro* and *in vivo* [106]. Finally, it has been discussed whether the lactate anion *per se*, metabolized to pyruvate after re-uptake in tumor cells, may stabilize HIF-1α and thus may induce VEGF expression [38].

In contrast to tumor cells, normal endothelial cells show no changes or even a reduced VEGF expression if they are exposed to low pH and the formation of vascular structures was reduced [90, 107]. However, if endothelial cells were kept at normal pH for 6 h after acidic priming, VEGF secretion and tubule formation was significantly increased *in vitro* and *in vivo* leading to an improvement of tumor perfusion [107].

Not only the formation of new blood vessels but also of lymphatics might be affected by the extracellular pH. Peppicelli et al. [108] showed that the expression of VEGF-C, an important lymphangiogenic growth factor, is induced in a pH-dependent manner. In normal lymphatic endothelial cells, a pH of 6.4 induced morphological changes and tubule formation [109].

Taking these data together, an acidic environment *per se*, independent on the presence of hypoxia, appears to induce the growth of new blood vessels and probably of lymphatics, which both can contribute to the increased metastatic spread of acidic tumors.

3 Sensing and signaling

The above-mentioned mechanisms open the question of how the acidic extracellular pH is sensed and which signaling cascades lead to transcriptional, post-transcriptional, or functional changes in tumor cells (Fig. 2).

3.1 Mechanisms of pH sensing

3.1.1 Acid-sensitive ion channels and ion transporters

Ion channels are well-studied structures that are known to be pH-sensitive and which influence the membrane potential. However, they could also activate secondarily intracellular signaling cascades. In all cells, cation fluxes can modulate biological behavior. Concerning metastatic processes, Ca^{2+} and Na^+ ions play predominant roles. Ca^{2+} either directly induces active cytoskeletal changes inducing cell migration

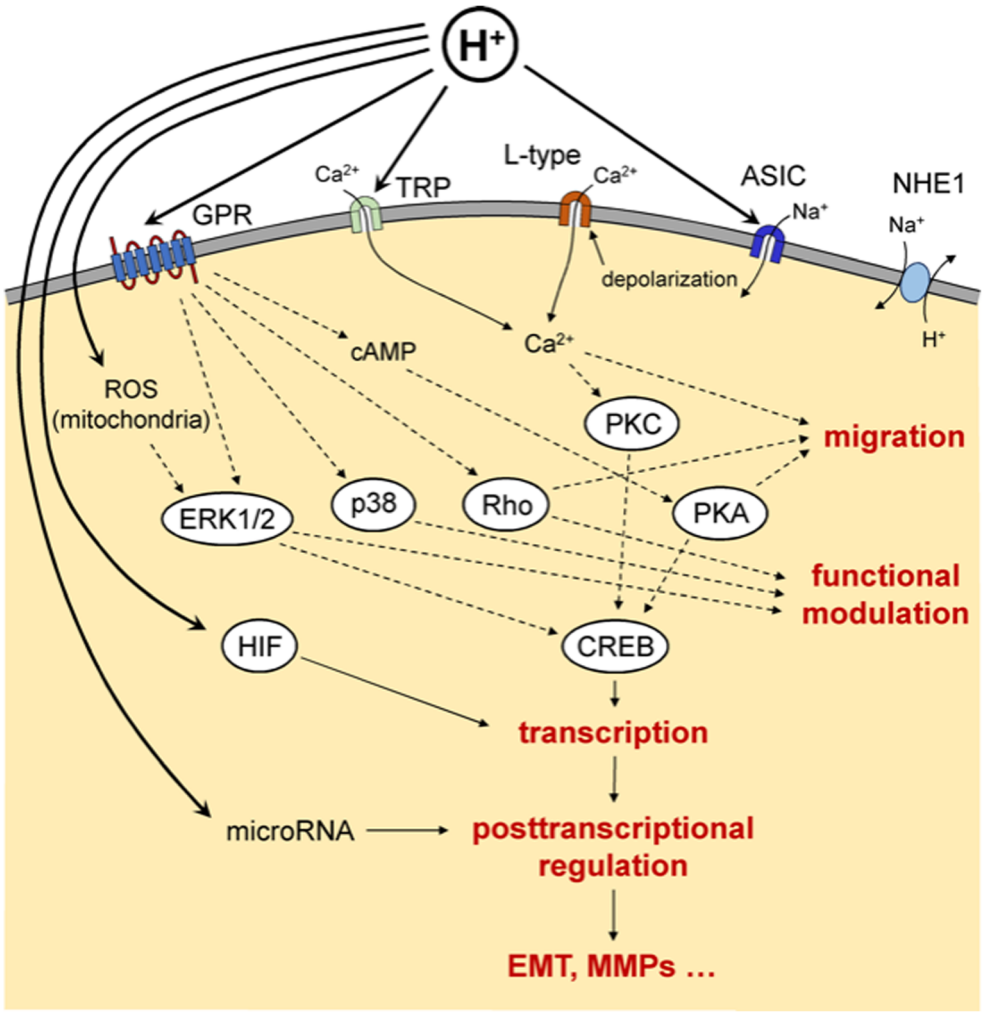

Fig. 2 Selection of important mechanisms for proton sensing and activation of secondary signaling cascades in tumor cells leading to transcriptional, post-transcriptional or functional changes relevant for metastatic spread. GPR, G protein coupled receptor (GPR4, GPR68, GPR132); ASIC, acid-sensitive ion channels; TRP, transient receptor potential channels (e.g., TRPV1, TRPM5); L-type, L-type Ca^{2+}-channel; NHE1, Na^+/H^+ antiporter 1; PKC, protein kinase C; PKA, protein kinase A; ERK1/2, ERK MAP kinase pathway (Ras-Raf-MEK-ERK pathway); p38, p38 MAP kinase pathway; Rho, RhoA GTPase depending signaling; CREB, cAMP response element-binding protein; HIF, hypoxia-inducible factor

[110] or can act as second messenger in different signaling cascades (e.g., protein kinase C, c-Src). Na^+ fluxes induce membrane potential depolarization (which can then secondarily influence the membrane conductance of other ions) and are essential for H^+ homeostasis by driving the Na^+/H^+-antiporter 1 (NHE1) [76, 111]. These ions can enter the tumor cells *via* different channels or transporters which are directly or indirectly pH-sensitive.

Acid-sensing ion channels (ASICs) are a family of sodium channels activated by low pH inducing a depolarization of the cells [76]. The expression of ASIC1 and ASIC2 has been shown to be induced by extracellular acidosis [85, 112] which was functionally correlated with an increase in cell migration [77, 85, 112]. This effect was also seen with normal osteoclasts which showed an ASIC1a-mediated increase in migration and reduced cell adhesion [113]. Overexpression of ASICs induced a higher rate of metastases in animal experiments and the overall survival of patients with colorectal cancer was negatively correlated with high ASIC2 expression [85]. As a possible mechanism, a Na^+-flux-induced depolarization of the cell leading to an increased Ca^{2+} influx and activation of secondary signaling cascades (e.g., Src or calcineurin/NFAT) was proposed [76, 85, 113]. This hypothesis is supported by the observation that inhibition of voltage-dependent Ca^{2+}-channels (L- or T-type) reduces the acidosis-induced activation of MAP kinases and prevents an upregulation of MMP-9 expression [93]. An alternative source of Ca^{2+} ions could be the activation of Ca^{2+}-dependent Ca^{2+} channels (ORAI1) which are notoriously sensitive to intra- and extracellular pH and which have also been linked to tumor cell migration [110, 111].

Another family of pH-sensitive ion channels are the TRPs (transient receptor potential channels) [76, 114]. Changes in the expression but also modulation of the functional properties of these channels alter the expression of MMP-9 and the formation of lung metastasis [115] as well as lymphangiogenesis [109]. High TRPM5 expression was correlated with poor overall survival in patients with different cancers. However, the exact mechanism by which TRPM5 affects metastasis is not fully understood.

The complex interaction between ion transport and pH has been analyzed in detail for the process of single cell migration.

NHE1 is an important component of the intracellular H^+ homeostasis [76]. However, in migrating tumor cells, these transporters are not homogenously distributed along the cell membrane, but are found at a much higher density at the leading edge of migrating tumor cells [116, 117]. Due to H^+ extrusion, the pH_e in these nanodomains is lower than at the rear part of the cell [118], which in turn leads to an activation of integrins at the front [70, 76, 116] that fosters the directed migration. Notably, treating tumors with an NHE1 inhibitor reduces their metastatic potential [119].

Finally, the metastatic behavior is also correlated with the presence of voltage-gated Na^+ channels (for a review see [120]). The mechanism could involve NHE1 regulating the local H^+ concentration at the cell surface.

3.1.2 G protein coupled H^+-sensors

In normal tissues, several proton-sensing G protein coupled receptors are known, including GPR4, GPR68 (OGR1), GPR132 (G2A), and GPR65 (TDAG8). These receptors are activated at pH below 7.0 and invoke different intracellular signaling cascades regulating protein function or gene expression. In normal cells (e.g., immune cells, macrophages, fibroblasts), low pH can induce cytokine or growth factor secretion and cell migration. These receptors are also expressed on many tumors [121]. However, the effects on malignant cells are heterogeneous [114, 122, 123].

GPR4, which is known to induce pH-dependent migration in normal cells, has been described to reduce tumor cell migration at low pH [124, 125]. This was attributed to an upregulation of focal adhesions on the tumor cell membrane. In addition, GPR4 overexpression reduced formation of distant metastases *in vivo* [124, 125]. On the other hand, low pH induced *via* GPR4 the secretion of pro-angiogentic cytokines (IL-6, IL-8, and VEGF-A) leading to an increased formation of new blood vessels [106]. If GPR4 is knocked-out in normal endothelial cells, angiogenesis in tumors was inhibited [105].

GPR68 (OGR1), a pH sensor that is upregulated by hypoxia in tumor cells [126], has been described to act as a tumor suppressor at least with respect to metastasis formation [127]. Overexpression of GPR68 reduced metastasis in different organs. Li et al. [128] found suppressed tumor cell migration with GPR68 overexpression but not with GPR4 overexpression.

Concerning GPR132 (G2A) an indirect mechanism has been described how acidosis may interfere with metastatic potential. Chen et al. [129] found that lactate produced by glycolytic metabolism directly acts on GPR132 in macrophages polarizing them towards a M2 phenotype. These activated cells then promoted cancer cell migration and invasion. These authors also found a correlation between GPR132 expression and metastasis formation in breast cancer patients.

3.2 Intracellular signaling cascades

After sensing extracellular acidosis, different signaling pathways are involved which regulate functional properties (e.g., migration, adhesion) or modulate protein expression on the transcriptional or posttranscriptional level (Fig. 2). The interplay between different second messengers and various signaling cascades is quite complex. However, some uniform mechanisms seem to play a relevant role for different steps of the metastatic process.

3.2.1 MAP kinases, ROS, and other signaling pathways

Mitogen-activated protein kinases (MAPK) play a central role in cancer cell proliferation, apoptosis, differentiation, or functional properties such as activity of multidrug-resistance transporter or tumor cell migration [130]. Important members of these kinases that can be activated by extracellular signals such as osmotic or oxidative stress and inflammatory cytokines are ERK1/2, p38, and JNK.

p38 signaling can be activated by extracellular acidosis, for instance directly *via* G protein coupled receptors, GPR4 [106]. Phosphorylation of p38 under moderate acidic conditions (pH 6.0–6.8) has been described for numerous experimental tumor lines [94, 131–135]. Phosphorylation of p38 was also observed if only the cytoplasm was acidified, with a normal extracellular pH [134]. pH-dependent changes in p38 activation are not only limited to malignant cells but were also observed in normal fibroblasts [136], mesenchymal stem cells [137], or (less pronounced) in normal epithelial cells [43]. Therefore, acidosis-induced p38 activation is not a tumor-specific response. Acidosis-induced p38 phosphorylation activates secretion of inflammatory cytokines in tumor cells [132] and induces cell proliferation [133]. Concerning metastasis, it has been shown that MMP-9 expression is induced *via* p38 and NFκB activation [94]. Jing et al. [106] showed that acidosis-induced activation of GPR4 leads to a phosphorylation of p38 and by this IL-6, IL-8, and VEGF-A secretion and angiogenesis. By time-lapse microscopy, it was shown that acidosis-induced p38 activation increased migratory speed of tumor cells [43].

Quite similar results were obtained when analyzing the MAP kinase ERK1/2. Several tumor cell lines, however not all, show a pronounced phosphorylation of ERK1/2 when incubated at low pH [94, 131–136, 138, 139]. This acidosis-induced ERK1/2 activation induced a significant increase in migration [43], an intensified MMP-9 secretion [94], angiogenesis by VEGF secretion [139], and increased local invasiveness of tumor cells [138].

Another signaling pathway related to extracellular acidosis is the Rho/Rho-kinase cascade. Acidosis has been shown to activate this pathway *via* proton sensor GPR4 which then regulates focal adhesion [124, 128]. By this, the Rho cascade

leads to an inhibition of migration. On the other hand, Li et al. [71] showed that acidosis-induced Rho activation promoted membrane protrusion, which could foster migration. Finally, it was shown that an acidic environment induced phosphorylation of Src kinase and by this led to a loss of ß-catenin from adherens junctions and increased invasiveness [69].

For the activation of MAP kinases, reactive oxygen species (ROS) can play an important role, since ROS are able to sustainably induce MAPK phosphorylation *via* protein kinase C, PKC [130] (see also Sonveaux, this volume). By activating MAP kinases, ROS can induce EMT and increase migration or invasion [140, 141]. Several studies have shown that increased ROS formation leads to intensified migration of tumor cells [130, 142, 143] and may therefore lead to higher metastatic spread. In normal epithelial cells, long-term exposure to ROS can induce EMT-like changes, such as downregulation of E-cadherin or induction of MMPs [144]. In tumor cells, ROS formation is increased by extracellular acidosis, which then leads to a stronger p38 phosphorylation [134, 145]. There is a clear indication that under acidic conditions, these ROS are originated from mitochondria [134]. The functional impact of ROS on metastatic potential can be demonstrated by scavenging ROS which leads to a reduced migration, invasion, and adhesion of tumor cells [43]. Besides MAP kinase activation, another possible mechanism by which ROS can affect tumor cell migration is the oxidation of membrane lipids which then leads to increased tumor cell migration and metastasis *in vivo* [146].

Taking these results together it becomes apparent that different signaling cascades (MAP kinases, ROS) are activated by extracellular acidosis and result in an activation of numerous processes, all related to an increased metastatic potential of tumor cells.

3.2.2 Transcriptional regulation

Activated signaling cascades can regulate gene expression by specific transcription factors. The intracellular level of two transcription factors was shown to be correlated with extracellular pH: (a) hypoxia-inducible factor 1 (HIF-1) and (b) cAMP response element-binding protein (CREB).

Canonically, HIF-1 is stabilized by low O_2 partial pressure in tissue and induces the expression of numerous genes responsible for angiogenesis, glucose transport, metabolism, and proton transport out of the cell. However, several studies using normal cells (neurons, endothelial, or epithelial cells) indicate that HIF can also be activated (hypoxia-independently) by an acidic extracellular pH [147–150] leading to the conclusion that the hypoxia-responsive element of genes can also function as an "acidosis-responsive element." For tumor cells, a direct influence of the acidic pH on HIF levels was also found [95, 151–153]. Possible mechanisms for a hypoxia-independent regulation include acidosis-induced inhibition of the VHL (von Hippel-Lindau) tumor suppressor [151], an upregulation of HSP90 chaperone [152], or an induction of 2-hydroxyglutarate [148]. Since HIF-1 expression is correlated with formation of metastases, these pathways could contribute to the impact of low pH on the malignant progression of tumors. However, Tang et al. [154] reported that incubation of cells with lactic acid repressed the hypoxia response by HIF. It remains unclear whether this was induced by the increased proton or lactate concentration.

CREB is a transcription factor binding to the CRE-element of genes and is known to induce metastases [155]. In normal epithelial cells and CNS pericytes, it was shown that CREB activation is induced by low extracellular pH [156, 157] *via* protein kinase A (PKA) or intracellular Ca^{2+} signaling. An acidosis-induced activation of CREB, as indicated by CREB phosphorylation and by CRE-reporter assays, has also been shown in different tumor cell lines [134, 135]. In these studies, CREB phosphorylation was induced by ERK1/2 activation. Additionally, the overexpression of the proton-sensitive GPR4 membrane sensor leads to CREB activation [158].

3.2.3 Post-transcriptional regulation

Protein expression is not only regulated by transcription factors but also by epigenetic processes or at the posttranscriptional level. Epigenetic processes like histone modifications and DNA methylation or microRNAs (miRNA) or long non-coding RNAs (lncRNA) can modulate the expression of proteins related to migration or metastasis. For instance, histone modifications like methylation, acetylation, or sumoylation mediate a hypoxia-induced EMT [159].

MicroRNAs are small (about 22 nucleotides) non-coding RNAs which bind to complementary sequences on mRNA strands and, by this, prevent this mRNA from being translated or lead to mRNA degradation. For many cancer types, it has been shown that hypoxia can induce different miRNAs, the most prominent being miR-210 or miR-101 [160–162]. Several hypoxia-dependent genes were identified to be targets of miR-210 [163, 164]. On the other hand, HIF-1 is also regulated by miR-210 [162, 165]. Therefore, miR-210 and HIF-1 form a control circuit to regulate the cellular response to hypoxia [160]. By this mechanism, upregulation of miR-210 fosters migration, invasion, and metastasis. However, these effects are cancer line-dependent [160, 166]. Besides microRNAs, long non-coding RNAs can promote hypoxia-induced EMT [167].

Recently, it was shown that several miRNAs are regulated by extracellular acidosis. Four miRNAs were consistently changed in different tumor lines: miR-183-5p (downregulation), miR-203a-3p (downregulation), miR-215 (downregulation), and miR-7a-5p (upregulation) [68, 168]. These miRNAs are related to metastatic spread. In several studies, it was shown that high levels of miR-203 inhibit tumor cell migration and invasion [169, 170] and that downregulation of

miR-203 promoted EMT [171]. MiR-183 has been found to inhibit mitochondrial function [172] and to increase migration [173], but also inhibition of invasion and metastasis was described [174, 175]. High levels of miR-215 reduced migration and invasiveness in several tumor entities [176, 177].

Taking these results together, extracellular acidosis might foster metastatic spread by regulation of specific miRNAs which then modulate the translation of proteins related to invasion or migration.

4 Conclusions

Extracellular acidosis, which is commonly found in many human tumors, and which results from the insufficient oxygen supply as well as from upregulated metabolic pathways in malignant cells ("Warburg effect"), modulates the metastatic behavior of tumor cells. There is clear evidence that the effects of acidic pH are independent from mechanisms induced by hypoxia. Tumor acidosis directly modulates local invasion by relaxing cell-cell-contact, by degrading extracellular matrix, and by fostering tumor cell migration. These acidosis-induced changes also facilitate the shedding of tumor cells into blood circulation. Due to priming of the cells in the acidic environment, elevated adhesion and invasion of circulating tumor cells in the new host tissue leads to distant metastasis. Several sensing mechanisms and signaling pathways can be activated by the extracellular H^+ concentration, leading to cellular changes of gene expression and/or functional properties.

The knowledge of these signaling cascades and pH-induced functional alterations offer the possibility to modulate the acidic environment, the cellular H^+ homeostasis or signaling as a therapeutic intervention and to counteract the switch to a metastatic phenotype of the tumor cells [55].

4.1 Therapeutical approaches

Based on the impact of acidic tumor pH on metastasis, some authors have suggested targeting the tumor pH by therapeutic interventions to improve long-term prognosis of patients. Most of the therapeutic approaches focus on targeting tumor pH either by reducing extracellular acidosis, and thus reducing metastasis, or by lowering intracellular pH, resulting in acidic stress for the cells [178]. Some studies addressed a normalization of the extracellular pH by substitution of buffer. Mathematical calculations showed that the substitution of bicarbonate (pK_a 6.1) should be able to increase the tumor pH substantially, however, using buffers with a pK_a closer to the physiological pH would be more effective [179]. For this reason, besides buffering with bicarbonate [79, 180], also other non-bicarbonate buffers have been considered [180] (see Ibrahim-Hashim, this volume). These hypotheses were tested in animal experiments where the buffers were supplemented orally. Using bicarbonate led to an increase of extracellular tumor pH from 6.95 to 7.4 which was correlated with a significant reduction of lung metastasis [181]. Also buffering with TRIS (tris(hydroxymethyl)aminomethane, $pK_a = 7.8$) or IEPA (2-imidazole-1-yl-3-ethoxycarbonylpropionic acid, $pK_a = 6.9$) increased tumor pH and reduced the metastasis rate [182, 183]. In a clinical pilot study, local bicarbonate infusion in patients with advanced hepatocarcinoma and transarterial chemoembolization led to an improved local tumor control but no significant change in extra-hepatic metastasis [184].

Besides adding buffers, other mechanisms of modulating cellular H^+ homeostasis have been suggested such as inhibition of H^+ transport through the cell membrane with inhibitors of proton pumps or the Na^+/H^+-exchanger [56, 79, 180]. This approach has been tested in animals where the proton pump inhibitor omeprazole reduced the expression of MMP-9 as well as invasiveness of tumor cells and reduced the formation of lung metastases after i.v. injection of pre-treated cancer cells [185].

Finally, a few approaches tried to interfere with the signaling cascades activated by low pH. Inhibition of TRPM5 channels (which are either activated by Ca^{2+} ions or by protons) under acidic conditions decreased MMP-9 expression and reduced the metastatic potential *in vivo* [115]. Finally blocking voltage-gated sodium channel $Na_v1.5$ (which is also pH dependent) by phenytoin reduced migration and invasion of tumor cells.

These data indicate that the acidic metabolic microenvironment of tumors is an independent process modulating different steps of the metastatic spread. A deepened knowledge of the mechanisms of pH sensing and the activation of secondary intracellular signaling cascades will open new therapeutic strategies to suppress tumor metastasis and improve long-term survival of the patient.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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