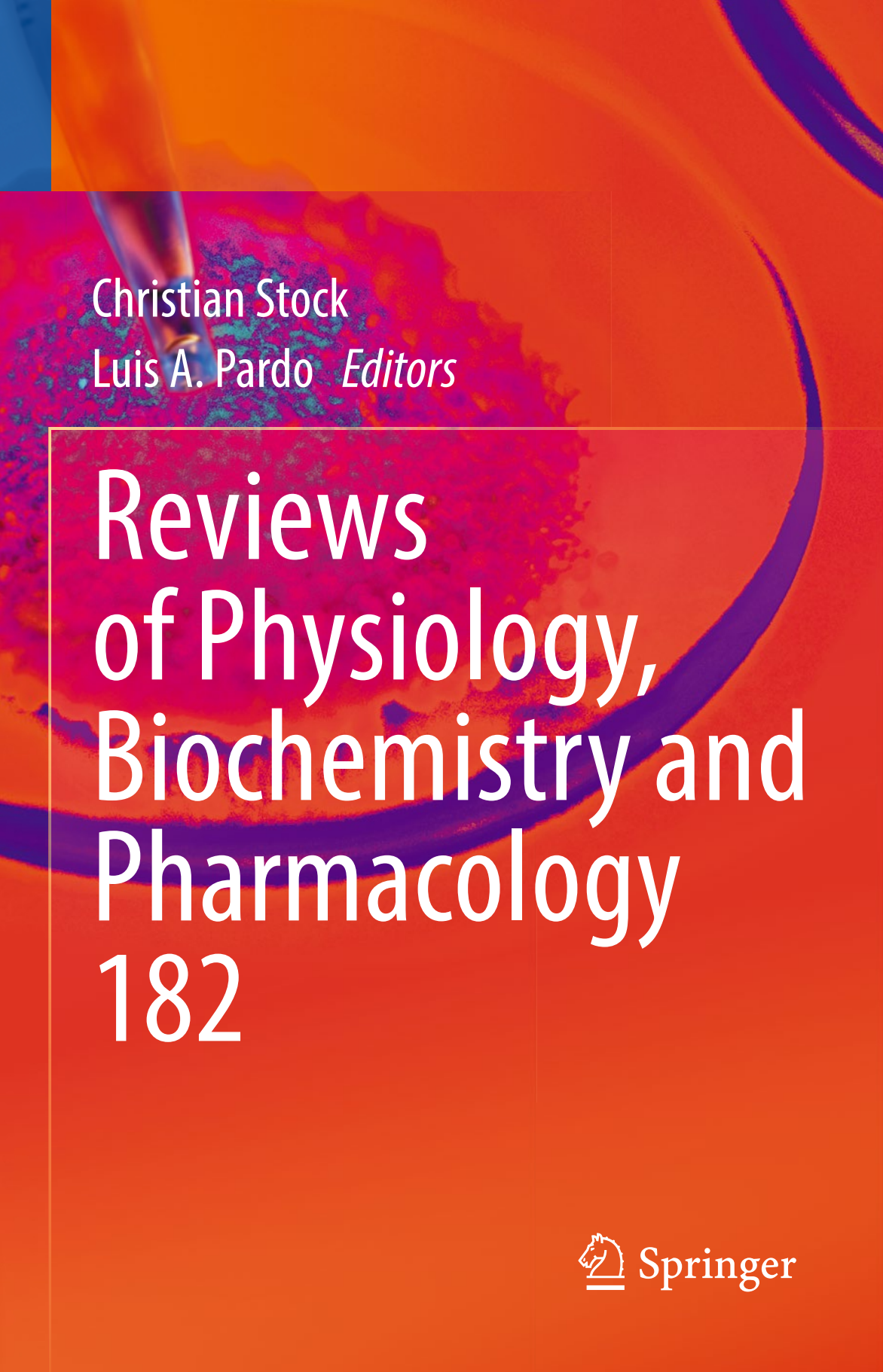
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# From Malignant Transformation to Metastasis

## Ion Transport in Tumor Biology

Springer

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# Preface

The first volume in the series "Ion Transport in Tumor Biology" dealt with the role of the transportome in different types of cancer. It gave a detailed description of the ion channels and transporters identified as significant players in virtually all of the epidemiologically relevant tumor entities. The contributors to that volume focused on the transport molecules identified in those tumor types, summarizing our current knowledge of how ion transport can contribute to tumor initiation and survival. The second volume deals with the role of ion transport molecules in the established tumor, how they contribute to the survival of cancer cells in a hostile environment, and how the cross-talk between the microenvironment and the transport proteins shapes tumor progression. It also describes the role of the transportome in essential events like tumor vascularization and along the metastatic cascade, from the invasion of the blood or lymphatic vessels to grafting in distant organs.

This volume does not explicitly present two relevant steps in the route to metastasis: epithelial to mesenchymal transition and cell migration. Several chapters in the first volume already covered the participation of some transportome molecules in cell migration, and because the role of ion channels in cell motility is a very well-established concept, several recent thorough reviews provide up-to-date information on this topic (e.g., Stock and Schwab 2015; Canales Coutino and Mayor 2021; Canales et al. 2019; Storck et al. 2017).

Epithelial to mesenchymal transition (EMT) represents a particularly complex situation. Carcinomas, derived from epithelial cells, are a majority of oncological diseases. The typical features of epithelial cells include basal-apical polarity, stable cell–cell junctions, and a tight attachment to the basement membrane, all features that render epithelial cells immotile. In contrast, mesenchymal cells show anterior-posterior polarity, are loosely attached to other cells or the extracellular matrix, and are motile, therefore able to migrate. Should cancer cells conserve epithelial properties, they would not be able to depart the primary tumor site, i.e. metastasis requires a mesenchymal phenotype to allow migration. Therefore, precluding the acquisition of a mesenchymal phenotype is a promising strategy for fighting

metastases (Lambert et al. 2017). Mesenchymal cells can also transition to epithelial cells in the reverse process (MET) (Dongre and Weinberg 2019), and this is also necessary for the tumor cells to colonize the distant site.

Due to its relevance, EMT is the subject of intense study also in the context of embryogenesis, morpho- and organogenesis, wound healing, and tissue regeneration. This broad spectrum of different disciplines leads to some degree of confusion. What different researchers term EMT does not always correspond to the same phenomenon, in physiological or molecular terms. To start with, different forms of EMT occurring in different contexts result in radically different cell types. The lineage resulting from EMT in development are mesenchymal cells, which are not only motile but also pluripotent, and form new epithelia with properties other than the cells of origin at the destination site. In scar formation and fibrosis, the cells resulting from EMT are fibroblasts, often misidentified as mesenchymal cells because they are motile, morphologically similar, and express fibrillar collagens and fibronectins (Zeisberg and Neilson 2009). Even taking this into account, a binary distinction between epithelial and mesenchymal phenotypes is, as in most biological processes, an oversimplification. Between the epithelial and the mesenchymal cell, there are many intermediate phenotypes whose importance is starting to be acknowledged. Thus, a subpopulation of cells showing a stable hybrid phenotype is responsible for high malignancy and invasive potential in breast cancer cells, which diminishes when these cells are forced into a fully developed mesenchymal phenotype (Kroger et al. 2019). Furthermore, in some cases, EMT appears dispensable for metastasis (Zheng et al. 2015; Fischer et al. 2015). There is likely no unique program of EMT in cancer progression, and the large amount of data available in cell culture is unlikely to represent the in vivo situation, which explains the discrepant results obtained (Francou and Anderson 2020). A large majority of the evidence of the relevance of ion channels and transporters has been obtained in cell lines and is likely to represent just a particular case in a defined tissue, making general statements at least risky. Recent efforts to unify criteria and definitions (Yang et al. 2020) will undoubtedly bring clarity to EMT research.

Keeping such warnings in mind, it is still clear that ion transport reprogramming is a crucial part of any phenotypic transformation of epithelial cells. This has been documented since at least two decades (Huber et al. 2000). The transportome is the workhorse of epithelial cells and is less prominent in the mesenchymal phenotype, but the process of EMT itself depends on ion fluxes, mainly $Ca^{2+}$ (e.g., Bhattacharya et al. 2018; Zhang et al. 2017; Schaar et al. 2016). Therefore, it is not surprising that $Ca^{2+}$ channels have been implicated in EMT in many types of cancer. Store-operated $Ca^{2+}$ entry is a relevant element in this process (e.g., Okeke et al. 2016; Stewart et al. 2015; Elzamzamy et al. 2020; Bhattacharya et al. 2018; Zhang et al. 2017; Schaar et al. 2016; Xia et al. 2016; Casas-Rua et al. 2015; Chan et al. 2016), but store-independent $Ca^{2+}$ entry, activated by a $Ca^{2+}$-transport protein (SPCA2), also plays a role at least in breast cancer (Feng et al. 2010; Dang et al. 2019; Makena et al. 2021). Individual $Ca^{2+}$-permeable channels have often been implicated in EMT in several tumor types. That is the case for TRPC1 (breast cancer (Azimi et al. 2017)), TRPM7 (breast (Davis et al. 2014), ovarian (Liu et al.

2019), prostate (Chen et al. 2017), and bladder cancer (Cao et al. 2016)), TRPM8 (breast (Liu et al. 2014)) or TRPP2 (larynx (Wu et al. 2016)). One out of the few TRPM channels that are not permeable to $Ca^{2+}$, TRPM4, appears to play a negative role for EMT in endometrial cancer (Li et al. 2020) while it favors EMT in breast cancer (Wong and Hussain 2020).

$Cl^-$ channels reportedly participate in EMT in certain tumors in a fashion in part related to $Ca^{2+}$. That is the case for bestrophin (Aldehni et al. 2009) in colon cancer or CFTR in breast (Zhang et al. 2013). CLIC4 negatively regulates EMT in gastric cancer (Wang et al. 2019), and the $Cl^-$ channel-associated proteins CLCA also counteract EMT (Chen et al. 2019; Walia et al. 2012; Yu et al. 2013; Sasaki et al. 2012).

In the case of $K^+$ channels, several reports reveal the implication of calcium-activated potassium channels of small ($K_{Ca}2.3$ in prostate (Bery et al. 2020)) and intermediate conductance ($K_{Ca}3.1$ in breast (Zhang et al. 2016; Thurber et al. 2017), hepatocellular carcinoma (Du et al. 2019), and thyroid cancer (Wen et al. 2020)). The list of channels implicated in EMT also contains voltage-gated $K^+$ channels, such as $K_V11.1$ (in breast (Breuer et al. 2019) and colorectal cancer (Fortunato 2017)), $K_V10.1$ (in lung cancer (Restrepo-Angulo et al. 2011)), and KCNQ1 (colon cancer (Rapetti-Mauss et al. 2017)). Other channels like the voltage-gated sodium channel $Na_V1.5$ (breast (Gradek et al. 2019)) or the zinc channel (also in breast (Hogstrand et al. 2013)), the acid-sensing channels ASIC 1 and 3 (in pancreatic cancer (Zhu et al. 2017)) or the aquaporin AQP1 (in lung (Yun et al. 2016)) are involved as well.

In addition, many ion transporters can be added to the list of molecules reportedly relevant for EMT, like NHE1 in pancreatic (Cardone et al. 2015) and breast cancer (Amith et al. 2016) or KCC3 in cervix carcinoma (Hsu et al. 2007).

This list does not attempt to be complete nor exhaustive, but it gives an idea of the large amount of ion transport molecules that could participate in the intriguing process of EMT. A better definition of EMT in different scenarios will undoubtedly confirm the potential of members of the transportome as targets to modify EMT and likely metastasis and bring clarity to the picture.

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# How Reciprocal Interactions Between the Tumor Microenvironment and Ion Transport Proteins Drive Cancer Progression


**Line O. Elingaard-Larsen, Michala G. Rolver, Ester E. Sørensen, and Stine F. Pedersen**

## Contents



**Abstract** Solid tumors comprise two major components: the cancer cells and the tumor stroma. The stroma is a mixture of cellular and acellular components including fibroblasts, mesenchymal and cancer stem cells, endothelial cells, immune cells, extracellular matrix, and tumor interstitial fluid. The insufficient tumor perfusion and the highly proliferative state and dysregulated metabolism of the cancer cells collectively create a physicochemical microenvironment characterized by altered nutrient concentrations and varying degrees of hypoxia and acidosis. Furthermore, both cancer and stromal cells secrete numerous growth factors, cytokines, and

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extracellular matrix proteins which further shape the tumor microenvironment (TME), favoring cancer progression.

Transport proteins expressed by cancer and stromal cells localize at the interface between the cells and the TME and are in a reciprocal relationship with it, as both sensors and modulators of TME properties. It has been amply demonstrated how acid-base and nutrient transporters of cancer cells enable their growth, presumably by contributing both to the extracellular acidosis and the exchange of metabolic substrates and waste products between cells and TME. However, the TME also impacts other transport proteins important for cancer progression, such as multidrug resistance proteins. In this review, we summarize current knowledge of the cellular and acellular components of solid tumors and their interrelationship with key ion transport proteins. We focus in particular on acid-base transport proteins with known or proposed roles in cancer development, and we discuss their relevance for novel therapeutic strategies.

**Keywords** Acidosis · Hypoxia · Lactate · Monocarboxylate transporters · $Na^+/H^+$ exchange · $Na^+/HCO_3^-$ cotransport · pH regulatory transporters · Tumor heterogeneity · Tumor stroma · V-type ATPases

## 1 Introduction

The first acknowledgment that tumor growth is dependent on a unique microenvironment is generally ascribed to the German physician Rudolf Virchow (1821–1902) and his observations of tumor inflammation (Virchow 1863). The key importance of both cellular and acellular components of the tumor microenvironment (TME) for essentially all aspects of cancer development has since been widely appreciated and is documented in numerous influential reviews (Kalluri 2016; Meads et al. 2009; Zou 2005; Joyce and Pollard 2009; De Palma et al. 2017).

In marked contrast to this, the roles of ion and nutrient transport proteins in the TME are still underappreciated: Transport proteins sense and regulate both the TME and the intracellular milieu and thereby serve as a very active interface between the various cells in the tumor and the physicochemical TME. Furthermore, they are excellent drug targets, rendering them highly interesting in a therapeutic context (Cesar-Razquin et al. 2015). The focus of this review is how the TME regulates and in turn is shaped by, transport proteins. We first provide an overview of the cell types and physicochemical conditions in the TME. This is followed by a discussion of key transporters at the interface between the acellular and cellular TME components and how their regulation by the TME may contribute to cancer development (Fig. 1).

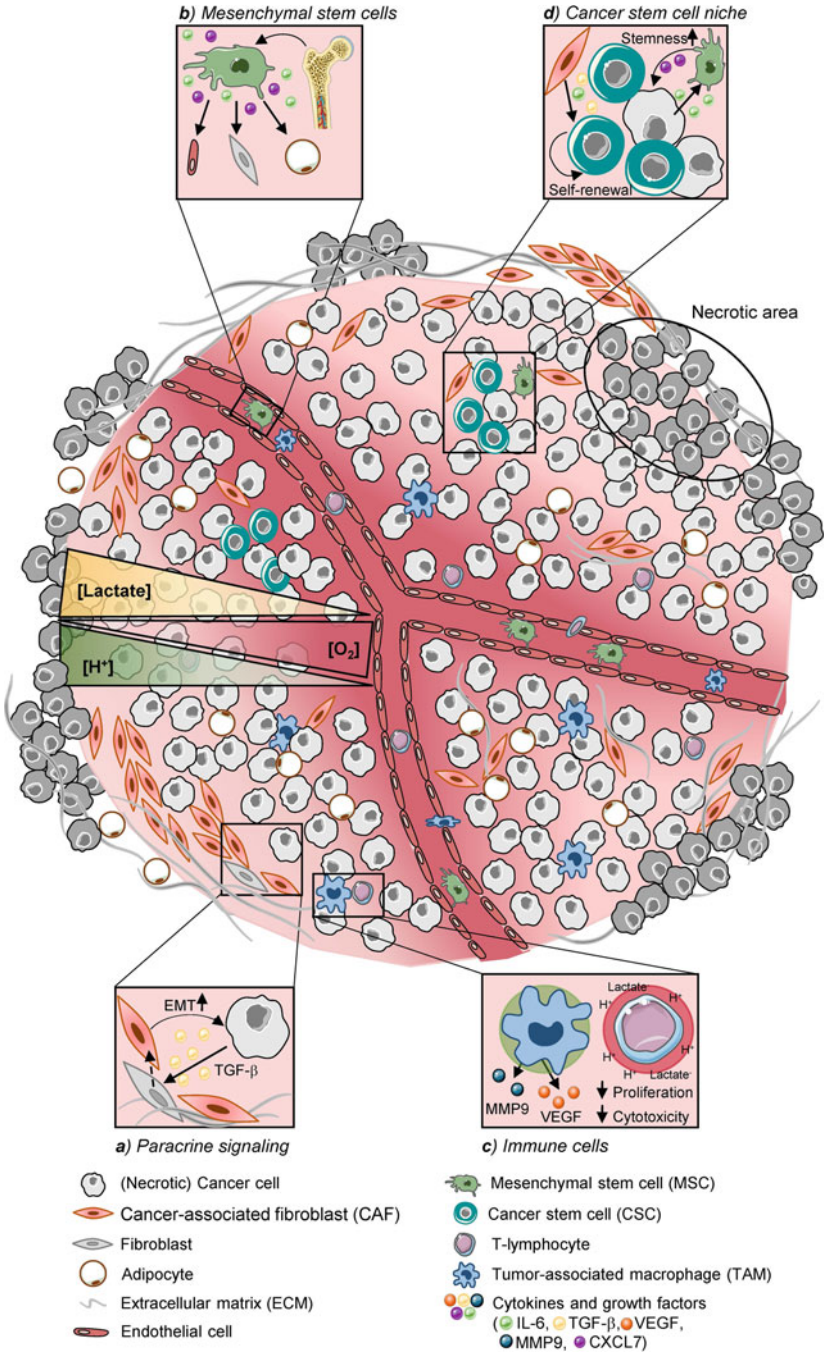


**Fig. 1** Overview of the key components of the TME. In addition to the cancer cells, tumors contain a large stromal component composed of extracellular matrix (ECM), tumor interstitial fluid (TIF), cancer-associated fibroblasts (CAF; **a**), mesenchymal stem cells (MSC; **b**), immune cells (**c**), cancer

## 2 The Tumor Microenvironment

### 2.1 The Cellular Component

*Cancer-associated fibroblasts (CAFs)* are a major component of the tumor stroma in essentially all types of cancer (Kalluri 2016; Raffaghello et al. 2015; Micke and Ostman 2005; Chen and Song 2019). Several studies have shown that factors secreted from cancer cells, such as transforming growth factor beta (TGF-β) and platelet-derived growth factor (PDGF), can transform normal fibroblasts into CAFs (Lieubeau et al. 1994; Ronnov-Jessen and Petersen 1993; Bronzert et al. 1987; Avgustinova et al. 2016) (Fig. 1a). Similarly, cancer cell-mediated activation of Myc in normal human fibroblasts drive their reprogramming into CAFs and thus the formation of a pro-tumorigenic environment (Minciacchi et al. 2017). CAFs differ from normal fibroblasts by increased proliferation and extracellular matrix (ECM) production, a unique cytokine secretion profile, and activation of stemness-related pathways such as Wnt and Notch signaling (Plaks et al. 2015; Anderberg and Pietras 2009). CAFs have been shown to induce epithelial-to-mesenchymal transition (EMT) in multiple types of cancer and thus contribute to cancer cell invasion and metastasis (Giannoni et al. 2010; Yu et al. 2014) (Fig. 1a). Reflecting their importance for the tumor-promoting properties of the TME, direct targeting of CAFs was shown to reduce tumor growth in a mouse HepG2 tumor model (Chen et al. 2016), and CAF-targeting therapies are currently being intensely studied (Chen and Song 2019). Fibroblasts, and presumably CAFs, were also recently assigned a role in absorbing and venting acid secreted from the tumor cells, at least in part via SLC4A2 (anion exchanger 2)-mediated net acid uptake and connexin-43-mediated cell-to-cell transmission (Hulikova et al. 2016).

*Mesenchymal stem cells (MSCs)* are multipotent stromal cells which are implicated in many cancer-promoting processes such as favoring cancer cell proliferation and metastasis, as well as angiogenesis and immunosuppression (Cuiffo and Karnoub 2012). MSCs are mainly recruited from the bone marrow into the tumor by soluble factors released from the cancer cells and are capable of differentiating into many different types of stromal cells such as endothelial cells, adipocytes, and fibroblasts (Cuiffo and Karnoub 2012) (Fig. 1b). MSCs can secrete a plethora of cytokines, chemokines, and growth factors, which through paracrine signaling regulate cancer growth and progression (Cuiffo and Karnoub 2012; Tsai et al. 2011; Hung et al. 2013; Karnoub et al. 2007) (Fig. 1b). As an example, secretion

**Fig. 1** (continued) stem cells (CSC; **d**), and adipocytes, among others. The interplay between these cell types occurs through secretion of, e.g., cytokines, growth factors, lactate, and protons and aids in tumor progression and development. A specific CSC niche exists within the tumor microenvironment which sustains stemness and the self-renewal potential of the CSCs (**d**). Heterogeneous gradients of oxygen, lactate, and $H^+$ arise according to the distance to the nearest blood vessels and affect the metabolism and phenotype of nearby cells. See text for further details

of the inflammatory cytokine interleukin 6 (IL-6) by MSCs enhances cancer cell malignancy in a paracrine manner in several cancer types, often via activation of the Janus kinase/signal transducers and activators of transcription-3 (JAK/STAT3) pathway (Tsai et al. 2011; Liu et al. 2011; Sasser et al. 2007). MSCs are also responsive to signals emanating from cancer cells, in turn increasing cancer cell aggressiveness (Karnoub et al. 2007; Borghese et al. 2013). For instance, upon physical contact between breast cancer cells and MSCs, the latter were shown to initiate de novo production of C-C motif chemokine ligand 5 (CCL5), an inflammatory chemokine which is normally minimally expressed in mammary epithelial cells (Soria and Ben-Baruch 2008). This in turn promoted the ability of the breast cancer cells to extravasate into lung parenchyma, strongly increasing their metastatic potential (Karnoub et al. 2007).

*Tumor-infiltrating immune cells*, including neutrophils, mast cells, and tumor-associated macrophages (TAMs), have been shown to play diverse and critical roles in tumorigenesis (Hanahan and Weinberg 2011; Aras and Zaidi 2017). Through secretion of epidermal growth factor (EGF), vascular epidermal growth factor (VEGF), matrix metalloproteinases (MMPs), and chemo- and cytokines, TAMs stimulate tumor cell proliferation, angiogenesis, and ECM reorganization and contribute to treatment resistance and suppression of the anti-tumor immune response (Hanahan and Weinberg 2011; Aras and Zaidi 2017). Conversely, early-stage infiltration by anti-tumor immune cells can limit tumor growth and reduce the frequency of metastasis and is associated with improved patient survival (Dranoff 2004; Naito et al. 1998). In the context of this review, it is particularly interesting that several studies have shown that the acidic extracellular pH ($pH_e$) in the TME inhibits this anti-tumor immune response and simultaneously enhances the activity of pro-tumorigenic TAMs (Boedtkjer and Pedersen 2020; Huber et al. 2017) (Fig. 1c). There are also multiple reports of lactate impacting immune cell response in a similar manner; however, as discussed below (Sect. 2.2.3), this is at least in some cases only seen in the simultaneous presence of acidification.

*Cancer stem cells (CSCs)*, while still incompletely understood and subject of controversy, are by now considered a crucial component of the TME in most if not all cancers. Similar to their normal counterparts, CSCs can self-renew and generate differentiated progeny, which in tumors is reflected in the heterogeneity of the cancer cells (Lau et al. 2017). CSCs can be identified based on their expression of stemness markers typical of the stem cells in their tissue of origin and on their ability to initiate tumor growth in vivo (Hanahan and Weinberg 2011). Due to their protection by multiple resistance mechanisms and their quiescent phenotype, CSCs are largely unaffected by conventional treatment strategies and are therefore tightly connected to tumor recurrence (Plaks et al. 2015; Lau et al. 2017). They reside in specific niches within the TME (see Sect. 2.3), and their phenotype relies heavily on secreted factors (e.g., IL-6, interleukin 8 (IL-8), and chemokine (C-X-C) motif ligand 7 (CXCL7)) and on stimulation of stemness pathways (e.g., Wnt and Notch) (Plaks et al. 2015; Liu et al. 2011). In fact, several studies have shown that cancer cells are capable of educating tumor stromal cells to become pro-tumorigenic and promote stemness properties of CSCs (Lau et al. 2017). For instance, in a breast cancer model, IL-6

secreted by cancer cells stimulated the expression of CXCL7 in MSCs, which in turn increased the pool of CSC (Liu et al. 2011) probably through downstream effects mediated via its G-protein-coupled receptor C-X-C motif chemokine receptor 2 (CXCR2) (Cheng et al. 2019) (Fig. 1d).

### 2.1.1 Other Cell Types in the TME

Dependent on the tissue in which the tumor develops, other TME-resident cells (Fig. 1) play more or less prominent roles in supporting tumor growth and progression.

By forming the tumor vasculature, *endothelial cells* are important for the progression of most solid tumors. Similar to other cell types in the TME, they both shape and are shaped by their environment, as both hypoxia, acidic $pH_e$ (Pedersen et al. 2017a), and lactate (Dhup et al. 2012) contribute to regulation of endothelial cell function.

In breast cancer, where adipocytes are particularly abundant, cross-talk between cancer cells and adipocytes, either by means of secreted factors or exosomal microRNAs, causes adipocyte metabolic reprogramming as well as a change in phenotype toward *cancer-associated adipocytes* (CAAs) (Wu et al. 2019a). Interestingly, the cancer cells can induce upregulation of monocarboxylate transporter 4 (MCT4) in the CAA. This facilitates a metabolic coupling between the cancer cells and CAA by creating a monocarboxylate shuttle through which the CAAs feed the cancer cells with lactate (see Sects. 2.2.3 and 3.3 and Fig. 3b) (Wu et al. 2019a, b).

Finally, the *stellate cells* (SCs) of the liver and pancreas, which are physiologically important for lipid storage and maintenance of normal stromal composition, contribute importantly to tumor progression, at least in part through production and remodeling of the ECM (Sherman 2018; Schnittert et al. 2019). For instance, activated pancreatic SCs share a similar transcription profile with CAFs (Sherman 2018) and constitute more than 50% of the tumor stroma in pancreatic ductal adenocarcinoma (PDAC), where they play a key role in the establishment of desmoplasia (Schnittert et al. 2019).

## *2.2 The Acellular Component*

Tumor development and growth cause profound and heterogeneous changes to the composition of the surrounding milieu, creating the complex, spatially and temporally variable, acellular TME component. We focus primarily on physicochemical factors such as hypoxia/oxidative stress; concentrations of protons, lactate, and other ions; and tumor pressure and mechanics. An in-depth description of other TME properties is beyond the scope of this review, and we refer the reader to excellent recent reviews providing further details on the roles of ECM architecture (Bonnans et al. 2014; Gilkes et al. 2014), nutrient deprivation (Gouirand et al. 2018;

Anastasiou 2017), and tumor cytokines and growth factors (Dranoff 2004; Landskron et al. 2014; Germano et al. 2008) in cancer progression.

### 2.2.1 Hypoxia and Oxidative Stress

***Hypoxia.*** Hypoxia, the deprivation of adequate oxygen supply, occurs within 50–100 μm from the nearest blood vessel and is a prominent feature of the often poorly vascularized TME and a key regulator of cancer cell phenotype (Robey et al. 2008; Papandreou et al. 2006). Hypoxia triggers the activation of hypoxia-inducible-factor 1 (HIF-1), a discovery awarded with the Nobel Prize to William Kaelin, Sir Peter Ratcliffe, and Gregg Semenza in 2019. When oxygen levels are adequate, HIF-1α is hydroxylated by the oxygen sensors prolyl hydroxylase domain-containing protein (PHDs) creating a binding site for the Von Hippel-Lindau (VHL) E3 ubiquitin ligase complex, leading to HIF-1α degradation by the proteasomal pathway (Papandreou et al. 2006). Conversely, under hypoxic conditions, HIF-1α is stabilized, allowing it to dimerize with HIF-1β, translocate to the nucleus, and bind to hypoxia response elements (HRE). The multiple downstream effects include activation of pyruvate dehydrogenase kinase 1, reducing mitochondrial oxygen consumption and inducing transcription of glycolytic enzymes (Papandreou et al. 2006). In fact, HIF-1 has been found to change the isoform expression pattern of glycolytic enzymes, making them less sensitive to physiological inhibitors and reducing affinities for their products, thereby increasing their catalytic capacity (Carnero and Lleonart 2016; Marin-Hernandez et al. 2009; Semenza et al. 1996). Furthermore, HIF-1-driven transcriptional changes contribute to the metabolic reprogramming of cancer cells from an oxidative to a more glycolytic phenotype and have also been found to be involved in angiogenesis, EMT, invasion, metastasis, and therapy resistance (Gilkes et al. 2014; Carnero and Lleonart 2016; Yang and Wu 2008; Wartenberg et al. 2003; Bhuria et al. 2019).

Interestingly, not all hypoxia-driven changes in the TME are downstream of HIF-1. Thus, VEGF upregulation in colon cancer was largely unaffected by HRE mutation or HIF-1α knockdown (Mizukami et al. 2004). Other transcription factors regulated by cellular oxygen levels in a manner independent of HIF-1 include nuclear factor kappa B (NF-κB) (Mizukami et al. 2005; Liao and Johnson 2007) and activating protein (AP)-1 (Cummins and Taylor 2005). Furthermore, as discussed below, lactate levels typically increase in hypoxic areas due to poor perfusion and high glycolytic rate. Interestingly, the intracellular protein N-myc downstream-regulated gene 3 (NDRG3) was recently found to be regulated by lactate in a manner similar to, yet independent of, the regulation of HIF-1α, leading to activation of the Raf-extracellular signal-regulated kinase (ERK) pathway to promote angiogenesis and cell growth (Lee et al. 2015). This led to the suggestion that NDRG3 serves as a lactate sensor and thus indirectly as a hypoxia sensor (Lee et al. 2015). However, as further detailed below, regions of primarily glycolytic activity and regions of hypoxia do not always fully overlap in tumors (Boedtkjer and Pedersen 2020; Rohani et al. 2019), and hence this correlation is not ubiquitous.

***Oxidative Stress and Redox Homeostasis*** While this may sound paradoxical at first, hypoxia and oxidative stress frequently co-exist in solid tumors. In fact, the pathways interact, as reactive oxygen species (ROS) derived from mitochondrial activity have been found to be necessary and sufficient to activate and stabilize HIF-1 under hypoxic conditions (Chandel et al. 1998), thereby contributing to cell proliferation and hypoxic adaptations. Early studies showed that cancer cells release large amounts of ROS (Szatrowski and Nathan 1991), and it is now known that ROS are produced by both cancer and stromal cells and can reach high levels in the TME, influencing multiple aspects of tumor development, including angiogenesis, anti-tumor immune response, cancer cell proliferation, and metastasis (Weinberg et al. 2019; Reczek and Chandel 2017). A major contributor to ROS generation is oxidative phosphorylation in the mitochondrial membrane, giving rise to the reactive species superoxide ($O_2\cdot$), which is converted into hydrogen peroxide ($H_2O_2$) by superoxide dismutase (SOD) and can be further reduced to the hydroxyl radical ($OH\cdot$). In high concentrations, these molecules are damaging to proteins, lipids, and DNA (Diebold and Chandel 2016). Hence, cancer cells need to keep a balance between production and removal of ROS to ensure redox homeostasis. This can be achieved by activating antioxidation pathways and upregulating the production of antioxidants such as SOD and catalase or the tripeptide antioxidant glutathione (GSH) (Vučetić et al. 2017). A mild oxidative state promotes many tumorigenic processes such as survival, growth, and proliferation, for example, via phosphoinositide 3-kinase (PI3K) signaling (Turrens 2003). However, once a certain threshold is reached, sustained oxidative stress will eventually promote cell death (Vučetić et al. 2017), in line with the concept of hormesis (Gems and Partridge 2008). Amino acid balance and redox homeostasis are closely linked, as cysteine and glutamine are essential for GSH biosynthesis. Notably, this – in addition to the metabolic importance of these amino acids – renders their transporters very important for tumor growth (Vučetić et al. 2017). These, which will be discussed below (Sect. 3.5), include the cystine/glutamate antiporter solute carrier family 7 member 11 (SLC7A11, also known as xCT) and alanine-serine-cysteine transporters (ASCTs).

### 2.2.2 Acidosis

The glycolytic shift in many cancer cells, together with a high proliferative rate and insufficient perfusion, causes the extracellular acidification characteristic of the TME in solid tumors (Boedtkjer and Pedersen 2020; Andersen et al. 2014; Kallinowski et al. 1988; Vaupel et al. 1989). Tumor acidosis exhibits spatial and temporal heterogeneity, with some regions displaying near-neutral to neutral pH values, while other areas are highly acidic, reaching levels as low as pH 6.0 (Boedtkjer and Pedersen 2020). In regions of low perfusion, oxygen delivery is limited, resulting in hypoxia and a change in metabolic preference toward glycolysis (Marin-Hernandez et al. 2009; Chiche et al. 2010). This leads to increased cellular production and extrusion of lactate and $H^+$ to the TME (Boedtkjer and Pedersen

2020) (Fig. 1). In better perfused peripheral regions of solid tumors, cancer cells exhibit an oxidative phenotype, in which $H^+$ is mainly produced through the hydration of $CO_2$ (Corbet and Feron 2017). However, as noted above, regions of hypoxia and acidosis do not fully overlap in in vivo tumors, a phenomenon at least in part reflecting the very high glycolytic activity of some well-perfused cancer cells (Boedtkjer and Pedersen 2020; Rohani et al. 2019).

The effect of extracellular acidity on cancer cell phenotype will depend on the duration and extent of acidity and on the cell type and its specific repertoire of transporters and receptors. For instance, through lowering intracellular pH ($pH_i$), acute acidosis will limit cell proliferation (Flinck et al. 2018), while the direct interaction of $H^+$ with acid-sensing receptors and ion channels can increase the free intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) conversely favoring proliferation (Boedtkjer and Pedersen 2020). On the other hand, several studies have shown that long-term adaptation to an acidic extracellular microenvironment selects for a more aggressive and invasive cancer cell phenotype, with profound changes in metabolism and an increased capacity to survive in extreme acidosis (Boedtkjer and Pedersen 2020; Damaghi and Gillies 2017; Pellegrini et al. 2018).

The often profound acidity of the TME is not only conducive to tumor aggressiveness by mechanisms acting directly on the cancer cells. Through protonation of weak base drugs, the acidic TME can contribute to drug resistance by limiting the access of drugs to their intracellular targets (see Sect. 3.1) (Raghunand and Gillies 2000). Furthermore, as noted above (Sect. 2.1), tumor acidosis reduces the effectiveness of anti-tumorigenic cytotoxic and helper T cells, while promoting recruitment of immune-suppressive pro-tumorigenic cells, overall facilitating immune escape and tumor progression (Huber et al. 2017; Nakagawa et al. 2015; Calcinotto et al. 2012). Thus, several studies have reported that extracellular acidosis is associated with impaired T-cell proliferation and reduced production of several important cytokines (interferon γ (IFNγ) and interleukin 2 (IL-2)) as well as of perforin and granzyme, all factors important for the cytotoxicity of T cells (Huber et al. 2017) (Fig. 1c).

### 2.2.3 Lactate

Besides contributing to TME acidification, the glycolytic shift in many cancer cells increases the lactate concentration in the tumor through the conversion of pyruvate to lactate that regenerates $NAD^+$ for continued glycolysis. In many cancer cells, this process is favored by HIF-1-induced upregulation of lactate dehydrogenase A (LDHA) (Semenza et al. 1996). The increased abundance of LDHA over LDHB favors the forms of the tetrameric LDH enzyme complex which preferentially catalyze the conversion of pyruvate to lactate. Lactate extruded from hypoxic or other primarily glycolytic cells can be used as metabolic fuel in oxygenated tumor regions or by oxidative cells. This is termed *metabolic coupling* and is proposed to occur both between cancer cells in hypoxic and oxygenated regions and between cancer cells and non-malignant cells (Ippolito et al. 2019; Doherty and Cleveland

2013) (Fig. 3b). The shuttling of lactate between cells and the TME is dependent on the monocarboxylate transporters MCT1 and MCT4 (Perez-Escuredo et al. 2016), as further described in Sect. 3.3. Additionally, recent work has shown a role for intercellular shuttling via connexin-43 gap junctions in moving lactate between hypoxic and oxidized tumor regions (Dovmark et al. 2017). Although the general importance of this pathway still needs to be further addressed, it is reasonable to assume that under conditions of profound extracellular acidity, a more alkaline $pH_i$, and relatively high extracellular lactate concentrations, MCT-mediated lactate-$H^+$ cotransport is not thermodynamically well poised to drive lactate export.

This raises the question of how high the lactate concentration in the extracellular space actually is in tumors. Reported lactate concentrations in tumors from patients and mouse models differ widely between cancers and between individual tumors, with mean values of 5–15 μmol/g tissue in metastatic human cervical tumors and 7–26 μmol/g in mouse xenograft models of human head and neck squamous cell carcinoma (Quennet et al. 2006; Walenta et al. 2000). However, such values are generally based on total tumor tissue, and do not distinguish between intra- and extracellular lactate. Furthermore, as recently pointed out by Rabinowitz and coworkers (García-Cañaveras et al. 2019), even measurements that specifically aim to determine the extracellular lactate concentration in tumors are often contaminated with intracellular fluid, suggesting that the high lactate concentrations in many tumors may in large part reflect intracellular lactate (García-Cañaveras et al. 2019). This is supported by recent careful measurements of metabolites in tumor interstitial fluid in mouse pancreatic tumors, arriving at lactate concentrations similar to those of plasma (Sullivan et al. 2019). Thus, the presumption that extracellular lactate concentrations are highly elevated in tumors may not be generally valid, and future studies should establish the precise intra- and extracellular concentrations of lactate in various tumors in vivo. Similar to acidosis, lactate has been shown to limit antitumor immune response, at least in part by blocking release of lactate and $H^+$ from the T cells, which, similar to tumor cells, are preferentially glycolytic in their activated state (Fischer et al. 2007). An elegant recent study showed that shRNA-mediated depletion of the *Ldha* gene in mouse melanoma cells led to reduced tumor growth in immunocompetent mice, whereas growth in immunosuppressed mice was similar, demonstrating the key role of immune cells in the tumor-suppressive effect of *Ldha* depletion in the tumor cells. Importantly however, this effect could be partially mimicked by extracellular acidification alone, but not by the sodium salt of lactate. It was furthermore shown that high extracellular lactate-$H^+$ levels were associated with intracellular acidosis and lactate accumulation of T cells, compromising their growth and activity and leading to tumor immune escape (Brand et al. 2016). On the other hand, in studies of chronic inflammation, also sodium lactate alone compromised T-cell function (Haas et al. 2015), suggesting that the role of lactate may differ between different immune cell subtypes and conditions.

In addition to its role as a metabolic fuel, accumulating evidence points to the importance of lactate as a ligand for bona fide sensors of intra- and extracellular lactate, although these remain incompletely described. Whereas NDRG3, as mentioned above (Sect. 2.2.1), appears to serve as an intracellular lactate sensor (Lee

et al. 2015), the recently described G-protein-coupled receptor 81 (GPR81; HCAR1) senses extracellular lactate (Husted et al. 2017). As an example of how the TME can directly regulate ion transporters important for cancer progression, GPR81 was recently assigned important roles in tumor progression, in part via regulation of MCT1 and MCT4 expression (Roland et al. 2014) (see Sect. 3.3). Furthermore, GPR81-mediated lactate sensing was shown to result in upregulation of the programmed cell death protein 1 ligand in lung cancer cells, protecting them from cytotoxic T-cell targeting (Feng et al. 2017). In vivo, GPR81 supports tumor growth and, in particular, metastasis, via effects on both tumor (Roland et al. 2014) and stromal (Brown et al. 2020) cells, although many open questions regarding the precise mechanisms remain (Brown et al. 2020). Finally, very recent work revealed that intracellular lactate can be a substrate for posttranslational modification of histones, denoted *histone lactylation*, activating transcription of genes involved in, for instance, wound healing responses (Zhang et al. 2019). While further work is needed to understand the impact and extent of this modification, these findings may shed further light on how lactate accumulation regulates gene transcription in tumors.

### 2.2.4 Other Physicochemical Factors Shaping the TME

While hypoxia, oxidative stress, acidosis, and elevated lactate concentrations as discussed above are probably the most widely studied, many other properties of the TME impact cancer progression. In this section we focus on how the ionic composition of the tumor interstitial fluid (TIF), the mechanical properties of the ECM, and the pressure within the tumor influence tumor growth.

***Tumor Interstitial Fluid*** The TIF, i.e., the fluid that surrounds the cancer and stromal cells, is formed by transcapillary filtration, which is determined by properties of the capillary wall, the blood pressure, the hydrostatic pressure, and the colloid osmotic pressure according to Starling's principles for fluid exchange (Starling 1896). Therefore, in healthy tissue, the interstitial fluid has a nutrient and ion composition comparable to that of plasma (Sullivan et al. 2019; Wagner and Wiig 2015). Interstitial fluid also functions as a transport medium for nutrients and waste products and contains regulatory factors and signalling molecules, either locally produced or supplied via the circulation (Wagner and Wiig 2015; Wiig et al. 2010). The composition of nutrients in TIF differs from that of the normal circulation, due to the altered metabolism and vasculature of tumors (Sullivan et al. 2019; Wagner and Wiig 2015). The altered expression and activity of many ion transporters (Sect. 3) and channels (reviewed in this volume by Halima Ouadid-Ahidouch and colleagues) in cancer cells (Monteith et al. 2017; Pardo and Stühmer 2014) also seem likely to contribute to TIF composition. However, to the best of our knowledge, direct studies of such contributions in a tumor setting are not available, presumably reflecting technical limitations (Sect. 2.2.3). A recent study comparing the composition of PDAC TIF to that of plasma in mice under conditions allowing good

separation of intra- and extracellular contributions demonstrated that TIF was enriched in glycine and glutamate, whereas the levels of glucose as well as arginine, tryptophan, and cysteine were reduced compared to plasma levels (Sullivan et al. 2019). Thus, metabolites used by cancer cells were generally low in the TIF and those extruded from cancer cells generally high, as a result of poor waste removal (Sullivan et al. 2019). By employing instrumental neutron activation analysis, Wu et al. demonstrated significantly elevated levels of, among others, $Na^+$, $K^+$, and iron in gastric cancer tissue (Wu et al. 1996). Furthermore, recent technical advances allowing a combination of $^{1}H$ and $^{23}Na$ MRI measurements have led to convincing demonstrations of an increase in total tumor tissue [$Na^+$] in both malignant gliomas (Ouwerkerk et al. 2003) and breast lesions (Ouwerkerk et al. 2007); however, also these measurements cannot conclusively distinguish between contributions from intra- and extracellular $Na^+$.

***ECM Stiffness and Tumor Mechanics*** The ECM, which together with the TIF comprises the tumor interstitium, is composed of collagen fibers, glycosaminoglycans (GAGs) such as hyaluronan (HA), proteoglycans, and fibroblasts (Wagner and Wiig 2015; Wiig et al. 2010). In tumors, the ECM is reorganized, typically with stiffer, more linear, crosslinked collagen fibers, and generally more disorganized than in normal tissues (Cox and Erler 2011; Mayorca-Guiliani et al. 2017). It commonly contains more collagen fibers, proteoglycans, and GAGs, often predominantly produced by the CAFs (Sect. 2.1) (Wagner and Wiig 2015; Wiig et al. 2010; Naba et al. 2012). This remodeling of the ECM in cancer plays important roles in both primary tumor growth, invasiveness, and establishment of the premetastatic niche (Cox et al. 2013; Peinado et al. 2017) (see also Sect. 2.3). An important, and still not widely studied, aspect of this is the key role of tumor mechanics in regulating cancer cell metabolism. For instance, cytoskeletal changes associated with changes in ECM stiffness regulate phosphofructokinase protein levels in a manner specifically altered in tumor cells (Park et al. 2020), and interaction with actin filaments regulates aldolase (Hu et al. 2016), both linking ECM properties to glycolysis. Furthermore, very recent work in PDAC elegantly showed that the creatine-phosphocreatine ATP recycling system is highly mechanosensitive via YAP-dependent regulation of creatine kinase B, in turn regulating invasion and metastasis (Papalazarou et al. 2020). Finally, as several ion transporters and channels are known to be mechanosensitive, it seems highly likely that the special architecture and stiffness of tumor ECM contribute to their regulation in tumors (Sect. 3).

***Tumor Pressure*** It has long been realized that high hydrostatic and oncotic pressure plays important roles in cancer progression and chemotherapy resistance, in particular in cancers with extreme desmoplasia such as PDAC (Dufort et al. 2016; Provenzano et al. 2012; Butcher et al. 2009; Heldin et al. 2004). The precise mechanisms are still debated, in part because of the difficulty of measuring the individual parameters (see, e.g., Dufort et al. (2016) vs Chauhan et al. (2014)). The increased pressure is the sum of several fluid and solid pressures and comprises altered composition and architecture of the ECM, changes in contractility of both cancer and stromal cells, and compromised vasculature. GAGs, and in particular

HA, are highly anionic and thus attract cations and water. Hydrated HA can swell to up to 1,000-fold of its solid volume forming a pressure-generating gel-fluid phase (Dufort et al. 2016) and has been mathematically shown to generate increased osmotic pressure in solid tumors (Voutouri and Stylianopoulos 2014). Leakage of plasma proteins to the TIF through the leaky tumor vasculature further increases colloid osmotic pressure and interstitial fluid pressure (Heldin et al. 2004), ultimately increasing the interstitial fluid volume (Leslie et al. 2019). In vivo evidence of the interactions between ion transporters and tumor pressure is, to our knowledge, still lacking. However, it seems reasonable to assume that because of osmotic effects of ion exchange with the intracellular compartment in a constrained extracellular space, the activity of ion transporters in the tumor may contribute to the tumor pressure, by analogy to the situation in, e.g., brain edema (Stokum et al. 2016). Conversely, several of the transporters relevant to cancer are sensitive to osmotic pressure, as discussed in Sect. 3.

### *2.3 Heterogeneity of the Primary Tumor and Metastatic Sites*

As tumors grow and develop, both the cellular and acellular components of the tumor stroma undergo dynamic changes, creating extensive spatiotemporal heterogeneity, including, but not limited to, gradients of oxygen, nutrients, and metabolic waste products between vessels and unperfused regions (Boedtkjer and Pedersen 2020) (Fig. 1). Fluctuations in regional levels of these parameters also occur as a result of the disordered and leaky nature of newly formed blood vessels and of the varying levels of oxidative and glycolytic metabolism in different tumor regions (Kallinowski et al. 1988; Vaupel et al. 1989; Dewhirst et al. 2008).

The cellular heterogeneity of tumors (see Sect. 2.1) aids in circumventing the limitation of low nutrient availability from the blood supply, in part because it gives rise to a substantial ECM deposition. This results in an abundance of macromolecules (e.g., hyaline, fibronectin, collagen, albumin), which can be exploited as a nutrient supply through micropinocytosis, a process occurring in many cancer cell types, but especially important in PDAC (Gouirand et al. 2018; Olivares et al. 2017).

Even within each class of cellular tumor components, heterogeneity is widespread. For instance, CAFs have been found to express various mesenchymal markers which in some cases overlap with other types of stromal cells (Sugimoto et al. 2006). Also the CSCs constitute a heterogeneous and dynamic cell population as both epithelial-like and mesenchymal-like CSCs have been found within a single tumor and the CSCs can even switch between these phenotypes (Biddle et al. 2011).

Further increasing the heterogeneity, the CSCs reside in an anatomically distinct microenvironment – often termed the stem cell niche – within the TME (Plaks et al. 2015; Carnero and Lleonart 2016). Beside the CSCs, other stromal cells reside in the niche, producing factors such as VEGF, TGF-β, Wnt, Notch, and Hedgehog ligands, which stimulate CSC self-renewal, angiogenesis, and further stromal cell recruitment (Plaks et al. 2015) (Fig. 1d). Several studies have shown that both acidosis

(Hjelmeland et al. 2011; Filatova et al. 2016) and hypoxia (Carnero and Lleonart 2016; Mao et al. 2013) promote the stem cell phenotype, suggesting that highly acidic and hypoxic tumor regions may form CSC niches, although CSCs may also be found in well-perfused perivascular regions (Plaks et al. 2015; Li et al. 2009; Li and Rich 2010). HIFs are frequently upregulated in CSCs compared to non-stem cancer cells (Li et al. 2009; Heddleston et al. 2009) and contribute significantly to CSC drug resistance, through maintenance of the quiescent, chemotherapy-insensitive state and upregulation of drug efflux transporters such as ATP-binding cassette family B member 1 (ABCB1) (Schoning et al. 2017). Interestingly, the hypoxia-induced carbonic anhydrase 9 (CAIX) was found to be of particular importance for the expansion and maintenance of the CSC pool due to its role in creating an acidic niche (Parks and Pouyssegur 2017), and consequently CAIX inhibition delayed tumor growth and reduced the CSC population in an in vivo model of breast cancer (Lock et al. 2013). Thus, also the CSC niche as well as the CSCs themselves highly contributes to the intratumor heterogeneity.

Although the great majority of cancer deaths can be attributed to metastasis, the TME at secondary tumor sites remains relatively poorly understood. The formation of the so-called premetastatic niche involves recruitment of hematopoietic progenitor cells from the bone marrow into organs that are targets of metastasis, driven by signals from the primary tumor, before the dissemination of the primary tumor cells (Kaplan et al. 2005). These cells then precondition the premetastatic niche, in a manner that is associated with release of cytokines and other signaling molecules, as well as with ECM reorganization (Peinado et al. 2017). Very few measurements of the properties of the TME at metastatic sites are available, reflecting the difficulty of such measurements in vivo and of recapitulating the secondary tumor in an in vitro setting. An interesting recent study showed that lactate concentrations were higher, and glucose concentrations correspondingly lower, in metastases of melanomas compared to the primary tumors. Again, however, these measurements were based on whole tissue and did not distinguish between intra- and extracellular concentrations (Brand et al. 2016). In contrast to the poor understanding of ion concentrations in premetastatic niches and metastases, the role of ECM reorganization in forming the premetastatic niche is relatively well studied. For instance, it has been shown that organ fibrosis plays a key role in favoring lung and liver metastasis of breast cancer cells, in a manner involving lysyl oxidase (LOX)-dependent ECM crosslinking (Cox et al. 2013; Peinado et al. 2017). Furthermore, CAIX stimulated granulocyte-colony stimulating factor production through NF-κB activation which promoted the establishment of the premetastatic niche (Chafe et al. 2015). Hence, emerging evidence shows that both the TIF and ECM of the metastatic niche differ from those of the primary tumor and further understanding of their properties and the transporters involved in shaping and responding to them may be beneficial in developing therapies aimed at limiting metastatic spread.

In summary, the TME is a regionally and temporally dynamic entity that changes as the tumor grows and continues to select for more aggressive phenotypes (Rohani et al. 2019; Damaghi and Gillies 2017; Moellering et al. 2008). The complexity is further increased by the formation of specific but dynamic niches, both within the

primary tumor and at secondary tumor sites. Understanding the mechanisms involved in the cross-talk between cancer cells, CSCs and other cells of the TME, will aid in designing promising therapies targeting these critical steps of cancer progression. By both sensing and shaping the TME, ion transporters expressed in these cell types are particularly important players in the cross-talk and therefore highly interesting potential therapeutic targets. Their regulation and roles are the topic of the following section.

## 3 Regulation of Transport Proteins by the TME

In this section, we discuss how specific transport proteins with key roles in tumor development are regulated by TME parameters, i.e., acidosis, lactate, hypoxia, tumor-derived cytokines, and ECM. We focus on transporters assigned important roles in transport of acid equivalents and/or lactate in cancer. These comprise mainly $Na^+/H^+$ exchangers, especially NHE1 (SLC9A1), $Na^+/HCO_3^-$ transporters, monocarboxylate transporters MCT1 and MCT4, and the vacuolar $H^+$ ATPases (V-ATPases of the ATP6 family). A brief section on how TME parameters regulate other key transporters of importance for cancer development is provided in Sect. 3.5.

Analysis of patient tumor data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (*GTEx*) databases shows that mRNA levels of different pH regulatory transporters are upregulated in different tumor types, NHE1 in esophageal cancer and PDAC, NBCn1 in acute myeloid leukemia (AML) and PDAC, and V-ATPases in PDAC and melanoma, while MCT4 is strongly upregulated in most human tumors (Fig. 2). mRNA levels do not necessarily translate to increased protein levels, let alone activity. However, this expression pattern is in congruence with the notion that increased net acid extrusion capacity can be supported by many different transporters, whereas lactate flux is specifically dependent on the MCTs, which may therefore be expected to be more ubiquitously upregulated. There are also examples of regulation of specific transporters by oncogenic signaling events, such as the upregulation of NBCn1 by the breast cancer oncogene human epidermal growth factor receptor 2 (HER2) (Gorbatenko et al. 2014; Lauritzen et al. 2010) and by constitutively active HRAS (Ramirez et al. 2019). Another interesting example is CAIX, which, although not a transporter per se, is very important for pH regulation in cancer cells and which is upregulated in PDAC by the combination of KRAS and HIF-1 signaling (McDonald et al. 2019).

### *3.1 $Na^+/H^+$ Exchangers*

The ubiquitously expressed $Na^+/H^+$ exchanger NHE1 (SLC9A1) is highly expressed in many cancer cells as well as in patient tissue from some cancers (Andersen et al. 2018a) (Fig. 2). Early studies demonstrated a key role of NHE1 in tumor

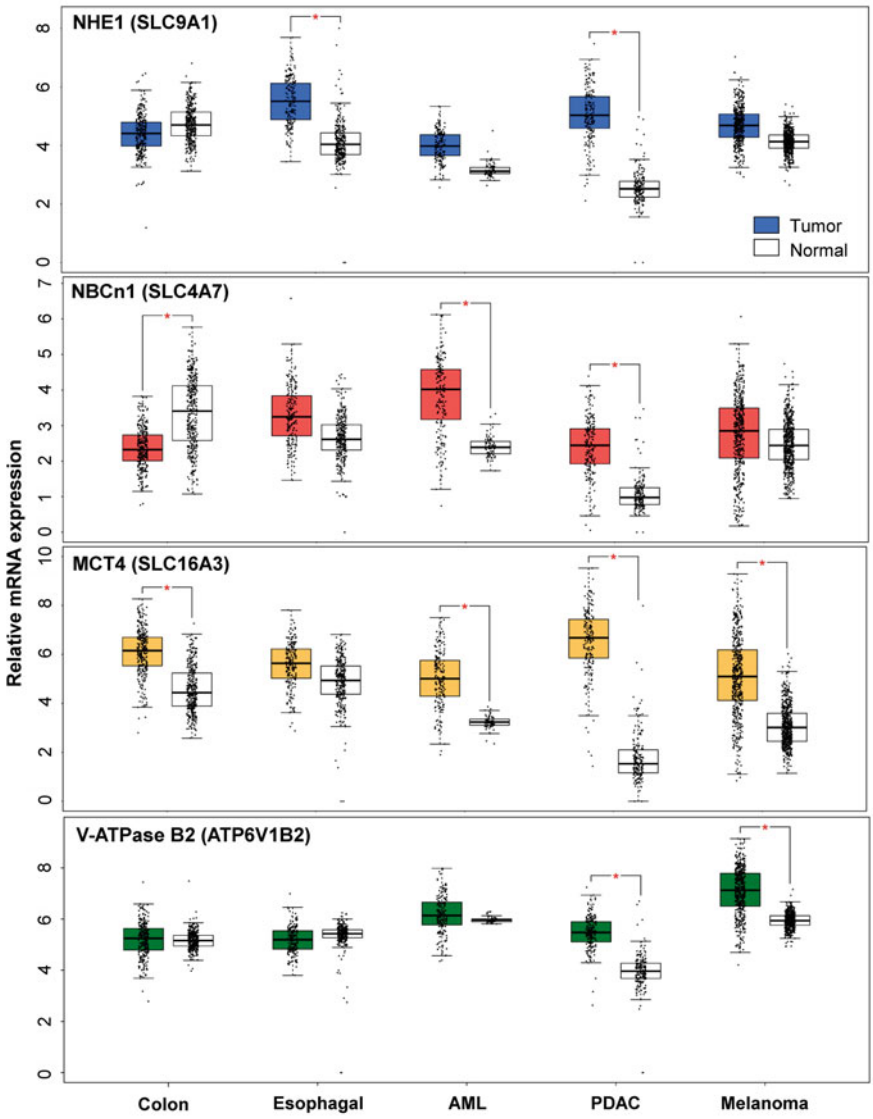


**Fig. 2** mRNA expression of NHE1, NBCn1, MCT4, and V-ATPases in patient cancer tissue. mRNA expression data for NHE1 (SLC9A1), NBCn1 (SLC4A7), MCT4 (SLC16A3), and the V-ATPase (using expression of the ubiquitous subunit ATP6V1B2 as representative of V-ATPase expression) in selected cancers was analyzed using the Gene Expression Profiling Interactive Analysis (GEPIA) server (http://gepia.cancer-pku.cn/) (Tang et al. 2017). Cancer data are from The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/tcga) (McLendon et al. 2008) and normal control values from TCGA and The Genotype-Tissue Expression (GTEx) project (Lonsdale et al. 2013), in order to ensure a balanced number of tumor and control samples for statistical validation (Tang et al. 2017). To minimize differences resulting from distinct sources, the GEPIA

development and metastasis in vivo, and numerous studies have addressed its importance for cancer cell growth, survival, and invasiveness (Rotin et al. 1989; Lagarde et al. 1988; Stock and Pedersen 2017). NHE1 also appears to contribute to chemotherapy resistance, as its pharmacological inhibition or knockdown sensitizes cancer cells to chemotherapeutics such as cisplatin and doxorubicin (Lauritzen et al. 2010; Miraglia et al. 2005). Although both studies were done in a buffered 2D growth setting, it has been demonstrated that even under such conditions, NHE1 activity plays an important role in regulating the pericellular pH in cancer cells (Stüwe et al. 2007; Lauritzen et al. 2012). The role of NHE1 may therefore at least in part reflect its important contribution to creating and maintaining an acidic pericellular TME, where protonation of weak base drugs and chemotherapeutics prevents their entrance into the cell, reducing their therapeutic effect (Raghunand and Gillies 2000) (Fig. 3a). Essentially all studies relating $Na^+/H^+$ exchangers to cancer involve NHE1. For a few studies on other isoforms, predominantly NHE6, see Pedersen and Counillon (2019).

Importantly, the role of NHE1 in the TME is not limited to the cancer cells themselves, as its activity is also very important for the function of immune cells (Shi et al. 2013), including tumor microglia (Zhu et al. 2016), as well as for endothelial cell function (Pedersen et al. 2017a). However, the knowledge on the specific roles of NHE1 in these and other stromal cells in a tumor setting remains minimal, highlighting the need for further research in this area. For instance, while roles of NHE1 in TAMs, as demonstrated in recent elegant studies of gliomas (Guan et al. 2018), support the therapeutic relevance of targeting this transporter, key roles of NHE1 in anticancer immunity could render its inhibition detrimental in some types of cancers and should be carefully evaluated.

In response to *hypoxia*, NHE1 is downregulated in various cancer cell lines (Hulikova et al. 2013), as is its expression in endothelial cells (Pedersen et al. 2017a). In contrast, NHE1 expression in pulmonary artery smooth muscle cells was increased by hypoxia in a HIF-1α-dependent manner (Shimoda et al. 2006). Interestingly, this apparent discrepancy may reflect a time dependency of regulation, as in astrocytes, NHE1 expression and activity were initially decreased, and subsequently increased, by $CoCl_2$ treatment to increase HIF-1α expression (Wang et al. 2016).

*Extracellular acidosis* acutely reduces NHE1-mediated acid extrusion by competition of $H^+$ with $Na^+$ for extracellular transport site binding. In contrast, an accompanying reduction in $pH_i$ conversely will favor increased NHE1 activity and

**Fig. 2** (continued) platform recomputes TCGA and GTEx gene expression data from raw RNA-Seq data by the UCSC Xena project (http://xena.ucsc.edu/) based on a uniform pipeline, and the choice of control values is based on consultation with medical experts (Tang et al. 2017). AML, acute myeloid leukemia. The y-axis of the boxplots shows $\log_2$ (transcript count per million (TPM) + 1) of tumor relative to normal expression levels. Statistical analysis was performed as one-way ANOVA, with a *p*-value cutoff of 0.01 (*). Colored boxes, tumor; open boxes, control tissue

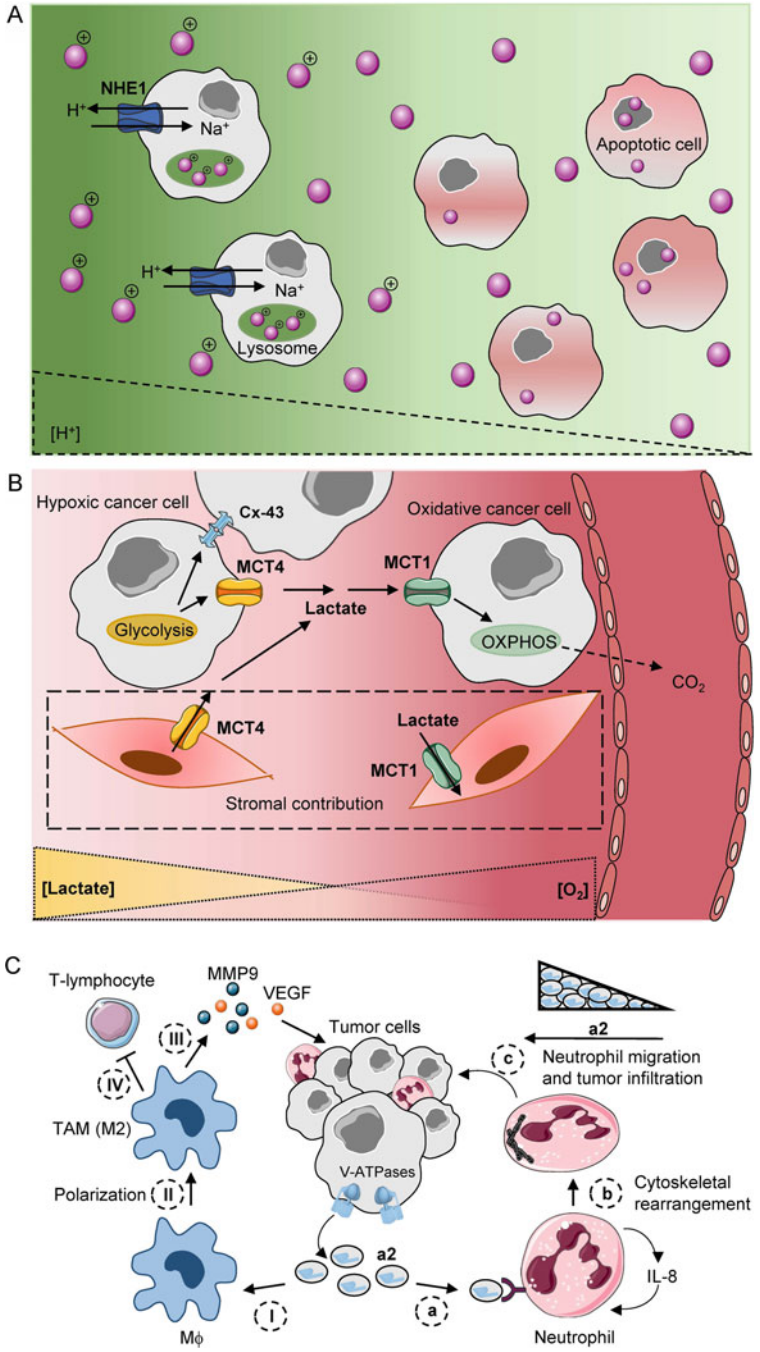


**Fig. 3** Examples of the interplay between transporters and the tumor microenvironment. (**a**) Acidosis contributes to drug resistance. At low pH, weak base chemotherapeutics and drugs (purple spheres) become protonated, hindering their entrance into the cell or causing their accumulation in acidic intracellular compartments, preventing therapeutic efficacy. In a more alkaline environment,

expression; thus net activity is a balance between the effects of $pH_e$ and $pH_i$ under these conditions (Pedersen and Counillon 2019). Therefore, since in tumor cells, $pH_i$ is often near-neutral or even alkaline despite the acidic $pH_e$, NHE1 may in fact not be the most consistent means of acid extrusion, especially in conjunction with the inhibition by hypoxia mentioned above (for a discussion, see Bonde and Boedtkjer (2017)).

Several *cytokines* present in the TME regulate NHE1 activity, including IFN-γ and tumor necrosis factor α (TNF-α) which activated NHE1 in astrocytes (Benos et al. 1994), as well as IL-6 which was shown to activate NHE1 in several human hepatocellular carcinoma cell lines (Xu et al. 2016). NHE1 protein expression was also significantly upregulated in prostaglandin-receptor-positive ($PR^+$) breast cancers (Andersen et al. 2018a). Although the mechanism is unknown, this may suggest the regulation of NHE1 by prostaglandins, which are also abundant in the TME (Kobayashi et al. 2018).

Also mechanical stimuli such as cell-matrix adhesion can activate NHE1 (Schwartz et al. 1991), suggesting that the increased *ECM stiffness* in the TME compared to normal tissues may contribute to the high NHE1 activity in tumors. As such, it is tempting to speculate that by regulating the cellular response to, e.g., integrin-mediated adhesion (Tominaga and Barber 1998), NHE1 may act as a transducer of mechanical signals from the ECM in tumor cells. Finally, NHE1, as well as several other members of the NHE family, are highly sensitive to osmotic cell volume perturbations (Pedersen and Counillon 2019). While the role of osmolarity in regulation of NHE1 in tumors has, to our knowledge, not been studied, it is interesting that increased extracellular osmolarity was shown to favor EMT and invasion of glioblastoma cells, in a manner associated with increased matrix degradation (Pu et al. 2020), a condition also supported by NHE1 activity (Greco et al. 2014).

**Fig. 3** (continued) weak base drugs will have a neutral charge, allowing entrance into the cancer cells. (**b**) Lactate shuttling between oxidative and hypoxic cells via MCTs or gap junctions. Lactate produced by hypoxic cancer cells as end product of their glycolytic metabolism is extruded to the TME through MCT4. This lactate can be taken up by oxidative cancer cells, probably predominantly via MCT1, and used to fuel oxidative phosphorylation. At least in some cancers, lactate may also be trafficked intracellularly from cancer cells to cancer cell via gap junctions. Stromal cells, such as CAFs, can likewise fuel this shuttle with lactate by extruding lactate via MCT4. Stromal cells can also use lactate as an energy source instead of glucose, leaving more glucose available for the cancer cells. (**c**) The N-terminal peptide of the a2 subunit of the V-ATPase (a2) regulates the immune cell composition of the TME. Hatched circles marked **a**–**c**: a2 peptide is secreted in microvesicles from tumor cells and stimulates neutrophil migration through autocrine IL-8 secretion. The a2 peptides can function as a chemo-attractant and recruit tumor-promoting neutrophils. Hatched circles marked I–IV: The a2 peptide can cause polarization of macrophages into the pro-tumorigenic M2 subtype and can facilitate immunosuppression, invasion, and angiogenesis through secretion of factors such as MMP9 and VEGF. See text for details. Abbreviations: connexin-43 (Cx-43), monocarboxylate transporter 4 (MCT4), monocarboxylate transporter 1 (MCT1), tumor microenvironment (TME), matrix metalloproteinase 9 (MMP9), vascular endothelial growth factor (VEGF)

### 3.2 $Na^+/HCO_3^-$ Cotransporters

Several $Na^+/HCO_3^-$ cotransporters have been linked with cancer development (Boedtkjer 2019). Most evidence is available for NBCn1 (SLC4A7), which is upregulated in tumor tissue from several cancer types (Andersen et al. 2018a; Boedtkjer et al. 2013) (Fig. 2) and has been shown to contribute to primary tumor growth in vivo using both whole animal knockout and xenograft models (Andersen et al. 2018a; Lee et al. 2016, 2018). It is also interesting to note that a single-nucleotide polymorphism (SNP) in the 3′ end of NBCn1 has been linked with breast cancer susceptibility in a genome-wide association study (GWAS) (Ahmed et al. 2009), although its relevance if any for NBCn1 regulation is unclear, as its reversion did not alter NBCn1 promoter activity (Gorbatenko et al. 2016). There are also notable exceptions, for instance, NBCn1 is downregulated in colon cancer tissue (Fig. 2). For other $Na^+/HCO_3^-$ transporters of the SLC4 family, the picture is frequently more complicated. For instance, NBCe1 (SLC4A4) seems to be either up- or downregulated depending on the cancer type (Boedtkjer 2019).

The roles of $Na^+/HCO_3^-$ cotransporters in the stromal cells in a tumor setting are essentially unstudied to date and will be important to address using orthotopic syngeneic in vivo models. It is, however, interesting to note that NBCn1 was strongly upregulated in M2 vs M1 macrophages (Martinez et al. 2006) and that a key role of NBCn1 in macrophage phagosomal acidification was recently reported (Sedlyarov et al. 2018). NBCn1 also plays important roles in maintaining endothelial cell $pH_i$ (Boedtkjer et al. 2011), but again, to our knowledge, this has yet to be studied in a tumor context.

*Hypoxia* has complex and perhaps tissue-dependent effects on $Na^+/HCO_3^-$ transporters. Thus, hypoxia inhibited NBCn1 expression in mouse brain (Chen et al. 2007), whereas $Na^+/HCO_3^-$-dependent net acid extrusion was unaffected by 48 h hypoxia in several cancer cell lines (Hulikova et al. 2013). Conversely, SLC4A4 and SLC4A9 expression was reported to be stimulated by HIFs in various cancer cells, and their downregulation or inhibition reduced cancer cell growth (McIntyre et al. 2016; Parks and Pouyssegur 2015).

NBCn1 activity is partially inhibited by *extracellular acidosis*, but not as extensively as is NHE1 (Bonde and Boedtkjer 2017). On the other hand, NBCn1 expression is strongly increased upon acid adaption of cancer cells to (partially) mimic the TME (Yang and Loh 2019), consistent with its upregulation by chronic metabolic acidosis in rat kidney (Kwon et al. 2002).

***Cytokines and Growth Factors*** As noted above, NBCn1 expression is increased by HER2 signaling via Krüppel-like factor 4 (Gorbatenko et al. 2014), and NBCn1 expression correlates with HER2 expression level in patient tissues (Andersen et al. 2018a) and was recently shown to be upregulated by constitutively active HRAS (Ramirez et al. 2019). Similar to NHE1, NBCn1 was upregulated in $PR^+$ breast cancers (Andersen et al. 2018a) although the mechanism remains unknown.

### 3.3 Monocarboxylate Transporters

The monocarboxylate transporters MCT1 (SLC16A1) and MCT4 (SLC16A3) mediate 1:1 transport of lactate and $H^+$ across the plasma membrane of most cancer cells and are of key importance for maintenance of lactate homeostasis. While the direction of transport is dictated by thermodynamics, MCT1 has high lactate affinity, whereas MCT4 is a low-affinity transporter and will thus only be efficient at high lactate concentrations. Accordingly, MCT4 is mainly expressed in glycolytic cancer and stromal cells (CAFs), whereas MCT1 is more widely expressed in oxidative cancer cells (Payen et al. 2019). On the other hand, some cancer cell types rely on only or primarily one of these isoforms. For instance, MDA-MB-231 breast cancer cells do not express MCT1 (see Andersen et al. (2018a)), and in glioblastoma stem cells, only MCT1 expression was significantly increased compared to that in normal glial cells, and its inhibition caused marked reduction in their proliferation and sphere formation efficiency (Takada et al. 2016). As noted above, lactate extruded by glycolytic cancer or stromal cells via MCT4 is shuttled through MCT1 into the oxidative cancer or stromal cells, which use the lactate to fuel the TCA cycle (Whitaker-Menezes et al. 2011; Sonveaux et al. 2008) (see Fig. 3b). Due to this metabolic coupling, lactate has been suggested to be the preferred oxidative fuel for oxygenated cancer cells compared to glucose, in turn leaving more glucose available for the hypoxic cancer cells (Sonveaux et al. 2008). This offers several advantages for the oxidative cancer cells (Sonveaux et al. 2008). Firstly, the cells save the initial energy required to generate lactate from glucose and thus produce 18 ATP per lactate molecule consumed (Sonveaux et al. 2008). Second, the cells spare energy for synthesis and maintenance of glycolytic enzymes and phosphorylation of glycolysis intermediates (Payen et al. 2019). Third, oxidation of lactate to pyruvate catalyzed by LDH-1 generates energy-rich NADH which can be used in the electron transport chain. As a fourth advantage for the oxidative cancer cells, the physical interaction of LDH-1 with the V-ATPase at the lysosomal membrane facilitates lysosomal acidification by directly supplying the V-ATPase with $H^+$ (Payen et al. 2019). Finally, intracellular lactate signaling following lactate uptake by MCT1 has been shown to promote glutamine uptake and metabolism in oxidative cancer cells by supporting c-MYC-driven upregulation of ASCT2 expression. Besides facilitating glutamine uptake, this could potentially also contribute to redox homeostasis through its importance for the function of xCT (Pérez-Escuredo et al. 2016) (Sect. 3.5). Hence, there seem to be multiple benefits from using lactate as oxidative fuel, even in well-perfused tumor areas. Finally, a transport-independent role of MCT1 in invasion, involving MCT1-dependent NF-κB activation, was also recently shown (Payen et al. 2017), although the general importance of this phenomenon remains to be seen.

The roles of MCT1 and MCT4 have thus been studied very extensively in cancer cells and CAFs. Much less is known about their roles in other stromal cells, but it is important to note that both MCT1 (Murray et al. 2005) and MCT4 (Tan et al. 2015) are important for immune cell function and that both transporters are highly

expressed in differentiated adipocytes and important for their metabolic function (Petersen et al. 2017).

MCT4 is upregulated by *hypoxia* through direct HIF-1α binding to the MCT4 promoter (Ullah et al. 2006). MCT1, on the other hand, appears to be regulated through a distinct mechanism, in which it is upregulated by hypoxia in p53-deficient cancer cells, yet repressed in cells expressing wild-type p53, through a direct binding of p53 to the *MCT1* gene promoter. Confirming its relevance in vivo, the same pattern was found in xenograft models and patient tissue (Boidot et al. 2012).

To our knowledge, no reports to date have directly investigated the impact of short- and long-term *extracellular acidosis* on MCT1 and MCT4 expression and function in tumors. In kidney, MCT1 mRNA expression was shown to be downregulated in a 2- and 7-day mouse model of metabolic acidosis (Becker et al. 2010). However, mechanistic insight is lacking, and MCT regulation may be very different in kidney vs tumors. As discussed above (Sect. 2.2.3), from a thermodynamic standpoint, acute extracellular acidosis will reduce the driving force for MCT-mediated lactate efflux because of the obligatory 1:1 cotransport with protons. It may be noted that this will be exacerbated at alkaline $pH_i$, which would thus be unfavorable for cancer cells in terms of getting rid of lactate, especially at high extracellular lactate concentrations.

Expression of both MCT1 and MCT4 is regulated by *lactate* through GPR81, which is expressed in various cancer cell lines including breast, lung, hepatocellular, and PDAC as well as in tumor samples from PDAC patients (Roland et al. 2014) (see also Sect. 2.2.3).

The tight functional relationship between MCT1 and MCT4 and the membrane glycoprotein CD147/basigin may link these transporters to the mechanical properties of the TME. CD147 expression predicts poor prognosis in many cancers (Xin et al. 2016). This is often ascribed to its key roles in CD44-hyaluronan regulation and ECM degradation; however, CD147 is also an essential chaperone for MCT expression and plasma membrane localization and links these transporters to CAIX (Ames et al. 2019). Notably, the relation is reciprocal as CD147 protein expression is also dependent on MCT expression (Le Floch et al. 2011; Andersen et al. 2018b). This suggests that in addition to their transport activity, the MCTs may impact regulation of ECM architecture through their physical interaction with CD147.

## *3.4 Vacuolar $H^+$-ATPases*

With the exception of a few specialized cell types where they are expressed at the plasma membrane, V-ATPases (ATP6 family pumps) are primarily expressed in the endosomes and lysosomes of normal cells, where they ensure the acidic pH required for the normal function of these compartments (Stransky et al. 2016). Various V-ATPase subunits are upregulated in several cancer types, in some cases leading to their translocation to the plasma membrane where they can play a role in facilitating invasiveness (McGuire et al. 2019; Nishisho et al. 2011). On the other

hand, in PDAC, V-ATPase transport activity at the plasma membrane is not detectable, and knockdown of the a3 subunit resulted in upregulation of migration and invasion in vitro (Flinck et al. 2020). Hence, the roles of V-ATPases in invasiveness remain poorly understood, likely complicated by the many possible subunit isoform combinations and frequent use of pharmacological tools that do not allow distinction between these isoforms (bafilomycin, concanamycin A), or even between V-ATPases and gastric $H^+/K^+$ ATPases (omeprazole-type compounds). It seems likely that a key role of V-ATPases in cancer is their involvement in macropinocytosis and autophagy and hence in nutrient scavenging (Ramirez et al. 2019), as well as in signaling processes such as regulation of mammalian target of rapamycin (mTOR)-dependent nutrient sensing, HER2, Wnt, and Notch signaling (Stransky et al. 2016; Lawrence and Zoncu 2019).

V-ATPase activity is, to a large extent, regulated by reversible assembly and disassembly of the $V_0$ and $V_1$ subunits, or by trafficking to and from the plasma membrane. Stimuli that increase V-ATPase assembly have been shown to do so via ERK and PI3K signaling (Marjuki et al. 2011; Sautin et al. 2005), or by EGF receptor ligands (Xu et al. 2012) or amino acid (AA) starvation (Stransky and Forgac 2015), conditions associated with the TME.

The effect of *hypoxia* on V-ATPase expression is largely unstudied; however it has been suggested that hypoxia can indirectly regulate V-ATPase transcription through downregulation of mTOR, which causes reduced activation of transcription factor EB (TFEB), a major regulator of V-ATPase expression (Hong et al. 2019). Conversely, genetic disruption of the V-ATPase and two of its assembly factors stabilizes HIF-1α under aerobic conditions by impairing its hydroxylation and proteasomal degradation (Miles et al. 2017; Hayek et al. 2019).

*Extracellular acidosis* was found to induce V-ATPase translocation to the plasma membrane of breast cancer cells, the extent of which correlated with invasiveness (Sennoune et al. 2004). Similarly, the V-ATPase activity at the plasma membrane was elevated in highly metastatic breast cancer cells, compared to poorly metastatic breast cancers (Sennoune et al. 2004). As cAMP regulates membrane trafficking of the V-ATPase in some cell types (Pastor-Soler et al. 2008), it may be speculated that adenylate cyclase-linked $H^+$-sensing receptors such as G-protein-coupled receptor 4 (GPR4) and ovarian cancer G-protein-coupled receptor 1 (OGR1; GPR68) (Glitsch 2011) could play a role in this response by increasing cellular cAMP. However, to our knowledge, this remains to be addressed, and it is not intuitively logical that a signal of extracellular acidity would activate the insertion of a proton pump – creating further extracellular acidosis – in the plasma membrane.

To our knowledge, there are no reports of regulation of V-ATPases by either lactate or by ECM stiffness. Interestingly however, knockdown of the a2 subunit of the V-ATPase was shown to reduce mechanical stiffness of mammary tumor ECM (Katara et al. 2018), suggesting that V-ATPases may, conversely, contribute to matrix stiffness in some cancers.

Interestingly, V-ATPases may play key roles in the interplay between tumor and immune cells in tumors. In several recent studies, the a2 subunit of the V-ATPase, which is uniquely expressed at tumor cell membranes, has been shown to regulate

the specific immune cell composition of the TME. The interplay between immune cells and the V-ATPase is facilitated by secretion of an N-terminal peptide of a2 into microvesicles. These vesicles have been reported to stimulate IL-8 production in neutrophils resulting in neutrophil migration and infiltration into the tumor (Ibrahim et al. 2016) (Fig. 3a-c). Furthermore, the a2 peptide induces polarization of macrophages toward a pro-tumorigenic subtype, which promotes cancer progression (Katara et al. 2016) (Fig. 3c, I-IV). Accordingly and strikingly, knockout studies revealed a decrease in helper and cytotoxic T-cell populations in tumors lacking a2 (Sahoo et al. 2018). Thus, it appears that also V-ATPases may play unconventional roles in the tumor microenvironment that differ from their roles as ion transporters.

## *3.5 Other Transport Proteins Regulated by the TME*

In addition to the above-described acid-base and lactate transporters, numerous other transporters that play essential roles in cancer development are also regulated by the physicochemical properties of the TME, and a few examples will be provided below. We focus here on transporters, but similar regulatory interactions with properties of the TME pertain to many ion channels (for references, see, e.g., Glitsch (2011) and Pedersen et al. (2017b), and the review in this volume by Halima Ouadid-Ahidouch and colleagues).

### 3.5.1 Drug Efflux Transporters Are Regulated by Hypoxia and Lactate

Several ATP-binding cassette (ABC) family transporters are regulated by hypoxia, including ABCB1 (multidrug resistance protein 1, MDR1), which is associated with resistance to doxorubicin, paclitaxel, and vincristine, as well as the two drug efflux pumps, ABCG2 and ABCC1, which have been implicated in chemotherapy resistance (Schoning et al. 2017). In fact, a strong correlation between HIFαs and expression of these transporters has been found in both human cancer samples and cancer cell lines (Schoning et al. 2017). Adding another dimension to the importance of lactate for tumor progression, ABCB1 was also recently shown to be upregulated by lactate acting as a ligand for GPR81 (Wagner et al. 2017).

### 3.5.2 Nutrient Transporters Maintain Redox Homeostasis and Are Regulated by Hypoxia

Nutrient transporters, which are often upregulated in human cancers, can play a dual role in supporting tumor growth by fueling the increased biosynthetic demand of the tumor cells while at the same time aiding in maintaining redox homeostasis (Koppula et al. 2018). This is often facilitated by increasing the production of key antioxidants, such as GSH. An important transporter in this regard is the xCT, which

promotes cystine uptake, and subsequent biosynthesis of GSH, resulting in protection from oxidative stress (Koppula et al. 2018). This transporter is overexpressed in several human cancers including non-small-cell lung cancer, breast cancer, and liver cancer (Lim et al. 2019), and its role in protection against oxidative stress was recently shown to support RAS-induced transformation and tumorigenicity (Lim et al. 2019). Another transporter involved in AA homeostasis and redox state is ASCT2, which through its import of glutamine impacts the activity of other AA transporters such as xCT and L-type amino acid transporter (LAT1), why this set of transporters is often highly upregulated in cancer (Vučetić et al. 2017).

Several nutrient transporters with key roles in cancer metabolism, such as glucose or AA transporters, are regulated by hypoxia (Parks et al. 2017). The best studied of these is the glucose transporter GLUT1, which is regulated by HIF-1 (Chen et al. 2001). Recently, the sodium-coupled neutral amino acid transporter SNAT2 (SLC38A2) was also found to be upregulated by hypoxia in breast cancer, primarily through HIF-1α, in both in vitro and in vivo xenografts. Finally, in estrogen-receptor-positive breast cancer cells, SNAT2 induction by hypoxia was shown to contribute to resistance to antihormonal therapy (Morotti et al. 2019).

### 3.5.3 Proton-Coupled Folate Transporters Are Thermodynamically Driven by Extracellular Acidosis

Tumor cells, like normal cells, require folate for biosynthesis of essential macromolecules, yet folates cannot be synthesized de novo by mammalian cells. Folate uptake in humans is mediated by two types of transporters, the reduced folate carrier (RFC, SLC19A1) and the proton-coupled folate transporter (PCFT, SLC46A1). In the latter, the proton gradient provides the driving force for folate transport, with an assumed stoichiometry of two protons per folate (Matherly et al. 2018; Hou and Matherly 2014). Notably, while SLC19A1 has a neutral pH optimum, SLC46A1 exhibits a pH optimum of pH 5–5.5, rendering it highly efficient at the extracellular acidity of the TME (Matherly et al. 2018; Hou and Matherly 2014). In this manner, the acidic TME thus favors tumor growth by facilitating SLC46A1-mediated uptake of an essential nutrient. SLC46A1 is accordingly expressed at high levels in many tumors (Kugel Desmoulin et al. 2011). Notably, this provides an interesting therapeutic possibility, as the transporter is an effective uptake pathway for antifolate drugs such as pemetrexed, used to treat many types of cancer (Matherly et al. 2018).

## 4 Conclusions and Perspectives

Transport proteins play a particularly important role in tumor development by serving dual roles as sensors and modulators of the TME and the intracellular milieu in both cancer and stromal cells. Here, we have discussed how TME properties regulate ion transporters of major importance for cancer development, and how some

of these in turn shape the TME. While the importance of acid and lactate extrusion from cancer cells for cancer development is now generally accepted, the transporters which mediate these fluxes remain understudied as potential drug targets in cancer (Cesar-Razquin et al. 2015). Much remains to be understood about the regulation and roles of individual acid-base transporters in the diverse niches within the TME, in different stromal and cancer cell types, and in various stages of tumor progression, such as in the (pre)metastatic niche or during regrowth. Dissecting these questions will be important for assessing the potential of, and the best strategies for, targeting these transporters in anticancer therapy.

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# Ion Channels, Transporters, and Sensors Interact with the Acidic Tumor Microenvironment to Modify Cancer Progression


**Ebbe Boedtkjer**

## Contents



**Abstract** Solid tumors, including breast carcinomas, are heterogeneous but typically characterized by elevated cellular turnover and metabolism, diffusion limitations based on the complex tumor architecture, and abnormal intra- and extracellular ion compositions particularly as regards acid-base equivalents. Carcinogenesis-related alterations in expression and function of ion channels and transporters, cellular energy levels, and organellar $H^+$ sequestration further modify the acid-base composition within tumors and influence cancer cell functions, including cell

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proliferation, migration, and survival. Cancer cells defend their cytosolic pH and $HCO_3^-$ concentrations better than normal cells when challenged with the marked deviations in extracellular $H^+$, $HCO_3^-$, and lactate concentrations typical of the tumor microenvironment. Ionic gradients determine the driving forces for ion transporters and channels and influence the membrane potential. Cancer and stromal cells also sense abnormal ion concentrations via intra- and extracellular receptors that modify cancer progression and prognosis. With emphasis on breast cancer, the current review first addresses the altered ion composition and the changes in expression and functional activity of ion channels and transporters in solid cancer tissue. It then discusses how ion channels, transporters, and cellular sensors under influence of the acidic tumor microenvironment shape cancer development and progression and affect the potential of cancer therapies.

**Keywords** Anion exchangers · Bicarbonate · Breast cancer · Carbonic anhydrases · G-protein coupled $H^+$ receptors · Ion carriers · Lactate · Monocarboxylate transporters · $Na^+$,$HCO_3^-$-cotransporters · $Na^+/H^+$-exchangers

## Abbreviations

| | |
|---|---|
| CA | Carbonic anhydrase |
| CHP | Calcineurin B homologous protein |
| EIPA | 5-(*N*-ethyl-*N*-isopropyl)-amiloride |
| PD-L1 | Programmed cell death 1 ligand 1 |
| $pH_i$ | Intracellular pH |
| $pH_o$ | Extracellular pH |
| PMCA | Plasma membrane $Ca^{2+}$ ATPase |
| RPTPγ | Receptor protein tyrosine phosphatase γ |
| SERCA | Sarcoplasmic endoplasmic reticulum $Ca^{2+}$ ATPase |
| TNF | Tumor necrosis factor |
| TRP | Transient receptor potential |
| VEGF | Vascular endothelial growth factor |
| WNK | With-no-lysine |

## 1 Introduction

In solid cancer tissue, regions of high cellular density alternate with sparsely packed stroma, infiltrating immune cells, and an irregular vascular network. This tissue architecture creates local heterogeneity in diffusion capacity, which disturbs the penetration of pharmacological agents into the tumor tissue (Rejniak et al. 2013) and contributes to the depletion of nutrients and accumulation of metabolic waste products in the tumor microenvironment (Sullivan et al. 2019). The ionic

composition of the interstitial fluid in solid cancer tissue differs drastically from that in corresponding normal tissue; e.g., with tumor extracellular pH ($pH_o$) ranging spatially and temporally between 6.5 and 7.6 (Vaupel et al. 1989; Rohani et al. 2019; Helmlinger et al. 1997; Chiche et al. 2010), the interstitial concentration of $K^+$ reaching up to 30 mM (Tan et al. 2020; Eil et al. 2016), and the extracellular level of ATP elevated to the hundreds micromolar range (Pellegatti et al. 2008). Such dramatic deviations in the chemical environment have a multitude of functional consequences with potential to modify disease progression, therapeutic efficacy, and development of treatment resistance.

In addition to specializations and adaptations in the cancer cells – influencing the expression and function of ion channels and transporters, intermediary metabolism, and cellular turnover – variability in the density, morphology, and function of intra- and extratumoral blood vessels affects the composition of the tumor microenvironment (Vaupel et al. 1989; Voss et al. 2019; Froelunde et al. 2018; Castañeda-Gill and Vishwanatha 2016). Although tumor blood flow is inadequate overall, the heterogeneity of the tumor vasculature induces marked regional variation in perfusion relative to the metabolic demand. The atypical blood vessels found within tumors combine characteristics of arterioles, capillaries, and venules; and areas of vessel wall discontinuity favor fluid filtration (Castañeda-Gill and Vishwanatha 2016; Dass and Choong 2008). The extratumoral feed arteries that supply breast carcinomas (Fig. 1) are thin-walled and functionally specialized toward enhanced tumor blood flow, showing depressed vasocontraction in response to sympathetic nerve activity and during influences from vasoactive signaling molecules (Froelunde et al. 2018).

## 2 $H^+$

The acidity of the extracellular tumor microenvironment has been documented for several decades and across many types of cancer (Vaupel et al. 1989). The $pH_o$ frequently reaches as low as 6.5 in cancer tissue, which corresponds to an approximate 10-fold increase in free $H^+$ concentration compared to normal tissue. Thus, the relative magnitude of the $H^+$ dynamics in cancer tissue is exceptional compared to most other pathophysiological disturbances influencing ion concentrations in bodily fluids. As discussed in detail below, we now increasingly understand the molecular mechanisms that establish the low $pH_o$ in breast carcinomas; and we are starting to appreciate the integrated consequences of the acidic environment for the malignant behavior of cancer cells and its impact on neighboring normal cells including those of the immune system (Boedtkjer and Pedersen 2020).

Net elimination of intracellular acid occurs through transporters that directly move $H^+$ across the plasma membrane or transfer other acid-base equivalents – such as $HCO_3^-$ or lactate – that are in chemical equilibrium with $H^+$ in the cytoplasm and extracellular space (Fig. 1). In cancer tissue, intracellular pH ($pH_i$) is frequently equal to or higher than $pH_o$ (Lee et al. 2015; Robey et al. 2009; Gallagher et al. 2008), and due to the inside-negative membrane potential, net elimination of $H^+$

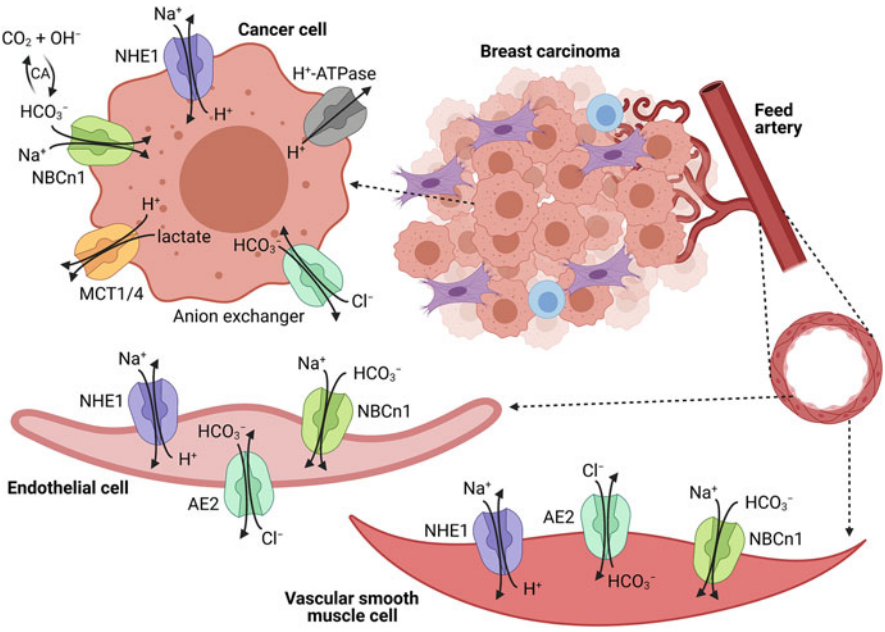


**Fig. 1** Schematic of acid-base transporters in the plasma membrane of breast cancer cells and in the wall of resistance arteries. For details, see the Sects. 2.1, 2.4, 3.1, 3.3, and 5.3. The most typical isoforms of the individual transporters are noted; but in some cases, other isoforms may also play a role. The figure was created with Biorender.com. *CA* carbonic anhydrase

from cancer cells requires energy input via primary or secondary active transporters that have implications for tumor growth (Boedtkjer and Pedersen 2020; Boedtkjer 2019; Andersen et al. 2018). In addition to transport across the plasma membrane, intracellular $H^+$ sequestration occurs via transporters in organelle membranes. Breast cancer cells are better protected against intracellular acidification than normal breast epithelial cells (Lee et al. 2015, 2016a); but due to the low $pH_o$ in solid cancer tissue, $pH_i$ of cancer cells can be below levels found in normal tissue where $pH_o$ is much higher. Under extreme levels of extracellular acidification, cancer cells can likely reach a $pH_i$ so low that it critically impedes, for instance, cell metabolism, proliferation, and survival.

## 2.1 $H^+$ Transporters in the Plasma Membrane

$Na^+/H^+$-exchangers expressed in the plasma membrane (Fig. 1) have been widely implicated in breast malignancies (Amith and Fliegel 2017; Lauritzen et al. 2010; Boedtkjer et al. 2012a). Protein expression of the ubiquitous $Na^+/H^+$-exchanger NHE1 (SLC9A1) varies between cancer models (Hulikova et al. 2013), but is generally maintained or moderately upregulated in human and murine breast cancer

tissue compared to normal breast tissue (Lee et al. 2015, 2016a; Boedtkjer et al. 2013a).

Especially the preferential expression of NHE1 in invadopodia plays a potentially crucial role in invasiveness of cancer cells by promoting acid-induced extracellular matrix degradation (Busco et al. 2010; Beaty et al. 2014). NHE1 also promotes directional migration of cultured cancer cells by controlling the cell surface pH and local cytoplasmic pH differentially at the leading and rear ends of migrating cells, thereby modifying integrin-dependent cell–matrix interactions and cytoskeletal dynamics (Stock et al. 2005, ; 2007; Grinstein et al. 1993; Bravo-Cordero et al. 2013; Martin et al. 2011). It should be noted that the pH of microdomains in proximity to the intra- and extracellular aspects of ion transporters depends not solely on acid-base transporter activity but also on the local effective $H^+$ diffusion, which in turn is influenced by the accessibility for mobile $H^+$ buffers (Villafuerte et al. 2014) and the local carbonic anhydrase activity (Boedtkjer et al. 2016a; Boron 2010).

NHE1 inhibition can limit cancer cell proliferation and enhance programmed cell death (Lauritzen et al. 2010; Friday et al. 2007; Kumar et al. 2009a; Reshkin et al. 2003). However, as the mitogenic effect of NHE1 appears to depend on a permissive, slightly alkaline $pH_i$ – that varies temporally and in response to growth factor signaling (Flinck et al. 2018) – the magnitude of this effect is likely exaggerated in previous studies performed under $CO_2/HCO_3^-$-free conditions where the influence of $Na^+/H^+$-exchange on $pH_i$ is artificially augmented. Also, pharmacological inhibitors of $Na^+/H^+$-exchangers have been shown to possess $pH_i$- and even NHE1-independent effects that under some conditions may be caused by toxicity due to intracellular accumulation (Rolver et al. 2020). In general, the pro-malignant effects of NHE1 have been most convincingly demonstrated in cultured cell lines; and the importance in cancer tissue retaining original phenotypic characteristics and in vivo needs confirmation. Particularly, the ability of NHE1 inhibitors to boost chemotherapeutic responses against cancer cells (Lauritzen et al. 2010; Reshkin et al. 2003; Amith et al. 2015; Jia et al. 2020; Chen et al. 2019) could represent a considerable therapeutic advancement if corroborated in human breast carcinomas in vivo.

Studies propose that vacuolar-type $H^+$-ATPases and $H^+/K^+$-ATPases expressed in the plasma membrane of cancer cells (Fig. 1) contribute to breast malignancy and pH control within tumors (Goh et al. 2014; Montcourrier et al. 1997; Martinez-Zaguilan et al. 1996). However, in human and murine breast carcinomas, omission of extracellular $Na^+$ lowers the capacity for net cellular elimination of $H^+$ during intracellular acidification by more than 90% (Lee et al. 2015, 2016a, 2018; Boedtkjer et al. 2013a; Voss et al. 2020), leaving little room for quantitatively relevant contribution from $Na^+$-independent transporters. Nonetheless, based on their influence on pH regulation in some breast cancer cell lines (Goh et al. 2014; Hinton et al. 2009; Cotter et al. 2015; Capecci and Forgac 2013), $H^+$- or $H^+/K^+$-ATPases may gain functional importance under pathophysiological circumstances influenced by particular oncogenic pathways or growth factors, or they may partake in pH regulation of subcellular or local extracellular compartments.

## 2.2 *Organellar $H^+$ Sequestration*

Sequestration of $H^+$ in membrane-bound intracellular organelles – particularly mitochondria and vesicles of the endolysosomal system (Fig. 2) – can temporarily protect against cytoplasmic acidification and in some cases promote elimination of $H^+$ via vesicular exocytosis (Chen et al. 2020; Poburko et al. 2011; Stappen and Krämer 1994).

The degree of vesicle acidification is controlled by the balance between several $H^+$ transporting membrane proteins: $H^+$ is pumped into endo- and lysosomes through vacuolar-type $H^+$-ATPases that are electrically compensated via electrogenic $2Cl^-/H^+$-exchange mediated by vesicular-type CLCs (CLC3–7) (Jentsch 2015; Jentsch and Pusch 2018). Vesicular acid loading can also occur via $Na^+/H^+$-exchangers driven by the higher $Na^+$ concentration inside endo- and lysosomes compared to the cytosol (Xu and Ren 2015). Lysosomes are internally acidified to pH 4.5–5.0 (Mindell 2012), which corresponds to an approximately 1,000-fold higher free $H^+$ concentration relative to the cytosol. This $H^+$ gradient can drive $H^+$ transport out of endo- and lysosomes through organellar-type $Na^+/H^+$-exchangers (NHE6–9) where the majority of studies show that $K^+$ can substitute for $Na^+$ to mediate $K^+/H^+$-exchange (Prasad and Rao 2018; Onishi et al. 2012; Kondapalli et al. 2015; Ohgaki et al. 2011), see Fig. 2. When the early endolysosomal $Na^+$ gradient has partly dissipated, the $H^+$-gradient will also be able to drive $Na^+/H^+$-exchange

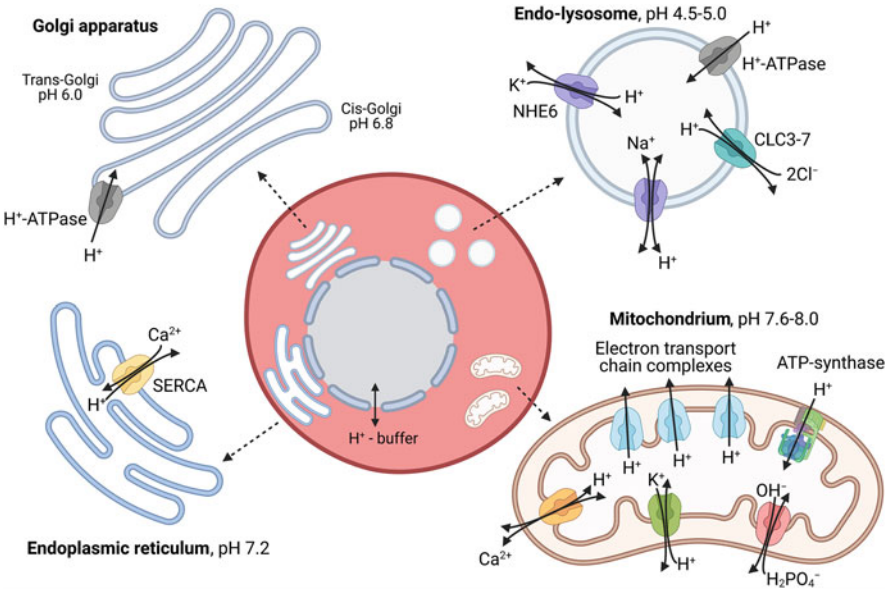


**Fig. 2** Schematic of acid-base transporters relevant for pH control in intracellular organelles. For details, see the Sect. 2.2. The most typical isoforms of the individual transporters are noted; but in some cases, other isoforms may also play a role. The figure was created with Biorender.com

leading to $Na^+$ sequestration coupled to endolysosomal $H^+$ extrusion (Kondapalli et al. 2014). The lysosomal acidification ensures optimal catalytic activity of the lysosomal hydrolases (Mindell 2012) and provides a potential mechanism for storing excess $H^+$ from the cytosol, particularly in cancer cells where the volume and acidity of lysosomes increase (Perera et al. 2015; Fehrenbacher et al. 2008). In response to low $pH_o$ – as encountered in the tumor microenvironment – lysosomes traffic from the perinuclear to the sub-plasmalemmal space of human breast cancer cells (Glunde et al. 2003), and exocytosis of the acidic, enzyme-rich lysosomal content accelerates extracellular matrix degradation and more permanently eliminates the cytoplasmic acid load. Proper autophagosome function also depends on lysosome acidification; and whereas an acidic extracellular environment inhibits autophagy in MCF7 breast cancer cells, low $pH_o$ stimulates autophagy in melanoma cells (Marino et al. 2012; Wojtkowiak et al. 2012; Xie et al. 2015). In line with these findings, inactivation or disrupted expression of vacuolar-type $H^+$-ATPases can inhibit cell survival and invasiveness in pancreatic and gastric cancer cells (Ohta et al. 1998; Nakashima et al. 2003; Liu et al. 2015).

Through the electron transport chain, $H^+$ is extruded from the mitochondrial matrix to the intermembrane space during reduction of oxygen to water (Fig. 2). Under normal circumstances, this process alkalinizes the mitochondrial matrix to the pH range 7.6–8.0, and the generated $H^+$ gradient across the mitochondrial inner membrane drives $H^+$ through the $F_0F_1$ ATP-synthase to energize ATP formation (Poburko et al. 2011; Llopis et al. 1998; Matsuyama et al. 2000; Abad et al. 2004). Due to the free permeability for $H^+$ across the outer mitochondrial membrane, $H^+$ extruded to the mitochondrial intermembrane space can also contribute to cytosolic acidification. Individual mitochondria are small, but measurements of the collective mitochondrial volume fraction (relative to cell volume) vary between 6 and 10% in cancer cells and can reach as high as 35% in cardiomyocytes with high aerobic capacity (Vazquez 2018). However, the intrinsic buffering capacity of the mitochondrial matrix is low, around 5–10 mM at pH 7.6–7.8 (Poburko et al. 2011) compared to 30–40 mM in the cytosol of human breast cancer cells at pH 7.1 (Lee et al. 2015). Considering the smaller volume and lower intrinsic buffering capacity of mitochondria compared to the cytosol, net $H^+$ extrusion from the mitochondrial matrix is expected to produce greater mitochondrial than cytosolic pH changes, even if the $CO_2/HCO_3^-$-dependent buffering capacity is somewhat increased in the alkaline mitochondrial compartment (Roos and Boron 1981). Still, for cells rich in mitochondria, the impact of mitochondrial $H^+$ transport on cytosolic pH appears functionally relevant as mitochondria-induced cytosolic acidification has been found important for caspase activation and induction of apoptosis (Matsuyama et al. 2000).

It is possible that the $H^+$ gradient across the inner mitochondrial membrane receives additional contribution from cytosolic acidification due to metabolic $H^+$ production in the cytosol or caused by ion transport across other organelle membranes or the plasma membrane (Poburko et al. 2011). However, acid loads imposed on the cytosol can relatively quickly be transferred to mitochondria, for instance, via $K^+/H^+$- or $H_2PO_4^-/OH^-$-exchange (Poburko et al. 2011; Stappen and Krämer 1994), see Fig. 2. Irrespective of whether the cytosolic $H^+$ is transferred via the

ATP-synthase or other $H^+$ permeation pathways in the inner mitochondrial membrane, this sequestration of $H^+$ in mitochondria potentially contributes to, at least temporary, recovery of cytoplasmic pH.

Interestingly, although ATP synthesis is usually fueled by the $H^+$ gradient across the inner mitochondrial membrane, there is evidence of $F_0F_1$ ATP-synthase expression in the plasma membrane of some, particularly aggressive, cancer cells (Dowling et al. 2007; Chi and Pizzo 2006). This could allow the cancer cells to exploit the inward electrochemical gradient for $H^+$ across the plasma membrane for ATP synthesis although at the cost of an increased cytoplasmic acid load.

It is not yet clear to what extent other intracellular organelles contribute to $H^+$ sequestration (Fig. 2). The lumen of the endoplasmic reticulum is near-neutral with a pH around 7.2 (Wu et al. 2000). The lumen of the Golgi apparatus is acidic with a typical pH ranging from around 6.8 in cis-Golgi to 6.0 in trans-Golgi, primarily controlled by a vacuolar-type $H^+$-ATPase (Llopis et al. 1998; Glickman et al. 1983; Kellokumpu 2019). There is evidence for defective acidification of the Golgi apparatus in breast cancer where trans-Golgi pH is higher than 6.75 in MCF7 human breast cancer cells and this abnormally elevated pH correlates with glycosylation alterations (Kellokumpu et al. 2002; Rivinoja et al. 2006, 2009). In colorectal CaCo-2 cancer cells, elevated Golgi pH is associated with abnormal protein sorting; and the associated loss of polarization can be mimicked in Madin-Darby Canine Kidney (MDCK) cells if Golgi pH is elevated by 0.8 in response to pharmacological inhibition of the vacuolar-type $H^+$-ATPase (Kokkonen et al. 2019).

$H^+$ bound to mobile buffers can also enter the nucleoplasm through nuclear membrane pores and may thereby influence both intracellular acid-base compartmentalization and gene expression (Hulikova and Swietach 2016).

In addition to the contribution from dedicated acid-base transporters, $H^+$ moves as counter-ions during $Ca^{2+}$-ATPase activity both in the membrane of the endoplasmic reticulum (SERCA, see Fig. 2) and in the plasma membrane (PMCA). In most cases, the transporters move $H^+$ to the cytosol from the lumen of the organelle or from the extracellular space, thus contributing an augmented cytosolic acid load (Bublitz et al. 2013; DeSantiago et al. 2007). As illustrated in Fig. 2, a similar phenomenon takes place during mitochondrial $Ca^{2+}/H^+$-exchange but the directionality of this transport depends on the electrochemical gradients and is particularly sensitive to cytosolic $Ca^{2+}$ dynamics (Jiang et al. 2009). Based on knockdown and overexpression studies, mitochondrial $Ca^{2+}$ extrusion coupled to $H^+$ uptake predominates under typical resting conditions (Shao et al. 2016). In support of $Ca^{2+}$-coupled $H^+$ transport processes influencing the local microenvironment, PMCA activity has been found to cause extracellular alkalinization and cytosolic acidification when the intracellular $Ca^{2+}$ concentration is elevated (Poburko et al. 2011; Naderali et al. 1997).

## 2.3 Cellular Sensors and Consequences of $H^+$

Many enzymatic and molecular interaction processes in the cytosolic, organellar, and extracellular space are sensitive to pH; and the involved enzymes and interacting partners can serve as $H^+$-sensors that adjust the function of the cells to the prevailing conditions. The degree of pH-sensitivity for various proteins spans from the functionally insignificant to almost complete activation or inactivation even in response to pH-shifts of a few tenth of a unit (Fleming et al. 1994; Fidelman et al. 1982; Trivedi and Danforth 1966; Boedtkjer et al. 2011; Srivastava et al. 2008).

G-protein coupled receptors (e.g., GPR4, TDAG8, OGR1) are increasingly recognized for their ability to sense extracellular $H^+$ (Ludwig et al. 2003; Wang et al. 2004; Ishii et al. 2005); and their capacity for initiating intracellular second messenger responses has relevance for development and progression of several types of cancer, including breast cancer (Justus et al. 2013; Zhang et al. 2019; Li et al. 2013a). Changes in $pH_o$ of 0.2 magnitude markedly alter receptor activity and lead to sizeable (~30%) changes in cAMP or $IP_3$ production (Ludwig et al. 2003; Wang et al. 2004). Thus, the $pH_o$ dynamics in the tumor microenvironment have potential to substantially influence cancer cell signaling and disease progression.

Cancer cells, endothelial cells, cancer-associated fibroblasts, and immune cells all express $pH_o$-sensitive G-protein coupled receptors (Wenzel et al. 2020; Horman et al. 2017; Yan et al. 2014; Onozawa et al. 2012; Huang et al. 2008); and due to this cellular complexity and insufficient data from realistic cancer models, we still have only rudimentary understanding of the integrated implications of $H^+$ sensing in the tumor microenvironment. As described below, the $H^+$-sensitive G-protein coupled receptors have direct effects on proliferation, migration, and survival of cancer cells but also modify the architecture of the vascular network and anti-cancer immune responses with possible indirect effects on the cancer cells. Variation in the magnitude of the local acid load (e.g., due to differences in relative dependency on fermentative vs. oxidative metabolism), tumor perfusion, and the degree of immune evasion also likely contribute to the oftentimes conflicting consequences of altered signaling through $H^+$-sensitive G-protein coupled receptors in different cancer types.

Only few studies have so far evaluated the malignant consequences of TDAG8/GPR65 with evidence provided for upregulation in a number of human cancers and support that TDAG8 can transform breast epithelial cell lines (Sin et al. 2004), accelerate tumor development, protect against acid-induced cell death, and promote proliferation (Ihara et al. 2010). GPR4 and OGR1 have been more comprehensively studied and – perhaps as a consequence – their functional ramifications for cancer development and progression appear more diverse. We presently know that GPR4 is overexpressed in multiple cancers, including breast cancer (Singh et al. 2007), and can malignantly transform NIH3T3 cells (Sin et al. 2004). Whereas GPR4 inhibits migration and invasion of melanoma and prostate cancer cells (Castellone et al. 2011), it has been found to promote colorectal carcinogenesis and metastasis (Yu et al. 2019) and stimulate ovarian cancer growth and invasion (Bai et al. 2021). The expression of GPR4 on vascular endothelial cells matches its proposed

role for vascular endothelial growth factor (VEGF)-dependent angiogenesis (Wyder et al. 2011), blood vessel morphology (Wyder et al. 2011; Yang et al. 2007), and inflammation (Dong et al. 2013; Chen et al. 2011). OGR1/GPR68 is upregulated in tumor tissue and by hypoxia (de Vallière et al. 2016) and has been found to promote growth of melanoma cell xenografts (Li et al. 2009). In MCF7 breast cancer cells, however, heterologous overexpression of OGR1 stimulates apoptosis and inhibits proliferation (Zhang et al. 2019). OGR1 also promotes murine prostate cancer development at least in part due to a shift from a pro-inflammatory M1 toward an anti-inflammatory M2 tumor-associated macrophage response (Yan et al. 2014). However, at the same time, OGR1 inhibits cancer cell migration (Li et al. 2013a; Singh et al. 2007; Ren and Zhang 2011) and reduces metastasis in models of prostate cancer (Singh et al. 2007), and OGR1 has also been found to mediate acid-induced inhibition of angiogenesis (Ding et al. 2019). Whereas, G2A/GPR132 shows homology to the $pH_o$-sensitive G-protein coupled receptors (Weng et al. 1998) and has been found to promote transformation of NIH3T3 cells (Zohn et al. 2000), the functional impact of $H^+$ on G2A signaling is still debated and needs clarification (Seuwen et al. 2006; Murakami et al. 2004).

As illustrated by the highlighted studies, $pH_o$-sensitive G-protein coupled receptors influence many fundamental processes relevant to cancer biology. It is also clear, however, that a comprehensive integrated understanding of how these receptors impact acid-adaptation, patient prognosis, and treatment resistance is needed before a cohesive model for their actions can be deduced. Due to the expression and functional involvement of $H^+$-sensitive G-protein coupled receptors in multiple cell types, future studies should concentrate on in vivo conditions or complex in vitro models covering multiple cell types organized in a relevant three-dimensional geometry.

The degree of extracellular acidification differs drastically between individual tumors and even spatially and temporally within single tumors, where metabolic activity and perfusion show substantial heterogeneity (Vaupel et al. 1989). The acidic and hypoxic conditions in tumors act as evolutionary selection forces and initiate adaptive responses in the cancer cells, thereby promoting malignant and invasive phenotypes that accentuate disease progression (Martinez-Zaguilan et al. 1996; Damaghi et al. 2021; Gatenby et al. 2007; Moellering et al. 2008). Acid-induced selection or adaptation may show particular impact, if the cancer cells subsequently encounter a less acidic environment (Boedtkjer and Pedersen 2020). For instance, the upregulated expression of acid-base transporters elevates $pH_i$ during breast carcinogenesis and likely further accelerates cell proliferation if the cellular acid load later decreases or net acid extrusion via NHE1 and NBCn1 is dis-inhibited in an area or at a time of less acidic $pH_o$ (Bonde and Boedtkjer 2017; Jean et al. 1985; Aronson et al. 1983; Paris and Pouyssegur 1983; Vaughan-Jones and Wu 1990).

## 2.4 *Effects of $H^+$ on Stromal Cells*

The acidified tumor microenvironment affects not only the neoplastic cancer cells but also the stroma, which is dominated by extracellular matrix and multiple resident and transient cell types including fibroblasts, vascular and lymphatic endothelial cells, vascular smooth muscle cells, pericytes, and innate and adaptive immune cells. All of these cell types are influenced by the abnormal ionic composition of the tumor microenvironment, can modify cancer prognosis, and are potential targets for therapeutic intervention (Dykes et al. 2018).

Extracellular acidification relaxes most resistance arteries (Boedtkjer 2018; Boedtkjer and Aalkjaer 2012), including breast cancer feed arteries (Froelunde et al. 2018). This net vasorelaxation is complex as it integrates pro-relaxant and pro-contractile stimuli elicited by low $pH_o$, low $pH_i$ in vascular smooth muscle cells and endothelial cells, and low extracellular $HCO_3^-$ concentrations (Boedtkjer et al. 2006, 2011, 2012b, 2016b; Boedtkjer 2018; Hansen et al. 2020). NHE1 helps defend vascular smooth muscle and endothelial cells against severe intracellular acidification (Fig. 1) as it mediates net acid extrusion mostly when $pH_i$ drops substantially below the normal physiological range (Boedtkjer et al. 2012b; Boedtkjer and Aalkjaer 2009). NHE1 activity in vascular smooth muscle cells is markedly attenuated at low $pH_o$ (Bonde and Boedtkjer 2017), and thus NHE1 is expected to play a smaller relative role in the acidic tumor microenvironment. Acidosis of the magnitude observed in solid cancer tissue also inhibits the rhythmic contractile activity of lymph vessels (Moeller et al. 2019; Lobov and Kubyshkina 2001). The functional implications of the acidic tumor microenvironment on the vasculature – e.g., in terms of local vascular resistance, endothelial permeability, and immune cell extravasation – need further evaluation if we are to harness the full therapeutic potential of selectively enhancing (e.g., to improve drug delivery or elevate tumor oxygenation during radiation) or reducing (e.g., to starve the cancer tissue) tumor perfusion. Previous studies using angiogenesis inhibitors have generally been unsuccessful for breast cancer treatment. Most likely, therapy was initiated too late – when blood vessels had already formed – or the expansion of the tumor vasculature relied predominantly on co-option (incorporation of existing blood vessels from surrounding tissue) rather than formation of new blood vessels (Donnem et al. 2013). Selectively targeting the function of the tumor vasculature requires identification of signaling pathways that are expressed and/or activated differentially in tumor blood vessels compared to normal blood vessels. Acid-base transporters, sensors, and effectors are interesting pharmacological targets due to their known implications for vascular resistance (Boedtkjer 2018; Boedtkjer and Aalkjaer 2012) and the abnormal acid-base composition of the tumor microenvironment.

Immune cells in cancer tissue receive growing interest due to the therapeutic success of immune system modulators, particularly in malignant melanoma and non-small cell lung cancer (Signorelli et al. 2019). Shaping the immune cell phenotype is critically important for determining whether the immune response becomes pro- or anti-malignant. As mentioned above (see the Sect. 2.3), the $H^+$-sensitive

G-protein coupled receptor OGR1 provides one example of how acid-base conditions in the tumor microenvironment can shift the immune involvement from M1 toward M2 tumor-associated macrophages (Yan et al. 2014). Additional immune components are affected by the acidic tumor microenvironment although the mechanisms whereby they contribute to immune evasion in cancer tissue are not yet fully elucidated. The activation threshold is higher (Bosticardo et al. 2001) and the expression of T cell receptor components reduced (Calcinotto et al. 2012) for T lymphocytes exposed to acidosis. In general, low tumor $pH_o$ inhibits anti-cancer immune effectors (e.g., M1 macrophages, T lymphocytes, dendritic cells, and natural killer cells) whereas it boosts immunosuppressive effectors (e.g., M2 macrophages and regulatory T lymphocytes) (Huber et al. 2017). Immunomodulatory therapy has not yet gained widespread clinical use for breast cancer but clinical studies are in progress (Signorelli et al. 2019). The large variation in response rates for different tumor types depends, amongst others, on the expression of immune check point signals (e.g., programmed cell death 1 ligand 1 (PD-L1)) and the degree of tumor inflammation (e.g., number of infiltrating lymphocytes) (Signorelli et al. 2019). Ongoing work focuses on the underlying molecular mechanisms that link the abnormal interstitial ion composition – and in particular, the interstitial acidosis – to immune evasion and the hitherto unsuccessful immune responses triggered against many solid cancers (Huber et al. 2017; Hanahan and Weinberg 2011).

## 3 $HCO_3^-$

$HCO_3^-$ plays a large number of functional roles throughout the body: it contributes to buffering of acute acid loads and facilitates $H^+$ mobility, for instance, between sites of intense metabolic activity and the plasma membrane; serves as substrate for acid-base transporters; and is sensed by intra- and extracellular sensors. Not all of these effects have yet been demonstrated in solid tumors, but they have potential to modify the malignant behavior of cancer cells and the accompanying responses of stromal cells.

Under physiological conditions, $CO_2$ and $HCO_3^-$ comprise the most important buffer pair in the interstitial space. Through the chemical reaction $CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$, the $CO_2/HCO_3^-$ buffer consumes and releases $H^+$ when pH decreases and increases, respectively. However, the quantitative importance of the $CO_2/HCO_3^-$ buffer system depends on the $HCO_3^-$ concentration and can be calculated as $\beta = 2.3 \cdot [HCO_3^-]$ (Roos and Boron 1981). Thus, the local $CO_2/HCO_3^-$ buffering capacity depends on the nature of the acid-base disturbance: it decreases during a simple metabolic acidosis, whereas it increases during a simple respiratory acidosis.

$CO_2/HCO_3^-$ equilibration is rate limiting under a number of circumstances, for instance, when $CO_2/HCO_3^-$ as a mobile buffer pair facilitates effective $H^+$ diffusion (Villafuerte et al. 2014; Boedtkjer et al. 2016a; Swietach et al. 2008, 2009), and when metabolic $CO_2$ is partly hydrated in erythrocytes to accelerate transfer from metabolically active tissue via the flowing blood to the lungs (Occhipinti and Boron

2019; Geers and Gros 2000). The expression of intracellular as well as extracellular-facing, membrane-tethered carbonic anhydrases (CAs) accelerates $CO_2/HCO_3^-$ buffer equilibration in solid cancer tissue (Fig. 1). Especially the exofacial carbonic anhydrases CAIX and CAXII have received intense interest; and in solid tumor-like models, they can facilitate acid elimination (Swietach et al. 2008, 2009). When studied in breast cancer patients as a whole unstratified group, high expression of CAIX is associated with poor prognosis (van Kuijk et al. 2016; Chen et al. 2018) whereas high expression of CAXII is associated with improved prognosis (Chen et al. 2018; Watson et al. 2003). However, this result is likely influenced by the predominant expression of CAXII in relatively indolent estrogen receptor-positive breast carcinomas (Barnett et al. 2008). Indeed, when breast cancer patients are stratified by molecular subtype, the patterns of prognostic influence even out: high expression of CAIX and CAXII associates with bad prognosis in basal-like/triple-negative breast cancer but with favorable prognosis in luminal A and B breast cancer (Chen et al. 2018). Yet, although the effects reach only borderline significance, and the mechanistic cause remains obscure, there is a strong tendency in HER2-positive breast cancer that high expression of CAIX improves overall survival whereas high expression of CAXII worsens overall survival (Chen et al. 2018). The different implications of carbonic anhydrases between breast cancer molecular subtypes probably relate to the generally higher metabolic activity – and thus greater dependency on acid elimination – in basal-like compared to luminal breast cancer (García Vicente et al. 2013). From the perspective of pH regulation in the interstitial space, CAIX and CAXII appear to perform similar functions by catalyzing exofacial $CO_2/HCO_3^-$ equilibration. However, studies suggest that the pro-malignant consequences of CAIX are modified through shedding of the extracellular domain in a process that is under control of the disintegrin and metalloproteinase ADAM17 (Zatovicova et al. 2005; Kajanova et al. 2020). The consequences of CAIX ectodomain cleavage may be related to the decrease in membrane-associated carbonic anhydrase activity but could also rely on changes in cell adhesion function associated with CAIX cell surface expression (Kajanova et al. 2020; Závada et al. 2000; Csaderova et al. 2013). This type of dynamic and isoform-specific regulation may explain potential differences in prognosis and therapy resistance between cancer cells expressing high levels of CAIX and CAXII (Vidlickova et al. 2016).

The role of the $CO_2/HCO_3^-$ buffer as a mobile shuttle for $H^+$ matters not only within the cytoplasm of individual cancer cells and across the interstitial space, but it also appears to improve intercellular diffusion of acid equivalents both along gap junction-coupled cancer cells (Dovmark et al. 2018) and in strands of stromal cells (Hulikova et al. 2016). This intracellular route of acid dissipation would share the burden of energy-dependent acid elimination across a larger group of cells and thus ease the load on the individual cells.

Tissue concentrations of $HCO_3^-$ are typically calculated based on measured pH values and assumed $CO_2$ levels that are, however, difficult to predict in tumors. Direct mapping of $HCO_3^-$ concentrations in the tumor microenvironment with information on organellar, cytosolic, and interstitial concentrations, and taking into consideration the spatiotemporal heterogeneity in each of these compartments,

would markedly improve our ability to evaluate the integrated impact of the intratumoral acid-base conditions. Despite technical limitations – that for now preclude measurements in realistic tumor models – the recent development of a genetically encoded intracellular $HCO_3^-$ sensor suited for living cultured cells (Bernhard et al. 2020) represents a potentially important step forward. Metabolic $HCO_3^-$ conversion in vivo can also be evaluated based on hyperpolarized $^{13}C$ magnetic resonance spectroscopic imaging (Bøgh et al. 2020). The concentration of $HCO_3^-$ will vary regionally depending on the local metabolic state: Generation of $H^+$ – for instance, from fermentative glycolysis – will lower the concentration of $HCO_3^-$, whereas oxidative metabolism liberates $CO_2$ and thereby raises the concentration of $HCO_3^-$. Thus, it is expected that the concentration of $HCO_3^-$ is particularly reduced in the most hypoxic regions of tumors and may even be raised in regions of intense oxidative metabolism. Direct and dynamic $HCO_3^-$ concentration measurements are required to appreciate fully the function of $HCO_3^-$ transporters and the impact of $HCO_3^-$ sensors within living cancer tissue.

## 3.1 $HCO_3^-$ Transporters in the Plasma Membrane

The predominant mechanism for cellular uptake of $HCO_3^-$ in breast cancer tissue is via the $Na^+$,$HCO_3^-$-cotransporter NBCn1/SLC4A7 (Lee et al. 2015, 2016a, 2018; Boedtkjer et al. 2013a), see Fig. 1. Studies based on breast cancer cell lines suggest that other $Na^+$,$HCO_3^-$-cotransporters may play a role mainly during hypoxia when NBCe1/SLC4A4, NBCe2/SLC4A5, and AE4/SLC4A9 are upregulated (Hulikova et al. 2013; McIntyre et al. 2016; Parks and Pouyssegur 2015). The gene locus *SLC4A7*, encoding NBCn1, is a human breast cancer susceptibility region based on genome-wide association studies in women of diverse ethnic backgrounds (Chen et al. 2012; Han et al. 2011; Long et al. 2010; Ahmed et al. 2009). NBCn1 uses the transmembrane gradient for $Na^+$, generated by the $Na^+/K^+$-pump, to overcome the unfavorable energetics of cellular $HCO_3^-$ uptake. Although NBCn1 expression is detectable, it plays a relatively minor role for $pH_i$ regulation in normal breast tissue from humans and mice (Lee et al. 2015, 2016a, 2018). However, NBCn1 protein expression is two- to threefold upregulated during breast carcinogenesis; and the associated $Na^+$,$HCO_3^-$-cotransport functionally dominates steady-state $pH_i$ regulation and net acid extrusion in breast cancer tissue particularly in the near-physiological $pH_i$ range (Lee et al. 2015, 2016a, 2018; Boedtkjer et al. 2013a). Consistent with a role of NBCn1 for protecting breast cancer cells against intracellular accumulation of harmful acidic waste products, mice with disrupted expression of NBCn1 show delayed breast cancer development in response to ErbB2/HER2 overexpression (Lee et al. 2018) and following carcinogen induction (Lee et al. 2016a). Disrupted NBCn1 expression also delays the rate of breast tumor growth and reduces cell proliferation particularly in tumor models and regions with prominent lactate accumulation and elevated need for elimination of acidic metabolic waste (Lee et al. 2016a, 2018).

NBCn1 was first shown to accelerate cell migration in vascular smooth muscle cells (Boedtkjer et al. 2016a) and this observation was later confirmed in a lung adenocarcinoma cell line (Hwang et al. 2020). Whereas the non-selective $Na^+$, $HCO_3^-$-cotransport inhibitor S0859 inhibited cell migration in vascular smooth muscle cells (Boedtkjer et al. 2016a), this effect was not observed in MCF7 human breast cancer cells (Lauritzen et al. 2012). Notably, however, the influence of acid-base transport in the MCF7 cells was unusual inasmuch as NHE1 inhibition with 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) accelerated rather than decelerated migration (Lauritzen et al. 2012). Thus, the influence of NBCn1 on cancer cell migration and metastasis deserves further evaluation.

The requirement for net base extrusion is typically low in cancer cells due to the net metabolic acid load. On the other hand, stromal cells – particularly cancer-associated fibroblasts – may contribute toward neutralizing the tumor microenvironment by export of $HCO_3^-$ or import of $H^+$, thereby serving as a sink for temporary storage of a locally increased acid load (Hulikova et al. 2016). The transport of base to the interstitial space of tumors occurs predominantly via anion exchangers of the SLC4 and SLC26 families that perform the dual role of extruding base and importing $Cl^-$ (Gorbatenko et al. 2014). Indeed, AE2 (SLC4A2) is expressed in cultured myofibroblasts that show high capacity for net base extrusion particularly following stimulation with tumor necrosis factor (TNF)-β1 (Hulikova et al. 2016).

## 3.2 Cellular Sensors and Consequences of $HCO_3^-$

Molecular mechanisms exist for $HCO_3^-$ sensing within and at the extracellular surface of cells. Still, we are in early phases of understanding the integrated consequences of cellular $HCO_3^-$ sensing in both cancer tissue and elsewhere in the body. The ability to sense $HCO_3^-$ in addition to $H^+$ provides the cells with richer information on the source and nature of an acid or alkaline load, and thus most likely allows for more appropriate and focused adjustments of cellular functions.

The soluble adenylyl cyclase catalyzes cAMP production in intracellular compartments, including the cytosol, nucleus, and mitochondria of many cells (Zippin et al. 2003). The activity of the soluble adenylyl cyclase is strongly modulated by the $HCO_3^-$ concentration with a sensitivity range that varies somewhat between enzymatic sources and experimental conditions (Chen et al. 2000) but covers the expected physiological and pathophysiological range of the cytosol and most intracellular compartments (see the Sect. 2.2). On this basis, the soluble adenylyl cyclase is considered an intracellular sensor for $HCO_3^-$ (Chen et al. 2000). In mitochondria, the soluble adenylyl cyclase contributes to regulation of metabolism and apoptosis pathways (Kumar et al. 2009b; Acin-Perez et al. 2009) that are fundamentally important for the development and progression of malignant disease. Indeed, the soluble adenylyl cyclase is a proposed tumor suppressor based on lowered mRNA and protein expression levels across a wide range of human cancers and a greater tendency to ex vivo transformation and in vivo tumor growth of fibroblasts isolated

from knockout mice (Ramos-Espiritu et al. 2016). In apparent contrast, the soluble adenylyl cyclase is overexpressed in prostate carcinomas (Ramos-Espiritu et al. 2016; Flacke et al. 2013), promotes proliferation of prostate cancer cells (Flacke et al. 2013), and favors dysplasia in neoplastic breast cell lines (Onodera et al. 2014). Interestingly, inhibition of the soluble adenylyl cyclase has also been found to enhance radiosensitivity of prostate cancer cells (Appukuttan et al. 2014). It is clear that cAMP signaling has substantial effects on malignancy, but also that the consequences vary between cell types and depend on subcellular compartmentalization of the adenylyl cyclase activity (Zippin et al. 2004).

Recent studies identify receptor protein tyrosine phosphatase (RPTP)γ, expressed in the plasma membrane of many cell types (Barnea et al. 1993), as a sensor of extracellular $HCO_3^-$ (Boedtkjer et al. 2016b; Hansen et al. 2020; Zhou et al. 2016). RPTPγ is a single-pass transmembrane protein that contains an extracellular carbonic anhydrase-like domain, which is expected to bind $HCO_3^-$ but predicted catalytically inactive (Barnea et al. 1993). The intracellular aspect of RPTPγ contains tyrosine phosphatase domains that can initiate intracellular signaling (Barnea et al. 1993), and they likely alter activity in response to monomerization-dimerization dynamics. Although RPTPγ is a proposed tumor suppressor in breast cancer (Panagopoulos et al. 1996; Shu et al. 2010; Sorio et al. 1995; Vezzalini et al. 2007), we need more evidence regarding its functional roles and implications for acid-base-dependent signaling.

Buffer therapy involves systemic supply of $HCO_3^-$ or other weak bases with the purpose of counteracting cancer-promoting consequences of the acidic extracellular tumor microenvironment. Whereas oral supplementation with $NaHCO_3$ is ineffective at decelerating growth of primary xenograft tumors (Robey et al. 2009) and carcinogen-induced breast carcinomas (Voss et al. 2020), it inhibits development of primary adenocarcinoma of the mouse prostate (Ibrahim-Hashim et al. 2012). More consistently, buffer therapy can dramatically inhibit metastasis and cancer recurrence (Robey et al. 2009; Robey and Martin 2011) and can also strengthen responses to immunomodulators (Pilon-Thomas et al. 2016) and chemotherapeutics (Raghunand et al. 2003). However, the consequences of systemic buffer therapy are widespread; and even within cancer tissue, the functional and structural complexity with multiple cell types and pronounced compartmentalization can make it difficult to predict the net effect. Pro-malignant consequences of buffer therapy – recently observed in breast tissue – likely reflect that increased $pH_o$ and the rise in interstitial $HCO_3^-$ concentration cause a concurrent rise in cellular net acid extrusion capacity and $pH_i$. Such intracellular alkalinization can accelerate cell proliferation and breast cancer development and progression (Voss et al. 2020). Clinical trials based on $NaHCO_3$ supplementation have so far been challenged by non-compliance with the treatment regimen due to adverse, primarily gastrointestinal, effects (Pilot et al. 2018).

### 3.3 *Effects of $HCO_3^-$ on Stromal Cells*

The balance of $HCO_3^-$ uptake via NBCn1-mediated $Na^+$,$HCO_3^-$-cotransport and $HCO_3^-$ extrusion via $Cl^-/HCO_3^-$-exchange is crucial for establishing the $pH_i$ of vascular endothelial and smooth muscle cells (Fig. 1) and thereby influences vasomotor responses (Boedtkjer et al. 2006, 2011, 2013b; Thomsen et al. 2014) and arterial morphology (Boedtkjer et al. 2016a; Boedtkjer and Aalkjaer 2013). Although not explicitly shown for tumor blood vessels, endothelial cells of resistance arteries widely express RPTPγ, which is important for promoting endothelium-dependent vasorelaxation, controlling tissue perfusion, and protecting against ischemia (Boedtkjer et al. 2016b; Hansen et al. 2020). The RPTPγ-dependent regulation of vascular tone covers a dynamic range that extends downwards from the normal extracellular $HCO_3^-$ concentration of around 22 mM (Boedtkjer et al. 2016b). Catalytically active carbonic anhydrases are also expressed in the vascular wall where they produce local pH transients that modify vascular tone in response to sudden $CO_2$ challenges (Rasmussen and Boedtkjer 2018). Carbonic anhydrase inhibitors generally induce vasorelaxation although the molecular mechanism in most instances remains unclear (Höhne et al. 2007; Torring et al. 2009; Pickkers et al. 2001).

$HCO_3^-$ has numerous functions in the immune system with potential implications for cancer progression. There is evidence that NBCn1 facilitates phagosome acidification in macrophages by protecting cytosolic pH homeostasis during phagocytosis (Sedlyarov et al. 2018) and that $Na^+/H^+$-exchangers, $H^+$-ATPases, and voltage-gated $H^+$-channels play a related role in neutrophils (Coakley et al. 2002; Morgan et al. 2009). The inhibitory effect of cytosolic acidification on phagocyte function is not yet clear but appears to involve reduced NADPH oxidase activity (Morgan et al. 2009). RPTPγ is expressed in monocytes and dendritic cells where it can influence the proliferative and secretory response of T lymphocytes (Lissandrini et al. 2006). The soluble adenylyl cyclase is expressed and mediates TNF-induced activation in neutrophils (Han et al. 2005) and plays a role for trans-endothelial leukocyte migration (Watson et al. 2015). More work is needed to elucidate the consequences of each of these $HCO_3^-$-related proteins for immune-mediated responses in cancer patients.

## 4 Lactate and Other Signaling Metabolites

Accelerated fermentative glycolysis in cancer tissue even under aerobic conditions – as described by Warburg in the mid-twentieth century (Warburg 1956) – generates chemical intermediates for anabolic processes and provides ATP for energy-requiring cellular activities. Despite the raised ATP demand, the intracellular ATP concentration is usually elevated in cancer cells compared to equivalent normal cells; but interventions that disrupt cellular ATP production can induce cancer cell death

and counteract acquired drug resistance (Zhou et al. 2012; Pecqueur et al. 2013; Qian et al. 2014).

The enzymatic machinery that catalyzes glycolysis is upregulated in breast cancer cells (Voss et al. 2020). Due to the accelerated glycolytic metabolism, interstitial concentrations of lactate are typically high in breast cancer tissue (Lee et al. 2016a, 2018; Voss et al. 2020); and hyperpolarized $^{13}C$ magnetic resonance spectroscopic imaging – which can provide non-invasive insights into lactate metabolism and transport (Woitek and Gallagher 2021) – reveals accentuated lactate accumulation in tumors with reduced perfusion (Bok et al. 2019). The fermentation of pyruvate to lactate maintains the cellular capacity for glycolysis as it replenishes the pool of the oxidized co-factor $NAD^+$. Typically, fermentative cancer cells secrete lactate, which is then taken up and metabolized by oxidative cells in the tumor microenvironment or elsewhere in the body (Pérez-Escuredo et al. 2016; Rattigan et al. 2012; Kennedy et al. 2013). However, recent evidence suggests that lactate may also be metabolized in the mitochondria of the fermentative cells themselves (Chen et al. 2016). In agreement with local metabolic production, measurements of interstitial lactate concentrations in tumors are in most cases elevated compared to plasma and normal tissue (Lee et al. 2016a, 2018; Voss et al. 2020; Burgess and Sylven 1962; Gullino et al. 1964; Ho et al. 2015); yet, there are examples of measurements suggesting that lactate concentrations in the tumor microenvironment can be similar to or even lower than in plasma (Sullivan et al. 2019). These findings underscore the heterogeneity of solid cancers and imply that some tumors rely mostly on oxidative phosphorylation and may even cover part of their energy demand through net lactate consumption.

Measurements of lactate and glucose in bulk tumor tissue have been rightfully challenged as surrogates for interstitial concentrations in the tumor microenvironment; and instead, microdialysis-based assessment has been suggested as a more accurate alternative (García-Cañaveras et al. 2019). The few microdialysis studies from human tumors report ~60% higher interstitial lactate concentration and trending lower glucose concentration in astrocytomas compared to adjacent brain tissue (Roslin et al. 2003) and an interstitial lactate concentration in head and neck cancer twice that of matched normal mucosa (Schroeder et al. 2013). When comparing microdialysis samples from murine breast cancer tissue to matched normal breast tissue, the lactate concentration is four- to fivefold elevated and the glucose concentration two- to fivefold reduced with the most extreme values observed in faster-growing carcinogen-induced breast tumors (Lee et al. 2016a; Voss et al. 2020) and more moderate values found in slower-growing ErbB2-induced breast carcinomas (Lee et al. 2018). Thus, the available evidence strongly supports the general conclusion that lactate accumulates and glucose is depleted in the tumor microenvironment.

### *4.1 Transport of Lactate Across the Plasma Membrane*

Lactate is primarily carried across cell membranes in symport with $H^+$ via monocarboxylate transporters (Halestrap 2013) although non-ionic diffusion of lactic acid or transfer of lactate via anion exchangers can also contribute (Deuticke et al. 1982). Irrespectively, the cellular extrusion of intracellular lactic acid will elevate $pH_i$ and lower the cytosolic lactate concentration to avoid inhibition of glycolytic activity. The relative contribution of the monocarboxylate transporters to net acid extrusion will depend on the source of the intracellular acid load and be most prominent under conditions of intense fermentative glycolysis. In congruence, the monocarboxylate transporters MCT1 (SLC16A1) and MCT4 (SLC16A3) markedly facilitate xenograft tumor growths based on cancer cells defective in oxidative phosphorylation (Le Floch et al. 2011). If the additional intracellular $H^+$ load stems from other sources – such as hydration of $CO_2$ from oxidative metabolism – the contribution of NBCn1 and NHE1 heavily outweighs that of the monocarboxylate transporters as is apparent from the distinct (~90%) $Na^+$-dependency of net acid extrusion in breast cancer tissue (Lee et al. 2015, 2016a, 2018; Boedtkjer et al. 2013a; Voss et al. 2020) experimentally acidified based on the $NH_4^+$ prepulse technique (Boron and De Weer 1976).

MCT1 and MCT4 are overexpressed in breast cancer tissue compared to normal breast tissue (Lee et al. 2016a; Pinheiro et al. 2010), and MCT4 is upregulated by hypoxia (Halestrap 2013; Ullah et al. 2006). Elevated MCT1 and MCT4 expression is associated with poor breast cancer prognosis (Li et al. 2018; Johnson et al. 2017; Doyen et al. 2014). There is additional evidence that MCT1 and MCT4 form protein complexes with CAIX in human breast cancer tissue but not in normal breast tissue, and that this interaction facilitates monocarboxylate transport activity, glycolysis, and cell proliferation (Ames et al. 2020). MCT1 overexpression results in larger xenograft tumors, greater microvascular density, and attenuated apoptosis (Levenson et al. 2005). In accordance, knockdown of MCT1 and MCT4 in breast cancer cells inhibits cell proliferation, migration, and invasion, and tumor formation and growth (Andersen et al. 2018; Morais-Santos et al. 2015). MCT1 is also expressed in macrophages, and infiltration with MCT1-positive M2 macrophages in breast cancer tissue correlates with recurrence-free survival (Li et al. 2020a).

Consistent with the abovementioned gene expression studies, the recently developed MCT1 inhibitors AZD3965 and AR-C155858 reduce proliferation in murine breast cancer models (Guan and Morris 2020; Guan et al. 2018). However, whereas AZD3965 lowers tumor volume, AR-C155858 does not, and AZD3965 increases lung metastasis (Guan and Morris 2020; Guan et al. 2018). AZD3965 shows a promising enhancement of radiosensitivity (Bola et al. 2014), but considering the overall mixed pharmacological responses, the therapeutic potential of MCT1 inhibitors needs further validation. The MCT inhibitors are expected to cause accumulation of lactate in cancer cells relying on fermentative glycolysis, and thereby to inhibit further metabolism. In addition, MCT inhibitors may lower interstitial lactate concentrations in the tumor microenvironment, which can further modify cancer

progression via cellular sensors (i.e., GPR81; see the Sect. 4.2). Combined with the variation in cellular dependency on oxidative vs. fermentative metabolism, these issues likely explain the variation in therapeutic responses.

Lactate has also been proposed to move through gap junctions (Dovmark et al. 2017). In this case, the transport is not directly coupled to $H^+$ although gap junctions are also permeable to mobile $H^+$ buffers (Dovmark et al. 2018). Thus, intercellular fluxes of lactate may lead to enhanced $pH_i$ disturbances. The permeability of gap junctions, composed of various connexin isoforms, is inhibited when $pH_i$ deviates substantially below or above the physiological range (Boedtkjer et al. 2013b; Turin and Warner 1977; Spray et al. 1981; Garciarena et al. 2018), which may further limit intercellular lactate diffusion in regions with a high cellular acid load.

## *4.2 Cellular Sensors and Consequences of Lactate*

It is increasingly clear that signaling in response to locally accumulating lactate plays roles in many physiological and pathophysiological processes. Cells are capable of sensing lactate in the extracellular space via the G-protein coupled hydroxycarboxylic acid receptor GPR81/HCA1 with an $EC_{50}$ in the low millimolar range (Cai et al. 2008). The proposed acid-sensing receptor G2A/GPR132 may also contribute to sensing of lactate by macrophages in breast cancer tissue (Chen et al. 2017) but considering the controversy regarding the ligand specificity of G2A (Seuwen et al. 2006; Murakami et al. 2004) more work is needed to settle its role (see the Sect. 2.3).

GPR81 expression is elevated by lactate (Xie et al. 1866; Soni et al. 2020) and in breast cancer tissue (Lee et al. 2016b; Stäubert et al. 2015) where it counteracts cell death in presence of extracellular lactate, enhances angiogenesis, and accelerates cancer growth (Lee et al. 2016b; Stäubert et al. 2015; Roland et al. 2014; Brown et al. 2020). Evidence supports that lactate per se – and not just the drop in pH associated with its production – inhibits immune responses (Gottfried et al. 2006; Nasi et al. 2013; Brand et al. 2016; Loeffler et al. 1991). GPR81 lowers T cell immune infiltration in tumors; and in accordance, lactate suppresses activation of GPR81-positive dendritic cells and their cell surface presentation of major histocompatibility complex class II proteins (Brown et al. 2020; Raychaudhuri et al. 2019). GPR81 also contributes to radio- and chemoresistance (Yang et al. 2020; Wagner et al. 2017). These studies provide a mechanism whereby enhanced metabolite-induced responses to the tumor microenvironment can provide cancer cells selective advantages that favor cancer progression.

### 4.3 Other Signaling Metabolites

In addition to lactate, several simple and modified carboxylates are generated during local and systemic metabolic challenges (Husted et al. 2017). With $pK_a$ values typically lower than five – and thus considerably below $pH_i$ and $pH_o$ even in tumors – these metabolites are generated in their deprotonated form, and their synthesis is thus associated with a metabolic acid load. As discussed above, the acidification can modify the function of both cancer and stromal cells, but the metabolites can also directly influence cells in the tumor microenvironment. For example, butyrate can increase MCT4 activity (Queirós et al. 2012); MCT1-mediated pyruvate transport promotes cell proliferation and xenograft tumor growth (Hong et al. 2016); succinate enhances interleukin 1β-mediated inflammatory responses (Tannahill et al. 2013) and can promote angiogenesis via its membrane receptor GPR91 (Mu et al. 2017); and ketone body production – systemically or locally in the tumor stroma – may fuel breast cancer cells and contribute to breast cancer progression (Martinez-Outschoorn et al. 2012). The carboxylates can act on membrane receptors (e.g., HCA2, FFA2, FFA3) – that are expressed widely, but particularly in immune cells (Husted et al. 2017). The diversity of metabolites and receptors allows for a plethora of biological effects with a so far greatly underexplored potential in malignancies.

## 5 Other Deregulated Ions in the Tumor Microenvironment

The following sections address the pathophysiology of $Ca^{2+}$, $Na^+$, $K^+$, and $Cl^-$ in malignancies, as these ions influence the cellular handling and functional consequences of acids and bases. Abnormal ion concentrations in the tumor microenvironment and altered expression and function of ion channels and transporters in cancer and stromal cells thus have potential to modify the impact of acid-base homeostasis on cancer development and progression.

### 5.1 Interactions Between $Ca^{2+}$ and Acid-Base Homeostasis

Transporters and channels in the plasma membrane and in intracellular organelles tightly control the free cytosolic $Ca^{2+}$ concentration, which is further stabilized by cytosolic buffering (Gilabert 2012). Because the cytosolic buffers for $Ca^{2+}$ and $H^+$ overlap, acute changes in pH can have dramatic effects on the intracellular free $Ca^{2+}$ concentration, which typically rises in response to abrupt intracellular acidification (Batlle et al. 1993; Abercrombie and Hart 1986). The interaction between $pH_i$ and the intracellular $Ca^{2+}$ concentration is bidirectional as increases in the cytosolic $Ca^{2+}$ concentration can also accelerate net acid extrusion. NBCn1 isoforms containing

splice cassette II – which is a variable domain of 123 or 124 amino acids in the *N*-terminus – bind the $Ca^{2+}$/calmodulin-activated serine-threonine phosphatase calcineurin A (Danielsen et al. 2013; Gill et al. 2014) that elevates NBCn1 activity in response to rises in the intracellular $Ca^{2+}$ concentration, for instance, during contractions of vascular smooth muscle cells (Danielsen et al. 2013). Binding of $Ca^{2+}$/calmodulin to the regulatory cytosolic domain of NHE1 during rises in the intracellular $Ca^{2+}$ concentration can also activate $Na^+/H^+$-exchange activity through the relief of an auto-inhibitory mechanism (Li et al. 2013b; Wakabayashi et al. 1994) and potentially via NHE1 dimerization (Sjøgaard-Frich et al. 2021). Additionally, the $Ca^{2+}$-binding calcineurin B homologous proteins (CHP1–3) – that are overexpressed in and promote proliferation of breast cancer cells (Zhao et al. 2018) – physically interact with NHE1 and thereby stabilize the expression and increase the $Na^+/H^+$-exchange activity (Di Sole et al. 2012). The activation of net acid extrusion, via both NBCn1 and NHE1, by rising intracellular $Ca^{2+}$ concentration provides a mechanism for accommodating the expected rise in metabolic acid production associated with cellular activation in both cancer and stromal cells.

Although the underlying pathophysiology of deregulated $Ca^{2+}$ signaling in breast cancer is multifaceted (Voss et al. 2020; Bruce and James 2020) and only partially uncovered, $Ca^{2+}$ is a key intracellular second messenger with fundamental impact on cell proliferation and programmed cell death (Varghese et al. 2019). Local $Ca^{2+}$ transients and intracellular $Ca^{2+}$ gradients are also important for directional cell migration (Wei et al. 2012). Voltage-gated $Ca^{2+}$ channels are overexpressed in breast cancer tissue and have been found to promote breast cancer development and metastasis (Wang et al. 2015; Kanwar et al. 2020). Whereas some studies suggest that $Ca^{2+}$ channel blockers increase breast cancer risk (Fitzpatrick et al. 1997; Davis and Mirick 2007) and facilitate breast cancer cell proliferation and migration (Guo et al. 2014), other studies report that $Ca^{2+}$ channel blockers inhibit breast cancer growth (Taylor and Simpson 1992). Transient receptor potential (TRP) channels are non-selective cation channels that play a prominent role for $Ca^{2+}$ influx across the plasma membrane in many cell types. Even though TRP channels have been implicated in multiple types of cancer (Bruce and James 2020), there is still a paucity of information regarding breast cancer. However, TRP channels have been found overexpressed in breast cancer cells (Mandavilli et al. 2012) and associated with enhanced proliferation, apoptosis, migration, and invasion (Jardin et al. 2018; Wang et al. 2019). Voltage-gated $Ca^{2+}$ channels are inhibited by extracellular acidification (West et al. 1992; Klockner and Isenberg 1994) whereas several TRP channels are activated at low $pH_i$ and $pH_o$ (Cao et al. 2012; Kawasaki et al. 2009; Kozak et al. 2005), but the consequences of acid-mediated regulation of $Ca^{2+}$ channel activity in breast cancer and stromal cells are not yet clear.

SERCA is responsible for $Ca^{2+}$ uptake into the endoplasmic reticulum and its expression is perturbed during breast carcinogenesis as assessed by quantitative mass-spectrometry (Voss et al. 2020). Ryanodine and $IP_3$ receptors that mediate release of $Ca^{2+}$ from the endoplasmic reticulum also link to breast cancer risk, cell proliferation, migration, and tumor grade (Abdul et al. 2008; Zhang et al. 2011; Mound et al. 2017). In various cell types, pH modifies the $Ca^{2+}$ release pattern from

the sarcoplasmic/endoplasmic reticulum at least in part via ryanodine receptor inhibition (Dabertrand et al. 2012; Ford et al. 2017; Xu et al. 1996; Rousseau and Pinkos 1990; Ma and Zhao 1994), but the interactions between the acidic tumor microenvironment, $Ca^{2+}$ store function, and cancer progression need further investigation.

Changes in the extracellular concentration of $Ca^{2+}$ can also influence cellular functions by regulating the activity of the $Ca^{2+}$-sensing receptor that is expressed in both normal breast epithelium and breast cancer cells (Cheng et al. 1998). The $Ca^{2+}$-sensing receptor is a G-protein coupled receptor that typically initiates intracellular signaling via $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ in response to changes in the extracellular $Ca^{2+}$ concentration with an $EC_{50}$ of approximately 4 mM (Das et al. 2020; Pearce et al. 1996). The heterotrimeric G-protein employed is ligand-dependent and it varies between cell types (Das et al. 2020); with the intriguing observation that whereas the $Ca^{2+}$-sensing receptor in normal breast epithelial cells inhibits the adenylyl cyclase via $G_i$-coupled signaling, it can stimulate the adenylyl cyclase in breast cancer cells via $G_s$-coupled signaling (Mamillapalli et al. 2008). The $Ca^{2+}$-sensing receptor links to breast cancer risk and aggressiveness (Campos-Verdes et al. 2018) and is a positive regulator of breast cancer cell proliferation and migration with importance for development of bone metastases (El Hiani et al. 2009a, b; Saidak et al. 2009). However, other studies show that the $Ca^{2+}$-sensing receptor is downregulated in breast cancer, that it inhibits proliferation and invasion, and that low expression of the $Ca^{2+}$-sensing receptor is associated with poor survival and accelerated metastasis (Li et al. 2014; Liu et al. 2009). The reason for these conflicting results is not yet clear. Extracellular acidification within the pathophysiologically relevant range inhibits the activity of the $Ca^{2+}$-sensing receptor as it lowers the sensitivity to (i.e., increases the $EC_{50}$ for) extracellular $Ca^{2+}$ (Campion et al. 2015; Quinn and Bai 2004). This acid-induced inhibition may reduce the influence of the $Ca^{2+}$-sensing receptor when the cancer cells reside in the primary tumor but relieve this brake once the cancer cells leave the tumor microenvironment during metastasis.

## 5.2 *Interactions Between $Na^+$, $K^+$, and Acid-Base Homeostasis*

The $Na^+/K^+$-pump fuels multiple secondary active transport processes as it maintains the low intracellular $Na^+$ concentration that drives, amongst others, the energetically unfavorable transport of $Ca^{2+}$ and $H^+$ out of cells and $HCO_3^-$ into cells (Fig. 1). The total tumor tissue $Na^+$ concentration is elevated in solid tumors, which may in part be explained by a larger extracellular volume fraction due to increased leakiness of tumor capillaries, promoting protein loss from the blood stream to the interstitial space and increasing net filtration (Wiig and Swartz 2012; Leslie et al. 2019). This effect may be further enhanced because of cell necrosis either spontaneously or due

to therapeutic interventions (Leslie et al. 2019). The extracellular volume expansion might also be exacerbated by reduced lymphatic drainage due to acid-mediated inhibition of lymphatic pumping (Moeller et al. 2019; Lobov and Kubyshkina 2001). There is also evidence for a more than twofold elevation of the intracellular $Na^+$ concentration in tumors compared to normal tissue and a higher $Na^+$ concentration in rapidly compared to slowly dividing cells (Cameron et al. 1980; Campbell et al. 2013; Roger et al. 2007). Although the $Na^+$ gradient drives net acid extrusion via NHE1 and NBCn1, it must decline dramatically (e.g., by experimentally reducing the extracellular $Na^+$ concentration below 70 mM) before the transporters are substantially inhibited (Bonde and Boedtkjer 2017).

It is increasingly evident that the $Na^+/K^+$-pump also serves as a scaffold for protein–protein interactions and as a receptor complex with signaling properties (Cui and Xie 2017). Together, these functions of the $Na^+/K^+$-pump are critically important for cell volume regulation, proliferation, motility, and invasion (Khajah et al. 2018; Lang 2007); and inhibitors of the $Na^+/K^+$-ATPase show promise against breast cancer as they can induce cell cycle arrest, decelerate tumor growth, promote a more benign cellular morphology, and lower the risk of cancer recurrence (Kometiani et al. 2005; Stenkvist et al. 1979, 1980, 1982). However, there are also suggestions that the cardiotonic steroid digoxin, possibly due to its structural similarity with estrogen, raises the longer-term risk of developing breast cancer (Ahern et al. 2014; Biggar et al. 2013). $Na^+/K^+$-ATPase activity requires a sufficient source of intracellular ATP, which can usually be covered by the accelerated aerobic glycolysis (Zhou et al. 2012; Pecqueur et al. 2013; Qian et al. 2014). The $Na^+/K^+$-ATPase activity is maximal around $pH_i$ 7.2–7.4, inhibited at both lower and higher $pH_i$, but unaffected by changes in $pH_o$ (Eaton et al. 1984; Breitwieser et al. 1987). Thus, considering the ability of cancer cells to maintain $pH_i$ in the neutral range despite extracellular acidification, the $Na^+/K^+$-ATPase activity is expected well maintained in the tumor microenvironment of solid tumors.

The extracellular concentration of $K^+$ is markedly elevated in the tumor microenvironment where it from the normal level around 4 mM can reach around 30 mM primarily due to cell necrosis (Tan et al. 2020; Eil et al. 2016). The depolarization associated with the elevation in interstitial $K^+$ concentration will not directly affect net acid extrusion from breast cancer cells, as it is almost exclusively via the electroneutral NBCn1 and NHE1 (see the Sects. 2.1 and 3.1). However, depolarization could exert indirect influences through changes in cellular activity, metabolism, and hence the cellular acid load. The magnitude of the $K^+$ accumulation determines the effect on the vascular smooth muscle cells and associated changes in vascular diameter. Smaller rises in $K^+$ concentration lead to vasorelaxation due to hyperpolarization via activation of inward-rectifier $K^+$-channels (Edwards et al. 1998), whereas larger rises in $K^+$ concentration – beginning at around 20 mM – cause vascular smooth muscle depolarization and vasocontraction (Voss et al. 2019; Froelunde et al. 2018). A large number of $K^+$ channels – including isoforms of $Ca^{2+}$-activated BK channels, voltage-gated $K_v$-channels, and inward-rectifier $K^+$-channels – are upregulated in breast cancer tissue and implicated in breast cancer development or progression (Mohr et al. 2020; Huang and Jan 2014). Intracellular

acidosis inhibits multiple $K^+$-channel isoforms from these families (Schubert et al. 2001; Ye et al. 2016; Lee et al. 2020), and the ability of cancer cells to defend against intracellular acidification may contribute to tumorigenesis and disease advancement by maintaining $K^+$-channel function.

## 5.3 Interactions Between $Cl^-$ and Acid-Base Homeostasis

Anion exchangers of the SLC4 and SLC26 families can mediate cellular extrusion of intracellular $HCO_3^-$ in exchange for extracellular $Cl^-$ in both cancer and stromal cells (Gorbatenko et al. 2014), see Fig. 1. Downregulation of $Cl^-/HCO_3^-$-exchangers is expected to lower the intracellular $Cl^-$ concentration and limit the cellular acid load. In accordance with the specialization of cancer cells toward enhanced net acid extrusion (see the Sect. 3.1), SLC26A3 (DRA) is a tumor suppressor in colon with predominant expression in well-differentiated epithelium (Gorbatenko et al. 2014; Antalis et al. 1998; Hoglund et al. 1996). Heterologous overexpression of SLC26A3 in cancer cell lines from various sources, including MCF7 human breast cancer cells, causes growth suppression (Chapman et al. 2002); and breast cancer patients with low SLC26A3 expression show decreased overall survival (Bhutia Yangzom et al. 2016). Furthermore, the related *SLC26A4* gene, encoding pendrin, is hypermethylated (Xing et al. 2003) and pendrin expression downregulated (Arturi et al. 2001; Porra et al. 2002) in thyroid cancer. Also, thyroid tumors, including follicular carcinomas, are observed in Pendred syndrome characterized by loss-of-function mutations in *SLC26A4* (Sakurai et al. 2013). However, the regulation and consequences of anion exchangers in cancer tissue are far from unequivocal. AE1 (SLC4A1) is upregulated in gastric and colon cancer, where it promotes cell proliferation and tumor growth (Suo et al. 2012; Tian et al. 2010; Xu et al. 2009). Likewise, AE2 (SLC4A2) is upregulated in ovarian cancer, hepatocellular carcinoma, and colon cancer; and reduction of AE2 expression induces apoptosis and suppresses tumor growth (Zhang et al. 2017; Wu et al. 2006; Hwang et al. 2009; Song et al. 2012). In esophageal cancer, depletion of AE1 inhibits cell proliferation, migration, and invasion and induces apoptosis (Shiozaki et al. 2017), whereas depletion of AE2 increases cell migration and inhibits apoptosis (Shiozaki et al. 2018). Interestingly, a recent preliminary report suggests that AE2 beyond the plasma membrane is expressed in membranes of the Golgi apparatus, where it provides a $H^+$ leak pathway that can promote cancer development (Khosrowabadi et al. 2021).

The dual impact of anion exchangers on acid-base and $Cl^-$ homeostasis makes it difficult to definitively establish the underlying mechanisms linking their expression and function to cancer development. In particular, the variable expression patterns – regarding cellular and subcellular localization in tumors – and the contrasting functional consequences of anion exchangers with seemingly similar transport functions highlight our current lack of understanding. The fundamental determinants of whether individual anion exchangers become pro- or anti-malignant in a given

cancer type or under a given condition need clarification. Notably, pro-malignant effects of anion exchangers are at least partly independent of associated changes in $pH_i$ (Shen et al. 2007) and may rather depend on protein–protein interactions, regulation of the local $Cl^-$ concentration, or cell volume. Correspondingly, $Na^+$, $K^+$,$2Cl^-$-cotransporters (NKCC) that work in parallel with anion exchangers to elevate intracellular $Cl^-$ concentrations (Boedtkjer et al. 2016c) are linked to glioma (Haas and Sontheimer 2010) and hepatocellular carcinoma where they promote proliferation and invasion (Zhou et al. 2017). The link between $Cl^-$ and cancer development is further strengthened by the observation that up- or downregulation of several different $Cl^-$ channels – including CFTR, TMEM16A, and CLCA2 – is associated with breast cancer development (Liu et al. 2020; Wu et al. 2017; Gruber and Pauli 1999). Still, as several of these $Cl^-$ channels are also permeable to $HCO_3^-$ (Tang et al. 2009; Poulsen et al. 1994; Qu and Hartzell 2000; Jung et al. 2013), cancer-modifying effects through altered $pH_i$ or $pH_o$ dynamics cannot be excluded. Interestingly, $Cl^-$ channels have also been proposed to influence gene regulation independently of their ion conductance, acting as transcriptional regulators, but for now this concept is not well developed (Dam et al. 2014).

Multiple cell types sense intracellular $Cl^-$ concentrations or extracellular tonicity based on with-no-lysine (WNK) kinases that are implicated in cancer development and progression (Gallolu Kankanamalage et al. 2018). WNK1 and WNK4 are sensitive to intracellular $Cl^-$ (Pacheco-Alvarez et al. 2020). WNK1 can stimulate tumorigenesis amongst others via angiogenesis (Sie et al. 2020; Li et al. 2020b) and heighten the invasive potential of MDA-MB-231 human breast cancer cells (Shyamasundar et al. 2016). WNK4 shows differential methylation amongst breast cancer patients (Cornen et al. 2014). WNK3 is predominantly sensitive to extracellular tonicity (Pacheco-Alvarez et al. 2020) and can stimulate glioma cell invasion through improved volume regulation (Haas et al. 2011).

## 6 Conclusions

Ion channels and carriers show variable expression and function between normal breast tissue and breast cancer tissue supporting their pathophysiological impact and promise as therapeutic targets. Pathways for transfer of acid-base equivalents across cell and organelle membranes are especially relevant, as they modify the local accumulation of acidic waste products from metabolism. The interactions between cellular ion transport and the tumor microenvironment are bidirectional, as changes in ionic concentrations – for instance, $H^+$, $HCO_3^-$, lactate, and $K^+$ – also influence the expression and function of ion channels and carriers. The $pH_i$ and $pH_o$ conditions are important for enzymatic activities; and ionic conductances in cell membranes furthermore modify local tonicity and membrane potential. These effects, and the contribution of membrane proteins as scaffolds for protein–protein interactions, can explain the influences of ion channels and carriers on cell proliferation, cell volume regulation, migration and invasiveness, secretory activity, immune responses, and

local perfusion. Thus, the abnormal $pH_i$ and $pH_o$ dynamics in the tumor microenvironment fundamentally influence the function of cancer and stromal cells, and their interactions, with the typical net effect of promoting cancer progression.

The cellular responses to the abnormal tumor microenvironment include signaling downstream of dedicated cellular sensors of intra- or extracellular ion concentrations. Cancer cells, cells of the vascular wall, and immune cells all express sensors sensitive to intra- and extracellular $H^+$, $HCO_3^-$, lactate, and $Cl^-$ that are important for the ability of the primary tumor to expand, the invasiveness of cancer cells, and development of metastases.

The intricate interplay between cancer cells and resident and circulating stromal cells in the tumor microenvironment adds a layer of complexity that can explain contradictory effects on cancer progression observed for related ion channels and carriers that otherwise perform seemingly similar transport functions. For instance, anion exchangers expressed in cancer cells typically increase the intracellular acid load, whereas anion exchangers in stromal cells have potential to facilitate acid elimination from the cancer cells by dissipating acute extracellular acid loads. The complex three-dimensional geometry of solid tumors, where multiple cell types are present in connected diffusion-restricted spaces, calls for more studies in cancer tissue and models that represent the in vivo situation and not just the biology of individual cancer cells.

Overall, the potential for developing new therapies is promising if we consider the possibility of modifying the tumor microenvironment (e.g., by manipulating cellular metabolism and ion transport) as well as the adaptations and consequences that it elicits (e.g., via cellular sensors). A broad range of pharmacological targets are available; and future investigations are required in order to identify new drug candidates and confirm how apparently positive therapeutic influences in one cell type (e.g., the cancer cells) are integrated with potential effects on other cell types (e.g., immune and vascular cells). Because the ion transporters, channels, and sensors are sensitive to the composition of the tumor microenvironment, they are particularly promising targets for therapies with tolerable adverse effects. In many cases, the expression of ion channels, carriers, and sensors is upregulated in cancer tissue. Even in cases with wide expression of the ion channel, transporter or sensor throughout the body, increased or decreased functional activity under influence of the local ion concentrations can render cancer or stromal cells in solid cancer tissue more sensitive to pharmacological targeting compared to cells residing under normal tissue conditions.

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# Role of pH Regulatory Proteins and Dysregulation of pH in Prostate Cancer


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## Contents



**Abstract** Prostate cancer is the fourth most commonly diagnosed cancer, and although it is often a slow-growing malignancy, it is the second leading cause of cancer-associated deaths in men and the first in Europe and North America. In many forms of cancer, when the disease is a solid tumor confined to one organ, it is often readily treated. However, when the cancer becomes an invasive metastatic carcinoma, it is more often fatal. It is therefore of great interest to identify mechanisms that contribute to the invasion of cells to identify possible targets for therapy. During prostate cancer progression, the epithelial cells undergo epithelial-mesenchymal transition that is characterized by morphological changes, a loss of cell-cell adhesion, and invasiveness. Dysregulation of pH has emerged as a hallmark of cancer with a reversed pH gradient and with a constitutively increased intracellular pH that is elevated above the extracellular pH. This phenomenon has been referred to as "a

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perfect storm" for cancer progression. Acid-extruding ion transporters include the $Na^+/H^+$ exchanger NHE1 (SLC9A1), the $Na^+HCO_3^-$ cotransporter NBCn1 (SLC4A7), anion exchangers, vacuolar-type adenosine triphosphatases, and the lactate-$H^+$ cotransporters of the monocarboxylate family (MCT1 and MCT4 (SLC16A1 and 3)). Additionally, carbonic anhydrases contribute to acid transport. Of these, several have been shown to be upregulated in different human cancers including the NBCn1, MCTs, and NHE1. Here the role and contribution of acid-extruding transporters in prostate cancer growth and metastasis were examined. These proteins make significant contributions to prostate cancer progression.

**Keywords** Acid extrusion · Carbonic anhydrase · Cell invasion · Metastasis · Monocarboxylate transporter · $Na^+/H^+$ exchanger · Prostate cancer · Proton transport · Sodium bicarbonate cotransporter · Vacuolar adenosine triphosphatases

## Abbreviations

| | |
|---|---|
| CA | Carbonic anhydrase |
| EMT | Epithelial-mesenchymal transition |
| MCT | Monocarboxylate transporter |
| MMP | Matrix metalloproteinase |
| NBC | Sodium bicarbonate cotransporter |
| NHE1 | $Na^+/H^+$ exchanger isoform 1 |
| pHe | Extracellular pH |
| pHi | Intracellular pH |
| uPA | Urokinase plasminogen activator |
| V-ATPases | Vacuolar-type adenosine triphosphatases |

## 1 Introduction

### *1.1 Prostate Cancer, General*

Prostate cancer is the fourth most commonly diagnosed cancer, and although prostate cancer is often a slow-growing malignancy, it is the second leading cause of cancer-associated deaths in men and the first in Europe and North America (Siegel et al. 2014). Therapy by androgen ablation results in tumor regression, but patients are still at risk of androgen refractory tumors (Zhu et al. 2018). In many forms of cancer, including prostate cancer, when the disease is a solid tumor confined to one organ, it is often readily treated. However, when the cancer transforms to an invasive carcinoma that is metastatic, it is more often fatal. It is therefore of great interest to identify mechanisms that contribute to the invasion of cells to identify possibly targets for therapy (Dykes et al. 2017a). During prostate cancer progression, the

epithelial cells undergo epithelial-mesenchymal transition (EMT) that is characterized by morphological changes from a cuboidal to a spindle-shaped cell (Grant and Kyprianou 2013). In prostate cancer and in several other types of cancers, there is a demonstrated downregulation of epithelial markers such as E-cadherin and occludins that leads to loss of cell-cell adhesion. Mesenchymal markers such as vimentin and N-cadherin are upregulated, and this allows the cells to migrate or metastasize to different organs (Lang et al. 2002; Sethi et al. 2010; Singh et al. 2003; Xu et al. 2009). EMT is affected by various growth factors and cytokines, and this induces various transcription factors such as Snail, Twist, and Zeb1/2 (Odero-Marah et al. 2018; Smith and Odero-Marah 2012). Most patients with prostate cancer are killed not by the cancer directly in the prostate but by the tumor metastasizing to critical organs such as the lungs or the liver. Prostate cancer in particular tends to metastasize to bone (Nauseef and Henry 2011). EMT and transcription factor expression play an important critical role in this progression and metastasis (Odero-Marah et al. 2018; Saha et al. 2008; Yuen et al. 2008).

## *1.2 Tumor Microenvironments, General*

Cells within a solid tumor are in a different microenvironment than cells in normal tissues. This affects their gene expression and their physiological properties. The tumor microenvironment is different from other cell microenvironments in that most tumors are hypoxic or anoxic, with reduced glucose and ATP levels, elevated extracellular lactate levels, and an acidic extracellular pH (pHe) (Andersen et al. 2014; Parks et al. 2011; Vaupel 2004). In addition, tumor cells have a very interesting shift in their metabolism called the Warburg effect. Here, aside from the hypoxic environment, and even in the presence of enough oxygen, they are shifted away from oxidative phosphorylation and favor use of glycolytic metabolism (Koppenol et al. 2011). This elevated level of glycolysis and ATP hydrolysis causes the highly proliferative and anabolic cancer cells to produce more acid than normal cells (Andersen et al. 2014). A more alkaline intracellular pH (pHi) is suggested to be a prerequisite for growth, proliferation, and motility (Fig. 1) (Schwab et al. 2012; Webb et al. 2011). For example, studies in neutrophils (Hayashi et al. 2008) have shown that cellular alkalinization of 0.26 pH units occurs despite increased acid production, and this plays a permissive role in cell motility. Additionally, in fibroblasts, a pH threshold exists of about 7.2, below which growth factors cannot set in motion a $G_0$ to S phase transition (Pouysségur et al. 1985), and intracellular pH controls protein synthesis and $G_0/G_1$ transition of fibroblasts (Chambard and Pouyssegur 1986). Conversely, inhibition of acid-extruding proteins, which raise intracellular pH, has been shown to be inhibitory to cell growth, migration, or invasion in several different cell types including in hepatocellular cancer, breast cancer, and gastric cancer (Amith et al. 2016a; Lu et al. 2005; Xie et al. 2017; Yang et al. 2010).

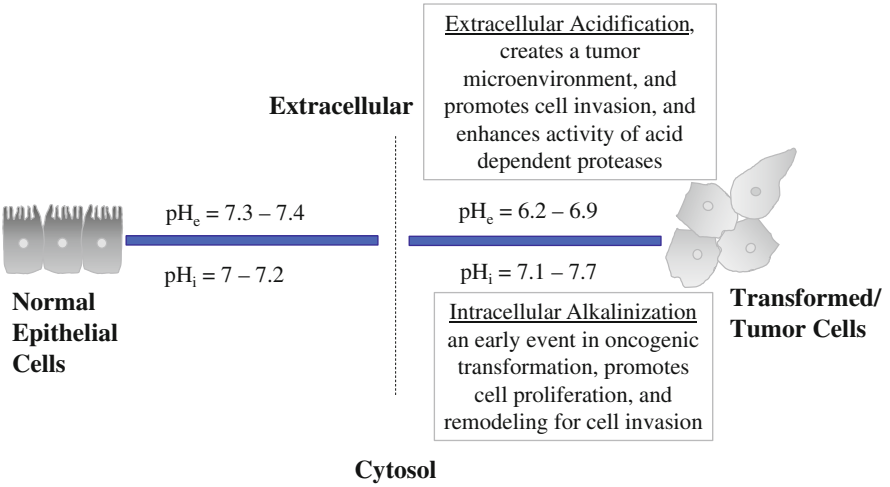


**Fig. 1** Dysregulation of intracellular and extracellular pH creates a "perfect storm" for cancer progression (Cardone et al. 2005; Webb et al. 2011). While tumor pH is not uniformly acidic (Helmlinger et al. 1997; Vaupel et al. 1981), with oncogenic transformation there is a general decrease in extracellular pH and an increase in intracellular pH (reviewed in Gillies et al. 2002). These events tend to promote cellular remodeling and invasive behavior that promote cell proliferation and metastatic behavior

Additionally, not only is an elevated pHi an important part of tumor cell growth and metastasis, a more acidic external microenvironment has recently been thought to be an important part of metastatic behavior (Fig. 1) (Reshkin et al. 2014; Stock et al. 2008), which may occur through more than one mechanism. The acidic external microenvironment may result in protonation of chemotherapeutic drugs through a mechanism called "ion trapping," which can result in decreased cellular uptake and decreased effectiveness (Lebelo et al. 2019; Mahoney et al. 2003; Peppicelli et al. 2017; Raghunand et al. 2003). Alternatively, acidification of the extracellular matrix may promote proteolysis of peri-invadopodial space (Fig. 2) (Busco et al. 2010; Greco et al. 2014).

The abnormalities of intracellular alkalinization along with extracellular acidification of tumors appear to be a specific hallmark of malignancy. Dysregulation of pH has emerged as a hallmark of cancer with a reversed pH gradient and with a constitutively increased intracellular pH that is elevated above the extracellular pH. In fact, this phenomenon has been referred to as "a perfect storm" for cancer progression (Fig. 1). It facilitates various characteristics of cancers such as being permissive for cell proliferation, helps evade apoptosis, and facilitates metabolic adaptation that is obligatory for cancer cells. Additionally, cellular migration is enhanced (Webb et al. 2011). Degradation of the extracellular matrix is a critical step of tumor cell invasion. It requires protease-dependent proteolysis concentrated at invadopodia, where proteolysis of the extracellular matrix occurs (Busco et al. 2010; Greco et al. 2014). The pH optimum of these extracellular proteases is acidic,

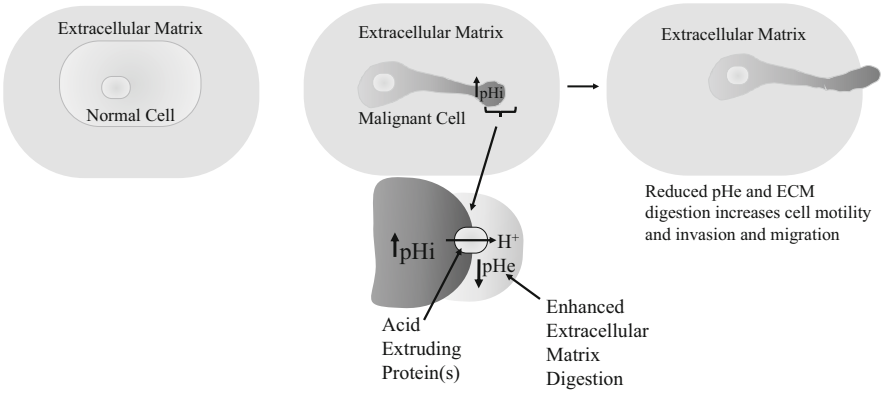


**Fig. 2** Proposed role of extracellular acidification facilitating proteolysis and metastasis of tumor cells. In malignant cells acid is extruded by various mechanisms, and this results in decreased extracellular pH in a localized leading-edge region. This causes enhanced extracellular matrix digestions and increased cell motility and migration through this region (Cardone et al. 2005). Left panel, normal cell surrounded by extracellular matrix. Centre panel, malignant cell surrounded by extracellular matrix and enlargement of the leading edge of the cell, indicating reversed pH gradient. Right panel, enhanced mobility of malignant cell leads to migration

and proton extrusion out of cancer cells makes an acidic external microenvironment that facilitates their activity (Cardone et al. 2005; Greco et al. 2014) (Fig. 2).

Therefore, various methods of producing intracellular acidification or inhibiting extracellular acidification are becoming a new therapeutic concept for cancer treatment sometimes referred to as "the pH-centric anticancer paradigm"(Harguindey et al. 2017, 2018; Harguindey and Reshkin 2017). While tumor pH is not uniformly acidic (Helmlinger et al. 1997; Vaupel et al. 1981), the general abnormality in cancer of intracellular alkalinization, along with extracellular acidification, results in "proton gradient reversal" of solid tumors and leukemic cells and is becoming recognized as a specific and selective hallmark of cell malignancy. Attempts to induce cellular acidification using treatments such as proton transport inhibitors are becoming a new therapeutic concept for treatment of cancers (De Milito et al. 2010; De Milito and Fais 2005; Harguindey et al. 2018; Koltai 2017).

By modifying the pH characteristics of cancer cells, it is suggested that it may be possible to decrease proliferation and invasion. The origin of extracellular acidity is the excess of protons generated by cancer cells' metabolism that are extruded from cancer cells by membrane proton transporters (or their equivalent). As noted above, the excess of protons is due to increased lactate and acid production because tumor cells metabolize more glucose than normal cells, through the glycolytic pathway, which is called the Warburg effect (Koltai 2017).

## 1.3 Acid-Extruding Ion Transporters

Mediators of increased acid extrusion in tumor cells include the $Na^+/H^+$ exchanger NHE1 (SLC9A1), $Na^+HCO_3^-$ cotransporters NBCn1 (SLC4A7) and NBCe1 (SLC4A4), anion exchangers, vacuolar-type adenosine triphosphatases (V-ATPases), carbonic anhydrases (CA), and the lactate-$H^+$ cotransporters of the monocarboxylate family (MCT1 and MCT4 (SLC16A1 and 3)) (Amith and Fliegel 2016, 2017; Boedtkjer et al. 2012; Koltai 2017; Lauritzen et al. 2010; Le Floch et al. 2011; Mcintyre and Harris 2016; Parks et al. 2011) (Fig. 3). Of these, several have been shown to be upregulated in different forms of human cancers including the $Na^+HCO_3^-$ transporter, MCTs, and NHE1 (Amith and Fliegel 2016; Boedtkjer et al. 2013; Chiche et al. 2012; Dhup et al. 2012; Parks et al. 2011).

## 1.4 Acid-Extruding Transporters in Prostate Cancer

Given that a number of different pathways can contribute to acid extrusion in various types of cancer (Fig. 3), here, the roles of various acid-extruding transporters in prostate cancer are examined, with a brief presentation of their role in some other

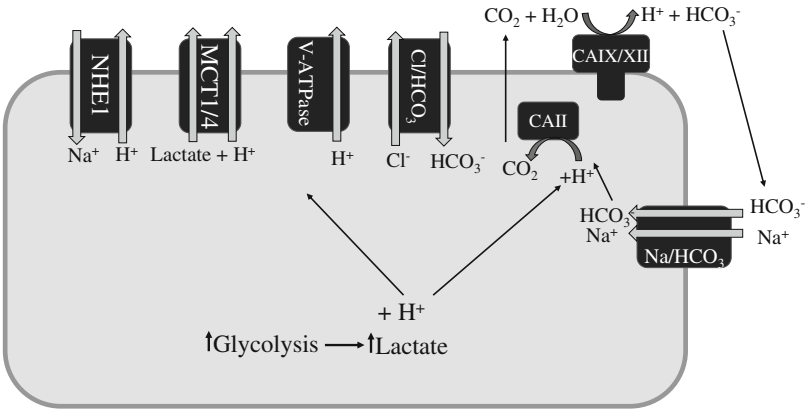


**Fig. 3** pH control systems in tumor cells for metabolically produced acid. Increased glycolysis leads to elevated lactate and proton production which are removed from the cell by various transporters. Carbonic anhydrases II, IX, and XII also make significant contributions to extracellular acidity (Harguindey et al. 2009; Webb et al. 2011). NHE1, $Na^+/H^+$ exchanger isoform 1; MCT1/4, monocarboxylate transporter 1 and 4; V-ATPase, vacuolar-type $H^+$-adenosine triphosphatase; $Cl/HCO_3^-$, chloride/bicarbonate anion exchangers; $NaHCO_3^-$, sodium-dependent bicarbonate cotransporters; CA, carbonic anhydrase with isoforms indicated. The putative cycle of proton release through the actions of CAs is shown. V-ATPase is shown at the plasma membrane where it is mistargeted in certain tumor types and contributes to pH regulation of tumor cells (Cotter et al. 2015; Martinez-Zaguilan et al. 1993)

types of cancer. Table 1 (supplementary) summarizes studies which have suggested a role for various acid-extruding proteins in prostate cancer.

## 1.5 $Na^+/H^+$ Exchangers

$Na^+/H^+$ exchangers have been implicated as playing key roles in a number of types of cancers. This has been extensively documented in several cell types, in notable detail in breast cancer cells. There, it has been shown that cytoplasmic alkalinization and extracellular acidification occur as a result of increased NHE1 activity (Amith and Fliegel 2013; Reshkin et al. 2013). The extracellular acidification leads to proteolytic digestion of the extracellular matrix (Busco et al. 2010). Knockout of overactive NHE1 protein in the triple negative breast cancer cell MDA-MB-231 leads to impaired in vivo xenograft tumor growth and impaired invasive behavior. In addition, NHE1 inhibition or knockout leads to increased sensitivity to paclitaxel treatment (Amith et al. 2015b). Several regulatory loci on the NHE1 regulatory cytoplasmic domain have been identified as modulating metastatic and invasive potential, including phosphorylation at serine 703. Additionally, NHE1 regulation, through phosphorylation of its regulatory cytosolic domain, modifies EMT of triple negative breast cancer cells (Amith et al. 2016b).

Inhibition of NHE1 prevents extracellular acidification in breast cancer cells and is suggested to be a treatment that may reduce chemotherapy resistance (Amith and Fliegel 2017; Harguindey et al. 2013). Conversely, increased expression of NHE1 is associated with poor outcomes in several forms of cancer including breast cancer (Amith et al. 2017), ovarian cancer (Wang et al. 2018), and gliomas (Guan et al. 2018).

NHE1 has been studied to some degree in prostate cancer, but much is unknown. NHE1 mRNA expression has been shown to be elevated in prostate cancer relative to normal tissue (Amith et al. 2015a). Expression of NHE1 in DU145 prostate cancer cells correlates with Zeb1 expression, a transcription factor that promotes EMT (Dykes et al. 2017a). The NHE3 isoform message has also been shown to be present in the prostate gland (Brant et al. 1995) and in prostate cancer cells (Brant et al. 1995). Additionally, the protein was also found on the cell surface of prostate cancer cells (Gonzalez-Gronow et al. 2005).

Hormonal regulation of NHEs in prostate cancer cells is of interest. Hepatocyte growth factor, which is found in the prostate tumor microenvironment, triggers invasion, metastasis, and EMT and induces NHE activity in DU145 prostate cancer cells (Steffan et al. 2010). Hepatocyte growth factor induced trafficking of lysosomes to the cell surface and increased extracellular acidification, which was thought to be important in promoting invasiveness (Fig. 4). In this study, NHE activity was suggested to directly extrude protons and was also shown to promote lysosomal migration to the cell surface in the DU145 prostate cancer cell line (Steffan et al. 2010). It should also be noted that two isoforms of NHE, NHE1 and NHE3, were shown to contribute to lysosomal trafficking and extracellular pH acidification in

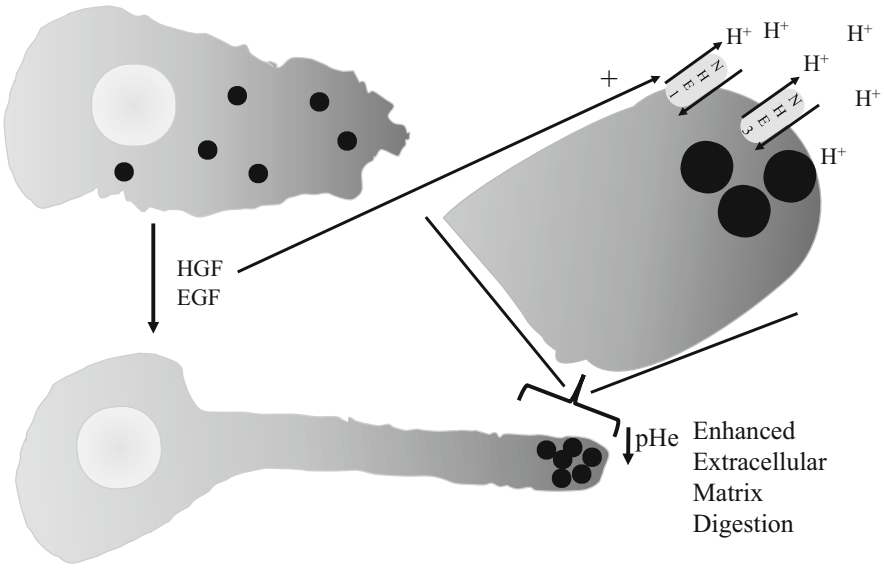


**Fig. 4** Lysosomal trafficking to the cell periphery is induced in prostate cancer cells by treatment with EGF (epidermal growth factor) or HGF (hepatocyte growth factor). The schematic diagram illustrates the effect of hormone treatment of prostate cancer cells (Dykes et al. 2017b; Steffan et al. 2010). Lysosome redistribution (and NHE concentration at the tip of pseudopods) is thought to result in local extrusion of acid and protease causing focalized proteolysis of the extracellular matrix. This is believed to compromise cell attachment resulting in increased cellular motility (Cardone et al. 2005). Lysosomes are represented by black filled circles. NHE1, NHE3, or other NHE isoforms may contribute (Steffan et al. 2010)

both DU145 and PC3 prostate cancer cells and inhibition of both together had an additive effect in preventing lysosomal exocytosis (Steffan et al. 2009). Steffan et al. (2010) suggested that other isoforms, aside from NHE1 and NHE3, may also be involved in HGF-induced lysosome movement in DU145 cells. However, this has yet to be investigated further.

Epidermal growth factor is another hormone that stimulates lysosomal trafficking and invasion by DU145 cells that is dependent on NHE activity. Epidermal growth factor was shown to stimulate lysosomal trafficking, protease secretion, and invasion via a p38MAPK regulated pathway that was different from hepatocyte growth factor stimulation. Hepatocyte growth factor required PI3K and ERK (Dykes et al. 2017b).

Several other forms of regulation of NHEs also stimulate prostate cancer cell invasion. The plasminogen activation system comprises the serine protease urokinase plasminogen activator (uPA), its cell surface receptor, and two endogenous serpin inhibitors, plasminogen activator inhibitors 1 and 2. Plasminogen II can be converted to the active serine protease plasmin upon binding to cell surface receptors. Plasminogen type II binding to dipeptidyl peptidase IV on the surface of highly invasive 1-LN (prostate cancer) cells stimulated a NHE3-mediated rise in intracellular pH that promoted cell invasiveness (Gonzalez-Gronow et al. 2005).

The uPA receptor controls plasticity of prostate cancer cell movement (Margheri et al. 2014). uPA expression is higher in patients with prostate cancer (Akudugu et al. 2015; Bohm et al. 2013; Lippert et al. 2016), and its receptor is a biomarker for prostate cancer aggressiveness (Skovgaard et al. 2017a, b). It is important to note that knockdown of uPA and its receptor inhibits prostate cancer cell invasion, survival, and tumorigenicity in vivo and uPA and its receptor levels correlate with invasive potential of prostate cancer cell lines. For example, knockdown of uPA and its receptor inhibits invasion and proliferation by PC3 cells (Pulukuri et al. 2005).

Membrane androgen receptors are expressed in several kinds of tumor cells including prostate cells. It is interesting to note that when stimulated with testosterone-albumin conjugates, membrane androgen receptors rapidly activate NHE1 in DU145 and LNCaP prostate cancer cells, an effect specifically blocked by NHE1 inhibitors and by inhibitors of glucocorticoid-inducible kinase (SGK1) and Rho-associated protein kinase (ROCK) (Chatterjee et al. 2014).

It should be noted that Chatterjee et al. (Chatterjee et al. 2014) also suggested a smaller amount of NHE2 is present in DU145 cells which agreed with Northern blot analysis of NHE2 distribution in whole tissues (Malakooti et al. 1999). However, NHE2's role in promoting invasiveness and acidifying extracellular pH is not well studied.

Overall, there is a large amount of evidence implicating $Na^+/H^+$ exchangers in prostate cancer metastasis, primarily the NHE1 isoform and also the NHE3 isoform, and possibly there is a contribution of other isoforms.

## *1.6 $Na^+HCO_3^-$ Cotransporter NBCn1 (SLC4A7)*

The $Na^+HCO_3^-$ transporter (NBCn1) is present in the prostate (Romero and Boron 1999; Sun and Bonanno 2003), but it has not been studied in the context of prostate cancer. It is worth briefly reviewing the role of NBCn1 in other forms of cancer, as a possible means of predicting possible roles in prostate cancer. In human MCF-7 breast cancer cells, NBCn1 mRNA and protein levels are increased by introduction of the constitutively active ErbB2 receptor, which is upregulated in cancers and associated with increased metastasis (Lauritzen et al. 2010), while in MCF-7 cells NHE1, not NBCn1, was important in enhancing cell motility in culture (Lauritzen et al. 2012). NBCn1 was critical in ErbB2-induced breast carcinogenesis in mice, and its knockout decelerated tumor growth by approximately 1/3 (Lee et al. 2018b). In other breast cancer types, NBCn1 may also be important. In primary breast carcinomas, it was notable that NBCn1 expression was upregulated and this protein was the major determinant of intracellular pH (Boedtkjer et al. 2013). In the triple negative breast cancer cell line MDA-MB-231 cells, shRNA-mediated knockdown of NBCn1 reduced pHi and the ability to form primary tumors in xenografts (as did knockdown of NHE1 and MCT4). Additionally, knockdown of NBCn1 (or MCT4) but not of NHE1 increased tumor-free survival and decreased cell proliferation and colony growth in soft agar (Andersen et al. 2018).

These studies indicate that NBCn1 can certainly have a critical role in some types of tumor growth and survival, though this seems to vary with the precise cell type, even in the same general cancer. Studies have also shown that other cancer types such as melanoma (Yang and Loh 2019), pancreatic (Kong et al. 2014), and renal cell carcinomas (Yamada et al. 2003) are dependent on bicarbonate transporters for intracellular pH regulation. The role of NBCn1 in various forms of prostate cancer would thus seem to be a priority for investigation.

The $Na^+HCO_3^-$ cotransporter NBCe1 (SLC4A4) is an electrogenic transporter with a stoichiometry of 1:2 or 1:3 $Na^+$:$HCO_3^-$. It has been less studied than NBCn1, but one interesting study examined its role in growth and migration of breast cancer and colon cancer cells. Hypoxia induced NBCe1 mRNA expression in colon adenocarcinoma cells. NBCe1 contributed to pH regulation, and knockdown of the protein reduced cell proliferation. In MDA-MB-231 breast cancer cells, knockdown of NBCe1 also decreased proliferation, migration, and invasion. These results suggested that NBCe1 contributes to $HCO_3^-$ transport in tumor cell phenotypes (Parks and Pouyssegur 2015). One preliminary publication has also suggested that hypoxia-induced acidification causes a small but significant upregulation of the protein (Lee et al. 2018a). Additionally, it is interesting to note that mutations in the SLC4A4 gene have recently been found in patients with prostate cancer (Liang et al. 2019). However, further studies in this area are needed.

## *1.7 Anion Exchangers*

Anion exchangers comprise a family of transport proteins typified by AE1 (anion exchanger type 1, SLC4A1). The protein functions in mediating the exchange of $Cl^-$ for $HCO_3^-$ and was originally studied as Band 3 in red blood cell membranes (Cordat and Casey 2009; Cordat and Reithmeier 2014). The bicarbonate transport proteins include over a dozen members, including a SLC26 family (Cordat and Casey 2009). AE1–3 are electroneutral transporters. There are few studies on the anion exchangers aside from those on NBCs as noted above. The SLC4A1 message is overexpressed in primary tumors and metastasis compared with normal prostate tissue as is the SLC26A8 ion transporter (Marin-Aguilera et al. 2015). However, there has been little to implicate most of these anion exchangers directly in promoting metastatic behavior in prostate cancers or other cancers, aside from studies on NBCs. In fact, the conclusive identification of the $HCO_3^-$ transporter as responsible for uptake is mostly unclear. The large diversity of the family, as well as lack of clear roles in tumors, plus the lack of specific inhibitors for the transporters, has delayed progress in this area. There have been earlier suggestions that $HCO_3^-$ uptake occurs via a $Cl^-/HCO_3^-$ exchanger, but more study is required in this area (Parks et al. 2013). In a tumor cell, where intracellular pH tends to be elevated, and extracellular pH tends to be decreased compared to normal cells, bicarbonate concentrations would tend to be more elevated, possibly because of reduced CA activity (Fig. 3).

Thus, there would be less of a propensity for uptake of bicarbonate through anion exchangers (Parks et al. 2013).

## *1.8 Lactate-$H^+$ Cotransporters (MCT1 and MCT4)*

Lactate-$H^+$ cotransporters (or symporters) are part of a fairly large SLC16 gene family. Monocarboxylate transporters, MCT1 and MCT4, are two of the four members of the family (MCT1 (SLC16A1), MCT2 (SLC16A7), MCT3 (SLC16A8), and MCT4 (SLC16A3)) that transport monocarboxylates such as L-lactate, pyruvate, and ketone bodies across the plasma membrane. The MCTs facilitate proton-linked transport of monocarboxylates across the plasma membrane, and L-lactate is the predominant substrate (Halestrap 2013). These proteins are driven mainly by substrate availability and were not thought of as normal pH regulatory proteins. Therefore, until recently they tended to be overlooked in relation to tumor pHi regulation. However, since tumors produce excess acid and protons, excretion of acid via this transport mechanism can provide important contributions to pHi regulation of tumors (Parks et al. 2013).

The discovery that MCT4 is induced by hypoxia provided part of the drive for studying these transporters in cancer (Parks and Pouyssegur 2017; Ullah et al. 2006). Combined genetic disruption of MCT1/2 and MCT4 caused significant decreases in pHi in colon adenocarcinoma and glioblastoma human cell lines (Marchiq et al. 2015). As noted above, MCT4 appears to also play an important role in breast cancer. MCT4 levels are elevated in breast cancer, and it is associated with poor patient survival correlating with lymph node status. Knockdown of MCT4 attenuated tumor growth in triple negative (MDA-MB-231) mouse xenografts and reduced proliferation rates, invasion, and anchorage-independent colony formation of MDA-MB-231 cells (Andersen et al. 2018). Similar results were found in invasive bladder cancer and urothelial carcinoma (Todenhofer et al. 2018).

In prostate cancer, MCTs are not well studied, but there are some interesting studies suggesting an important role for MCT4. Elevated MCT4 expression was associated with castration-resistant prostate cancer (Pertega-Gomes et al. 2011). Additionally, knockdown of MCT4 levels with antisense oligonucleotides in PC3, DU145, and C4-2 cells decreased proliferation of these prostate cancer cell types. Antisense oligonucleotides against MCT4 also inhibited tumor growth in PC3 tumor-bearing mice (Choi et al. 2016). Another group (Pertega-Gomes et al. 2015) showed the presence of MCT1 and MCT4 in human prostate samples. Of course, cancer cells exhibit high glycolytic rates with consequent lactate production. When a large cohort of human prostate tissue cancers were examined, increased glycolytic rates correlated with a poor prognosis, and MCT4 was elevated in metastatic tumors, supporting a role for MCT4 as a target in the disease (Pertega-Gomes et al. 2015). Further, a recent study examined a murine model of prostate cancer examining extracellular pH of low- and high-grade tumor regions. A low extracellular pH correlated with elevated MCT4 gene expression, which suggested that MCT4 is a

contributor to extracellular acidosis. Low-grade tumors also possessed low lactate dehydrogenase activity (lactate dehydrogenase catalyzes the production of lactate from pyruvate) (Korenchan et al. 2019). In summary, several studies have implicated MCT4 as being an important contributor to tumor effectiveness and as a cause of extracellular acidosis.

## *1.9 Vacuolar-Type $H^+$-ATPase*

In addition to NHEs, MCTs, and bicarbonate transporters, vacuolar-type $H^+$-ATPases (V-ATPases) are also proposed to contribute to pH regulation of tumors (Parks et al. 2011). V-ATPases are multi-subunit, ATP-driven proteins that play roles in processes such as endocytosis, trafficking, and lysosomal acidification. They are comprised of 13 distinct subunits that are part of the membrane integral or cytosolic domain. They are oriented to either proton pumps from the cytosol to organelles or, if on the plasma membrane, to the extracellular space (Whitton et al. 2018). Aside from acidification of organelles, they play a role in acid secretion at the plasma membrane (Fig. 3) in a variety of cell types including renal intercalated cells, osteoclasts, and epididymal cells (Sennoune et al. 2004b). Certain tumor types express V-ATPases at the plasma membrane, possibly because of missorting to the plasma membrane (Cotter et al. 2015; Martinez-Zaguilan et al. 1993; Smith et al. 2016).

Overexpression of V-ATPases in tumor cells has been documented in several cases, and these $H^+$ pumps are thought to be important removers of acid in these tumor cells (Parks et al. 2013). In human breast cancer cells, highly metastatic cells have more V-ATPase than lowly metastatic cells (Sennoune et al. 2004a). Similarly, when comparing highly metastatic melanoma cells to lowly metastatic cells, the highly metastatic cells expressed V-ATPases at the plasma membrane, while the lowly metastatic cells did not (Nishisho et al. 2011). Overexpression of V-ATPase was demonstrated in breast cancer; cervical cancer; gastric, lung, esophageal, and oral squamous cell carcinoma; and ovarian and pancreatic cancer (Whitton et al. 2018). In many cases, elevated expression in patients was correlated with poorer survival. Inhibition of V-ATPase has been associated with inhibition of EMT (Merk et al. 2017). Activity of plasma membrane V-ATPases is important in invasion of MDA-MB231 breast cancer cells (Cotter et al. 2015).

As V-ATPases have many subunits, for physiological analysis by knockdowns, a multitarget approach may be required. For example, disruption of subunit a4, or a3 subunits, inhibited migration and invasion of MDA-MB-231 breast cancer cells (Hinton et al. 2009). Similarly, another study examined MCF10CA1a cells and found that knockdown of a3 (but not a1, a2, or a4 subunits) decreased invasion of these cells (Capecci and Forgac 2013). The a3 isoform has also been localized specifically to the plasma membrane of breast cancer cells (Cotter et al. 2016). Recently however, it should be noted that invasion and migration of 4T1-12B breast cancer cells was disrupted by ablation of the a4-encoding gene alone. This also

inhibited targeting of the V-ATPase to the leading edge of the cell, suggesting it is important in the invasive cancer phenotype in these cells. Knockdown or knockout of other isoforms (a1, a2, a3) did not inhibit cell invasion (Mcguire et al. 2019). Clearly, there may be some cell-specific dependencies on different isoforms of the V-ATPase.

Studies on the role of V-ATPase in prostate cancer are fewer and further between than for other types of cancer but support a role for the V-ATPase in disease. It was recently demonstrated that expression of the C subunit of the V-ATPase was higher in prostate cancer cell lines with greater metastatic potential. Further, downregulation of the V-ATPase C subunit using siRNA, in the PC-3M-1E8 human prostate cancer cell line, decreased V-ATPase activity and invasion in vitro by these cells (Zou et al. 2018). Conversely, upregulation of V-ATPase activity was thought to be responsible for increased invasion and metastasis which occurred with downregulation of the tumor metastasis suppressor gene LASS2/TMSG1 of PC-3M-2B4 prostate cancer cells (Xu et al. 2014a, b). No database information appears to be available linking overexpression or amplification of the V-ATPase subunits to prostate cancer; however there is some such linkage for one subunit in breast cancer. No doubt this type of analysis is complicated by the presence of the 13 distinct subunits (Mcconnell et al. 2017; Whitton et al. 2018).

It is worth mentioning that proton pump (V-ATPase) inhibitors have been tried as a therapeutic target, for various forms of cancers, with mixed success. The chemical inhibitors such as bafilomycin A and concanamycin A bind to the subunit responsible for proton translocation. V-ATPase inhibition results in reduced cancer cell growth in a number of cell types (reviewed in (Whitton et al. 2018)). There has been a suggestion that the effects of bafilomycin on tumor cells are through induction of HIF-1α (hypoxia-inducible factor) expression and subsequent p21 induction and cell cycle arrest, rather than through effects on pH regulation (Lim et al. 2006; Parks et al. 2011). However, despite this possibility, proton pump inhibitors are being pursued to reduce tumor growth and have showed promise in many studies (Parks et al. 2011; Whitton et al. 2018). Proton pump inhibitors have an interesting advantage for cancer therapy as their activation requires acidity which is found in the tumor microenvironment (Parks et al. 2013), so they may be cancer specifically targeted to tumors. Proton pump inhibitors have been shown to inhibit tumor cell growth by disrupting acidosis in vivo in several models and may improve the efficacy of chemotherapy agents (Avnet et al. 2016; De Milito et al. 2010; Spugnini and Fais 2017). Of interest, Martins et al. recently found a plant-derived monoterpene that inhibits V-ATPases and had therapeutic potential in malignant melanoma (Martins et al. 2019).

Studies on the effect of proton pump inhibitors on prostate cancer are rarer. The proton pump inhibitors bafilomycin A and concanamycin A have been shown to decrease migration and invasion of the PC-3 prostate cancer cell line, though in this study it was suggested that the effects were mediated through intracellular pumps, and plasma membrane levels of V-ATPase were negligible (Licon-Munoz et al. 2017). A different study examined LNCaP and C4-2B cells and found that the same inhibitors reduced invasion by both cell types, regardless of the different levels of

V-ATPase in these cell lines. The V-ATPase was predominant at the plasma membrane in the androgen-independent C4-2B cells, and only traces were found on the plasma membrane of androgen-dependent and low metastatic LNCaP cells (Michel et al. 2013). Clearly, more studies in this area would be of interest, along with a characterization of the subunits of the V-ATPase subunits that are present in prostate cancer cells.

A class of proton pump inhibitors (including omeprazole, esomeprazole, lansoprazole, pantoprazole, and rabeprazole) inhibit gastric acid pump ($H^+$, $K^+$-ATPase) but also directly inhibit vacuolar V-ATPases ($H^+$-ATPase). In 2005 it was suggested that these compounds may be of use to reduce tumor resistance (De Milito and Fais 2005). Of these lansoprazole was investigated in some detail by the Fais group. A small study examined the effect of adding high doses of lansoprazole to their chemotherapy regimen in cats and dogs with advanced neoplasms. The drug was generally well tolerated with an indication that tumor chemoresistance was reduced by the treatment (Spugnini et al. 2011, 2014). Additionally, lansoprazole pretreatment enhanced the sensitivity of human melanoma cells to suboptimal doses of paclitaxel in vitro, and also, in SCID mice lansoprazole increased the sensitivity of subcutaneously injected human tumor cells to paclitaxel (Azzarito et al. 2015). A similar effect was shown with combination treatment using the reverse transcriptase inhibitor efavirenz and lansoprazole (Lugini et al. 2017). Lansoprazole also had an antitumor effect, a cytotoxic action, against human multiple myeloma cells (Canitano et al. 2016), and the combination of lansoprazole and CA inhibitors inhibited cell proliferation and induced cell death in human melanoma cells (Federici et al. 2016). Lansoprazole appears to have greater cytotoxicity toward tumor cells than other proton pump inhibitors of this type (rabeprazole, pantoprazole, omeprazole, esomeprazole) both in vitro and in xenografts (Lugini et al. 2016). A review in 2017 summarizes clinical studies in this area. In one study, patients receiving proton pump inhibitors experience the highest response rates to chemotherapy and longest survivals (Spugnini and Fais 2017; Wang et al. 2015). While these reports strongly support the idea that proton pump inhibitors may be useful in clinical treatment of cancers, a recent large-scale study examined individuals with incidences of breast cancer, prostate cancer, and malignant melanoma in Iceland (Halfdanarson et al. 2019). Here there was no correlation between exposure to proton pump inhibitors and any chemoprotective effect. It should be noted however that this study examined a chemopreventive effect, and not the effect of combination therapies in disease. Studies in that area would still be of high interest.

## *1.10 Carbonic Anhydrases*

Carbonic anhydrases are a family of 15 proteins that catalyze the hydration of $CO_2$ to generate basic $HCO_3^-$ and $H^+$ (Fig. 3) (Pastorekova and Gillies 2019). Of the family members, CAIX is rapidly induced by hypoxia-inducible factor and is present on the extracellular surface of tumor cells. Normally, it is only expressed in limited parts of

the gastrointestinal tracts. It, together with CAXII on the extracellular surface, can acidify extracellular pH of tumor cells and promote tumor cell growth (Parks et al. 2013; Parks and Pouyssegur 2017; Svastova et al. 2004), in addition to raising intracellular pH (Chiche et al. 2009). CAIX has been localized to tumor invadopodia where it promotes matrix degeneration (Debreova et al. 2019). Studies have looked at the effect of knockdown of CAs on tumor growth. In 4T1 breast cancer cells, depletion of CAIX resulted in regression of orthotopic mammary tumors and metastasis formation. Stable depletion of CAIX also resulted in attenuation of primary growth in MDA-MB-231 cells (Lou et al. 2011). Knockout of CAIX can reduce xenograft tumor growth of LS174Tr colon carcinoma cells, and knockout of both CAIX and CAXII gives a much better reduction in tumor growth (Chiche et al. 2009). Similarly, another group showed knockout of CAIX in LS174 cells reduced xenograft tumor growth which was more pronounced in hypoxic conditions (Parks et al. 2017), and a third group showed that knockout of CAIX in MDA-MB-231 breast cancer cells also attenuated xenograft tumor growth (Lou et al. 2011). It is of note that examination of the effects of CAIX on tumor extracellular pH in vivo has shown that CAIX acidifies the extracellular environment (Lee et al. 2018c). CAs are also known to bind to some of the other proton transport proteins such as MCT4 (Noor et al. 2015) and NHE1 (Li et al. 2002). This enables rapid $H^+$ availability and enhances transport of protons. The presence of intracellular CAII was shown to enhance MCT transport (Klier et al. 2014) and NHE1 activity (Li et al. 2006). CAs are associated with poor prognosis in various cancer types, and this is through regulation of EMT (Hyuga et al. 2017; Lock et al. 2013).

Aside from these direct, experimental studies, there have also been links to elevated CAIX expression and various forms of cancer. In human glioblastoma, elevated CAIX expression level was associated with a significant decrease in survival time (Proescholdt et al. 2012), and a large-scale meta-analysis confirmed that CAIX is a marker of poor tumor prognosis in the majority of tumor types studied (Pastorekova and Gillies 2019).

There appears to be fewer studies on CAs and prostate cancer, compared with some other cancer types, but there is some key work that linked CAs with prostate cancer. Lin et al. have recently shown that there is a genetic polymorphism of CAIX that is associated with increased risk of lymph node invasion and metastasis in prostate cancer (Lin et al. 2019). While another study showed an association with CAIX expression levels and with expression levels in high-grade tumors, the localization of the protein was mostly nuclear in these tissue samples (Ambrosio et al. 2016). In contrast, a different earlier study could not find an association of CAIX expression and prostate cancer (Smyth et al. 2010). CAXII and CAIX mRNA are found in PC3 prostate cancer cells but not in DU145 cells (Ivanov et al. 2001). CAIX in PC3 cells was inducible by hypoxia (Li et al. 2009). Interestingly, the pivotal work of Fiaschi et al. (Fiaschi et al. 2013) showed that CAIX is upregulated in cancer-associated fibroblasts from prostate cancer cells, and this leads to extracellular acidification that is blocked by CAIX inhibition. Also, exposure of prostate cancer cells to conditioned medium from cancer-associated fibroblasts increased expression of CAIX. It is of note that CAIX expression was dependent on hypoxia-

inducible factor-1 expression. Elevated CAIX activity enhanced matrix metalloproteinase (MMP) activity which is thought to be a trigger for metastatic behavior. Further, the cancer-associated fibroblasts enhanced EMT in prostate cancer cells, as indicated by decreased E-cadherin levels. Silencing of CAIX prevented the ability of conditioned medium from cancer-associated fibroblasts, to decrease E-cadherin levels (Fiaschi et al. 2013).

There are a large number of publications that have explored translation into the clinic of CA work in various cancer types (reviewed in (Pastorekova and Gillies 2019)). Different families of sulfonamides, sulfamates, or related compounds show anticancer potency in animal models (Lou et al. 2011). Briefly, in a breast cancer model of 4T1 mammary tumor cells, 4-{[(3′-nitrophenyl)carbamoyl]amino} benzenesulfonamide significantly inhibited the formation of metastases (Pacchiano et al. 2011). Similarly, a small molecule CAIX inhibitor treatment of mice with 4T1 tumors resulted in attenuation of tumor growth and metastasis formation (Lou et al. 2011). The sulfamide drug DH348 was also shown to reduce hypoxia-induced extracellular acidosis and to sensitize tumors to radiation therapy in CAIX-expressing tumors (Dubois et al. 2013). It should be noted however that adverse side effects and compensation by other proteins are a concern in these studies (Pastorekova and Gillies 2019).

In prostate cancer cells, little has been done in this regard. However, a CAIX isoform-specific selenazole compound recently showed antitumor activity against human prostate cancer (PC3) cells (Angeli et al. 2018).

## 2 Summary and Future Directions

The above review of the literature makes it clear that with regard to prostate cancer, there has been a significant amount of important work in the area of the tumor microenvironment and the pH-centric hypothesis, but still there are a lot of unanswered questions. Clearly some of the acid transport proteins have been shown to play notable roles in prostate cancer cells, especially $Na^+/H^+$ exchangers, MCTs, V-ATPases, and CAs. They have all been shown to be present in prostate cancers, and when expression is reduced or knocked out, or inhibited, there are positive effects on prostate tumor growth, or invasive properties can occur. For NHE1 and MCTs, databases have shown an association with expression and poor prognosis in prostate cancers. CA expression is also known to be associated with poor prognosis in several types of cancer.

Inhibitors of these acid transport proteins have been used in many studies and are being developed for clinical use. For example, CA inhibitors are being developed as are V-ATPase inhibitors. NHE1 inhibitors have potential in this area (Amith et al. 2016a) but still require further refinement. As noted above, one of the critical steps in cancer invasion is the acidification of the microenvironment at the leading edge of invasive cancer cells. This leads to activation of MMP in that microenvironment and subsequent matrix digestion (Fiaschi et al. 2013; Greco et al. 2014). If that is truly a critical key, then it seems obvious that whether the acidifying protons originate from

NHEs, V-ATPases or other transport mechanisms might be irrelevant to the disease, presuming a similar acid load is present in the same extracellular location. Additionally, it supports the suggestion that combinations of proton transport inhibitors might create a very hostile environment for tumor growth, invasion, and metastasis (Harguindey et al. 2018). Thus, it seems that combinations of proton transport inhibitors might be a path to explore in the search for the prevention of metastatic behavior.

Several of the proton transport systems have more than one isoform of transport protein. For example, MCT1 and MCT4 are both thought to be important in cancer cells, NHE1 and NHE3 sometimes coexist in the same tumorigenic cell type, and V-ATPases have the involvement of multiple subunits and subtypes. It was also of interest that the presence and importance of different isoforms of the same transport proteins varies, even in the same general type of cancer. For example, CAIX and CAXII mRNA were detectable in one prostate cancer cell line and not another though induction by hypoxia may be required to demonstrate expression. And, as noted above, knockdown of CAIX can reduce xenograft tumor growth of LS174Tr colon carcinoma cells, while knockout of both CAIX and CAXII gives a much better reduction in tumor growth (Chiche et al. 2009). Thus, it is evident that different isoforms of the same proton transport proteins can contribute more, or less, and both can have significant contributions in tumorigenic cells.

Variations in the importance of different isoforms of proton transport proteins also exist. For example, disruption of the a4 subunit of V-ATPase inhibited invasion by MDA-MB-231 cells, but did not in another breast cancer cell type (Capecci and Forgac 2013; Hinton et al. 2009). Complicating this is the knowledge that some different isoforms of the same proton transport activity are insensitive to inhibitors that affect other isoforms. For example, in the case of $Na^+/H^+$ exchanger inhibitors, many are isoform specific, inhibiting NHE1 and not NHE3 (Pedersen et al. 2007). This makes the prospect of targeted therapies, based on knockout or inhibition of one type of proton transporter, difficult. It would seem that a general catalog of the various isoforms of proton transporters in many examples of prostate (and other) cancers could prove of use. It may be that in the era of "personalized medicine," if clinical targeting of proton transporters comes to a fruition, it might require characterization of the proton transporters present in tumors, prior to treatment. An interesting related study of Meehan et al. (Meehan et al. 2017) examined three different types of breast cancer cell lines. They found that induction of expression of CAIX with hypoxia was cell line dependent. In the breast cancer cell lines MDA-MB-231 and HBL-100, CAIX was highly induced by hypoxia in 2D or 3D cultures, whereas in MCF-7 cells it was not. In contrast, NHE1 was induced in MCF7 cells by hypoxia but not in the other cell types. Differences in effects of CA inhibition on invasion were also noted between the cell lines. In another study comparing MCF7 and MDA-MB-231 cells, inhibition or knockdown of NHE1 had no effect on spheroid growth of MCF7 cells, while in contrast, these treatments decreased spheroid growth of MDA-MB-231 cells (Andersen et al. 2016). These results highlight the differences that can occur between cell lines from the same general type of cancer and emphasize the putative need for individual assessment.

If more than one proton extrusion pathway is important in prostate cancers, it may be that a cocktail of proton transport inhibitors is required to be effective in treatment (Iessi et al. 2017). In this regard one group examined the effect of dual admission of lansoprazole (V-ATPase inhibitor) and CA inhibitors against malignant melanomas. The combination of use of the two inhibitors was more effective than use of one alone (Federici et al. 2016). Presently, V-ATPase inhibitors and CA inhibitors are more developed as therapeutic agents, and the combination of these inhibitors seems a more likely starting point than some other proton transporters (Iessi et al. 2017). Other putative combination therapies could involve NHEs, MCTs, or even uPA inhibitors as anticancer drugs (Buckley et al. 2018). A recent study investigated whether use of proton pump inhibitors by humans is associated with a decreased risk of breast cancer, prostate cancer, and malignant melanoma. The results did not show any chemopreventive effect (Halfdanarson et al. 2019). The authors of this paper noted that the lack of a protective effect could be due to continued extracellular acidification by other means such as NHEs, MCT, CA, and $HCO_3^-$-based transporters, supporting the idea that a cocktail of inhibitors may be an improved alternative for treatment. It should be noted that one recent study found that knockout of NHE1 and CAIX led to induction of expression of CAXII in colon cancer cells (Parks et al. 2017). Thus, care needs to be taken to check for induction of expression of potentially compensating proteins during knockouts of other such proteins. Compensation by other isoforms does occur in prostate cancer cells. For example, knockout of both forms of lactate dehydrogenase, A and B, was necessary to ablate lactate production in DU145 tumors (Liu et al. 2018).

With the above in mind, it might be useful to suggest some more immediate future avenues of research in prostate cancer. It would be useful to examine the isoforms of the various proton transport proteins in different prostate cancer cell lines and compare that to the content of patient samples. Additionally, the effect of combinations of inhibitors of different proton transport proteins could be examined in vitro and in vivo. This could be coupled with an examination of the effect of knockout or downregulation of these proteins on invasion and metastatic behavior. Together these studies would provide useful information on potential clinical treatments for the disease.

While the challenges remain considerable, the background knowledge about the proteins involved in regulation of intracellular and extracellular pH has increased greatly in the area and points to many future areas of research that can be explored, and it has great potential to develop clinically useful strategies for treatment.

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# Calcium-Permeable Channels in Tumor Vascularization: Peculiar Sensors of Microenvironmental Chemical and Physical Cues


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## Contents



**Abstract** Calcium ($Ca^{2+}$)-permeable channels are key players in different processes leading to blood vessel formation via sprouting angiogenesis, including endothelial cell (EC) proliferation and migration, as well as in controlling vascular features which are typical of the tumor vasculature.

In this review we present an up-to-date and critical view on the role of $Ca^{2+}$-permeable channels in tumor vascularization, emphasizing on the dual communication between growth factors (mainly VEGF) and $Ca^{2+}$ signals. Due to the complexity of the tumor microenvironment (TME) as a source of multiple stimuli acting on the

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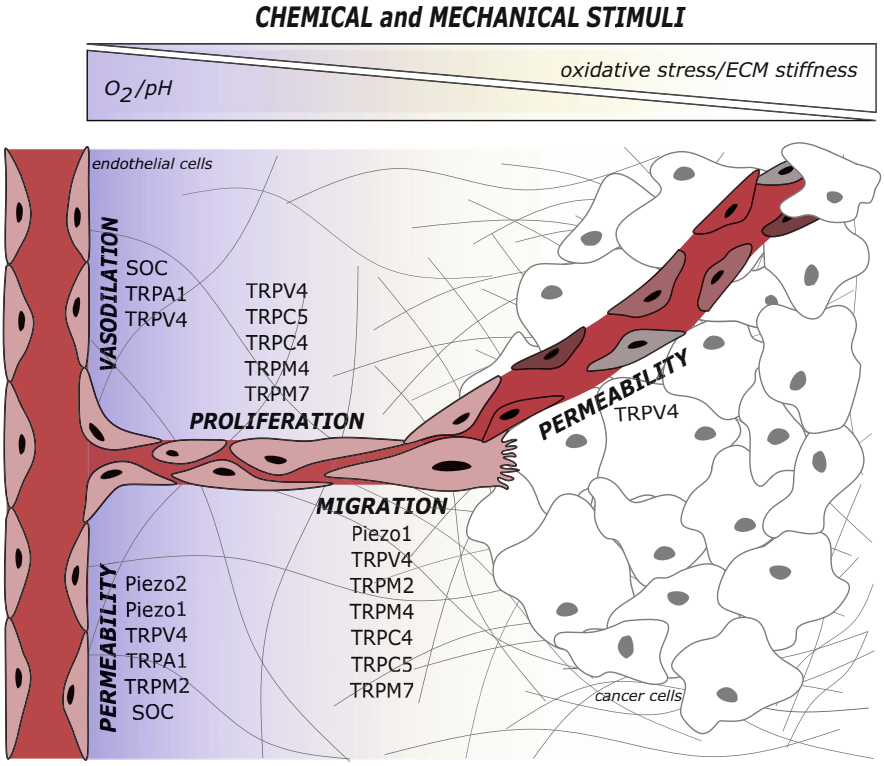
endothelium, we aim to discuss the close interaction between chemical and physical challenges (hypoxia, oxidative stress, mechanical stress) and endothelial $Ca^{2+}$-permeable channels, focusing on transient receptor potential (TRP), store-operated $Ca^{2+}$ channels (SOCs), and mechanosensitive Piezo channels. This approach will depict their crucial contribution in regulating key properties of tumor blood vessels, such as recruitment of endothelial progenitors cells (EPCs) in the early steps of tumor vascularization, abnormal EC migration and proliferation, and increased vascular permeability.

**Graphical Abstract** Graphical abstract depicting the functional role of $Ca^{2+}$-permeable TRP, SOCs and Piezo channels in the biological processes regulating tumor angiogenesis in presence of both chemical (oxidative stress and oxygen levels) and mechanical stimuli (ECM stiffness). SOCs store-operated $Ca^{2+}$ channels, *TRPA* transient receptor potential ankyrin, *TRPV* transient receptor potential vanilloid, *TRPC* transient receptor potential canonical, *TRPM* transient receptor potential melastatin, *TRPM* transient receptor potential vanilloid, $O_2$ oxygen, *ECM* extracellular matrix.




**Keywords** $Ca^{2+}$ channels · Hypoxia · Mechanical stress · Microenvironment · Piezo · SOC · TRPs · Tumor vascularization

## 1 Tumor Vascularization

Tumors, as any tissue, rely on blood vessels which supply oxygen ($O_2$) and nutrients, and discard catabolites through the systemic circulation (Carmeliet and Jain 2011). Moreover, tumor blood vessels provide a route for metastatic cancer cells to spread over the body and invade different districts (Carmeliet and Jain 2000). The formation of the vessel network in tumors mainly occurs via sprouting angiogenesis, a process by which new blood vessels grow from existing ones (Carmeliet and Jain 2011). During this process, endothelial cells (ECs) undergo several sequential modifications which ultimately lead to the formation of dysfunctional and abnormal vessels. Angiogenesis starts with the activation of ECs by specific growth factors, such as vascular endothelial growth factor (VEGF). In response to a VEGF gradient, endothelial tip cells elongate actin-rich filopodia and migrate towards the source of VEGF, giving directionality to the sprout (Potente et al. 2011). The invasion of the leading tip cell through the tissue occurs upon digestion of the extracellular matrix and basement membrane by specific proteases. Neighboring stalk cells proliferate behind the tip cells allowing elongation of the sprout (Potente et al. 2011). The resulting immature vessels are stabilized by the recruitment of mural cells (smooth muscle cells and pericytes), a feature that is missing in tumor vessels (Carmeliet and Jain 2011). Tumor vessels are surrounded by only few pericytes and therefore are highly immature. Moreover, the loss of the adhesion molecule VE-cadherin (vascular endothelial cadherin) weakens the endothelial intercellular junctions and leads to the formation of a leaky endothelium associated with increased interstitial fluid and solute accumulation, as well as transmigration of cancer cells (Cantelmo et al. 2016; Carmeliet and Jain 2000). These structural abnormalities render the tumor vessels highly dysfunctional, overall contributing to impaired oxygen and drug delivery (Jain 2014).

Beside angiogenic sprouting, the recruitment and differentiation of bone marrow-derived endothelial precursors (EPCs) and endothelial colony forming cells (ECFCs), a process known as vasculogenesis, is a critical determinant of tumor vessel formation (Poletto et al. 2018; Naito et al. 2016; Gao et al. 2009; An et al. 2018).

The current description of the tumor angiogenic sprouting takes into account the complexity of the TME as source of several triggers acting on the endothelium. In particular, both chemical and physical stimuli contribute to tumor blood vessel dysfunctions. In addition to secreted signaling molecules and growth factors by stromal and cancer cells, the lack of $O_2$ in growing tumors (hypoxia) is the major driver of cancer aggressiveness, as well as vessel sprouting (see also Sect. 3). Moreover, oxidative stress contributes to the remodeling of the tumor vascular wall by favoring EC activation (Vaupel and Mayer 2007; LaGory and Giaccia 2016; De Bock et al. 2011). Regarding physical stimuli, mechanical stress is generated by tumor extracellular matrix (ECM), an intricate structure made by fibrous proteins, glycosaminoglycans, and matricellular proteins responsible for the increased stiffness observed in tumors. Beside the well characterized role of

ECM molecules as direct or indirect regulators of angiogenesis (Mongiat et al. 2019), the peculiar biophysical and mechanical properties of ECM influence cancer growth via mechanical activated signaling (Pethő et al. 2019; Dupont et al. 2011; Bonnans et al. 2014; Zanotelli and Reinhart-King 2018) (see also Sect. 4).

## 2 $Ca^{2+}$-Signals and $Ca^{2+}$-Permeable Channels in Activated ECs

The role of $Ca^{2+}$ permeable ion channels in vascular physiology has been extensively reported as the focus of several reviews (Nilius and Droogmans 2001; Moccia et al. 2018; Thakore and Earley 2019). This is not surprising due to the multifaced role of intracellular $Ca^{2+}$ as universal messenger tuning different cellular functions (Berridge et al. 2003). On the other hand, several evidences are now accumulating showing a critical role in the physiopathological angiogenic processes in vitro and in vivo (Moccia et al. 2019; Yokota et al. 2015; Smani et al. 2018; Munaron et al. 2012; Brossa et al. 2019). ECs are non-excitable cells and as such, $Ca^{2+}$ signals are mediated mainly via non-voltage gated ion channels, including ionotropic receptors (purinergic or nicotinic primarily), second messenger-dependent ion channels (such as TRP channels), or mechano-activated channels (such as several TRP channels or PIEZO). The well characterized mechanism regulating $Ca^{2+}$ entry in several cell types, the so-called Store-Operated $Ca^{2+}$ Entry (SOCE), is a biphasic process which primarily involves inositol trisphosphate (IP3)-mediated intracellular $Ca^{2+}$ release from endoplasmic reticulum (ER). The decrease in ER $Ca^{2+}$ content following $IP_3$ receptor ($IP_3R$) activation stimulates the clusterization of STIMs proteins, thus promoting extracellular $Ca^{2+}$ influx via the activation of plasma membrane $Ca^{2+}$-permeable channels (Parekh and Putney 2005). The main channels involved in SOCE are ORAI1 (responsible of the so-called CRAC currents), as well as TRPC1 and TRPC4 channels (SOC), which belong to the TRP superfamily of channels (Ambudkar et al. 2017). SOCE mechanism has been described as one of the main $Ca^{2+}$ entry modes in several ECs and EPCs where it exerts different roles, including regulation of EC proliferation, migration, and tube formation. The molecular nature of vascular SOCE varies depending on the cell types including ORAI1, TRPC1, or TRPC4 and will be discussed in depth in the following sections (Abdullaev et al. 2008; Li et al. 2011; Sundivakkam et al. 2012; Scarpellino et al. 2019; Freichel et al. 2001; Lodola et al. 2012) (Table 1).

Since their discovery, TRP channels provided putative candidates for a non-voltage-gated $Ca^{2+}$ entry mechanism in non-excitable cells due to their ubiquitous expression and polymodal activation mechanisms (Birnbaumer 2009; Bernd Nilius et al. 2007). TRP channels are indeed recruited and modulated by a variety of chemical stimuli, including ions ($H^+$ or $Ca^{2+}$), intracellular ligands (diacylglycerol (DAG) and phosphoinositide-4,5-bisphosphate (PIP2)), extracellular natural or

**Table 1** Modulation and function of TRP, SOC, and Piezo channels by chemical and mechanical microenvironmental triggers in EPCs and ECs from different origins

| CHANNEL | TRIGGER | FEATURE/RESPONSE | CELL TYPE/MODEL | Refs |
|---|---|---|---|---|
| **TRPA1** | Hypoxia | Adaptive response to ischemic damage | Cerebral ECs | (Pires and Earley 2018) |
| | | Cell sprouting and directional migration | Retina ECs; PTECs | (Bernardini et al. 2019) |
| **TRPC1** | ATP | Increased channel expression and SOC activity | ECFCs from renal cellular carcinoma | (Lodola et al. 2012) |
| **TRPC3** | VEGF | (Increased) channel expression modulating angiogenic responses by intracellular $Ca^{2+}$ oscillations | PTECs; EPCs; HUVECs | (Bernardini et al. 2019; Dragoni et al. 2013) |
| **TRPC6** | VEGF | Increased vascular permeability, | Mesenteric vessels | (Pocock, Foster, and Bates 2004) |
| | | Increased proliferation, migration, sprouting | HMEC | (Hamdollah Zadeh et al. 2008) |
| **TRPC4** | Hypoxia | Increased channel expression and $Ca^{2+}$ entry | HPAE | (Fantozzi et al. 2003) |
| | | Increased channel expression and SOC activity | Human retina microvascular ECs | (Song et al. 2015) |
| | | | ECFCs from breast cancer | (Lodola et al. 2017) |
| **TRPM2** | $H_2O_2$, ADP-ribose | Increased permeability, migration | Murine artery ECs | (Mittal et al. 2015; Hecquet et al. 2008) |

(continued)

**Table 1** (continued)

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| **TRPM7** | Oxidative stress (by low $Mg^{2+}$ medium) | Increased expression associated with reduced cell growth, migration and adhesion | HUVECs | (Baldoli, Castiglioni, and Maier 2013; Zeng et al. 2015) |
| | | Decreased expression associated with reduced cell proliferation and migration | HMECs; ECFCs | (Baldoli and Maier 2012) |
| **TRPM8** | Icilin | Altered channel expression associated with reduced cell migration | HMEC; BTECs | (Genova et al. 2017) |
| **TRPC5** | Hypoxia, NO, $H_2O_2$ | Nitrosylation of TRPC5 and Ca2+ entry | Vascular ECs | (Yoshida et al. 2006) |
| | | Increased angiogenesis and sprouting *in vitro* and *in vivo* | MMEC; HMECs | (Zhu et al. 2019) |
| **TRPM4** (non-Ca2+ permeable TRP) | Hypoxia, ATP depletion, increased intracellular Ca2+ levels, tPA | Increased expression and channel activity; secondary hemorrhage following brain injury; modulation of angiogenesis | HUVECs; *in vivo* spinal cord injury model | (Loh et al. 2014; Gerzanich et al. 2009, 2018) |
| **TRPV2** | Lysophosph-atidylcholine (LPC) | Increased channel expression and modulation of angiogenesis | PTECs | (Bernardini et al. 2019) |
| **TRPV4** | $H_2O_2$ | Increased permeability, abnormal cell migration and proliferation | Murine and human lung microvascular ECs | (Suresh et al. 2018, 2015) |

(continued)

**Table 1** (continued)

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|---|---|---|---|---|
| | Mechanical stress | Arterial vasodilation | Human coronary arterioles | (Bubolz et al. 2012) |
| | | Spatial reorientation and cell migration upon channel expression | BTECs | (Fiorio Pla et al. 2012) |
| | | Vascular normalization *in vivo;* VE-Cadherin stabilization | Murine normal and tumor ECs; *in vivo* syngeneic tumor models | (Thodeti et al. 2009; Adapala et al. 2015; Cappelli et al. 2019) |
| | | Altered vascular permeability *in vivo* and *in vitro* upon channel expression | HrMVEC; MRCEC; HMECs; *in vivo* mouse lung model; HUVECs | (Phuong et al. 2017; Arredondo Zamarripa et al. 2017; Thorneloe et al. 2012; Villalta, Rocic, and Townsley 2014; Pairet et al. 2019) |
| **SOCE** | Hypoxia, HIF-1$\alpha$ | Regulation of vascular tone by impaired SOCE inhibition | Rat pulmonary ECs; intact carotid arteries | (Paffett et al. 2010; Liu et al. 2018) |
| **Piezo 1** | Mechanical stress | Spatial reorientation and vascular development; altered migration and capillary-like formation *in vitro* and *in vivo* by Ca2+ entry | Murine ECs; FpECs; HUVECs | (Li et al. 2014; Ranade et al. 2014; Morley et al. 2018) (Zhang et al. 2017) |
| | | Pulmonary edema by endothelial barrier breakdown | *In vivo* genetic mouse models | (Friedrich et al. 2019) |

(continued)

**Table 1** (continued)

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|---|---|---|---|---|
| **Piezo 2** | Mechanical stress | Tumor angiogenesis and vascular permeability *in vitro* and *in vivo;* hyperalgesia | HUVECs; murine ECs | (Yang et al. 2016; Ferrari et al. 2015) |

*PTECs* prostate-derived TECs, *ECFCs* endothelial colony forming cells, *EPCs* endothelial progenitors cells, *HPAE* human pulmonary artery ECs, *HUVECs* human umbilical ECs, *HMECs* human dermal microvascular ECs, *BTECs* breast-derived TECs, *MMECs* mesenteric ECs, *HrMVEC* human retinal microvascular ECs, *MRCEC* murine retinal capillary, *FpECs* human fetoplacental ECs

synthetic compounds (such as menthol and capsaicin), or by physical stimuli (such as mechanical forces and temperature) (Gees et al. 2010; Smani et al. 2018). TRP channels are largely expressed in ECs, as well as in EPCs, although their expression levels vary according to the different vascular beds (Smani et al. 2018; Earley and Brayden 2015; Fiorio Pla and Gkika 2013). Moreover, tumor ECs (TECs) and ECFCs have a specific TRP signature. In particular, we and others have demonstrated that TRPV4 and TRPM8 are deregulated in breast cancer TECs (BTECs) (Fiorio Pla et al. 2012; Genova et al. 2017), while TRPA1, TRPV2, and TRPC3 are strongly upregulated in prostate-derived TECs (PTECs) (Bernardini et al. 2019). Regarding ECFCs, studies from Moccia's group showed that TRPC1 is upregulated in renal cancer-derived ECFCs, thereby enhancing SOCE amplitude and stimulating ECFC proliferation (Lodola et al. 2012). Furthermore, TRPC4 is upregulated in breast cancer-derived ECFCs, although its role in SOCE regulation is unclear (Lodola et al. 2017) (Table 1). TRP deregulation in TECs leads in turn to a deregulation of intracellular $Ca^{2+}$ signaling that is quite different from that observed in normal human microvascular EC (Fiorio Pla and Munaron 2014; Genova et al. 2017) (Table 1).

The most evident link between $Ca^{2+}$-permeable channels and angiogenesis comes from studies describing the pro-angiogenic $Ca^{2+}$ signals mediated by growth factors in ECs that interestingly are significantly altered in TECs compared to normal ECs (Fiorio Pla and Munaron 2014; Avanzato et al. 2016).Growth factors and cytokines induce angiogenic switch by promoting intracellular $Ca^{2+}$ increase, which in turn stimulates EC proliferation, adhesion, migration, and sprouting (Moccia et al. 2019). In particular, VEGF-mediated $Ca^{2+}$ signals have been extensively studied in last decades both in vitro and in vivo (Faehling et al. 2002; Dragoni et al. 2011; Noren et al. 2016; Savage et al. 2019; J. Li et al. 2011). Depending on its concentration, VEGF is able to promote an initial peak of $Ca^{2+}$ influx with a persistent increase (higher VEGF concentrations) or $Ca^{2+}$ oscillations (suboptimal VEGF concentrations). These signals activate two distinct signaling pathways, myosin light chain kinase (MLCK) activation, modulated by the amplitude of $Ca^{2+}$ signals and coupled to cell migration, or nuclear factor of activated T-cells (NFAT) translocation coupled to cell proliferation and modulated by the frequency of $Ca^{2+}$ signals (Noren et al. 2016). VEGF-mediated $Ca^{2+}$ oscillations are also present in budding ECs observed

in the zebrafish angiogenesis model (Yokota et al. 2015; Noren et al. 2016). Interestingly, the maximum velocity in the migration of tip cells has been shown to inversely correlate to the spike frequencies in in vitro observations (Noren et al. 2016).

The molecular nature of ion channels associated with VEGF-mediated $Ca^{2+}$ oscillations varies in ECs from different origin. In human umbilical vein ECs (HUVECs), the activation of SOCs (ORAI1, TRPC1, and TRPC4) requires both $Ca^{2+}$ entry from extracellular medium and $Ca^{2+}$ release form the ER (J. Li et al. 2011; Jho et al. 2005; Fantozzi et al. 2003). Beside ER release, in HUVECs VEGF also activates the release of $Ca^{2+}$ from nicotinic acid adenine-dinucleotide phosphate (NAADP) and TPC-2-dependent acidic intracellular stores which support angiogenic responses (Favia et al. 2014). In macro and microvessels, VEGF may also induce $Ca^{2+}$ signals via a non-SOCE mechanism involving TRPC3 and TRPC6 (Dragoni et al. 2013), resulting in increased vascular permeability and in vitro migration and proliferation (Pocock et al. 2004; Cheng et al. 2006; Hamdollah Zadeh et al. 2008) (Table 1). VEGF signaling and TRP channels are also connected at transcriptional level: on one hand, VEGF-activated NFkB mediates the transcription of different TRP genes, and on the other hand, $Ca^{2+}$ entry via TRP channels in turn induces the transcription of different growth factors including VEGF (Smani et al. 2018).

## 3 Role of $Ca^{2+}$ Permeable Channels in Hypoxia-Induced Angiogenesis

The reduction of physiological $O_2$ levels known as hypoxia, regulates several aspects of cancer progression, including chemoresistance, angiogenesis, and cancer aggressiveness (Chen et al. 2009). The hypoxic environment, and the subsequent activation of the hypoxia-inducible factor 1α (HIF-1α), is a common feature of advanced solid cancers (Chen et al. 2009). Due to the aberrant vascularization and poor blood supply, most solid tumors are characterized by permanent or transient hypoxia (Petrova et al. 2018). Adaptation to these hostile conditions is a key escape mechanism responsible for cancer progression. Under hypoxic conditions, cancer cells consume glucose and secrete lactate producing an acidic environment. Moreover, ECs internalize lactate via lactate importer monocarboxylate transporter 1 (MCT1), thus promoting angiogenesis via NFkB pathway (De Palma et al. 2017). One of the main outcomes of HIF-dependent signaling is the expression of a myriad of growth factors and cytokines including VEGF, epidermal growth factor (EGF), and stromal cell-derived factor 1 α (SDF-1α), which deliver a sustained pro-angiogenic signal to ECs residing in close proximity to the primary tumor, and for EPCs recruitment to the sites of neovessel formation (Poletto et al. 2018). As consequence, ECs become activated and acquire abnormal proliferative and migratory capabilities, thus leading to tumor vessel dysfunction (Potente et al. 2011; De

Palma et al. 2017; Hanahan and Weinberg 2011). Beside growth factors and cytokines, HIF-dependent signaling promotes TRP channels and STIM1 expression itself in cancer cells, supporting cancer progression (Azimi et al. 2017; Li et al. 2015). HIF-1α-mediated release of growth factors contributes to tumor angiogenesis by stimulating $Ca^{2+}$ influx through SOCE. On the other hand, genetic or pharmacological targeting of SOCE inhibits VEGF secretion by cancer cells and thus tumor neovascularization (Chen et al. 2011), suggesting the existence of a positive loop regulating tumor vascularization by the cross-talk with the surrounding microenvironment.

Another example of hypoxia-regulated TRP channel is TRPC4 upregulated by low $O_2$ levels in several endothelial districts, such as retina and pulmonary arteries, where it is also implicated in the regulation of the $Ca^{2+}$-dependent activating protein-1 (AP-1), and thus growth factor synthesis and release (Song et al. 2015; Fantozzi et al. 2003) (Fig. 1, Table 1).

Beside the transcriptional modulation by hypoxia or the regulation of their activity by hypoxia-mediated release of growth factors, several $Ca^{2+}$ channels are directly sensitive to $O_2$ levels, or rather their signaling can be controlled by local $O_2$. Numerous studies suggested changes in channel activity in presence of acute hypoxia, and alterations in channel expression upon prolonged hypoxic challenge (chronic hypoxia) (Shimoda and Polak 2011). The ability of ion channels to sense and react to changes in environmental $O_2$ levels is a crucial player of tumor vascularization.

Among the TRP family members, many candidates are sensitive to redox species associated with the hypoxic environment (Mori et al. 2016). In particular, TRPA1 has emerged as a sensor receptor able to detect modifications of $O_2$ availability in vivo. TRPA1 has an inverted bell shape $O_2$ dependence with a minimum around 137 mmHg which is slightly lower than atmospheric $O_2$ (152 mmHg), and it is therefore activated in both hyperoxic and hypoxic conditions (Takahashi et al. 2011). The $O_2$ sensitivity is due to proline hydroxylation by specific prolyl hydroxylases (PHDs) and oxidation of cysteine residues. In normoxia, PHDs are active and proline residues are hydroxylated, inactivating TRPA1. In hypoxic states, due to lack of $O_2$, PDHs are less active relieving TRPA1 from its inhibitory state. In hyperoxia, the overload of $O_2$ directly oxidases cysteine residues and activates TRPA1 (Takahashi et al. 2011; Mori et al. 2016). Endothelial TRPA1 expression is restricted to cerebral arteries and retina vasculature, while it is absent in HUVECs, mesenteric vessels, dermal and renal arteries (Earley and Brayden 2015). Interestingly, recent evidences described TRPA1 as an early sensor of hypoxia in cerebral endothelium, suggesting its key role in the adaptive responses to ischemic damage in the brain (Pires and Earley 2018) (Fig. 1, Table 1).

The regulation of sprouting angiogenesis by TRPA1 has been recently demonstrated in vivo in the mouse postnatal retinal model (Bernardini et al. 2019). Moreover, TRPA1 acts as chemoattractant in PTECs (Bernardini et al. 2019). This effect closely correlates with specific TRPA1-mediated $Ca^{2+}$ signals in migrating PTECs (Bernardini et al. 2019) (Table 1).

Other two TRP candidates for $O_2$ sensing belong to the melastatin TRP family members (TRPM). TRPM2 was one of the first TRP redox-sensitive channels

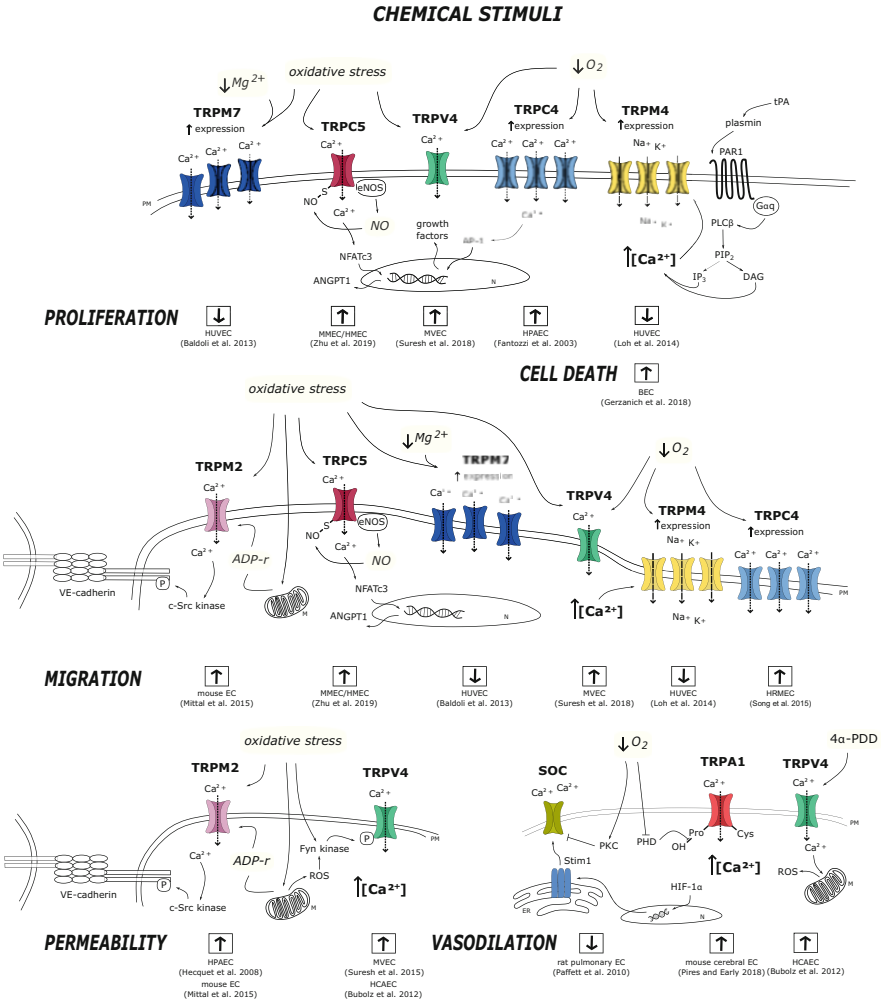


**Fig. 1** Impact of chemical stimuli on TRP and SOC expression and activation. Arrows indicate channel-mediated effects on EC phenotype (proliferation and migration), as well as on vessel permeability and vasodilation by the different triggers (oxidative stress, $O_2$ levels, $Mg^{2+}$, ADP-ribose, 4α-PDD)

identified. It can be exogenously activated by hydrogen peroxide ($H_2O_2$), or endogenously by stress-induced ADP-ribose accumulation in the mitochondria (Perraud et al. 2005; Hara et al. 2002). The involvement of this channel in mediating vascular permeability has been extensively described (Hecquet et al. 2008; Dietrich and Gudermann 2008; Hecquet and Malik 2009) (Table 1). Treatment of endothelial monolayers with $H_2O_2$ leads to $Ca^{2+}$ influx and decreases transendothelial electrical resistance (TEER) (indicative of barrier integrity) via a direct interaction of TRPM2 with VE-cadherin following VEGF stimulation (Mittal et al. 2015) (Fig. 1).

Moreover, TRPM2-silenced ECs showed reduced EC migration upon VEGF stimulation (Mittal et al. 2015) (Fig. 1, Table 1). Nevertheless, the precise role of TRPM2 in regulating endothelial barrier function remains unresolved as in vivo investigations have provided conflicting results. TRPM2 knock-out mice challenged with lipopolysaccharide (LPS) show no changes in vascular barrier properties or increased pulmonary edema (Hardaker et al. 2012; Di et al. 2012), suggesting the involvement of other mechanisms.

TRPM7 activity has been initially identified to be regulated by $O_2$ and glucose deprivation in primary cultured neurons, revealing a key role for this channel in mediating anoxic death (Aarts et al. 2003). TRPM7 sensitivity to hypoxia was confirmed by Mori's group who showed that, similarly to TRPA1, TRPM7 activity is increased in hypoxia (Takahashi et al. 2011). Notably, TRPM7 expression is significantly increased in HUVECs cultured under oxidative stress conditions (low $Mg^{2+}$ medium) where it is proposed as marker for EC dysfunction. Indeed, TRPM7 pharmacological or genetic inhibition promotes HUVEC cell growth and migration, as well as adhesion (Baldoli et al. 2013; Zeng et al. 2015) (Fig. 1, Table 1). This behavior seems to be specific for macrocirculation since opposite function was observed in human dermal microvascular ECs (HMECs) or ECFCs where TRPM7 inhibits cell migration and proliferation (Baldoli and Maier 2012). The authors discussed these differences based on the lack of $Mg^{2+}$-mediated oxidative stress induction in HMECs as shown by the fact that antioxidant treatments do not prevent low $Mg^{2+}$ effects in these cells (Baldoli and Maier 2012).

Among the canonical TRP family (TRPC), TRPC5 is activated by $H_2O_2$ and nitric oxide (NO). In particular, endothelial TRPC5 is positively regulated by NO via nitrosylation of two cysteine residues (Cys 553 and Cys 558) located in the N-terminal side of the pore region, promoting $Ca^{2+}$ entry and thus triggering a positive feedback (Yoshida et al. 2006; Mori et al. 2016) (Table 1). In agreement, TRPC5 improves EC sprouting and angiogenesis under hypoxia/ischemia conditions by activating NFAT signaling pathway (Zhu et al. 2019) (Fig. 1, Table 1). Interestingly, TRPC5 induces HIF-1α expression associated with VEGF release and increases angiogenesis in human breast cancer after chemotherapy (Zhu et al. 2015). In this context, another member of the TRPC family, TRPC1, indirectly regulates HIF-1 protein expression via Akt-HIF1 axis in breast cancer cells (Azimi et al. 2017).

Other TRP channels with relevant role in hypoxia-induced angiogenesis are not directly gated by $O_2$ or oxidative stress. This is the case for TRPM4 whose expression and function is increased in vascular endothelium under hypoxic conditions following $O_2$ and glucose deprivation (Loh et al. 2014) (Fig. 1). TRPM4 is a non-selective monocationic ($Na^+$ and $K^+$) channel, and therefore not permeable to $Ca^{2+}$, however it is modulated by increased intracellular $Ca^{2+}$ levels and ATP as occurring during hypoxia and reactive oxygen species (ROS) release (Vennekens and Nilius 2007). TRPM4 expression in ECs has been associated with secondary hemorrhage after spinal cord or brain injury: TRPM4 is indeed overexpressed after spinal cord injury and its blockade significantly reduce the hemorrhage. Although the molecular mechanism is still unclear, the capillary damage is probably due to TRPM4 sensitization by high ATP release at site of injury and the following oncotic

capillary death (Gerzanich et al. 2009). In addition to ATP, the pathological role for TRPM4 has been broadened by recent studies showing that the co-assembly of the sulfonylurea receptor 1 (Sur1) and TRPM4 is involved in hemorrhagic transformation following brain ischemia. The Sur1-TRPM4 heteromers are activated by the G protein-coupled receptor PAR-1 and $Ca^{2+}$ influx, leading to a biphasic MMP-9 secretion in NFkB-activated brain ECs (Gerzanich et al. 2018). On the same line, TRPM4 is upregulated in ECs following hypoxia/ischemia in vitro and in vivo, and in HUVECs following $O_2$–glucose deprivation; blocking TRPM4 activity leads to enhanced angiogenesis in vitro and in vivo upon hypoxia, suggesting that its inhibition could provide protective effects against $Na^+$ mediated cell toxicity (Loh et al. 2014) (Fig. 1, Table 1).

A second example of indirect regulation is TRPV4, whose role in ECs and (tumor) angiogenesis is well established (for reviews, see Thakore and Earley 2019; Smani et al. 2018; Fiorio Pla et al. 2012). TRPV4 promotes vasodilation and increases vascular permeability in several vascular districts both in vitro and in vivo (see also Sect. 4). TRPV4 is indeed a polymodal channel activated by both physical stimuli, such as temperature and stretch, and chemical stimuli. On the other hand, it is also indirectly activated by exogenous ROS ($H_2O_2$ or mitochondrial ROS) via Fyn kinase activation that in turn promotes TRPV4 phosphorylation and opening: the final effect is an increased vascular permeability in vitro (Suresh et al. 2015, 2018), as well as arterial dilation (Bubolz et al. 2012) (Fig. 1, Table 1).

As reported in Sect. 2, SOCE is a crucial $Ca^{2+}$ entry pathway in ECs. The molecular identities involved in this process are the $Ca^{2+}$ sensors STIM proteins in the ER, and ORAI1 or TRPC1 channels on the plasma membrane (Parekh and Putney 2005; Ambudkar et al. 2017). Paffett et al. showed that chronic hypoxia reduces intracellular $Ca^{2+}$ levels in freshly isolated intrapulmonary ECs, with a PKC-dependent SOCE inhibition mechanism (Paffett et al. 2010) (Table 1). Moreover hypoxia mediated ORAI1 expression and SOCE regulation in colon cancer indirectly mediate tumor angiogenesis in vitro (Liu et al. 2018).

## 4 Role of $Ca^{2+}$-Permeable Channels in Stiffness-Induced Angiogenesis

Tumor ECM undergoes continuous remodeling by tumor itself or stromal cells, becoming stiffer than normal tissues. As a consequence, the high interstitial pressure generated within the tumors acts as an important mechanical stimulus in cancer progression by modulating angiogenesis. To convert mechanical forces and biophysical cues in biochemical signaling pathways, ECs take advantage of the interconnected mechanosensory networks which include cytoskeleton, membrane proteins (such as integrins, cell–cell adhesion receptors, ion channels, receptor tyrosine kinases, G-protein-coupled receptors), and transcription factors (Zanotelli and Reinhart-King 2018). Capillary formation is strongly dependent on ECM

stiffness, which promotes sprouting angiogenesis and increases vascular permeability by altering the VE-cadherin localization (Bordeleau et al. 2017). In this context, in vitro data on hydrogels showed that 25 kPa surface ECM provides ideal conditions toward the formation of healthy and confluent endothelial layer (Wood et al. 2011). ECM stiffness modulates VEGFR2 expression via a mechanosensitive signaling transduction pathway that tunes the balance between TFII-I:GATA2 transcription factors (Mammoto et al. 2009). Similarly, in vitro tissue contractility and deformation assays using microfabricated platforms can induce the formation of VEGF gradients, contributing to the long-range patterning of the vascular system (Rivron et al. 2012). More recently, Zanivan and co-workers demonstrated that tumor stiffness alters the CCN1/b-catenin/N-cadherin pathway in ECs, thus facilitating cancer cells adhesion to the endothelium and contributing to the metastatic cascade (Reid et al. 2017).

In the most general definition, mechanosensitive ion channels are channels gated by mechanical forces changing their biophysical state between close and open state (Sachs 2010). As such, mechanosensitive channels do not refer to a unique protein but rather a large group of channels including $Na^+$ and $K^+$ channels, as well as $Ca^{2+}$-permeable channels (Pethő et al. 2019). Here, we will mainly focus on $Ca^{2+}$-permeable channels among which the noteworthy are Piezo1/2 and several TRP channel family members.

Mechanical signals involve different types of stimuli such as extracellular fluid shear stress, lipid bilayer tension, or local membrane changes in curvature and composition. Moreover, the channels can be indirectly activated by mechanical forces that trigger signaling pathways leading to the gating of the channel (Pethő et al. 2019). Shear stress in vascular endothelium, defined as the frictional force due to blood flow (Chiu and Chien 2011), has been extensively described (for review see (Davies 1995)) and may evoke vasorelaxation counteracting the constitutive vasoconstriction that controls the basal vessel tone. On the other hand, more relevant for the TME, tissue stiffness and consequent mechanical forces may promote EC migration and gene expression (Chien 2007; Thakore and Earley 2019). Notably, intact blood vessels combine both local mechanical and agonist-evoked stimuli to create a common output. As an example, the release of IP3 and consequent $Ca^{2+}$ mobilization form ER is strongly inhibited by pressure-dependent mechanical forces in selective regions of ECs resulting in global $Ca^{2+}$ perturbation even without direct mechanosensitive channels involvement (Wilson et al. 2015).

Piezo1/2 are large trimeric and non-selective $Ca^{2+}$-permeable channels known as the main sensors of mechanical stress. Their discovery is relatively recent (Coste et al. 2010), and since then their molecular nature and functional role have been subject of interest in many research fields (Wu et al. 2017) (for a recent overview see also (Dance 2020)). The main question that still needs to be addressed is related to the exact mechanism by which Piezo senses mechanical forces, although the use of cryo-electron microscopy (CRYO-EM) provided compelling information (Ge et al. 2015; Wang et al. 2019). In addition to mechanical stimulation, Patapoutian's group identified an alternative activation of Piezo1 by using the chemical agonist Yoda1 which caused an intracellular elevation of $Ca^{2+}$ concentration (Syeda et al. 2015).

Piezo1 is definitely playing a pivotal role in vascular development and physiology as clearly illustrated by the delay in yolk vasculature development in endothelial specific Piezo1 knock-out (KO) mice. Piezo1 is directly involved in shear stress-evoked $Ca^{2+}$ signaling in several mouse and human ECs, including human placental arteries (J. Li et al. 2014; Ranade et al. 2014; Morley et al. 2018) (Table 1). The endothelial ability to sense mechanical stimuli is crucial for the development and function of vascular as well as lymphatic systems, during both embryogenesis and adult life (Lucitti et al. 2007; Hahn and Schwartz 2009; Nonomura et al. 2018). The alignment of ECs towards the direction of blood flow is the result of an important adaptive mechanism promoted by shear stress acting on the cellular membrane which affects the cytoskeleton (Hahn and Schwartz 2009). In this context, Piezo1 proved to be a key sensor of the frictional force determining the spatial reorganization of ECs towards the polarity of the applied force in a $Ca^{2+}$-dependent manner (Ranade et al. 2014; J. Li et al. 2014). The modulation of membrane tension detected by Piezo channels leads to intracellular $Ca^{2+}$ entry and following calpain involvement, leading to focal adhesion remodeling and EC alignment to the flow (J. Li et al. 2014). On the other hand, Piezo1 plays a role in endothelial migration in the absence of shear stress: indeed, its downregulation significantly inhibits HUVECs migration toward VEGF. Accordingly, pharmacological blockade of Piezo1 prevents capillary-like formation in vitro and in vivo (Li et al. 2014). A recent study proposed the sarcoplasmic/endoplasmic $Ca^{2+}$ ATPase (SERCA) as key modulator of Piezo (Zhang et al. 2017). The authors identified a specific peptide in the linker region at the C-terminus of Piezo1, which is essential for its interaction with SERCA2. Interestingly, the disruption of this linker region affects EC migration, pointing out to a critical for SERCA-Piezo coupling in the regulation of mechanosensitive transduction (Fig. 2). The role of Piezo1 in vascular permeability has been recently described by Malik's group. Indeed, EC Piezo1 is responsible for pulmonary edema: this event is due to the endothelial barrier breakdown which occurs secondarily to a reduced VE-cadherin homotypic interaction leading to adherens junctions disruption and increase in permeability. On the other hand, the native expression of Piezo1, and the consequent pressure induced by pulmonary edema, could act physiologically as an "escape valve" preserving vascular endothelium from a catastrophic pulmonary barotrauma, as speculated by the authors (Friedrich et al. 2019) (Fig. 2, Table 1).

Piezo2 has been mainly implicated in touch and pain linked to sensory neurons, referred as the main component in touch-sensitive cation current in vitro (Woo et al. 2014). However, recently it was demonstrated to be expressed in murine ECs (Ferrari et al. 2015; H. Yang et al. 2016). Interestingly, Piezo2 downregulation in HUVECs significantly contrasts VEGF-induced increased permeability, suggesting its role in this process. Accordingly, VEGF and interleukin-1 (IL-1b)-mediated Evans Blue extravasation is significantly dampened in Piezo2 KO mice (H. Yang et al. 2016) (Fig. 2, Table 1). However, further experimental evidences are required since this is the only study reporting a role for Piezo2 in vascular permeability.

TRPV4 is considered a mechanosensitive channel: several reports showed that it is activated by extracellular osmotic changes (upon hypotonicity) or mechanical stress (White et al. 2016). However, the exact mechanism of its gating is less clear

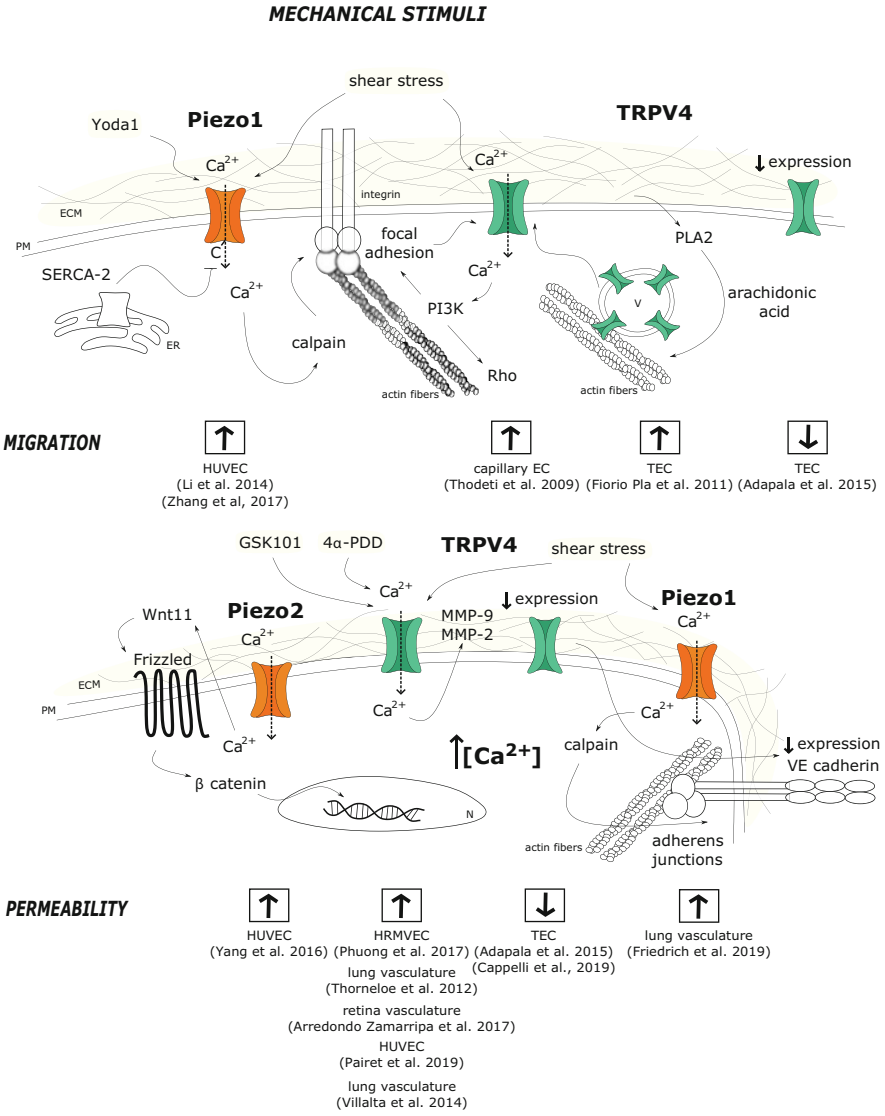


**Fig. 2** Impact of mechanical stimuli and related $Ca^{2+}$-dependent regulatory mechanisms on the regulation of TRPV4 and Piezo channels. Arrows indicate channel-mediated effects on EC migration and vessel permeability by the different triggers (ECM stiffness, shear stress, Yoda1, GSK101, 4α-PDD)

compared to other ion channels, such as TRAAK and TREK for which it has been shown that the gating relies on lipid bilayer modification (Brohawn et al. 2014). TRPV4 mechanosensitivity could occurs directly or indirectly via cell membrane

interacting structures (cytoskeleton or ECM proteins), or via second messengers responding to mechanical forces (for a detailed review refer to White et al. 2016).

In the context of the tumor vasculature, recent studies have reported a TRPV4 involvement in the regulation of vessel integrity. TRPV4 mediates EC reorientation to shear stress in integrin dependent manner, and its inhibition targets b1 integrin activation under static stretch condition (Thodeti et al. 2009) (Fig. 2, Table 1). Moreover, TRPV4-mediated $Ca^{2+}$ influx stimulates TEC migration and angiogenesis via membrane stretch-induced arachidonic acid activation, which in turn promotes actin remodeling and TRPV4 membrane expression (A Fiorio Pla et al. 2012) (Fig. 2, Table 1). Interestingly, Thodeti's group proposed TRPV4 mechanosensitivity as a regulator of tumor angiogenesis and vessel maturation. TECs lacking TRPV4 exhibit abnormal mechanosensing towards ECM stiffness, dysfunctional angiogenesis, and enhanced migration (Adapala et al. 2015). In addition, TRPV4 regulates tumor angiogenesis through the modulation of Rho-dependent mechanosensitivity, and it has been proposed as a target for developing a novel mechanotransduction-based therapy for vascular normalization (Adapala et al. 2015) (Fig. 2, Table 1). In line, the authors showed that tumors implanted in TRPV4 KO mice have highly disorganized and permeable vessels. Conversely, TRPV4 is downregulated in TECs compared to healthy ECs, with a subsequent decrease in endothelial $Ca^{2+}$ influx and vessel abnormalities. Activation of TRPV4 with the chemical agonist GSK1016790A promoted tumor vasculature normalization (Adapala et al. 2015). Recently, the same group showed that in tumors TRPV4 regulates tumor vessel integrity by stabilizing VE-cadherin expression at cell–cell contacts (Cappelli et al. 2019) (Fig. 2, Table 1). At a first glance, these evidences are counterintuitive since, despite few exceptions (Ke et al. 2015; Akazawa et al. 2012), TRPV4 activity has been associated with barrier breakdown and increased permeability by massive $Ca^{2+}$ influx (Phuong et al. 2017; Arredondo Zamarripa et al. 2017; Thorneloe et al. 2012) (Fig. 2, Table 1). The disrupting effects on the vascular barrier are $Ca^{2+}$-influx dependent, as observed suggested by pharmacological interference. The TRPV4 agonist GSK1016790A strongly decreases TEER in HUVECs as a result of the robust increase of intracellular $Ca^{2+}$ concentrations. In contrast, the less potent 4α-phorbol 12,13-didecanoate (4α-PDD) agonist leads to a small increase of intracellular $Ca^{2+}$ concentrations and reduced TEER only at high concentrations (Pairet et al. 2019) (Fig. 2, Table 1). In vivo, GSK1016790A increases blood vessels permeability in wild type (WT) but not in TRPV4 KO (Phuong et al. 2017), and causes endothelial dysfunction and circulatory collapse in WT mice, rats, and dogs (Willette et al. 2008). Stimulation of TRPV4-mediated $Ca^{2+}$ entry by 4α-PDD improves the permeability of mouse lungs without affecting the expression of the adhesion molecule P-selectin (Wu et al. 2009). $Ca^{2+}$ influx via TRPV4 enhances vascular permeability and it is required to recruit matrix metalloproteinases-2 and -9 leading to the proteolytic disruption of cell–cell and cell–matrix adhesion (Villalta et al. 2014) (Fig. 2, Table 1).

Several other studies highlighted the contribution of TRPV4 activation to lung vasculature damage and pulmonary edema formation. In a rat model of heart failure, pulmonary edema is attenuated by TRPV4 blockade (Thorneloe et al. 2012).

Moreover, chemically-induced lung injury in mice leads to vascular leakage via the production of an endogenous TRPV4 channel agonist (Balakrishna et al. 2014).

Beside Piezo and TRPV4, particularly noteworthy is the sensitivity of SOCE to the membrane-stretching (Gottlieb et al. 2008). SOCE-induced intracellular $Ca^{2+}$ elevation is engaged in development and progression of different cancer types (Chen et al. 2019). For example, SOCE is required for breast cancer-derived cells migration in vitro and metastasis formation in mice (Yang et al. 2009). There is a tight connection between $Ca^{2+}$ signaling and cytoskeleton reorganization associated with cell migration suggesting that SOCE-induced calpain activation may modulate cell motility. Interestingly, calpain-2 recruitment by SOCE is related to enhanced adhesion, spreading, and migration of ovary cancer and osteosarcoma-derived cells (Huttenlocher et al. 1997; Saraiva et al. 2013). Moreover, previous studies using different substrate rigidities suggest that cancer cells favor rounded morphologies on soft substrate and more polarized morphologies on stiff substrates where their migration is enhanced (Ni and Chiang 2007). In this context, TRPC1-mediated SOCE plays a crucial role in cell polarization which is essential for directional migration (Huang et al. 2015). TRPC1 acts as a mechanosensor rising the intracellular $Ca^{2+}$ levels near high tension compartment (Fabian et al. 2008; Anke Fabian et al. 2012; Huang et al. 2015). However, all the studies reported referred to cancer cells and need to be confirmed in ECs.

## 5 Conclusions

In the current review we provided an up-to-date view of the involvement of different $Ca^{2+}$-permeable channels in tumor angiogenesis, and discussed the close interaction between chemical and physical challenges (hypoxia, oxidative stress, mechanical stress) and voltage-independent endothelial $Ca^{2+}$-permeable channels, with a particular focus on TRP, SOC, and Piezo channels (Table 1). The relative low abundance of these channels in non-excitable ECs has raised several questions about their functional role. However, growing evidences suggest a much more complex contribution to the (tumor) vasculature than previously anticipated. We are far from considering ion channels as targets for pathological angiogenesis, but these molecular machines are promising tools since they could act as driving forces of EC dysfunctions in many diseases.

Although different channels were discussed separately, it is important to notice that in the TME, ion channels are likely to function as a network rather than individual functional units, forming microdomain of "signalplexes." For example, TRPC3/NCX1 complex is necessary to translate VEGF-mediated signaling pathway and sustained angiogenesis in primary ECs (Andrikopoulos et al. 2017) (Table 1). Very recently, a functional interaction between Piezo1 and TRPV4 channels has been reported in pancreatic exocrine cells (Swain et al. 2020). $Ca^{2+}$ entry following Piezo1 activation by acinar pressure triggers PLA2 (phospholipase A2) recruitment

and the consequent TRPV4 activity, thus sustaining aberrant $Ca^{2+}$ entry (Swain et al. 2020).

Another piece of complexity that should be taken into account is the ability of some channels described in this review to be engaged by both chemical and physical stimuli acting as "coincidence" sensors (Glitsch 2019). This is indeed the case for TRPV4 that can be activated by mechanical forces and indirectly by oxidative stress (Table 1). Similarly, TRPA1, one of the best known $O_2$ sensors, is sensitive to mechanical stretch (Glitsch 2019; Pethő et al. 2019). Finally, Piezo1, which is strongly implicated in mechano-activated events, is also modulated by protonation: indeed, extracellular protonation affected the inactivation gating of the channel without altering the mechano-gating (Bae et al. 2015).

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# Circulating Tumor Cells: Does Ion Transport Contribute to Intravascular Survival, Adhesion, Extravasation, and Metastatic Organotropism?


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## Contents



**Abstract** Survival in the circulation, extravasation from vasculature, and colonizing new tissues represent major steps of the metastatic cascade and pose a big challenge for metastasizing tumor cells. Tumor cells circulating in blood and lymph vessels need to overcome anoikis, cope with mechanical stimuli including shear stress, and defeat attacks by the immune system. Once adhered to the vessel wall, a circulating tumor cell (CTC) can trick the endothelial cells into loosening their intercellular junctions so that the endothelium becomes penetrable for the tumor cell. Since tumor cells tend to metastasize to predestinated target organs and tissues, called organotropism, the distribution of metastases is anything but random. The molecular-physiological mechanisms underlying CTC survival, extravasation, and

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organotropism are very likely to include the presence and activity of ion channels/transporters due to the latter's key function in cytophysiological processes. To date, a very limited number of studies explicitly show the involvement of ion transport. This review describes the contribution of ion channels and transporters to CTC survival, extravasation, and organotropism where known and possible. In addition, supposed connections between ion transport and CTC behavior are demonstrated and imply the potential to be therapeutically taken advantage of.

**Keywords** $Ca^{2+}$ · Homing · Intravascular milieu · Mechanosensitivity · Premetastatic niche

## 1 Introduction

The degree of malignancy of a tumor disease is determined by the tumor cells' propensity to invade surrounding tissue, to spread and metastasize. These steps of the metastatic cascade also include the cells' long-distance transport by blood and lymph flow as well as their ability to adhere to the vessel wall in order to extravasate at a distant organ site far away from the primary tumor (Valastyan and Weinberg 2011). During the course of these events blood cells play a double-edged role. While natural killer (NK) cells represent serious opponents of circulating tumor cells (CTCs), platelets, neutrophils and monocytes/macrophages may even help them to survive the intravascular milieu, extravasate and colonize a new tissue or organ.

In respect of rolling and adhesion to the vessel wall, CTCs quite often mimic or avail themselves of the mechanisms used by leukocytes (Strell and Entschladen 2008). The receptor-ligand pairs involved in rolling are mostly the same in leukocytes and tumor cells, with E- and P-selectins expressed on endothelia as well as (peritoneal) mesothelia being the major receptors (Gebauer et al. 2013; Köhler et al. 2010). In contrast, the receptor-ligand pairs that mediate tumor cell adhesion to the endothelium are quite different from those involved in leukocyte adhesion (Strell and Entschladen 2008). Specific interactions between structures on the tumor cell surface and tissue-/organ-specifically expressed proteins on the endo-/mesothelium, including locally released chemokines (please see Sect. 5.1), contribute significantly to the organ distribution of metastases which is anything but random (Langley and Fidler 2011; Paget 1989). The preference of tumor cells to metastasize to predestinated target-organs is called "organ-specific metastasis" or "metastatic organotropism."

The present review article describes the travel route of metastasizing tumor cells from the moment of intravasation through to the colonization of the target-organ, including indispensable survival mechanisms. There is hardly any direct evidence for the contribution of ion transport to these steps of the metastatic cascade. However, due to their pivotal role in regulating cellular functions, ion channels and transporters must be inevitably involved. Their involvement will be described

and explained where known and possible. In other cases, presumed links between ion transport and the survival of metastasizing cells are pointed up. Central modulators affecting, and being affected by, ion channels and transporters are pH and cytosolic $Ca^{2+}$ concentrations together with signaling events.

## 2 Surviving the Intravascular Milieu

Being swept away by the blood flow represents a major challenge for tumor cells. From thousands up to millions of cells that come off the primary tumor every day (Butler and Gullino 1975; Swartz et al. 1999), less than one out of ten thousand circulating tumor cells (<0.01%) may eventually end up as a metastasis (Fidler 1970, 2003; Strilic and Offermanns 2017). In breast cancer patients, the half-life of circulating tumor cells (CTCs) was found to be 1–2.4 h (Meng et al. 2004). Most of these CTCs die due to hemodynamic shear stress in the circulation (Fan et al. 2016) or anoikis, i.e. the loss of cell–cell or cell–matrix contacts including the absence of extracellular matrix-derived survival signals (Kim et al. 2012). A third obstacle to be overcome by CTCs is the immune surveillance, particularly the clutches of natural killer (NK) cells of the innate immune system (Morvan and Lanier 2016).

To cope with all these challenges, CTCs use a number of (molecular) mechanisms (Strilic and Offermanns 2017).

### *2.1 Coping with Mechanical Stress*

In order to resist mechanical destruction by hemodynamic forces, CTCs activate both the RhoA/actomyosin axis and actin-nucleating formins in response to fluid shear stress which, including the activity of myosin II, protects them from plasma membrane damage (Moose et al. 2020). Accordingly, short-term inhibition of myosin II delays metastasis of circulating prostate cancer cells in a mouse model (Moose et al. 2020). Since the CaM-dependent activity of myosin II needs $Ca^{2+}$, and the resistance to fluid shear stress requires the presence of extracellular $Ca^{2+}$ (Barnes et al. 2012), CTC adaptation to mechanical stress definitely involves $Ca^{2+}$ transport across the plasma membrane. In general, a number of mechanosensitive ion channels have a share in $Ca^{2+}$ signaling of tumor cells: while direct $Ca^{2+}$ influx can be mediated by $Ca^{2+}$ conducting channels such as Piezo or TRP channels, $K^+$ outward currents carried by, inter alia, mechanosensitive members of the two-pore domain $K^+$ channel family keep up the electrochemical gradient essential for $Ca^{2+}$ influx (Pethö et al. 2019). Albeit there is no study to date explicitly proving the nature of the $Ca^{2+}$ channels and transport mechanisms that are involved in CTCs' shear stress resistance, exposure to fluid shear stress does trigger $Ca^{2+}$ influx accompanied by an increase in cell stiffness. Transformed prostate cancer cells (PC-3) show a graduated increase in stiffness in response to the level of shear stress whereas non-transformed

prostate epithelial cells (PrEC LH) do not show a significant change (Chivukula et al. 2015). In addition to channels and transporters mediating $Ca^{2+}$ influx provoked by fluid shear stress, the $Na^+/H^+$ exchanger NHE1 may contribute to the increase in stiffness and thus facilitate CTC survival. Its overexpression, typical of a multitude of tumor entities, leads to a reorganization of the cortical cytoskeleton accompanied by a significant increase in cortical stiffness of human melanoma (MV3) cells (Keurhorst et al. 2019). This effect is based on the mere presence of NHE1 as a structural element independently of its ion transport function.

An additional strategy by which single CTCs can protect themselves from mechanical stress-induced death is the recruitment of thrombocytes (platelets) and monocytes/macrophages in order to form a physical shield (Schlesinger 2018; Stegner et al. 2014). To this end, CTCs express tissue factor at their surface (Bourcy et al. 2016; Hisada and Mackman 2019). The tissue factor triggers the coagulation cascade including the activation of platelets which results in the formation of a protective platelet clot around the tumor cells. The clot then recruits monocytes/macrophages to the CTCs (Gil-Bernabé et al. 2012, 2013), and the accruing clusters or microaggregates not only protect the CTCs from mechanical stress but also help them adhere to the endothelium and extravasate at a distant site (Strilic and Offermanns 2017). According to this, an inhibition of mechanisms underlying tumor cell–platelet interaction causes a significant decrease in metastasis (Labelle and Hynes 2012; Mammadova-Bach et al. 2020; Takagi et al. 2013).

Another survival mechanism has been found in highly metastatic human breast cancer cells expressing significant amounts of a truncated form of the channel protein Pannexin 1 (PANX1) (Furlow et al. 2015). PANX1 is an ATP-permeable channel and, under normal cellular conditions, auto-inhibited because it is plugged by its C-terminal tail. During apoptosis, cleavage of the C-terminus by caspase 3 or 7 activates PANX1 and allows ATP release (Chekeni et al. 2010; Ruan et al. 2020; Sandilos et al. 2012). In highly metastatic breast cancer cells, however, co-expression of a truncated form of PANX1 with full-length wild-type PANX1 protects from apoptosis (Furlow et al. 2015). The presence of truncated PANX1 is accompanied by an elevated ATP release through mechanosensitive full-length PANX1 activated by membrane stretch during deformation in the microvasculature. By autocrine binding to purinergic P2Y receptors the released ATP induces a signaling cascade that suppresses deformation-induced apoptosis of the circulating breast cancer cell. Consequently, therapeutic inhibition of PANX1 by small-molecule inhibitors can reduce breast cancer metastasis (Furlow et al. 2015).

## *2.2 Resistance to Anoikis*

A loss of integrin-mediated cell adhesion to extracellular matrix proteins normally induces anoikis, a special type of apoptosis (Tajbakhsh et al. 2019). CTCs utilize a variety of mechanisms to counteract anoikis (Buchheit et al. 2014). An efficient way to avoid anoikis is the retention of cell–cell or even fragmented cell–matrix

adhesions within the circulating tumor cell clusters, also termed circulating microemboli. These circulating microemboli can either originate from collectively migrating tumor cells that enter the blood stream via chaotically structured and leaky tumor vessels typical of highly angiogenic tumors (Hou et al. 2011) or they arise from the disintegration of the primary tumor into the vasculature (Liotta et al. 1976). Although circulating tumor cell clusters are rather rare compared to single CTCs, these clusters have a 23–50-fold increased metastatic potential (Aceto et al. 2014).

Since the focal adhesion kinase (FAK) is a central player in integrin-mediated adhesion signaling, single CTCs establish alternative ways of FAK phosphorylation or even bypass FAK signaling. Thus, FAK phosphorylation and signaling in non-adherent cells may be ensured by endosomes that carry integrin dimers while containing integrin-binding extracellular matrix components such as fibronectin (Alanko et al. 2015). Another way to maintain FAK signaling may be integrin-mediated self-stimulation by self-secreted fibronectin or collagen. Stimulation of β1 integrin by fibronectin or collagen causes activation of $K_v11.1$ (*human ether-a-go-go-related gene* potassium channel hERG, KCNH2), which is essential for direct FAK phosphorylation (Cherubini et al. 2005; Jehle et al. 2011). FAK phosphorylation in response to $K_v11.1$ activation may enable detached cells to resist anoikis. In fact, overexpression of both FAK and $K_v11.1$ has been shown to enhance dissemination and invasiveness of tumors (Kornberg 1998; Lastraioli et al. 2004).

Moreover, fibronectin can promote cell survival, mediate chemo- and radioresistance, and inhibit apoptosis in breast and lung cancer cells (Aoudjit and Vuori 2012; Naci et al. 2015). In pancreatic cancer cells, an increased *Wnt2* expression correlates with a TGFβ-activated kinase 1 (TAK1; MAP 3 K7)-dependent upregulation of fibronectin, suppresses anoikis, and facilitates adhesion-independent sphere formation (Yu et al. 2012).

Aside from fibronectin, CTCs could potentially also make use of serum vitronectin and other serum proteins, e.g. osteopontin, thrombospondin or reelin, as ligands in order to keep up integrin-mediated signaling and thus resist anoikis (Bera et al. 2020; Cooper et al. 2002; Lal et al. 2009; Rouanne et al. 2016).

Beyond that, FAK-mediated anoikis resistance has been found to correlate with the expression of carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), also known as CD66c (Duxbury et al. 2004; Johnson and Mahadevan 2015; Lee et al. 2018). As a bypass or an alternative to missing FAK signaling, anti-apoptotic, pro-survival pathways are upregulated or tumor suppressors and suppressing pathways are downregulated. For instance, the PI3/Akt signaling pathway, which normally is inducible by FAK as well, or the MAPK/ERK pathway is stimulated by overexpressed receptor tyrosine kinases and downregulation of the tumor suppressor PTEN (Paoli et al. 2013). A moderately increased ROS production is often found in tumor cells (Perillo et al. 2020) and helps to counteract anoikis by modulating the activities of redox-sensitive proteins of the PI3/Akt and MAPK signaling pathways and prominent transcription factors such as p53, NF-κB, HIF, AP-1, and Nrf2 (Groeger et al. 2009).

Finally, although not shown explicitly in CTCs, the detachment from the extracellular matrix could induce autophagic and antioxidant effector pathways whose

concerted action might (i) enable increased survival in the bloodstream and (ii) facilitate the formation of metastases (Dey et al. 2015). In more detail, cells react to the loss of substrate adhesion by activating a cytoprotective ER stress response consisting of three pathways that are normally kept inactive by the ER-located chaperone GRP-78 (also known as "binding immunoglobulin protein" (BiP) or "heat shock 70 kDa protein 5" (HSPA5)) (Korennykh and Walter 2012): the ATF6 (transmembrane activating transcription factor *6*), the IRE1 (iron responsive element 1), and the PERK (transmembrane protein kinase RNA-like endoplasmic reticulum kinase; located in the ER membrane) pathway (Dey et al. 2015; Wakabayashi and Yoshida 2013). Activated PERK directly activates transcription factor Nrf2 and phosphorylates eIF2α (eukaryotic (translation) initiation factor 2α). peIF2α leads to upregulated translation of the cAMP-dependent transcription factor ATF4. ATF4 then triggers a cytoprotective program by upregulating key genes of autophagy, and, by cooperating with Nrf2, activates the antioxidant protein HO-1 (heme oxygenase 1) in order to antagonize the increasing oxidative stress induced by the loss of cell-matrix adhesion (Dey et al. 2015).

In breast cancer cells of the MCF-7 line, incorporation of the STAT3 (signal transducer and activator of transcription 3)-controlled zinc transporter ZIP6 (SLC39A6) into the plasma membrane induces EMT (epithelial-mesenchymal transition), cell detachment, resistance to anoikis and an ongoing proliferative activity of cells in suspension (Hogstrand et al. 2013). ZIP6-mediated $Zn^{2+}$ influx inactivates the glycogen synthase kinase 3β (GSK-3β) leading to activation of the transcription factor Snail. Snail then oppresses the transcription of E-cadherin resulting in cell rounding and detachment (Hogstrand et al. 2013). Snail is generally considered to be one of the key players inducing EMT accompanied by resistance to anoikis (Paoli et al. 2013; Peyre et al. 2021; Smit et al. 2009).

The $Ca^{2+}$ activated $Cl^-$ channel regulators 1 and 2 (CLCA1, 2; also called $Cl^-$ channel accessory 1, 2) are secretory, self-cleaving, $Zn^{2+}$-dependent metalloproteases that activate $Ca^{2+}$-dependent $Cl^-$ currents (Liu and Shi 2019; Yurtsever et al. 2012). They are involved also in apoptosis (Hutchings et al. 2019; Winpenny et al. 2009). Their downregulation, however, results in resistance to anoikis (Elble and Pauli 2001). While CLCA2 overexpression leads to increased $Cl^-$ currents accompanied by a decrease in intracellular pH, a reduced expression of CLCA2 is associated with increases in proliferation, migration, and invasion, and a higher risk of metastasis (Walia et al. 2009, 2012).

## *2.3 Defeating Attacks by the Immune System*

Once in the circulation, tumor cells encounter a huge number of immunosurveilling cells such as natural killer (NK) cells. NK cells express NKG2D (NK group 2d) receptors on their surface in order to recognize and bind their ligands (NKG2DL) which are primarily the cell surface glycoproteins MICA, MICB (MHC class I chain-related molecules A and B), and ULBPs 1–6 (Duan et al. 2019; Ghadially

et al. 2017; Molfetta et al. 2017). Basically, the transcription factor Sp1 mediates an upregulation of NKG2DL-expression during EMT resulting in an increased immunogenicity (Huergo-Zapico et al. 2014). However, NKG2DL-expression decreases as the tumor cells continue to dedifferentiate and is completely absent in poorly differentiated human colorectal cancer samples (López-Soto et al. 2013). For camouflage purposes, i.e. in order to elude immune surveillance, CTCs can either shed their NKG2DLs to (1) remain undetected and (2) misdirect the immune system (Dhar and Wu 2018), or they even avoid surface expression of NKG2DLs (Liu et al. 2019a; Schmiedel and Mandelboim 2018) as shown for leukemic stem cells in patients with acute myeloid leukemia (Paczulla et al. 2019).

*At transcriptional level*, aberrant methylation of the genes encoding NKG2DLs or low acetylation of histones can lead to NKG2DL silencing in tumor cells of different origin (Li et al. 2011a; Ritter et al. 2016). In glioma cells with mutations of the isocitrate dehydrogenase (IDH), loss-of-function mutations induce 2-hydroyglutaric acid-mediated epigenetic and metabolic reprogramming, eventually silencing ULBPs 1 and 3 (Zhang et al. 2016a). In other malignant glioma cells, TGF-β suppresses the transcription of *MICA*, *ULBP2*, and *ULBP4* without affecting the mRNA levels of *MICB*, *ULBP1,* and *ULBP3* (Eisele et al. 2006). *MICA* mRNA expression can be decreased also by IFN-γ as shown for both solid (cervical) and hematological (erythroleukemia and lymphoma) cell lines (Zhang et al. 2008).

*At translational level*, miR-10b, miR-20a, mir-34a, miR-93, or miR-106 either destabilize the NKG2DLs' mRNAs or inhibit their translation in a number of tumor cell lines such as melanoma, breast, prostate, or colorectal cancer (Codo et al. 2014; Heinemann et al. 2012; Stern-Ginossar et al. 2008; Tsukerman et al. 2012; Yang et al. 2018). In contrast, miR-889-overexpression protects hepatocellular carcinoma cells from NK cell-mediated lysis, because it significantly inhibits MICB expression (Xie et al. 2018).

*At post-translational level*, proteolytic enzymes, shedding and secretion help to reduce NKG2DL surface expression in tumor cells (Duan et al. 2019). Thus, IFN-γ not only regulates MICA expression at the transcriptional level but also promotes its hydrolysis by matrix metalloproteinases (MMPs) (Zhang et al. 2008). "A disintegrin and metalloproteases" (ADAMs) 10 and 17 mediate shedding of MICA and MICB from human mammary, pancreatic, and prostate carcinoma cells (Chitadze et al. 2013). A significant amount of soluble NKG2DL is found in sera of leukemia patients where it impairs antileukemia reactivity of NK cells by downregulating their NKG2D (receptor) expression (Hilpert et al. 2012). Similarly, glioblastoma cells secrete lactate dehydrogenase 5 (LDH5) to trigger NKG2DL expression in myeloid cells including monocytes, which then results in the downregulation of NKG2D in NK cells (Crane et al. 2014).

NK cells' effective antitumor activity requires direct, physical contact. Consequently, physical shielding does not only protect tumor cells from mechanical stress (please see Sect. 2.1) but also helps them to escape from NK cell attacks as coating with tumor cell-activated platelets impedes lysis of tumor cells by NK cells (Nieswandt et al. 1999) and facilitates metastasis (Palumbo et al. 2005). The formation of stable platelet/tumor cell aggregates needs fibrinogen or fibrin

crosslinking factor FXIII. Loss of these coagulation factors causes a strong decrease in metastasis in an NK-cell dependent manner (Palumbo et al. 2005, 2008). The adhesion molecule P-selectin is expressed on platelets and mediates platelet/tumor cell adhesion by binding to sialylated, fucosylated glycans on the tumor cell surface (Borsig et al. 2002; Mannori et al. 1995), mostly in a $Ca^{2+}$-dependent way (Erpenbeck and Schön 2010). Furthermore, by releasing TGFβ, also the platelets cause a reduction of NKG2D receptors on NK cells (Kopp et al. 2009). Finally, platelets can furnish tumor cells with both platelet-derived GITRL (glucocorticoid-induced TNF-related ligand; TNFSF18) which inhibits NK cells' antitumor reactivity (Placke et al. 2012b) and with normal MHC class I molecules which help the tumor cells to hide from immunosurveillance (Placke et al. 2012a).

While CTC clusters and CTCs surrounded by platelets or leukocytes can easily travel through the macrovasculature as silent emboli, these virtually conglomerate structures need to regroup before entering microvessels and capillaries with diameters of $\leq$10 μm, so that the single cells can pass through sequentially (Au et al. 2016). In capillary beds, even single CTCs can be halted within <30 min after entering the blood stream (Aceto et al. 2014; Micalizzi et al. 2017). Hence, it seems plausible that extracellular vesicles, exosomes or microparticles released from platelets/leukocytes rather than the actual, intact cells would confer the above-mentioned ligands/receptors to CTCs and thus enable them to camouflage and remain undetected by the immune system.

## 3 Adhesion to the Vessel Wall

In addition to simply being physically stuck inside small capillaries at the secondary site, CTCs need to adhere to and interact with the endothelium in order to eventually extravasate (Azevedo et al. 2015; Foss et al. 2020; Osmani et al. 2019). While the attachment of CTCs to endothelial cells can be mediated by a variety of ligands and receptors such as selectins, cadherins, integrins, CD44 and immunoglobulin superfamily receptors (Bendas and Borsig 2012; Reymond et al. 2013), CD44 and β1 integrin have been identified as key mediators of CTC adhesion. They counteract the shear forces that otherwise would cause the detachment of CTCs from the endothelial cell layer (Follain et al. 2018, 2020; Osmani et al. 2019). In addition to mediating CTC adhesion to the endothelial cell layer or being a biomarker for cancer cells with stem-like properties (Mani et al. 2008) CD44 may enhance metastatic potential by effectuating homophilic CTC interactions, possibly resulting in the formation of CTC clusters even post-intravasation (Chaffer and Goetz 2018; Liu et al. 2019b; Rodrigues and Vanharanta 2019).

Melanoma cell adhesion molecule (MCAM; also known as MUC18 or CD146) is expressed on both melanoma and endothelial cells, and it is believed that homophilic interactions promote tumor cell extravasation and metastasis because antibodies against MCAM inhibit human melanoma growth and metastasis (Mills et al. 2002), and B16 wild-type cell metastasis to the lungs is drastically reduced in

MCAM knockout mice (Jouve et al. 2015). In human melanoma cells of the MV3 cell line, MCAM expression correlates with the expression of the $Na^+/H^+$ exchanger NHE1 (SLC9A1), and MV3 cell–cell adhesion is pH-sensitive and depends on NHE1 expression (Hofschröer et al. 2017). This observation together with the aforementioned homophilic interaction of MCAM expressed on melanoma and endothelial cells (Mills et al. 2002) points to a potential contribution of NHE1 to the adhesion of tumor cells to the vessel wall.

## 4 Extravasation

Specific ligand-mediated interactions between tumor and endothelial cells do not necessarily result in adhesion but are nonetheless required for extravasation. Thus, the homophilic interaction between junctional adhesion molecules C (JAM-C) expressed on melanoma and endothelial cells does not impact adhesion but clearly abets lung metastasis (Langer et al. 2011). Also soluble ligands secreted by endothelial cells, e.g. CXCL12, mediate tumor extravasation by binding to chemokine receptors such as CXCR4 expressed particularly on gastrointestinal tumor cells which then stimulates the small GTPases Rho, Rac, and Cdc42 required for cell migration (Gassmann et al. 2009). The latter is consistent with the observations that (1) Cdc42 depletion in various tumor cells leads to a significant decrease in both β1 integrin-dependent interaction with endothelial cells and experimental lung metastasis (Reymond et al. 2012), and (2) that transient RhoC depletion in prostate cancer (PC3) cells reduces early PC3 cell retention in the lungs and in vivo metastasis formation (Reymond et al. 2015).

Paracellular diapedesis, i.e. squeezing through the endothelial cell layer by moving between endothelial cells, is the prevalent mode of extravasation and requires loosening of inter-endothelial cell junctions (Leong et al. 2014; Schumacher et al. 2013). Transcellular diapedesis, i.e. crossing the endothelium by penetrating individual cell bodies, has been shown in vitro, but seems rather rare and most likely requires both endothelial myosin II activity and E-selectin mediated activation of ERK and p38 MAPKs in endothelial cells (Khuon et al. 2010; Tremblay et al. 2008; Wettschureck et al. 2019). A recent study confirms that the microvascular endothelium reorganizes its membranes and cytoskeletal structures in order to directly contribute to the extravasation of tumor cells into the brain, and that melanoma cells primarily migrate paracellularly while breast cancer cells are able to migrate transcellularly (Herman et al. 2019). However, it needs to be stressed that up to now transcellular extravasation in vivo has been found only in microvascular endothelia, possibly because they are typically characterized by a lack of smooth muscle cells.

Endothelial reorganization is usually induced by the CTCs themselves. Breast cancer cells secrete angiopoietin-like 4 (ANGPTL4) or its C-terminal fibrinogen-like domain (cANGPTL4). cANGPTL4 weakens endothelial cell–cell contacts by activating an α5β1 integrin-mediated Rac1/PAK/β-catenin pathway. In a subsequent step, cANGPTL4 directly interacts with VE-cadherin and claudin-5 which causes

disruption of intercellular adhesion, thus allowing for transendothelial tumor cell migration (Huang et al. 2011; Padua et al. 2008). Melanoma cells secrete osteonectin (SPARC). SPARC binds to VCAM1 which triggers actin remodeling and loosening of endothelial junctions, mediated by a ROS-MKK3/6-p38MAPK-MLC2 signaling pathway and promoting extravasation and metastasis (Tichet et al. 2015). Other soluble factors that are secreted by metastatic cells and increase vascular permeability by modulating endothelial tight and adherens junctions include lipid 12(S)-hydroxyeicosatetranoic acid (12(S)-HETE), angiopoietin 2 (Ang-2), the chemokine CCL2 (C-C motif chemokine ligand 2, monocyte chemotactic protein 1), CXCL12 (stromal cell-derived factor 1α, SDF-1α), fibrinogen, HGF/SF, VEGF, PCB 104 (2,2′,4,6,6′-pentachlorobiphenyl), and a group of heat-stable, trypsin-sensitive, O-glycosylated glycoproteins ranging from 10 to 50 kD (García-Román and Zentella-Dehesa 2013).

Instead of gently loosening endothelial cell–cell junctions, a variety of human and murine tumor cells act more ruthlessly by inducing necroptosis in endothelial cells in order to locally perforate the endothelium and hence facilitate extravasation and metastasis (Strilic et al. 2016). To this end, CTCs express amyloid precursor protein (Pandey et al. 2016; Tsang et al. 2018) which binds to its receptor, death receptor 6 (DR6), on endothelial cells to induce necroptotic signaling pathways (Strilic et al. 2016). Additionally, necroptotic endothelial cells could possibly reinforce the opening of the endothelial barrier by releasing damage-associated molecular patterns (DAMPs) such as high-mobility group protein 1 (HMGB1) or ATP (Kaczmarek et al. 2013; Pilzweger and Holdenrieder 2015; Strilic and Offermanns 2017).

## *4.1 With the Assistance of Blood Cells*

### 4.1.1 Platelets

Also blood cells contribute to CTCs' extravasation. For instance, platelets normally assist immune cells with their extravasation (Gros et al. 2015). They – like the metastatic CTCs (see above) – release HGF, fibrinogen, VEGF, and 12(S)-HETE, and, in addition, platelet-derived activating factor (PAF), thrombin, ATP and serotonin in order to increase vascular permeability. Indeed, there is evidence that platelets recruited by CTCs occasionally promote CTC extravasation (Foss et al. 2020; Labelle et al. 2014; Schumacher et al. 2013). Dense granule-derived ATP released from tumor cell-activated platelets acts on endothelial junctions and the cytoskeleton, mediated by $P2Y_2$ receptors and with the objective of opening the endothelial barrier to facilitate transendothelial migration and metastasis (Schumacher et al. 2013). Upon activation by ATP, the G-protein coupled $P2Y_2$ receptor leads to (1) $Ca^{2+}$ release from intracellular stores via stimulation of phospholipase Cβ including the generation of IP3 (Raqeeb et al. 2011) and (2) activation of the PKC/Src pathway (Bilbao et al. 2010). The activated $P2Y_2$ transiently associates with VEGFR-2 and VE-cadherin at endothelial cell–cell adhesions

while Src phosphorylates VEGFR-2, VE-cadherin, VE-cadherin-bound p120-catenin, and probably also β- and γ-catenins in order to ensure a coordinated release of endothelial adherens junctions (Liao et al. 2014; Liu et al. 2004; Seye et al. 2004; Zou et al. 2015). Subsequent binding of p120-catenin to the guanine nucleotide exchange factor Vav2 activates Rac1 (Valls et al. 2012) which may induce cytoskeletal rearrangements to further facilitate the passage of CTCs through the newly formed intercellular space (Liao et al. 2014; Spindler et al. 2010). At the same time, the $P2Y_2$ mediated $Ca^{2+}$ release from intracellular stores results in the activation of $SK_{Ca}$ and $IK_{Ca}$ channels. The concomitant membrane hyperpolarization causes additional $Ca^{2+}$ influx via store-operated channels (SOCs, consisting mainly of TRPC1 & 4 and requiring TRPC4 subunits (Cioffi et al. 2005)) further promoting $K_{Ca}$ channel activity (Raqeeb et al. 2011; Sheng and Braun 2007). The elevated cytosolic $Ca^{2+}$ concentration also stimulates the activities of CaM (calmodulin) and eNOS (endothelial nitric oxide synthase) which considerably contributes to the increase in endothelial permeability (Sheng and Braun 2007; Thibeault et al. 2010). On the whole, CTCs usurp the physiological mechanism by which platelets assist neutrophils in extravasating at inflamed sites. Although a number of ion channels and transporters passing $Ca^{2+}$ and $K^+$ are involved, they just fulfill their regular functions. In this context, their expression and activity cannot be considered to be pathophysiological so that they are barely usable as therapeutic targets. The actual pathological step is the platelet activation by CTCs via either direct physical interaction between mucin-like glycoprotein podoplanin or galectin on the CTC cell surface and CLEC-2 or glycoprotein VI on the platelet surface, respectively, or via ADP, thromboxane A2 or high-mobility group box 1 (HMGB1) released by the CTC to bind to the toll-like receptor 4 (TLR4) on the platelet (Schlesinger 2018).

In addition, the podoplanin, expressed on tumor cell surfaces, stimulates the release of TGFβ from platelets (Takemoto et al. 2017). The TGFβ then activates Smad and NF-κB signaling pathways in the tumor cells leading to a more mesenchymal and invasive phenotype which may contribute to extravasation (Labelle et al. 2011).

### 4.1.2 Neutrophils

Although neutrophils are known to play pro-metastatic roles, their short half-life makes it difficult to precisely analyze the underlying mechanisms. Nevertheless, it has been shown that granulocyte-colony stimulating factor (G-CSF) mediates conversion of neutrophils into immunosuppressive cells that block the antitumor functions of $CD8^+$ T (Coffelt et al. 2015, 2016) and NK cells (Spiegel et al. 2016). Furthermore, platelets promote tumor cell extravasation indirectly by recruiting granulocytes specifically to the vicinity of platelet/tumor cell aggregates. To this end, tumor cell-activated platelets release CXCL5 and CXCL7 both of which bind to CXCR2 chemokine receptors on granulocytes co-expressing granulocyte marker Ly6G, integrin α-M (=CD11b), and matrix metalloproteinase 9 (MMP9) (Labelle et al. 2014). Releasing MMPs 8 and 9, neutrophils facilitate extravasation by

disintegrating the extracellular matrix such as the basement membrane (Cools-Lartigue et al. 2014; Spiegel et al. 2016). The tumor-activated platelets can also trigger neutrophil degranulation including the formation of neutrophil extracellular traps (NETs) (Cedervall et al. 2018). NETs are netlike structures that (1) consist of expelled neutrophil DNA with associated proteolytic enzymes, (2) function as a pathogen trap, and (3) can also sequester circulating tumor cells and thus promote local adhesion and metastasis (Cools-Lartigue et al. 2013; Demkow 2021; Park et al. 2016). The capture of CTCs in NETs can be mediated by NET-associated β1-integrin or CEACAM1 (carcinoembryonic Ag cell adhesion molecule 1) as shown for lung (A549; Najmeh et al. 2017) and colorectal cancer (HT-29, MC38; Rayes et al. 2020) cells. Accordingly, preventing the formation of NETs or disintegrating them by application of DNase I-coated nanoparticles reduces lung metastases in mice (Park et al. 2016), and impeding NET formation with the peptidylarginine deiminase 4 (PAD4) inhibitor BMS-P5 can slow down the progression of multiple myeloma in mice and humans (Li et al. 2020).

### 4.1.3 Monocytes/Macrophages

In addition to neutrophils and platelets, monocytes/macrophages contribute to CTC extravasation as well. Metastatic CTCs recruit monocytes/macrophages to the site of extravasation by releasing the CC-chemokine ligand 2 (CCL2) which attracts circulating monocytes expressing CC-receptor 2 (CCR2) and 6C2 (LY6C; in mice) or $CD14^{high}CD16^{negative}$ (in humans) (Cassetta and Pollard 2018; Qian et al. 2011), or indirectly by inducing local endothelial activation which results in E-selectin expression (Häuselmann et al. 2016). The endothelial E-selectin mediates the adhesion of the attracted monocytes to the endothelium, and the bond between E-selectin and its ligand triggers signaling in both the monocytes and the endothelial cells, eventually leading to (1) a stronger, integrin-mediated adhesion, (2) the retraction of endothelial cells, and (3) a subsequent loosening of the endothelial tight junctions through de-phosphorylation of VE-cadherin (Häuselmann et al. 2016). Beyond that, extravasated monocytes in the underlying tissue can differentiate into metastasis-associated macrophages, which then release VEGF to increase vascular permeability and thus promote tumor cell extravasation (Cassetta and Pollard 2018; Qian et al. 2009, 2011).

## 5 Organotropism

Already in 1889, Stephen Paget postulated that metastasis formation requires both cancer cell-intrinsic properties (“seed”) and a congenial microenvironment (“soil”) (Paget 1989). Accordingly, different cancers show different preferences with regard to the organs they metastasize to (Gao et al. 2019). Renal, thyroid, and liver cancer cells metastasize preferentially to the lungs; ovarian, colon, and gastric cancer cells

to liver and peritoneum; pancreatic cancer cells to lungs and liver; lung cancer cells to bone and brain. Breast and prostate cancer share the same preferences with the highest incidence of metastases in bone and lungs. In addition, breast cancer often metastasizes to liver and brain. Melanoma can be considered an all-rounder because it spreads nearly everywhere with the highest incidences of metastases in lungs, liver, brain, bone, and peritoneum (Gao et al. 2019). CTCs can also colonize the primary tumor, i.e. their tumor or origin. This process, called "tumor self-seeding," may select for cancer cells that are more aggressive than those originally in the primary tumor, and may – at least partly – explain local recurrence after tumor excision (Kim et al. 2009). However, it needs to be stated that so far there is not sufficient clinical evidence to substantially support this concept.

Independently of different cancer types' preferences including the unique characteristics of each target organ, they share a number of general principles underlying organotropism (Gao et al. 2019). At first, a premetastatic environment is formed. Both soluble factors and exosomes containing (mi)RNA are released from the primary tumor. They interact directly with cells residing at a prospective metastatic site. At the same time, they trigger the release of progenitor cells from bone marrow and conduct their target-oriented travel to a prospective metastatic site. Thus, the soluble factors and exosomes released by the primary tumor in combination with bone marrow-derived cells are significantly involved in arranging the premetastatic niche for later colonization (Fig. 1a; Izraely and Witz 2021; Wang et al. 2021).

In a second step, CTCs are then decoyed into this premetastatic niche by inflammatory chemokines released from local cells (Moore 2001; Zlotnik et al. 2011).

## *5.1 Locally Released Chemokines Lure CTCs*

The involved chemokine receptor-ligand pairs include, among others, CXCR1/2-CXCL8 (Ha et al. 2017; Li et al. 2014), CXCR4-CXCL12 (Guo et al. 2016; Iwasa et al. 2009; Müller et al. 2001), CCR6-CCL20 (Ghadjar et al. 2006; Kadomoto et al. 2020), and CCR7-CCL21 (Mashino et al. 2002; Rizeq and Malki 2020). Thus, in patients with axillary node positive primary breast cancer, the expression of chemokine receptors determines the target organ of metastasis. CXCR4 expression increases the risk of metastasis to the liver, CX3CR1 expression favors metastasis to the brain, CCR6 expression causes metastasis to the pleura, and CCR7 expression can be associated with the occurrence of skin metastases (André et al. 2006).

Lung tropism of osteosarcoma is mediated primarily by CXCL8 and IL-6 (Gross et al. 2018). CXCL8 triggers the release of $Ca^{2+}$ from intracellular stores (Joseph et al. 2010) and causes phosphorylation of Akt and Erk1/2 (Hosono et al. 2017), i.e. two signaling pathways known to drive cell migration and invasion. To date, ion channels and transporters potentially involved in this CXCR1/2-CXCL8-dependent organotropic process, such as $K^+$ or $Ca^{2+}$ channels, have not been identified.

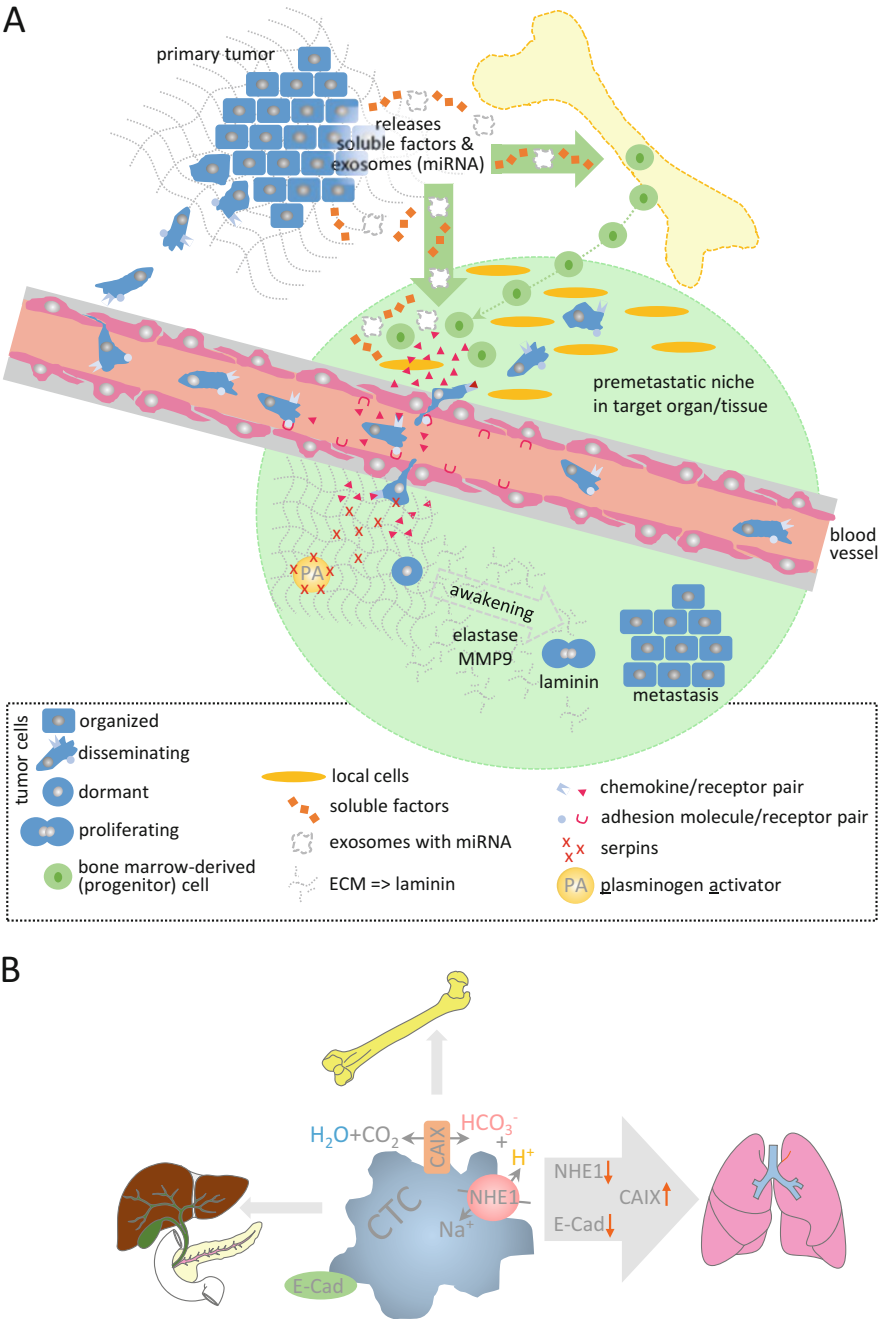


**Fig. 1** Major steps and known factors in organotropism. (**A**) The primary tumor releases soluble factors and miRNA-containing extracellular vesicles and exosomes that arrive at prospective target organs/tissues and at the same trigger the release of progenitor cells from bone marrow. A premetastatic niche is established by a concerted action of the bone marrow-derived cells, exosomes, and soluble factors from the primary tumor, and, not shown for the sake of clarity,

### 5.1.1 CXCL12/CXCR4

Another example is CXCL12 (= stromal cell-derived factor 1 (SDF-1)) which is preferentially expressed in lung and liver and thus attracts CXCR4-carrying melanoma, breast cancer (Minn et al. 2005; Müller et al. 2001), and pancreatic cancer cells (Saur et al. 2005). Interestingly, the water/glycerol channel aquaporin-3 (AQP3) is required for CXCL12/CXCR4-dependent, directional breast cancer cell migration, including spontaneous metastasis of orthotopic xenografts to the lungs (Satooka and Hara-Chikuma 2016). CXCL12 induces the membrane NADPH oxidase 2 (Nox2) to generate $H_2O_2$. $H_2O_2$ then enters the breast cancer cell through AQP3. It oxidizes the phosphatases PTEN (phosphatase and tensin homolog) and PTP1B (protein tyrosine phosphatase 1B), resulting in the activation of the Akt pathway which drives directional cell migration (Satooka and Hara-Chikuma 2016). Similarly, CXCL12-activated Akt and Erk1/2 pathways propel endothelial colony-forming cell (ECFC) migration, homing and incorporation into neovessels, thus re-establishing perfusion in ischemic tissues and promoting tumor vascularization and metastasis (Zuccolo et al. 2018). The activation of the Akt and Erk1/2 pathways requires a CXCL12-induced increase in the intracellular $Ca^{2+}$ concentration which is initiated by an $InsP_3$-mediated $Ca^{2+}$ release from the ER and maintained by subsequent store-operated $Ca^{2+}$ entry across the plasma membrane (SOCE) (Zuccolo et al. 2018).

In invasive glioblastoma, CXCL12 causes the activation of $K_{Ca}3.1$ ($IK_{Ca}$; KCNN4) channels including their long-term functional upregulation. $K_{Ca}3.1$ channel activity mediates glioblastoma cell migration and chemotaxis depending on CXCR4 expression (Sciaccaluga et al. 2010). Accordingly, a combined, simultaneous use of the anti-fungal $K_{Ca}3.1$ blocker clotrimazole, the CXCR4 inhibitor plerixafor (AMD3100), and the histamine 1 (H1) receptor antagonist mirtazapine has been suggested for cytotoxic glioblastoma treatment. The H1 receptor needs to be inhibited because it also can mediate $K_{Ca}3.1$ activation and thus represents a potential bypass of CXCR4 inhibition (Kast 2010).

$K_v11.1$ (hERG) channels mediate CXCL12/CXCR4-stimulated migration and invasion in leukemia cells (Li et al. 2009). In their plasma membranes, $K_v11.1$, CXCR4 and β1 integrin assemble to form a multiprotein complex (Pillozzi et al.

**Fig. 1** (continued) local fibroblasts, mesenchymal stem cells, neutrophils, and macrophages. Chemokines released by local cells decoy the circulating tumor cells into the premetastatic niche, supported by specific, local adhesion events at the endothelial surface often mediated by selectins or integrins. Once in the target tissue, single tumor cells can fall dormant and eventually be woken up by the presence of extracellular matrix components such as laminin emerging from locally induced extracellular matrix digestion events. Metastasizing tumor cells can also repulse local attacks, for instance by releasing serpins to prevent the plasminogen activator (PA) from generating plasmin which otherwise would lead to the release of apoptosis-inducing soluble Fas Ligand. (**B**) Circulating tumor cells (CTCs) such as metastasizing melanoma, breast or pancreatic cancer cells are directed predominantly to the lungs when their NHE1 activity is reduced, their CAIX expression is high, or when E-cadherin expression is low. Please see text for additional, more detailed information

2011). Targeting CXCL12 or the CXCL12/CXCR4 axis with peptides and small molecules induces pro-apoptotic effects and may thus help to overcome chemoresistance in leukemia that is induced by CXCL12-releasing bone marrow mesenchymal stromal cells (Pillozzi et al. 2019).

### 5.1.2 CCL20/CCR6

In general, the CCL20 chemokine/CCR6 chemokine receptor pair contributes to cancer cell motility and metastasis (Korbecki et al. 2020). This has been shown for breast cancer (Muscella et al. 2017), prostate cancer (Ghadjar et al. 2008), ovarian cancer (Liu et al. 2020), lung cancer (Wang et al. 2016), esophageal squamous cell carcinoma (Liu et al. 2017), gastric cancer (Han et al. 2015), pancreatic cancer (Campbell et al. 2005; Kimsey et al. 2004), hepatocellular carcinoma (Huang and Geng 2010), colorectal cancer (Frick et al. 2016), and renal cell carcinoma (Kadomoto et al. 2019).

In patients with primary lung cancer, the production of CCL20 in adrenal glands is most likely to recruit CCR6-expressing lung cancer cells which then leads to the development of adrenal metastases (Raynaud et al. 2010).

Multiple myeloma cells trigger the upregulation of both CCL20 and CCR6 in the bone microenvironment and thus contribute to osteoclast formation and eventually to osteolytic bone lesions (Giuliani et al. 2008).

The expression of CCL20 within the periportal area of the liver is likely to attract CCR6 expressing colorectal cancer (CRC) cells (Dellacasagrande et al. 2003; Frick et al. 2016). Accordingly, liver metastases can be found in approximately 50% of CRC patients (Jemal et al. 2008). Here, too, as described above for the CXCR1/2-CXCL8 and the CXCR4-CXCL12 pairs, Erk1/2 and Akt signaling pathways are activated and promote proliferation and motility of CRC cells (Brand et al. 2006). Furthermore, CCL20 stimulation of CCR6 expressing human colon carcinoma cells causes phosphorylation of BCAR1/p130$^{Cas}$ (Yang et al. 2005), a scaffolding protein overexpressed also in breast, ovarian, prostate, lung, and colorectal cancers as well as in hepatocellular carcinoma, glioma, melanoma, anaplastic large cell lymphoma, and chronic myelogenous leukemia (Barrett et al. 2013). BCAR1/p130$^{Cas}$ is a key component of the pathway by which the focal adhesion kinase (FAK) can drive cell migration (Tikhmyanova et al. 2010). In a monolayer of polarized human colon adenocarcinoma cells, CCR6 stimulation has been associated with cAMP-stimulated electrogenic chloride secretion as CCL20 inhibits forskolin-stimulated cAMP production (Yang et al. 2005). The nature of ion transporters and channels possibly involved has not yet been identified. A potential candidate would be the cAMP-dependent CFTR (cystic fibrosis transmembrane conductance regulator). NKCC1 ($Na^+$, $K^+$, $2Cl^-$ cotransporter 1) could also be involved. NKCC1 activity is sensitive to cytoskeletal dynamics (Hecht and Koutsouris 1999; Matthews et al. 1994), and the BCAR1/p130$^{Cas}$, phosphorylated in response to CCL20 stimulation, associates with cytoskeletal complexes (Sawada et al. 2006; Defilippi et al. 2006) and could thus be an integrative module linking NKCC1 activity with cytoskeletal dynamics.

### 5.1.3 CCL19 and 21/CCR7

The CCL21/CCR7 chemokine axis contributes to a metastatic phenotype in a wide variety of cancer types (Rizeq and Malki 2020), including breast (Müller et al. 2001; Weitzenfeld et al. 2016), prostate (Maolake et al. 2018), urinary bladder (Xiong et al. 2017), cervical (Kodama et al. 2007), esophageal (Shi et al. 2015; Goto and Liu 2020), gastric (Ma et al. 2015; Ryu et al. 2018), pancreatic (Hirth et al. 2020; Zhang et al. 2016b), colorectal (Li et al. 2011b), and lung cancer (Zhong et al. 2017), as well as melanoma (Cristiani et al. 2019; Takeuchi et al. 2004), lymphoma (Fleige et al. 2018; Li et al. 2018; Yang et al. 2011), and oral, head, and neck squamous cell carcinoma (Chen et al. 2020; González-Arriagada et al. 2018).

Generally, the binding of CCL19 and CCL21 to the GPCR CCR7 induces the activation of a $G_{\alpha}$-subunit and a $Gi_{\beta\gamma}$ heterodimer which then triggers downstream signaling effectors and signaling cascades. As a result, the activation of ERK1/2, PI3K/Akt, Rho GTPases, MAPK, and JAK/STAT can lead to the transcription and expression of different genes including MMPs and thus promote chemotaxis, cytoskeletal remodeling, extracellular matrix degradation, cell adhesion, migration, invasion, angiogenesis, and proliferation (Rizeq and Malki 2020). To date, it has not been shown explicitly that CCL19, 21/CCR7 mediated changes in tumor cell behavior involve ion channels and transporters. However, the signaling pathways sparked by CCR7 stimulation most likely address ion transport mechanisms as well, also in tumor cells. In CCR7 expressing mature dendritic cells, CCL19 and CCL21 trigger $Ca^{2+}$ influx from the extracellular space. This $Ca^{2+}$ influx is accompanied by KCa3.1 mediated $K^+$ efflux and, in presence of a yet undefined $Cl^-$ conductance, propels cell migration (Shao et al. 2015).

## 5.2 *Given Factors at the Premetastatic Niche*

In addition to being attracted by chemokines CTCs can be retained at the premetastatic niche by specific, local adhesion events. E-selectin, for instance, supports hematogenous metastasis of estrogen-receptor negative ($ER^-$) $CD44^+$ breast cancer cells (Kang et al. 2015). Furthermore, characteristic vascular structures in target organs are associated with special requirements for cancer cell extravasation (Gao et al. 2019; Minami et al. 2019; Nguyen et al. 2009; Weidle et al. 2016), so that the particular architecture of a blood barrier, typical of an organ or a tissue, may select for cancer cells that are able to break down the local endothelial junctions and the appendant basement membrane. This interplay between metastasizing cell and local environment is continued by the cancer cells' interaction with the unique resident cells and their secretome including the extracellular matrix. However, the initiation of proliferation and growth in the secondary organ appears to be another obstacle for disseminating tumor cells (Chambers et al. 1995).

### 5.2.1 Falling Asleep and Awakening

Some of the disseminating tumor cells enter a dormant phase, induced by a lack of a sufficient, integrin-mediated adhesion to the extracellular matrix in the secondary organ (Barkan et al. 2010). In order to survive without proper anchorage, detached breast cancer cells autocrinally secrete laminin-5, a component of the basement membrane, which induces their own survival through α6β4-mediated NFκB activation (Zahir et al. 2003). As soon as the biomolecular composition of the surrounding microenvironment changes, for example by the release of membrane receptor-ligands from a locally degrading extracellular matrix or by inflammatory events, dormant cancer cells can be awakened by induction of various signaling pathways leading to the revival of proliferative activity (Park and Nam 2020). Sustained lung inflammation, for instance, can provoke the formation of neutrophil extracellular traps (NETs). Two NET-associated proteases, neutrophil elastase and MMP9, then successively fragment laminin, and the proteolytically remodeled laminin awakens dormant breast cancer cells (Fig. 1a), i.e. induces their proliferation, by activating α3β1 signaling (Albrengues et al. 2018).

### 5.2.2 Local Nutrient Supply

Furthermore, the nutrient composition in the target organ may differ considerably from that around the primary tumor and thus force the disseminating tumor cells to adapt their metabolic pathways to the new environment (Elia et al. 2018). Accordingly, brain metastases originating from various tissues drive their oxidative TCA cycle utilizing acetate rather than glucose or glutamine (Maher et al. 2012; Mashimo et al. 2014), and breast cancer-derived lung metastases change over to a pyruvate carboxylase-dependent replenishment of the TCA cycle (anaplerosis) due to an elevated bioavailability of pyruvate in the lung environment (Christen et al. 2016).

### 5.2.3 Defeating the Local Defense System

On the other hand, tumor cells are capable of repulsing attacks by the tissues that they are going to populate. Normally, plasmin from the reactive brain stroma represents a defense against metastatic invasion. Plasmin is generated from plasminogen by plasminogen activator (PA) which in brain is released mainly by astrocytes. Plasmin cleaves off soluble Fas Ligand (sFasL) from the membrane-bound FasL, also expressed on astrocytes. The sFasL then induces apoptosis in metastatic cells and inactivates the axon pathfinding molecule L1CAM, a cell adhesion molecule expressed by metastatic cells for spreading along brain capillaries and for metastatic outgrowth. However, metastasizing breast and lung adenocarcinoma cells express high levels of PA inhibitory serpins (serin-protease inhibitors) to

prevent plasmin generation and thus its metastasis-suppressive effects (Valiente et al. 2014).

### 5.3 *Lack of E-Cadherin, Reduced NHE1 Activity, and the Presence of CAIX Each Contribute to Lung Tropism*

The epithelial-mesenchymal transition (EMT) does not only confer on epithelial cells the abilities to detach from the cell layer/tissue, migrate, invade the surrounding tissue and degrade components of the extracellular matrix (Lambert et al. 2017), but it can also play a considerable role in metastatic organotropism as shown for pancreatic cancer (Reichert et al. 2018). One characteristic of EMT is a decreased expression of E-cadherin, the main component of adherens junctions. Adherens junction protein p120 (P120CTN) stabilizes E-cadherin at the adherens junctions (Ishiyama et al. 2010; Thoreson et al. 2000). A complete loss of *p120ctn* in metastatic pancreatic ductal adenocarcinoma (PDAC) cells shifts their organotropic preference from the liver to the lungs. Rescue with a p120ctn isoform restores liver organotropism (Reichert et al. 2018). According to this, and independently of the presence of P120CTN, E-cadherin-expressing PDAC cells prefer to metastasize to the liver while E-cadherin-negative metastases are found predominantly in the lungs (Fig. 1b; Reichert et al. 2018). Analogously, the inhibition of NHE1 by cariporide seems to direct the metastatic spread of murine melanoma (B16V) cells to the lungs (Vahle et al. 2014). NHE1 activity is affected by the NHE regulatory factor (NHERF1), and NHERF1 expression is upregulated in a variety of cancers where its expression level correlates with malignancy (Georgescu et al. 2008; Greco et al. 2019; Ma et al. 2016; Saponaro et al. 2014; Vaquero et al. 2017). The phosphorylation state of NHERF1 on serines S279 and S301 differentially controls NHE1 activity and metastatic organotropism of breast cancer (MDA-MB-231) cells (Greco et al. 2019). Replacing both S279 and S301 by alanine results in a significantly increased NHE1 activity and, in a xenograft mouse model, drives a shift from the predominantly lung colonization to a predominantly bone colonization. This led the authors (Greco et al. 2019) to conclude that NHERF1 phosphorylation can act as a signaling switch in metastatic organotropism.

Also the carbonic anhydrase IX (CAIX) contributes indirectly to organotropism (Fig. 1b). Bone marrow-derived cells (BMDCs), including myeloid-derived suppressor cells (MDSC), macrophages, dendritic cells, and hematopoietic progenitor cells are recruited to potential metastatic sites where they act in concert to establish the premetastatic niche prior to the arrival of metastasizing tumor cells (Gabrilovich et al. 2012; Kaplan et al. 2005; Psaila and Lyden 2009; Quail and Joyce 2013). The production of chemokines and cytokines that mobilize granulocytic MDSCs to a potential (pre)metastatic niche requires the hypoxia-induced expression of CAIX by cancer cells in the (primary) tumor (Chafe et al. 2015). Hypoxic breast cancer cells

express significant amounts of CXCL10, CCL5, and the granulocyte colony stimulating factor G-CSF when, and only when, CAIX is expressed. Hypoxia-induced CAIX is needed for the activation of the NF-κB pathway which then results in the generation of G-CSF and eventually promotes breast cancer metastasis to the lungs (Chafe et al. 2015).

## 6 Conclusion and Outlook

Even though there is hardly any direct evidence proving it, the literature suggests that ion channels and transporters do contribute to both extravasation and organotropism of metastasizing tumor cells. Table 1 summarizes the channels and transporters potentially involved in (1) surviving the intravascular milieu, (2) adhesion to the vessel wall, (3) extravasation, and (4) metastatic organotropism.

NHE1 may be considered as a kind of "all-rounder" due to its dual function. (1) In its role as a structural element contributing to the organization of the cortical actin cytoskeleton and tying it to the plasma membrane, NHE1 possibly protects CTCs from mechanical stress. (2) In its role as $H^+$ extruder, NHE1 may promote both CTC adhesion to the vessel wall and subsequent, organ-specific extravasation by generating pH-nanodomains that modulate not only pH-dependent cell–substrate and MCAM-mediated cell–cell (melanoma-endothelium) adhesions but also the activity of matrix metalloproteases. Finally, there is evidence to suggest that NHE1 activity, regulated by NHERF1, has a hand in organotropism.

Regulation of the intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$ is interwoven with the modulation of $K^+$ conductances. $K^+$ channels including mechanosensitive K2P channels stabilize the membrane potential required for $Ca^{2+}$ influx, e.g. through mechanosensitive channels (TRPs, Piezo), while increases in $[Ca^{2+}]_i$ activate $Ca^{2+}$ sensitive $K^+$ channels ($K_{Ca}$s). This interplay, especially the controlled $Ca^{2+}$ influx, may strengthen the actin cortex of CTCs, accompanied by an increase in cortical stiffness, and thus protect them from shear forces in the blood vessels. In endothelial cells, an elevation of $[Ca^{2+}]_i$ (1) can be induced by binding of ATP released from tumor cell-activated platelets to endothelial $P2Y_2$, (2) is mediated by SOC channels, and (3) results in an increased endothelial permeability which facilitates extravasation (Table 1).

In addition to pH and $Ca^{2+}$ including the affected signaling pathways (e.g., $Ca^{2+}$/CaM signaling), the FAK signaling and the Akt pathway are major variables being modulated by ion channels/transporters and involved in organotropism and surviving the intravascular milieu. Permanent activation of FAK can prevent anoikis. Some CTCs secrete fibronectin or collagen and thus "autostimulate" their β1 integrin leading to activation of $K_v11.1$ concomitant with FAK phosphorylation. Another mechanism by which CTCs avoid anoikis is the adoption and perpetuation of mesenchymal features with the help of the $Zn^{2+}$ transporter ZIP6.

AQP3 in cooperation with the Akt pathway is likely to play a role in organotropism by directing CXCR4 expressing breast cancer cells to the lungs

**Table 1** Involvement of ion channels/transporters in CTC survival, extravasation, and organotropism

| | | Ion channel/transporter | Function/mechanism |
|---|---|---|---|
| Surviving intravascular milieu | Mechanical stress | TRPs & PIEZO (mechanosensitive) | $Ca^{2+}$ influx => cell stiffness↑ => strengthens cell cortex => protects plasma membrane from shear stress damage |
| | | Mechanosensitive two-pore $K^+$ channels (K2P) | Stabilize the electro-chemical gradient required for $Ca^{2+}$ influx |
| | | NHE1 | Expression↑ => cortical stiffness↑ |
| | Anoikis | $K_v11.1$ (hERG, KCN2) | β1 integrin, stimulated by self-secreted fibronectin or collagen, activates $K_v11.1$ which is essential for FAK-phosphorylation |
| | | ZIP6 ($Zn^{2+}$ transporter, SLC 39A6) | $[Zn^{2+}]_i$ ↑=> GSK-3β↓ => E-cadherin↓ => EMT↑ => anoikis↓ |
| | | CLCA2 ($Cl^-$ channel *accessory*) | ↓Expression => proliferation↑, motility↑, metastasis↑ |
| Adhesion to vessel wall | | NHE1 | Regulation of cell surface pH => pH affects cell-cell adhesion => homophilic interaction between MCAM expressed on melanoma *and* endothelial cells↑ |
| Extravasation | | SOC (TRPC1 & 4) channels and $K_{Ca}$s *of endothelial* cells | ATP released from tumor cell-activated platelets binds to endothelial $P2Y_2$ => $[Ca^{2+}]_i$↑ => $K_{Ca}$ activity↑, CaM activity↑, eNOS activity↑ => endothelial permeability↑ |
| Organotropism | | AQP3 | CXCL12 activates Nox2, Nox2-generated $H_2O_2$ enters via AQP3 & oxidizes PTEN & PTP1B => Akt pathway ↑ => cell migration => CXCR4 carrying breast cancer cells directed to the lungs |
| | | ? CFTR and/or NKCC1 ? | CCL20 in periportal area attracts CCR6 expressing CRC cells to the liver<br>1. CCL20/CCR6 affects cAMP-stimulated electrogenic $Cl^-$ secretion => CFTR?<br>2. CCL20/CCR6 => phosphorylation of BCAR1/$p130^{Cas}$ => affects FAK pathway & cytoskeleton => NKCC1 activity sensitive to cytoskeletal dynamics |
| | | NHERF1/NHE1 axis | • NHE1 inhibition with cariporide directs metastasizing melanoma to the lungs<br>• NHE1 activation by modified NHERF1-phosphorylation state shifts metastatic breast cancer spread from lungs to bone |

Please bear in mind that several of these interrelations have not been shown directly and, therefore, are partially hypothetical. Please see text for more details
↑ = stimulation; ↓ = inhibition; => = causal connection

where local CXCL12 stimulates $H_2O_2$ production via membrane-bound Nox2. $H_2O_2$ crosses the plasma membrane through AQP3 in order to activate the Akt pathway by oxidizing PTEN and PTP1B which eventually stimulates directional cell migration.

Altogether the literature strongly suggests that several ion channels and transporters have a hand in CTC survival, extravasation, and organotropism, which points to their potential usefulness as therapeutic target(s) during and after resection of the primary tumor. Given the great potential to be exploited as therapeutic targets on the one hand, yet the insufficient hitherto existing knowledge and unsatisfying data availability on the other, it becomes apparent that far more efforts need to be made in order to identify and characterize the mechanistic roles of ion channels and transporters in the behavior of CTCs including extravasation and organotropism. Provided that an experimental setting includes chemokines, extracellular matrix (proteins and structure), and preferably also immune cells typically found in the organ of interest, advanced microfluidic models of cancer cell extravasation (Mondadori et al. 2020; Offeddu et al. 2021) may be a suitable tool to validate the involvement of ion channels/transporters in extravasation and organotropism, e.g. by using genetically modified tumor cell lines, and to test their responsiveness to antimetastatic drugs.

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