Review

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The role of the Na^+/Ca^{2+}-exchanger (NCX) in cancer-associated fibroblasts

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Abstract: Cancer is characterized by uncontrolled growth, invasion, and metastasis. In addition to solid cancer cells, cancer-associated fibroblasts (CAFs) play important roles in cancer pathophysiology. They arise from "healthy" cells but get manipulated by solid cancer cells to supply them and develop a tumor microenvironment (TME) that protects the cancer cells from the immune defense. A wide variety of cell types can differentiate into CAFs, including fibroblasts, endothelial cells, and epithelial cells. Precise Ca^{2+} regulation is essential for each cell including CAFs. The electrogenic Na^+/Ca^{2+} exchanger (NCX) is one of the ubiquitously expressed regulatory Ca^{2+} transport proteins that rapidly responds to changes of the intracellular ion concentrations. Its transport function is also influenced by the membrane potential and thereby indirectly by the activity of ion channels. NCX transports Ca^{2+} out of the cell (forward mode) or allows its influx (reverse mode), always in exchange for 3 Na^+ ions that are moved into the opposite direction. In this review, we discuss the functional roles NCX has in CAFs and how these depend on the properties of the TME. NCX activity modifies migration and leads to a reduced proliferation and apoptosis. The effect of the NCX in fibrosis is still largely unknown.

Keywords: Ca^{2+}; cancer; cancer-associated fibroblasts (CAF); migration; Na^+/Ca^{2+}-exchanger (NCX); tumor microenvironment (TME).

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Ion transport proteins sensing the tumor microenvironment

Precisely functioning ion channels and transporters are essential for the physiology of all cells. In recent years, the function of ion transport proteins has been investigated in quite some detail in many cancer entities. Ion transport proteins contribute to different "hallmarks of cancer" which were originally described in 2000 by Hanahan and Weinberg (2000) and recently updated for a second time by Hanahan (2022) These include among others proliferation (Prevarskaya et al. 2018), migration (Schwab et al. 2012), and apoptosis (Bortner and Cidlowski 2014).

Due to their location in the plasma membrane, ion transport proteins serve as sensors of the cellular microenvironment and confer the cells with the ability to react to their microenvironment. The tumor microenvironment (TME) comprises soluble factors, extracellular matrix proteins, and several non-tumorous cell types. Thus, cells in a solid tumor are exposed to a markedly decreased pH (Pethő et al. 2020) and hypoxia (Jing et al. 2019). Moreover, the mechanical properties of tumors are very different from those of healthy tissue: Pressure and stiffness are usually strongly increased (Liu et al. 2020; Northcott et al. 2018). Cancer-associated fibroblasts (CAFs) are a very prominent cell type in the tumor microenvironment (Bagaev et al. 2021; Dominguez et al. 2020). Messenger substances secreted among others by tumor cells force resident cell types such as fibroblasts to transform into CAFs. CAFs respond to the altered physico-chemical properties of their environment by means of ion transport proteins. The altered microenvironment provides cues for the activation of CAFs (Fels et al. 2016). These cells then begin to produce extracellular matrix (ECM), resulting in fibrosis of the TME (Apte et al. 2004, 1999; Fels et al. 2016). The tumor tissue develops a desmoplastic phenotype in some cancer entities such as pancreatic ductal adenocarcinoma which causes a change from a cell-rich to an ECM-rich stroma. Thus, CAFs are important elements in solid tumors. Notably, in addition to matrix production distinct CAF subtypes may also elicit other functions within

the TME that will be outlined in more detail in the section "The role of cancer-associated fibroblasts in tumors" below.

Intracellular ion concentrations are determined by the activity of ion transport proteins, which in turn are influenced by the physico–chemical properties of the tissue. A precisely regulated Ca^{2+} homeostasis is necessary for every cell, but the regulation of the intracellular Ca^{2+} concentration is altered in cancer. In order to maintain the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) under resting conditions at ~100 nM, each cell needs efficient transport mechanisms to protect itself from an uncontrolled increase of the ($[Ca^{2+}]_i$). The Na^+/Ca^{2+} exchanger (NCX) is an important transport protein to achieve this goal. It is an electrogenic transporter, whose transport mode depends on the membrane potential and on the concentration gradients of Na^+ and Ca^{2+}. In any given transport cycle, one Ca^{2+} ion is always exchanged for three Na^+ ions (Reeves and Hale 1984). In the forward mode, Ca^{2+} is transported out of the cell, and in the reverse mode Ca^{2+} is transported into the cell (Blaustein and Lederer 1999).

The importance of the NCX for Ca^{2+} homeostasis and its function in CAFs will be discussed in this review. The relation of the tumor microenvironment with CAFs and the NCX is summarized in Figure 1. To date, the mechanisms of NCX transport itself are quite well understood. However, the functional roles of NCX for tumor cell behavior and its modulation by the tumor microenvironment are far less well known.

The role of the Na^+/Ca^{2+}-exchanger in Ca^{2+} signaling

Precise regulation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is critical for each cell because cytosolic Ca^{2+} acts as a second messenger in intracellular signaling. In mammalian cells, the free cytosolic Ca^{2+} concentration is very low (~100 nM Ca^{2+}), while the extracellular free Ca^{2+}

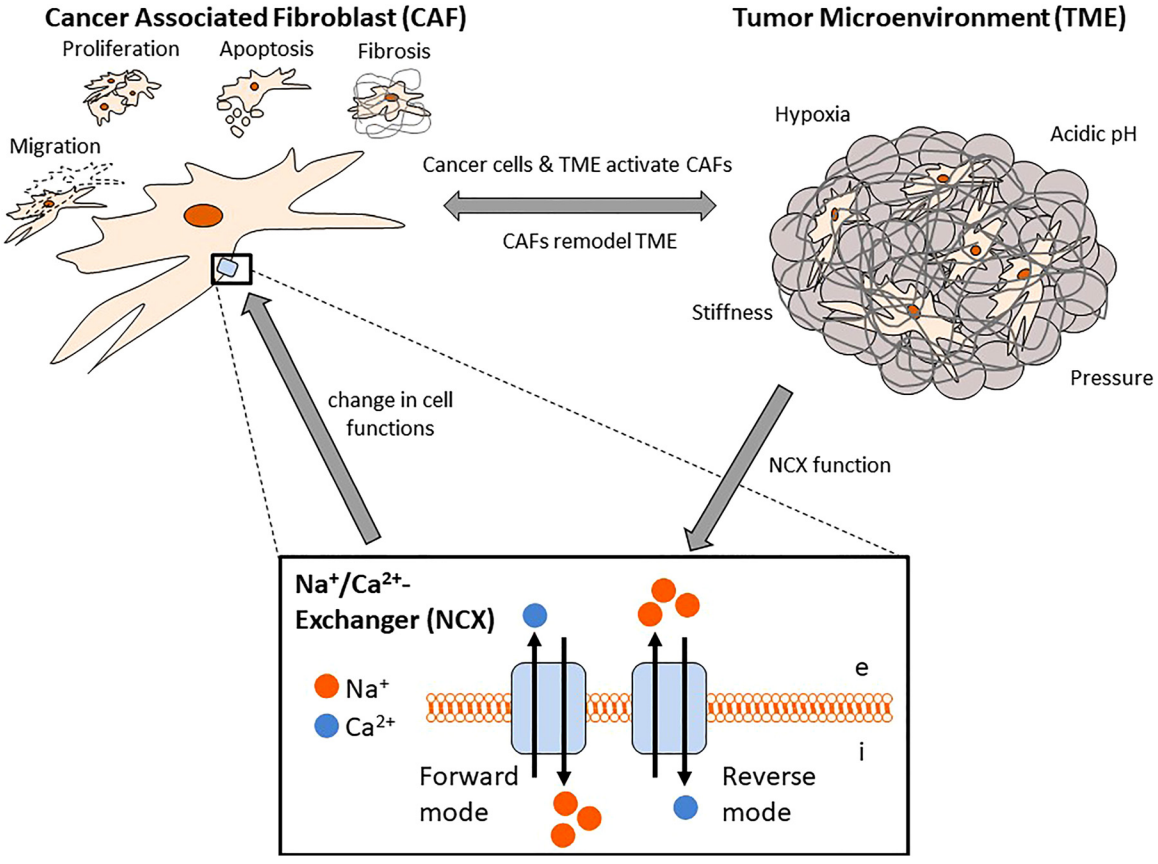

Figure 1: Cancer associated fibroblast (CAFs) and the tumor microenvironment (TME) are dependent on each other. The CAFs are activated by soluble factors and altered mechanical properties of the TME. Ion transport proteins act as sensors in the plasma membrane. They are modulated by the conditions of the TME (hypoxia, acidic pH, stiffness and pressure). Essentially all cell functions such as migration, proliferation, apoptosis and fibrosis can be modified by the Na^+/Ca^{2+} exchanger (NCX). NCX transports 3 Na^+ into the cytosol (i) and one Ca^{2+} in the extracellular space (e) in the forward mode. The transport direction is switched in the reverse mode.

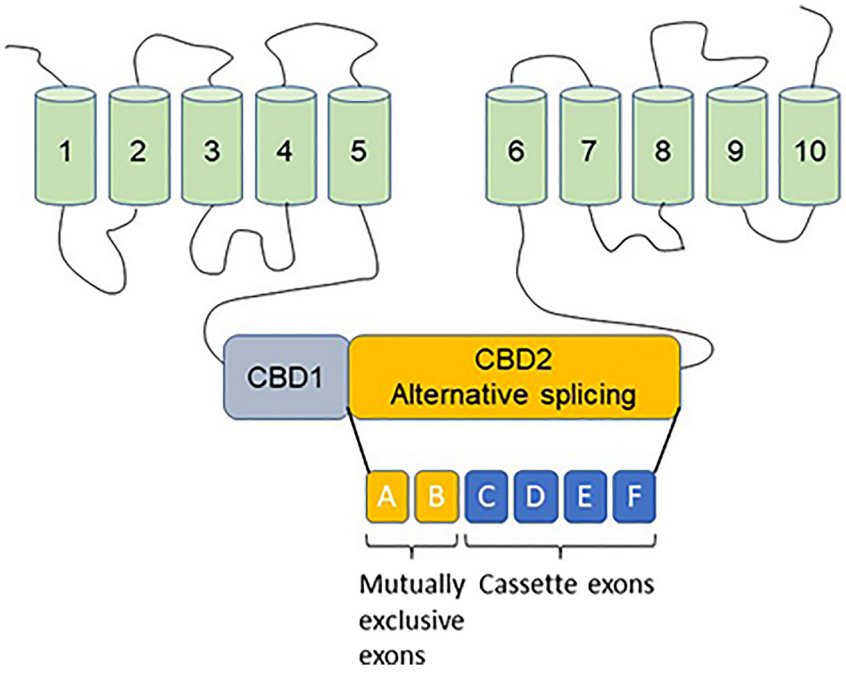

Figure 2: Molecular topology of the NCX1. NCX has 10 membrane helices which have a large intracellular loop between transmembrane domains five and six. This loop contains different regulatory regions such as the Ca^{2+}-binding domains (CBD) one and two. The CBD2 can be spliced in NCX1 and NCX3. The exons A and B are mutually exclusive exons.

concentration is in the 1 mM range, i.e. 10,000-fold higher. An effective control of the intracellular Ca^{2+} homoeostasis is essential for Ca^{2+} signaling. The proteins involved in Ca^{2+} regulation must be temporally and spatially precisely controlled, respond to (transient) signals by increasing and decreasing the ($[Ca^{2+}]_i$), and adapt to the conditions of the cell (Berridge et al. 2003; Clapham 2007).

A specifically controlled network of different families of ion channels orchestrates the Ca^{2+} release from intracellular stores and entry from the extracellular environment. The extrusion of Ca^{2+} into the extracellular space is performed by the plasma membrane Ca^{2+}-ATPase (PMCA) and by the NCX. The transport of Ca^{2+} from the cytosol to the sarcoplasmic or endoplasmic reticulum is regulated by the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) (Cui et al. 2017).

NCX is a transport protein that links Ca^{2+} and Na^{+} signaling. The three NCX isoforms are encoded by *SLC8A1* (NCX1), *SLC8A2* (NCX2), and *SLC8A3* (NCX3) (Quednau et al. 1997). It was first identified and characterized by Baker et al. (1969). This antiporter belongs to the superfamily of Ca^{2+}-cation antiporters (CaCA), which all share a similar topology (see Figure 1) (Linck et al. 1998). Members of the CaCA superfamily have 2 clusters of five–six transmembrane domains which are connected with an intracellular loop. The NCX protein has 10 transmembrane helices (Ren and Philipson 2013). The large cytoplasmic f-loop between transmembrane domains five and six contains different regulatory regions like the Ca^{2+}-binding domains (CBD) one and two (Figure 2) (Liao et al. 2012). Ion transport occurs via a conserved sequence motif (α1 and α2), each of which is found in a cluster of transmembrane domains (Khananshvili 2014; Liao et al. 2012). The eucaryotic NCX isoforms contain 930–970 amino acids (Lytton 2007). The discovery of the crystal structure of archaebacterial *Methanococcus jannaschii* (NCX_Mj) has provided new insights into the structure and function of NCX (Liao et al. 2012). However, the crystal structure of the eukaryotic NCX is not available until now.

The different isoforms and splice variants are expressed in a tissue-specific manner to regulate Ca^{2+} fluxes. While NCX1 is ubiquitously distributed, NCX2 is mainly expressed in the brain and spinal cord. NCX3 has so far been found in the brain and skeletal muscle (Khananshvili 2020; Quednau et al. 1997). NCX1 and NCX3 can be spliced but not NCX2. NCX1 has 17 splice variants formed from a combination of six small exons (A–F). So far, five splice variants of NCX3 have been discovered. However, there are only three exons (A–C) that generate different splice variants (Figure 2). The mutually exclusive exons A and B are expressed in all NCX variants. All the splice variants modify the CBD2 domain in their response to Ca^{2+} binding. The binding of Ca^{2+} at the CBD2 stops the Na^{+} induced inactivation (Hilge et al. 2006). Via this binding site, the response kinetics and affinity of allosteric NCX sensors are regulated. Depending on the splice variants the regulating Ca^{2+} can modify the activity of the NCX (Boyman et al. 2011). NCX2 has no Na^{+}-dependent inactivation (Li et al. 1994). This could be an explanation for why no splice variants exist of NCX2. The different isoforms play a

role in tissue-specific Ca^{2+} and Na^+ dependent allosteric regulation. NCX is activated by binding Ca^{2+} to CBD1. Inhibition of NCX occurs via binding of Na^+ to an unknown site in NCX1 and NCX3 (Khananshvili 2020). Inactivation by Na^+ can be reversed by binding Ca^{2+} to CBD2 in NCX1 and NCX3. Up to now, there is a lack of knowledge about the expression of the NCX different splice variants in cancer.

Driving forces set the transport direction of the NCX

Understanding and interpreting NCX function requires a precise understanding of the driving forces and under which conditions NCX reverses its transport direction. NCX has four binding sites to which 3 Na^+ ions and one Ca^{2+} ion can bind (Liao et al. 2012). Findings from the *NCX_Mj* model showed that two conformational changes are necessary for a transport process and that a simultaneous occupation of the binding sites is thermodynamically not possible (Giladi et al. 2017; Marinelli et al. 2014).

NCX is a high capacity/low affinity system, which means that it can rapidly exchange many ions. In cardiomyocytes NCX cycles at a rate of 2500–5000/s (Niggli and Lederer 1991). The directionality of the transport mode depends on three factors: the membrane potential, and the intracellular as well as the extracellular concentrations of Ca^{2+} and Na^+ (Blaustein and Lederer 1999). Under resting conditions, the reversal potential E_m is in the order of −30 mV. When the membrane potential is more negative, the thermodynamically favored transport mode is the forward mode. NCX changes its transport direction when E_{NCX} is equal to or more positive than E_m. The reversal potential can be calculated according to (Khananshvili 2014):

$$E_{NCX} = 3E_{Na} - 2E_{Ca} = \left(\frac{RT}{F}\right)\ln\left\{\left([Na_o]^3[Ca_o]\right)\Big/\left([Na_i]^3[Ca_i]\right)\right\}$$

with R, T, and F being the gas constant, the absolute temperature, and the Faraday's constant, respectively.

An influence on the NCX reversal potential is exerted indirectly by other ion channels, whose activity can also significantly alter the intracellular ion concentrations. Some studies have investigated the functional interplay of TRP channels with NCX. For example, in human pancreatic duct cells, it has been shown that NCX interplays with TRPC1 and shows a role in TGF-β-mediated cell mobility (Dong et al. 2010). In vascular smooth muscle cells, the involvement of TRPC4 and TRPC6 was investigated in the context of intracellular Ca^{2+} store emptying and activating the reversal of NCX (Zhang et al. 2018). In another example, different TRPC channels have been shown to be responsible for cation influx that trigger the reverse mode of NCX in human cardiac fibroblasts (Ikeda et al. 2013). Moreover, it has been shown that Na^+ influx via TRPM4 regulates NCX in goblet cells. Both proteins are required in the production of mucus, which plays a role in diseases like the cystic fibrosis (Cantero-Recasens et al. 2019). In addition, transporters such as Na^+/K^+-ATPase (Balasubramaniam et al. 2015) and NHE1 (Liskova et al. 2019) also influence NCX. All these studies show that when considering the NCX and determining the direction of transport, a variety of other ion channels and transporters also have an impact on NCX kinetics. Table 1 gives a summary of transport proteins that functionally cooperate with NCX.

Table 1: NCX and its interaction partners in different tissues.

Cell type	Functional cooperation partner	Function	Reference
Human pancreatic duct cells	TRPC1	TGF-β mediated cell mobility	Dong et al. (2010)
Vascular smooth muscle cells	TRPC6 & TRPC4	Ca^{2+} depletion by TRPC6/TRPC4 from ER activates the reverse mode of NCX	Zhang et al. (2018)
Human cardiac fibroblast	TRPC1, TRPC3, TRPC5, TRPC6	Na^+ entry by TRPC channels enhances the reverse mode of NCX	Ikeda et al. (2013)
Goblet cells	TRPM4, TRPM5	NCX and TRPM4/TRPM5 are both required for mucin secretion	Cantero-Recasens et al. (2019)
Human cervical carcinoma cell line SiHa, colon adenocarcinoma cell line DLD1, ovarian cancer cell line A2780, renal cell carcinoma cell line RCC4	NHE1, carbonic anhydrase IX	pH control in hypoxic tumors	Liskova et al. (2019)
Renal epithelial cells	E-cadherin	Knockdown of NCX 1 induces epithelial-to-mesenchymal transition	Balasubramaniam et al. (2017)
Renal epithelial cells	Na^+/K^+-ATPase	Cell migration	Balasubramaniam et al. (2015)

Pharmacological inhibition of NCX

To better explore the functions of NCX, various inhibitors have been developed. In general, it has been difficult until now to develop an inhibitor that has a high affinity for NCX and is free of unspecific binding to other channels or transporters at the same time. First, divalent and trivalent cations such as Cd^{2+}, $Ni^{2+,}$ and La^{3+} were used to block the NCX. The disadvantage of these cations is their unspecificity, and their effect is only seen when other Ca^{2+} channels were inhibited. Another major problem is the toxicity of these substances (Kimura et al. 1987; Shimizu et al. 1997).

Other substances were initially used, analogs of amiloride, with hydrophobic substitutions on the acylguanidinium nitrogen. These substances have only a low affinity for NCX (IC_{50}: 10–100 μM) (Kaczorowski et al. 1985; Kleyman and Cragoe 1988), and they are not specific as they also block other Ca^{2+} channels and Na^{+} transporters (Floreani et al. 1987). The development of benzyloxyphenyl NCX inhibitors led to more affine and specific NCX inhibitors. Initially, KB-R7943 was developed in 1996. This inhibitor enjoyed great popularity in NCX research for many years. KB-R7943 preferentially inhibits the reverse mode (Ca^{2+} influx) (IC_{50}: 1.2–2.4 μM), while the forward mode is only weakly inhibited (IC_{50}: >30 μM) (Iwamoto et al. 1996). However, some NCX1 splice variants, e.g. NCX1.3, are equally sensitive in their forward mode (Hamming et al. 2010). Yet, its specificity is not ideal and a number of ion channels and transporters are also inhibited KB-R7943 (Kraft 2007; Pintado et al. 2000; Sobolevsky and Khodorov 1999). The problem of unspecificity is also shared by further blockers such as SN-6 (Iwamoto et al. 2004) or SEA0400 although SEA0400 has a ~100-fold higher affinity than KB-R7943 (Matsuda et al. 2001; Tanaka et al. 2002) and preferentially inhibits NCX1, whereas KB-R7943 primarily inhibits NCX3 (Iwamoto et al. 2008). The next generation of NCX inhibitors was developed in 2013 and modified in the following years. ORM-10103 (Jost et al. 2013), ORM-10962 (Kohajda et al. 2016) and ORM-11372 (Otsomaa et al. 2020) block all three NCX isoforms and also inhibit forward and reverse mode with similar IC_{50} values. The potential side effects of ORM blockers have not yet been well characterized.

The role of cancer-associated fibroblasts in tumors

Tumor cells are in close contact with their environment. They can activate other cells by secreting various growth factors and cytokines. The tumor secretome targets essentially all cells of the TME, in particular CAFs. In addition, cancer stem cells are stimulated in an autocrine manner (Ping et al. 2021). A common definition of CAFs states that they are negative for epithelial, endothelial, and leukocyte markers, have an elongated morphology, and are devoid of mutations that are typically found in cancer cells (Sahai et al. 2020). CAFs are genetically stable and accordingly do not develop chemoresistance, which makes them interesting targets for new drugs.

Up to date, there is no specific marker found for CAFs. However, some proteins are upregulated in CAFs, and thus activated CAFs can be distinguished from their cells of origin. Common markers for fibroblast and myofibroblast activation include α-smooth muscle actin (αSMA) and fibroblast activation protein (FAP). In addition, vimentin, desmin, platelet-derived growth factor receptor-α and -β (PDGFR-α and -β), and fibroblast specific protein-1 (FSP-1) also serve as markers for CAFs (Ping et al. 2021). An increased presence of CAFs generally indicates a worse prognosis for the patient, as this is a sign of an already advanced stage of the disease. The activation or transformation of fibroblasts and other CAF progenitor cells is due to the excessive secretion by tumor cells of a wide variety of growth factors, such as transforming growth factor β (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor 2 (FGF2), and C-X-C motif chemokine ligand (CXCL) 12 (Tao et al. 2017). Moreover, the involvement of microRNA in gene regulation of CAFs has also been previously demonstrated for certain cancers, such as ovarian cancer (Mitra et al. 2012).

In recent years, various CAF subtypes have been characterized. These are distinguished by their localization and their function: Some CAFs support tumor growth while others inhibit it. Thus, four subtypes of CAFs were found in breast cancer (Costa et al. 2018) and three subtypes in pancreatic ductal adenocarcionoma (PDAC) (Elyada et al. 2019). In PDAC, they are named myofibroblast CAF (myCAF), inflammatory CAF (iCAF), and antigen-presenting CAF (apCAF) (Elyada et al. 2019; Öhlund et al. 2017). These subgroups are distinguished by their cells of origin, their expression profile of αSMA, FAP, and IL-6 (Öhlund et al. 2017). iCAFs contribute to immunosuppression by secreting hyaluronic acid synthase 1 (HAS1) and HAS2 (Öhlund et al. 2017). Less is known about myCAFs. They resemble antigen-presenting cells and likely play a role in immune regulation (Elyada et al. 2019).

CAFs promote tumor cell growth by supporting angiogenesis (Tang et al. 2016). Without an adequate blood supply, cancer cells cannot grow and will die. CAFs do this by secreting vascular endothelial growth factor (VEGF), CXCL12, fibroblast growth factor (FGF), and PDGF (Sahai et al. 2020).

These growth factors stimulate the formation of blood vessels. CAFs also support cancer cell metastasis by secreting proteases such as matrix-metalloproteases (MMPs), which degrade the fibrotic environment and provide a pathway for tumor cells. Co-metastasis of CAFs with cancer cells has also been observed (Gonzalez-Zubeldia et al. 2015).

One other key function of CAFs is the remodeling of the tumor microenvironment (TME). Thereby, CAFs create niches for cancer cells that protect them from the immune defense and chemotherapeutic agents. This occurs through the secretion of extracellular matrix (ECM) proteins (Kaur et al. 2019) mainly collagen I and III, fibronectin, proteoglycans, matricellular proteins, and matrix-degrading enzymes such as MMPs and its inhibitor (tissue inhibitors of metalloproteinases, TIMPs) (Bachem et al. 2006; Erkan et al. 2012; Hessmann et al. 2020). Moreover, the cells of this unique network secrete growth factors and different cytokines (Bachem et al. 2006). The constituents of this secretome amplify the activation of CAFs by autocrine and paracrine signaling loops (Hessmann et al. 2020). This in turn has dramatic effects on the original tissue by changing its physico–chemical properties. It changes from a cell-rich to a fibrotic stroma, which is also called desmoplasia. For the remaining cells, desmoplasia means a significantly altered environment to which they must adapt in order to survive. In many tumors, this fibrotic stroma makes up a large proportion of the tissue, e.g. in pancreatic ductal adenocarcinoma (PDAC) this can be up to 90% (Erkan et al. 2012; Xie and Xie 2015) and in melanoma up to 80% (Simiczyjew et al. 2020).

Chemical–physical properties of the TME

Tumor cells communicate with their environment and activate CAFs, which in turn can modulate the environment. The chemical-physical properties of a desmoplastic tumor microenvironment change in a way that can impact on NCX function. In the following section the different properties of the TME are discussed in more detail and in light of their impact on NCX function.

Acidic pH modifies NCX activity

A typical characteristic of cancer cells is their metabolic rate, in which energy is obtained mainly from glycolysis and lactate production. During this process of energy production, the concentration of free protons is up to 10 times higher in the TME as compared with the corresponding healthy tissue. In PDAC, extracellular pH values can be lower than pH 6.5, which is very different from the physiological pH 7.4 (Pedersen et al. 2017; Pethő et al. 2020; Swietach et al. 2014). Due to the acidification of the environment, ion channels and transporters are restricted in their functioning. So far, it has not yet been investigated how tumor acidosis affects NCX function in CAFs. However, we can draw some analogies from studies related to the cardiac ischemia-reperfusion injury which is also accompanied by a marked acidification (Lemasters et al. 1996). The cardiac NCX1.1 variant is strongly inhibited by intracellular proton binding. It is an allosteric regulatory mechanism distinct from Na^+ and Ca^{2+} regulation. Proton sensitivity of NCX is conferred by His124 and in particular by His165 (John et al. 2018).

NCX is not the only ion transport protein whose pH sensitivity is of relevance for the tumor pathophysiology. This applies to many other transport proteins as well. Depending on the transport protein an acidification can either lead to their inhibition or their activation. This has been summarized in a recent review (Pethő et al. 2020). In many cases the tumor acidosis may cause a depolarization because of the inhibition of pH-sensitive K^+ channels. A depolarized membrane potential would favor the reverse mode of NCX activity. Indeed, it is reported that the NCX preferentially works in the reverse mode in some tumor cells (e.g. prostate cancer cells, melanoma, leukemia) (Chovancova et al. 2020; Long et al. 2016). However, this interpretation has to be viewed with some caution. In prostate cancer cells it is entirely based on the use of KB-R7943 (Long et al. 2016) whose lack of specificity was discussed above.

Hypoxia

Hypoxia is a common hallmark of the tumor microenvironment of nearly all solid tumors. It is defined by a decrease in the fractional oxygen concentration from 2 to 9% to less than 2% (Jing et al. 2019). The cause of hypoxia in cancer is due to uneven oxygen consumption and supply. Tumor cells adapt to the low oxygen level. In most cases, this leads to a more aggressive tumor cell behavior. Hypoxia results in the hypoxia-inducible factor 1α (HIF-1α) not being degraded and being present in increased amounts in the cell (Carmeliet et al. 1998; Lee et al. 2020; Zhong et al. 1999).

Hypoxia is frequently accompanied by an increase of the ($[Ca^{2+}]_i$). This is due to the increased expression of TRP and ORAI channels (Azimi et al. 2017; Girault et al. 2020). For example, increased TRPC6 expression or activity was found in hepatic (Iyer et al. 2015) or pancreatic stellate cells, respectively (Nielsen et al. 2017). Similarly, TRPC1 is overexpressed in several breast cancer cell lines (MDA-MB-

468, HCC1569, MDA-MB-231) (Azimi et al. 2017). One study with PC12 cells proposed that hypoxia causes NCX to switch to the reverse mode because of the depolarized membrane potential. An opening of TRPC channels together with NCX reversal was said to induce an Ca^{2+} accumulation, which promotes apoptosis (Meng et al. 2008). This may be an example of how hypoxia and other ion channels impact on NCX activity. Yet, the conclusions should be viewed with some caution because it relied on the use of inhibitors with low specificity. Another study had shown that the NCX in BHK (baby kidney hamster) cells responds isoform-specifically to hypoxia. The authors suggested that the NCX3 might provide a protective function during chemical hypoxia (Secondo et al. 2007).

Mechanical properties of the TME affect the function of ion transport proteins

The modified mechanical properties in the TME may cause a malignant transformation of the tissue. The increased stiffness and pressure in the tumor tissue lead to an activation of fibroblasts and the production of more ECM (Lachowski et al. 2017, 2019). The ECM advances fibrosis. Finally, the cells are in a rigid and dense environment. These effects lead to a loss of cell polarity that causes dedifferentiation and transformation into CAFs (Paszek et al. 2005). Moreover, the mechanical properties can promote tumor progression by activating epithelial-to-mesenchymal transition (Rice et al. 2017).

Cells sense the changes in their environment by ion channels and integrins. This leads to a modification of signaling and to an inhibition of tumor suppressor genes (Nagelkerke et al. 2015). In the context of this review, it is relevant to note that Piezo1 and TRPC1 channels contribute to the activation of pancreatic stellate cells by a pressure load (Fels et al. 2016; Kuntze et al. 2020). Pancreatic stellate cells are residing in the pancreas. Once activated by the TME they secrete abundant amounts of ECM proteins and thereby underly fibrosis that is a typical feature of pancreatic ductal adenocarcinoma. Piezo1 and TRPC1 are both non-selective cation channels whose activity will at first lead to a depolarization of the membrane potential. Depending on whether or not the cells are also expressing Ca^{2+}-activated K^+ channels this may be followed by a hyperpolarization. In either way the change of the membrane potential and the accompanying changes of the intracellular Ca^{2+} concentration will influence the driving forces of NCX and potentially its direction of transport. Thus, mechanical stimuli will indirectly also affect the function of NCX.

The role of the NCX in cancer-associated fibroblasts and their progenitor cells

The role of the Ca^{2+} regulator NCX in CAFs will be considered in more detail in the following sections. However, this field of research is quite new so that the knowledge on the function of NCX in CAFs is rather limited. Nevertheless, we will relate the most important cell behavioral traits to NCX function in CAFs.

Migration – invasion

The migration of tumor cells is a prerequisite for metastasis. CAFs support the directional migration of cancer cells (Erdogan et al. 2017) and co-metastasize with them (Gonzalez-Zubeldia et al. 2015).

Cells migrate in a coordinated process. First, migrating cells have to adopt a polarized morphology with lamellipodia forming in the direction of migration. Protrusion of the leading cell pole, formation of new and dissolution of older focal adhesions, and retraction of the posterior cell pole, represent the basic steps of cell migration (Trepat et al. 2012; Zanotelli et al. 2021). These processes are complemented and regulated by fine-tuned local ion and water movement (Schwab et al. 2012). To maintain cell polarity, the cell rearranges the positioning of its cell organelles, which is coordinated among others by the microtubule organizing center in the anterior pole of the cell and motor proteins, which carry organelles and vesicles with them (Ridley et al. 2003). Ca^{2+} plays an important role by causing actin-myosin II-mediated cell contraction via calpain and calcineurin-dependent signaling pathways. Myosin II is the central motor protein of migration (Betapudi et al. 2011). The activation of myosin II is initiated by an increase of the intracellular Ca^{2+} concentration. The highest Ca^{2+} concentration is located at the posterior cell pole where it contributes to the retraction of the cell posterior. Pulse-like Ca^{2+} increases in the anterior part of the cell are also crucial for directed migration (Tsai et al. 2014).

Up to now, a few studies investigated the role of NCX in the migration behavior of CAF progenitor cells. Their results led to different conclusions about the NCX in migration of these cells. Sakamoto et al. performed wound healing scratch assays with rat tendon fibroblasts. Under control conditions, they discovered that wound healing is suppressed upon NCX blockade with KB-R7943 and SEA0400. This result could be confirmed in transwell assays following

NCX1 knockdown with siRNA. The same group could also show that contraction is suppressed upon NCX blockade. From these results, they concluded that NCX is probably important for wound healing (Sakamoto et al. 2009).

Kemény et al. studied the migratory behavior of human gastric myofibroblasts. Myofibroblasts are predominantly fibroblasts that have undergone a phenotype transition. Moreover, they can arise from other cells like smooth muscle cells by a phenotype switch (Pakshir et al. 2020). Therefore, they have properties of both cell types. They are contractile but also electrically non-excitable. Blocking NCX with CB-DMB and $NiCl_2$ inhibits migration in wound healing scratch assays (Kemény et al. 2013). However, it has to be kept in mind that these inhibitors are of limited specificity. Therefore, the confirmation with other NCX blocking technics, e.g. with siRNA, would have strengthened the relevance of these results.

Similar to fibroblasts, other cells can also differentiate into CAFs. Epithelial cells are able to differentiate into CAFs via the epithelial-to-mesenchymal transition (EMT). The blockade of NCX by KB-R7943 leads to a marked inhibition of migration in transformed renal epithelial cells (Madin-Darby canine kidney cells; MDCK-F) (Dreval et al. 2005). The authors observed that ($[Ca^{2+}]_i$) is crucial for migration. The ($[Ca^{2+}]_i$) being too high or too low leads to a reduced migration. Moreover, they saw a dose-dependent ($[Ca^{2+}]_i$) increase by KB-R7943 administration and an intracellular alkalinization both of which are consistent with NCX operating in the forward mode before the inhibition with KB-R7943 (Dreval et al. 2005).

NCX is also cooperating with the Na^+/K^+-ATPase. Knockdown of the regulatory Na^+/K^+-ATPase β-subunit in MDCK cells decreases NCX expression and function but the cell migration is increased. Moreover, the increased Ca^{2+} influx and reduced NCX1 expression or its reduced function led to upregulation of ERK1/2 (Balasubramaniam et al. 2015). In a follow-up study the same authors showed that NCX1 knockdown induced EMT in MDCK cells. The epithelial MDCK cells acquire a fibroblastic morphology with non-compact tight junctions and a reduction of epithelial makers. The loosening of cell-cell contacts resulting in an increase of the intercellular distance between epithelial cells was interpreted as the beginning of EMT. Moreover, they MDCK cells loose their apico-basal polarity. These changes are due to reduced E-cadherin stabilization and induction of β-catenin signaling (Balasubramaniam et al. 2017).

The above cited studies indicate that up to now, it is difficult to make a general statement whether NCX activity promotes or impairs migration. This is most likely due to the different experimental conditions and cell types. This is further complicated by a lack of specific inhibitors. As outlined above, many of the inhibitors used in these studies are notorious for multiple off-target effects (Iwamoto et al. 2008; Kraft 2007; Pintado et al. 2000). Nonetheless, we can conclude that NCX is involved in migration but the complexity of CAFs and the TME conditions are likely to make its impact very context-specific.

Proliferation – cell cycle

Ca^{2+} is involved in the proper regulation of cell cycle progression and cell proliferation. Thus, it has already been demonstrated in human fibroblasts some 30 years ago that Ca^{2+} influx is essential for proliferation and thus also for the cell cycle. Without Ca^{2+}, the transition from G1/G0 to S phase is reduced (Wahl and Gruenstein 1993). The growth factor TGF-β, which is also secreted by tumor cells, promotes proliferation. In cardiac fibroblasts, the effect of TGF-β was found to be reduced in the presence of KB-R7943 (Ikeda et al. 2013). In flow cytometric analysis of the cell cycle, no major differences were found in cardiac fibroblasts between untreated control cells and cells treated with KB-R7943. But cells treated with TGF-β and KB-R7943 showed a reduced cell cycle progression compared with TGF-β treatment alone (Ikeda et al. 2013). Cardiac myofibroblasts were also found to have decreased proliferation upon PDGF stimulation and concurrent KB-R7943 treatment (Raizman et al. 2007). The findings are relevant for CAFs since cancer cells are known to secrete a variety of growth factors, such as TGF-β, or PDGF, to stimulate other cells. In conclusion, the inhibition of the NCX impairs growth factor-stimulated proliferation and cell cycle progression. Further studies with other inhibitors and knockdown models are needed to confirm the results of KB-R7943. If this is the case, it should be considered to what extent tumor growth could be reduced via NCX inhibition.

Apoptosis

In addition to cell proliferation, apoptosis also occurs during development or tissue renewal. Apoptosis prevents degeneration and tumor cell development in healthy cells. Various stimuli can trigger apoptosis. For example, extracellular ligands can bind to cell membrane receptors (e.g. Fas ligand) and trigger a death signal, which results in a cleavage of caspases leading eventually to a caspase-3 mediated cell death. Alternatively, intracellular processes involving mitochondria can trigger apoptosis. The Bcl-2 family members are crucial for this death pathway. In its final stage, an

apoptosome is formed which activates caspase-3. Bcl-2 proteins are also known to regulate the intracellular Ca^{2+} compartmentalization and signaling (Orrenius et al. 2003).

Other intracellular processes triggering apoptosis include DNA damage and also a disturbed intracellular Ca^{2+} homeostasis through endoplasmic reticulum Ca^{2+} stress as well as cytoplasmic or mitochondrial Ca^{2+} overload (Dubois et al. 2016; Elmore 2007). Ca^{2+} can enter the cell by a multitude of transport proteins including store-operated Ca^{2+} entry channels or by other Ca^{2+} permeable channels (e.g. TRP channel, Ca_V channels) from the extracellular space. Uncontrolled Ca^{2+} influx is leading to cell death.

The NCX could be both anti and pro-apoptotic depending on its working mode. If it removes Ca^{2+} in the forward mode, it will likely be anti-apoptotic. However, if the conditions of the tumor microenvironment force the NCX to work in the reverse mode (Ca^{2+} entry), it would be pro-apoptotic because an accumulation of intracellular Ca^{2+} can induce apoptosis.

The role of NCX in apoptosis was investigated during hypoxia-reoxygenation in adult guinea pig ventricular myocytes. The reverse mode (Ca^{2+} entry mode) results in a Ca^{2+} overload, leading to enhanced apoptosis. There is enhanced activity of NCX during hypoxia-reoxygenation. Accordingly, apoptosis is reduced after NCX knockdown (Eigel et al. 2004). Another study confirmed the results that the reverse mode of NCX plays a role in triggering apoptosis (Li et al. 2014). Moreover, the effect of H_2S on apoptosis and the NCX was analyzed. H_2S is a gasotransmitter which regulates a variety of processes including Ca^{2+} transport. It could be shown that NCX is upregulated by sulfide signaling and induces apoptosis in Hela cells (Markova et al. 2014). In a follow up study, the authors detected an intracellular acidification of tumor cells after treatment with a H_2S donor. They also observed an increased NCX and NHE1 expression in colorectal cancer DLD1 cells in nude mice. The authors supposed that the intracellular acidification is due to an uncoupling of NCX1/NHE1 (Szadvari et al. 2019).

Too few studies have been published so far to make a clear judgment on the role of NCX in apoptosis of CAFs. We still rely on extrapolating results obtained in other cell types. However, the available studies show that when the NCX is involved in apoptosis, it is likely to operate in the reverse mode.

Fibrosis

The tumor microenvironment of many tumors is characterized by massive fibrosis. Traditionally, fibrosis is defined as a strong repair response after inflammatory or chronic injuries (Gerarduzzi and di Battista 2017). The involvement of NCX in tissue fibrosis was already shown in 1998 in association with the development of liver fibrosis. When hepatic stellate cells become activated, they differentiate into myofibroblasts. They are responsible for fibrosis by secreting large amounts of extracellular matrix proteins. Interestingly, NCX becomes only detectable in hepatic stellate cells when they are activated (Nakamura et al. 1998).

So far, the role of NCX function in fibrosis is still largely unknown. A little bit more is known about the Na^+/K^+-ATPase in fibrosis. This is relevant in the context of this review since blocking the Na^+/K^+-ATPase with ouabain will change the driving forces for NCX. The attenuated Na^+ gradient will favor the reverse mode (Ca^{2+} influx) of the NCX. The effects of Ouabain and SEA0400 were analyzed in a model of cardiac fibrosis. Ouabain treatment promotes fibrosis through reverse (Ca^{2+} entry) mode of the NCX. Another experiment analyzed the effect of SEA0400 on fibrosis. The administration of SEA0400 reduces fibrosis and myocardial stiffness as well as increases survival (Kamimura et al. 2012). These findings indicate an involvement of the NCX in the development of fibrosis by activating the reverse mode. Thus, these findings can be viewed of proof-of-principle for the contribution of NCX activity to the development of fibrosis. Future experiments will have to show whether the antifibrotic effect of NCX inhibition can be reproduced in solid tumors.

Conclusions

The development of solid cancer is closely related to the development of the TME and thus to a complex change in tissue properties. Various studies pointed towards the involvement of NCX in processes related to the properties of the TME. These properties, in turn, are importantly determined by the function of CAFs. In this context, the mechanical properties, fibrosis, hypoxia and tumor acidosis are particularly relevant. All of these properties have been shown to modify NCX function or to be modified by NCX function. However, one important conclusion that can be drawn from this review is that much of the existing knowledge has been gained in other (disease) models. Much of this extrapolation to CAFs and the TME has yet to be verified.

In general, interpreting NCX function is not straightforward because the NCX is also highly dependent on other ion transport proteins. A network of different ion channels and transporters, the so-called transportome, orchestrates

the regulation of ion concentrations in a fine-tuned and interdependent way. Moreover, the available inhibitors have varying degrees of side effects on other proteins. This is the case for the commonly used inhibitor KB-R7943, which preferentially blocks in the reverse mode (Iwamoto et al. 2008). Therefore, data obtained from pure pharmacological interventions without any further validation (e.g. siRNA, $[Ca^{2+}]_i$ measurements etc.), should be regarded with some caution. Newer inhibitors are available which inhibit the forward and reverse mode in a similar level and are not isoform specific. Further studies have to prove their specificity. Moreover, restitution of the complex physico-chemical properties of the TME is often limited *in vitro.* Therefore, the interpretation is dependent on the different *in vitro* settings. However, it should be noted that studying ion transport proteins in the context of the tumor microenvironment is still a fairly new field of research. There are still many gaps of knowledge to be filled. It was one of our aims to identify open questions with respect to NCX function in cancer-associated fibroblasts and its modulation by the tumor microenvironment.

In conclusion, the presented studies show that NCX affects various cellular functions. The NCX modifies migration. The blockade of the NCX leads to reduced proliferation and reduced apoptosis. The effect on fibrosis is still largely unknown, first hints indicate an involvement of the reverse mode of the NCX and inhibition with pharmacological inhibitors results in less fibrosis in the heart. However, a clearer picture of the effects of NCX is required. Partially contradictory results in the literature need clarification and also properties not mentioned here could be affected by NCX, such as the stiffness of the ECM. Finally, the field would largely profit from the development of further isoform- and transport mode-specific pharmacological modulators. Possibly, the characteristic properties of the tumor microenvironment could further increase the tissue specificity and thereby limit adverse side effects mediated for example by targeting cardiac NCX.

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