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ION TRANSPORT IN CELL CYCLE AND CANCER

Topic Editors
Andrea Becchetti, Annarosa Arcangeli and Luca Munaron

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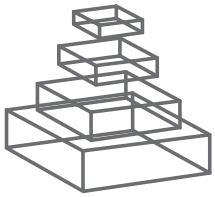

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ION TRANSPORT IN CELL CYCLE AND CANCER

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Increasing evidence points to the role of membrane transport in the regulation of cell cycle, differentiation and other aspects of cell physiology that shape the multistep tumor progression, such as resistance to apoptosis and cell invasiveness. Ion channels and other transporter proteins can regulate these mechanisms in many ways, by controlling membrane potential, calcium fluxes, intra and extracellular pH, the release of growth factors, interaction with the extracellular matrix and cytoskeleton, and so forth. Some of these actions may occur through non-conductive mechanisms, such as conformational coupling with other membrane proteins (e.g. growth factor receptors or integrins) or intrinsic enzyme activity. Other transporters exert their effects in the intracellular membrane systems, e.g. the endoplasmic reticulum, mitochondria and other organelles. This Research Topic aims at collecting papers on all of the manifold aspects of this growing field. Hence, we will consider experimental papers, essays and reviews concerning the function of membrane transporters in cell cycle, differentiation, apoptosis, adhesion/migration, the metastatic process, the transition from stem cell to cancer cell and angiogenesis. Use of ion channels and other transporters as possible diagnostic tools or therapeutic targets will also be considered.

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EDITORIAL
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The role of ion channels and transporters in cell proliferation and cancer

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After the early wavering steps tracing back to at least the 1960's (Cone, 1974; Binggeli and Cameron, 1980), the study of ion transport in cell proliferation and neoplasia is on its way to become a mature research field (Arcangeli et al., 2009). Wide evidence is now available about the regulatory roles exerted by ion channels and transporters on the cell cycle phases (Becchetti, 2011) and other aspects of cell physiology that shape the multistep neoplastic progression, such as resistance to apoptosis (Lehen'kyi et al., 2011), cell invasiveness (Becchetti and Arcangeli, 2010), and angiogenesis (Fiorio Pla et al., 2012). Ion transport is implicated in these cell functions in many ways, from the classic mechanisms relating membrane potential (V_m) to Ca^{2+} homeostasis, to the control of pH, cell volume, growth factor release, interaction with the extracellular matrix, and so forth. Some of these actions probably occur through non-conductive mechanisms, such as intrinsic enzyme activity or conformational coupling with other membrane proteins (e.g., Arcangeli and Becchetti, 2006; Hegle et al., 2006). A major function of ion channels is to mediate the cell interaction with its environment. In the case of cancer cells, the interaction with the tumor milieu' has relevant implications for therapy (Arcangeli, 2011).

The Research Topic Issue on *Ion Transport in Cell Cycle and Cancer* addresses classic issues in the field as well as it points to novel perspectives. One example of the latter is the role of Ca^{2+} in autophagy (Rizzuto et al., 2012), which has not received as much attention as Ca^{2+} signaling in the cell cycle machinery and apoptosis (Roderick and Cook, 2008; Munaron, 2012). As discussed by Kondratskyi et al. (2013), modulating autophagy has a great potential in cancer diagnosis and treatment. The review discusses the related Ca^{2+}-dependent mechanisms and their meaning in the context of cancer progression and therapy. The role of Ca^{2+} in autophagy is related to the increasing recognition of the multiple functions of intracellular ion channels, which regulate ion transport across the membranes of cellular organelles. Leanza et al. (2013) provide an updated overview of the role of ion channels in mitochondria, endoplasmic reticulum, nucleus, endosomes, and lysosomes, with special attention to the implications for the biology of cancer.

A general picture of the role of V_m in cell proliferation and differentiation is provided by Yang and Brackenbury (2013), with a special focus on voltage-gated channels. The paper reviews what is known about the V_m changes during cell cycle and the implications of different types of ion channels in the cell cycle stages, cancer cell migration and the differentiation of cancer stem cells. That a wide variety of ion channel types affect the main hallmarks of cancer is also suggested by Crottès et al. (2013), who focus on the role of the sigma-1 receptor (Sig1R) in cancer cells. Sig1R is a stress-activated chaperone associated with both the plasma membrane and the interface between the mitochondria and the endoplasmic reticulum. Sig1R is often expressed in tumors and several lines of evidence suggest that it is implicated in regulating Ca^{2+} homeostasis as well as some of the major types of ion channels. The authors suggest that Sig1R contributes to regulate the ion channel expression and function in cancer cells in response to environmental signals.

Following the lead of the seminal studies in lymphocytes (DeCoursey et al., 1984) and early embryos (Day et al., 1993), K^+ channels continue to have the lion's share in the field. An overview of the general role exerted by K^+ channels in cell cycle progression is provided by Ouadid-Ahidouch and Ahidouch (2013), whereas the specific contribution of the voltage-gated $K_V1.3$ and $K_V1.5$ channels in human cancer is reviewed by Comes et al. (2013). A group of voltage-gated K^+ channels frequently found to be involved in cancerogenesis comprises the *ether-à-go-go* (K_V10 or Eag; Pardo and Stühmer, 2008) and *ether-à-go-go-related* (K_V11 or Erg; Arcangeli, 2005) subtypes, whose cell biology is under intense investigation. In this issue, Herrmann et al. (2013) show that the surface expression of the oncogenic $K_V10.1$ channel is regulated by the Golgi-resident protein PIST (also known as GOPC), by an interaction mediated by PDZ domain.

In addition, growing evidence points to the role of ligand-gated channels in cancer cells. In particular, the nicotinic acetylcholine receptors (nAChRs) are homo- or heteropentamers of α and β subunits. These were originally identified in the nervous system, but are now increasingly recognized to be widely expressed outside the nervous system (Egleton et al., 2008; Schuller, 2009; Ambrosi and Becchetti, 2013). Thus, Improgo et al. (2013) discuss the role of the nAChR genes in lung cancer and propose an interesting mechanism whereby signaling mediated by α3/α5/β4-containing nAChRs (which genome-wide analyses correlate with increased smoking dependence and risk of developing lung tumors) promote carcinogenesis in small cell lung carcinoma cells.

Another central aspect of the function of normal and neoplastic cells is the regulation of cell volume, as reviewed by Pedersen et al. (2013). This applies especially to the secretive and absorbing

epithelia, in which the massive transport of ions, organic compounds, and fluid must be tightly matched to the control of cell volume. Such regulatory processes are generally disrupted in cancer cells and the general oncologic relevance of these observations turns on the fact that most cancers derive from epithelial cells. The authors give a thorough overview of the interaction between the tumor microenvironment and the altered regulation of ion transport. Moreover, they discuss the role of cell volume in cell proliferation and apoptosis, and the involvement of ion transport in tumor drug resistance, with special focus on the implication of chloride, calcium, and pH regulation.

A key step of the neoplastic progression is the regulation of cell migration, whose derangement is implicated in the metastatic cascade (Becchetti and Arcangeli, 2010) and tumor vascularization (Fiorio Pla et al., 2012). Some subtypes of the Transient Receptor Potential (TRP) channels have been found to participate to the regulation of cell migration, as is reviewed by Fiorio Pla and Gkika (2013). The authors especially focus on the implication of TRP channels in cell migration during the neoplastic progression. A different and novel aspect of the regulation of cancer cell migration is the implication of the tumor-associated carbonic anhydrase IX (CA IX) on focal contacts during cell spreading and migration. CA IX provides intracellular bicarbonate and extracellular H^+ to support cancer cell survival and invasiveness and is the only human CA isoform containing an extracellular proteoglycan domain (Monti et al., 2012). In their paper, Csaderova et al. (2013) show the regulatory interaction of CA IX with the focal contact sites, and provide the first evidence that CA IX localizes in the focal adhesion structures.

Several of the above papers describe the potential therapeutic applications of targeting specific ion channels and transporters. Cancer treatment is however more specifically addressed by Huber et al. (2013), who illustrate the use of ionizing radiation. This causes double-strand DNA breakages and thus cancer cell death, but also targets the plasma membrane. The ensuing modifications of ion channels and transporters can contribute to the survival of the irradiated cells. The authors discuss what is known about the mechanisms of the radioresistance dependent on ion transporters and suggest possible ways to make tumor cells more sensitive to radiation by proper targeting of ion channels and transporters.

Besides illustrating some of the hot topics in the field, the papers of the present Research Topic Issue constitute a most useful introduction to a literature that has already become too vast to be mastered by a single investigator.

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The sigma-1 receptor: a regulator of cancer cell electrical plasticity?

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Originally mistaken as an opioid receptor, the sigma-1 receptor (Sig1R) is a ubiquitous membrane protein that has been involved in many cellular processes. While the precise function of Sig1R has long remained mysterious, recent studies have shed light on its role and the molecular mechanisms triggered. Sig1R is in fact a stress-activated chaperone mainly associated with the ER-mitochondria interface that can regulate cell survival through the control of calcium homeostasis. Sig1R functionally regulates ion channels belonging to various molecular families and it has thus been involved in neuronal plasticity and central nervous system diseases. Interestingly, Sig1R is frequently expressed in tumors but its function in cancer has not been yet clarified. In this review, we discuss the current understanding of Sig1R. We suggest herein that Sig1R shapes cancer cell electrical signature upon environmental conditions. Thus, Sig1R may be used as a novel therapeutic target to specifically abrogate pro-invasive functions of ion channels in cancer tissue.

Keywords: sigma-1 receptor, chaperones, ion channels modulation, stress, physiological, cancer

INTRODUCTION

The concept of "Sigma receptors" arose 40 years ago in a pharmacological study postulating the existence of three types of opioid receptors on the basis of the psychomimetic effects induced by several opioid compounds (μ, κ and σ receptor respectively accounting for the effects produced by morphine, ketacyclazocine and SKF 10,047) (Martin et al., 1976). Further pharmacological studies revealed the existence of two binding sites, namely Sigma 1 (Sig1R) and Sigma 2 receptors (Sig2R) (Quirion et al., 1992). The Sig1R was cloned in 1996 (gene names: SIGMAR1 or OPRS1) and the gene is located on 9p13 (Hanner et al., 1996; Prasad et al., 1998). Sig1R is a 25-kDa protein anchored in the endoplasmic reticulum (ER) with no similarity with other known mammalian proteins, thus definitely ruling out any connection with a classical receptor family. Sig1R possesses two transmembrane regions and two steroid binding domains (SBD). These domains form a pocket which is the binding site for cholesterol, steroids, sphingolipids (Palmer et al., 2007; Fontanilla et al., 2008), and also for a wide panel of synthetic or natural compounds (sigma ligands) from different classes such as opioids, antipsychotics, psychostimulants, alkaloids or antidepressants (Pal et al., 2008; Maurice and Su, 2009) (**Figure 1**). *In vivo*, endogenous dimethyl tryptamine (DMT) interacts with Sig1R in the brain but its physiological significance as an endogenous sigma ligand is not yet clarified (Fontanilla et al., 2009; Mavlyutov et al., 2012).

The molecular nature of Sig2R has long been questioned. A recent work proposed the progesterone receptor membrane component 1 (Pgrmc1) as the putative sigma 2 binding site (Xu et al., 2011). This cytochrome-related protein binds several P450 proteins and various chemical compounds and it participates to cholesterol synthesis. However, while this putative Sig2R shares some pharmacological properties with Sig1R, the two proteins belong to distinct families. This review will focus on Sig1R.

Sig1Rs have been associated with many diseases including stroke, cocaine addiction, Alzheimer's disease, amnesia, amyotrophic lateral sclerosis, retinal degeneration, and cancer (Romieu et al., 2004; Aydar et al., 2006; Renaudo et al., 2007; Maurice and Su, 2009; Luty et al., 2010; Mavlyutov et al., 2011; Ruscher et al., 2011; Kourrich et al., 2013). Nonetheless, the way Sig1R operates in such diseases is still poorly understood. Su and colleagues'work on neurons and CHO cells have shed light on the molecular mechanisms underlying Sig1R functions. Sig1R is mainly located at the ER, in close contact with the mitochondria, in the so-called mitochondria-associated-ER membrane domains (MAM). In resting condition, Sig1R resides in ceramide- and cholesterol-rich lipid microdomains associated with the ER-resident chaperone GRP78 (BiP) (Hayashi and Su, 2007; Hayashi and Fujimoto, 2010). Under cellular stress leading to ER injury, Sig1R dissociates from BiP and binds IP_3 receptors, enhancing in turn cell survival through the control of calcium signaling between the ER and mitochondria. In addition, Sig1R translocates to other cell compartments and binds to different membrane proteins. The stimulation with sigma "agonists" mimicks stress-induced Sig1R dissociation from BiP and Sig1R delocalization, while sigma ligands classified as "antagonists" impede this process (Hayashi and Su, 2007). Altogether, these results have led to a model in which Sig1R is "silent" in normal physiological conditions, whereas in case of a disease, Sig1R behaves as a chaperone that binds client protein to the benefit of cell survival (Su et al., 2010). This exciting hypothesis has been validated by recent studies demonstrating that Sig1R molecular silencing reduces brain

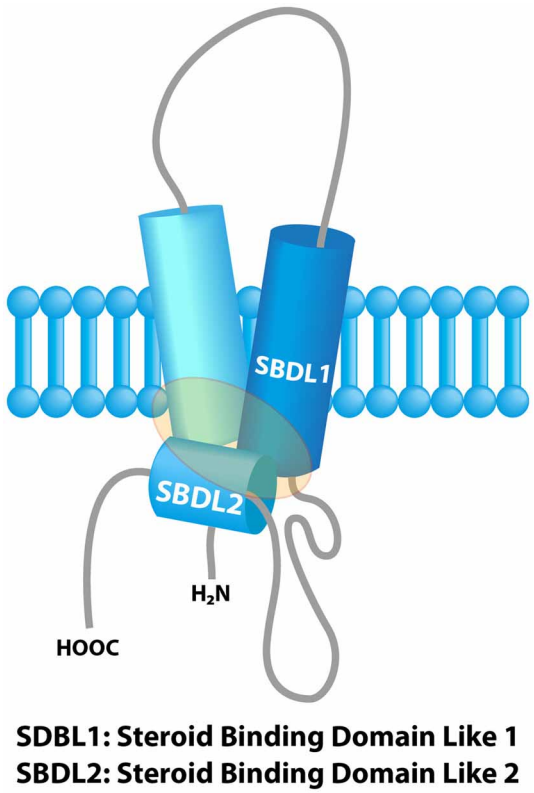

FIGURE 1 | Model of the sigma-1 receptor binding region from previous photolabeling studies. The shaded area represents the ligand binding region. Adapted with permission from Chu et al. (2013).

recovery after experimental stroke (Ruscher et al., 2011) and promotes retina degeneration after acute damage to the optic nerve (Mavlyutov et al., 2011).

The question of client proteins targeted by Sig1R is of importance. Beyond the coupling with IP3 receptors, a number of studies mainly based on the effects of exogenous sigma ligands have shown that Sig1R interferes with dopamine and acetylcholine systems and modulates the function of ion channels belonging to various families. Recent studies have described a molecular interaction between Sig1R and ion channels, suggesting that ion channels represent a major client protein family for the Sig1R chaperone (Carnally et al., 2010; Crottes et al., 2011; Balasuriya et al., 2012; Kourrich et al., 2012). Over the past decades, ion channels have been integrated to the main cellular processes underlying the hallmarks of cancer: tumors often express ion channels and transporters that are absent from the corresponding tissue. It is suggested that these channels and transporters enhance the cell's capacity to adapt themselves to restraint metabolic conditions encountered within the tumor tissue (low pH and PO_2, poor nutrient supply, etc. . .) (Wulff et al., 2009; Prevarskaya et al., 2010; Arcangeli, 2011). Transport proteins therefore participate to the adaptive cancer cells'response to environmental stress, conferring them with greater aggressiveness. This review attempts to draw together the knowledge about ion channel regulation by Sig1R and the recent discoveries on the function of ion channels in cancer. We suggest that upon environmental challenging conditions within the tumor, Sig1R may participate in the electrical remodeling of cancer cell electrical properties to enhance their survival and aggressiveness.

SIGMA 1 RECEPTORS IN CANCER

Binding experiment studies realized in the 90's revealed that sigma receptors are highly expressed in many human and rodent tumor cell lines including breast, lung, prostate, colon, melanoma, neuroblastoma and glioma (John et al., 1995; Vilner et al., 1995b; Aydar et al., 2004). However, most of the sigma ligands used in these studies are not selective enough between Sig1R and Sig2R to draw a definitive conclusion on the density of each binding site in the explored cancer cell types. Using a specific Sig1R antibody, a high expression of Sig1R was found in lung, breast and prostate cancer cell lines whereas low levels were found in normal counterpart cells. Interestingly, the Sig1R density was increased in high metastatic potential cancer cells suggesting a link between Sig1R expression and aggressiveness (Aydar et al., 2006). In another study, the expression of Sig1R was explored by imunohistochemistry in 58 breast cancer patients and 51 normal breasts. Sig1R positive epithelial cell staining was detected in 60 or 41% of invasive or *in situ* cancers respectively, in 75% of ductal hyperplasia and in 33% of normal breast (Wang et al., 2004). Accordingly, scintigraphy with a moderately-selective Sig1R ligand (*N*-[2-(1′-Piperidinyl) Ethyl]-3-^{123}I-Iodo-4-Methoxybenzamide) on patients with primary breast cancer revealed that the ligand was specifically retained within the tumor site, but not in healthy tissues (Caveliers et al., 2001). Several reports indicate that the use of Sig1R ligands to target therapeutic nanoparticles dramatically enhances the delivery of siRNA or drugs at the tumor site in melanoma, prostate, lung and breast cancer (Li and Huang, 2006; Chen et al., 2010; Guo et al., 2012; Kim and Huang, 2012).

Altogether, these studies strongly suggest that Sig1R is over expressed in many cancer cells and an extensive exploration of Sig1R expression in biopsies from various cancers is now required to determine whether Sig1R could be proposed as a diagnosis or prognosis marker.

The effects of sigma ligands on cancer cells' behavior have been assessed by many groups *in vitro* and *in vivo*. Early descriptive works showed that cell treatment with sigma ligands causes rounding, detachment and growth inhibition of C6 glioma (Vilner et al., 1995a), breast and colon carcinoma and melanoma cells (Brent and Pang, 1995; Aydar et al., 2004). Further works by Spruce and coll. showed that the moderately selective ligand rimcazole provokes *in vitro* and *in vivo* (mouse xenograft model) a tumor-selective, caspase-dependent apoptosis of breast and colon cancer (Spruce et al., 2004; Achison et al., 2007). Rimcazole was shown to antagonize a Sig1R-dependent mechanism involving a calcium-dependent activation of phospholipase C, a calcium-independent inhibition of phosphatidylinositol 3′-kinase pathway signaling and the accumulation of HIF-1α. While Sig1R agonists (+) pentazocine and (+) SKF10,047 had no effect *per se*, these ligands could abrogate rimacazole-induced apoptosis, suggesting that in cancer cells, Sig1R is in an activated state and enhances survival. In agreement with this hypothesis, transfection of Sig1R in

HEK293 cells reverses apoptosis induced by the over-expression of Bax or by staurospaurine (Spruce et al., 2004; Achison et al., 2007; Crottes et al., 2011). However, whether Sig1R protects cancer cells from death through a chaperoning activity has not yet been addressed.

Sig1R has also been connected to cell/matrix interaction. Aydar et al. have demonstrated that in breast cancer cells, Sig1R is associated with β1 integrin in lipid cholesterol-enriched rafts. Silencing Sig1R with siRNA chased β1 integrin from lipid rafts, reducing cell adhesion to matrix component such as fibronectin and vitronectin. Interestingly, treatment with the Sig1R agonist SKF10,047 also reduced β1 integrin density within lipid rafts and cell adhesion, an effect that was mimicked by the depletion of membrane cholesterol by methyl-β-cyclodextrin (Palmer et al., 2007).

From these data, it is clear that Sig1R participates on several facets of cancer cell biology. Recently, mutations in Sig1R have been found to cause a form of ALS and frontotemporal lobar degeneration (Luty et al., 2010; Al-Saif et al., 2011; Prause et al., 2013). Whether mutations in Sig1R also occur in cancer tissues is a question that remains to be explored. So far, the common mechanism by which Sig1R or sigma ligands drives cancer cell behavior is not clear. An exciting hypothesis arises from converging studies describing Sig1R as a sterol-dependent, stress-activated chaperone controlling lipid raft formation in the ER and the plasma membrane (PM) [extensively reviewed in Tsai et al. (2009), Hayashi and Su (2010)]. In response to environmental conditions encountered in cancer tissue (hypoxia, nutrient and growth factor deprivation) Sig1R may dynamically trigger various adaptation mechanisms, the nature of which being tightly dependent on the client protein available in a given tumor cell type. At this stage, it is noteworthy that ion channels emerge from the literature as the main client protein family for Sig1R (Hayashi and Su, 2007; Crottes et al., 2011; Balasuriya et al., 2012; Kourrich et al., 2012, 2013).

SIG1R: A MODULATOR OF ION CHANNELS

VOLTAGE-GATED ION CHANNELS

Voltage-gated ion channels (VGIC) are mainly involved in the initiation and shaping of action potentials and global cell excitability (Hodgkin and Huxley, 1952; Hille, 1984). The progress made during the past decade in characterizing the electrical signature of cancer cell has intriguingly extended the initial function of VGIC far beyond the field of exciting cells. Indeed, VGIC are involved in a number of tumor cell processes including mitosis (Weber et al., 2006; Becchetti, 2011), migration (Gillet et al., 2009; Becchetti and Arcangeli, 2010), apoptosis (Lang et al., 2004), adhesion to ECM (Pillozzi and Arcangeli, 2010), angiogenesis (Pillozzi et al., 2007), homing and drug resistance (Pillozzi and Arcangeli, 2010). Interestingly, Sig1R has been shown to interact with K^+, Ca^{2+}, Cl^- and Na^+ channels (Renaudo et al., 2004; Kourrich et al., 2012). Very recent studies have provided some clues about these interactions.

1- Voltage-gated K+ channels (VGKC)

Numerous studies have reported the inhibition of VGKC by sigma ligands in a wide range of cell types (Kennedy and Henderson, 1990; Soriani et al., 1998, 1999a,b; Lupardus et al., 2000; Kourrich et al., 2012). In particular, sigma ligands decrease current density and provoke a leftward shift in the voltage-dependency inactivation (Zera et al., 1996; Soriani et al., 1999a; Aydar et al., 2002). In a study performed in frogs'pituitary cells, it was nonetheless shown that sigma ligands depress the M-current by a rightward shift of the activation curve (Soriani et al., 1999b). The mechanism by which sigma ligands modulate Kv channels has been proposed to be either direct or indirect, depending on the model used. Inside-out patch clamp experiments suggested a direct effect of sigma ligands on Kv channels in rodent neurohypophysal terminals and in small cell lung carcinoma (Wilke et al., 1999; Lupardus et al., 2000). However, in frogs'pituitary cells, the inhibitory effects of the selective Sig1R ligand (+) pentazocine, on both delayed-rectifier and I_A currents, were abolished in the presence of cholera toxin, GTPγS or GDPβS suggesting the involvement of a Gs-protein dependent pathway (Soriani et al., 1998, 1999a). How could these two sets of observation be interpreted? In a recent study, Mei et al. showed that the sigma ligand Cyproheptadine stimulates the Kv2.1-dependent current in cortical neurons in a Sig1R and $G_{i/o}$-dependent manner. The study indeed describes a functional interaction between Sig1R, Kv2.1 and a G-protein coupled receptor (GPCR): the mu-opoid receptor (He et al., 2012). It can then been proposed that sigma ligands, either alter directly the Sig1R/VGKC coupling or modulate functional complexes that integrate Sig1R, VGKC and GPCR. This last hypothesis is strengthened by recent reports demonstrating that Sig1R modulates several GPCR in the brain (i.e. opioid and muscarinic acetyl choline receptors) (Kim et al., 2010), and forms a complex with D1 and D2 dopamine receptors (Navarro et al., 2010, 2013).

Whether Sig1R requires an endogenous/exogenous sigma ligand to modulate VGKC is a crucial question that has been addressed in a few but important reports focusing on the molecular interaction between Sig1R and its partners. In *Xenopus oocytes*, it has been shown that the co-expression of Sig1R with Kv1.4 or Kv1.3 accelerates the inactivation kinetic parameters (Aydar et al., 2002; Kinoshita et al., 2012). In human leukaemic cells, our group found that the silencing of Sig1R by shRNA reduces the endogenous human ether-à-gogo-related gene (hERG; Kv11.1) current density without altering channel voltage dependency or kinetic parameters. Delving into the molecular mechanisms, we observed that the silencing of Sig1R decreases hERG maturation efficiency and diminishes the α-subunit channel stability at the plasma membrane, in turn reducing the number of ion channels available (Crottes et al., 2011). Inasmuch Sig1R co-immunoprecipitates with hERG, these observations are consistent with the idea of a Sig1R protein behaving either like a chaperone or a channel regulatory β-subunit through a protein/protein interaction (Aydar et al., 2002; Crottes et al., 2011; Kinoshita et al., 2012). This hypothesis was further strengthened by a recent report showing that cocaine exposure induces in nucleus accumbens a persistent protein/protein association between Sig-1Rs and Kv1.2 channels. This phenomenon is associated with a redistribution of both proteins from the intracellular compartments to the plasma membrane (Kourrich et al., 2013).

The presence of VGKC is linked to cell proliferation in various cancer types, through the regulation of both the resting (controlling the Ca^{2+} driving force) and cell volume, both phenomenon participating in the cell cycle checkpoints (for review; Wulff et al., 2009; Becchetti, 2011; Felipe et al., 2012). The connection between VGKC, Sig1R and cancer cell proliferation has been first addressed by Renaudo et al. (2004). We observed that selective Sig1R ligands provoke a cell cycle arrest in small cell lung carcinoma and T-ALL cells by blocking the delayed-rectifier and Kv1.3 channels, respectively. In both cases, Sig1R-dependent inhibition of potassium currents resulted in an accumulation of the cyclin inhibitor $p27^{Kip1}$ and a reduction in cyclin A contents, leading to an arrest at the end of the G_1 phase of the cell cycle (Renaudo et al., 2004).

VGKC of the ether-à-gogo family, i.e., hERG and EAG channels, represent a source of highly promising therapeutic targets for cancer. hERG is mainly expressed in the heart, the central nervous system and the endocrine system were it regulates the frequency of action potentials (for review: Vandenberg et al., 2012). In a series of excellent papers, Arcangeli's group has demonstrated that hERG is a tumor marker of myeloid and lymphoid leukaemias, colon and breast carcinoma, ovarian cancer and glioblastoma (Pillozzi et al., 2007, 2011; Pillozzi and Arcangeli, 2010; Arcangeli, 2011). Importantly, they demonstrated that upon β1 integrin stimulation, hERG forms signaling macro-complexes with β1-integrin, the VEGF receptor Flt-1 or the cytokine receptor CXCR4 in lipid rafts. The channel in turn participates in a crosstalk between cancer cells and their microenvironment to promote invasive processes such as motility, angiogenesis, homing and chemoresistance (Pillozzi et al., 2007; Pillozzi and Arcangeli, 2010). As stated above, we recently showed that Sig1R expression stimulates hERG maturation and membrane stability in the chronic myeloid leukaemia cell line K562. Inasmuch Sig1R co-immunoprecipitates with both immature and mature forms of the channel α-subunits, it is suggested that Sig1R not only associates with hERG in the ER, but also drives it to the plasma membrane (Crottes et al., 2011). The question of whether Sig1R is involved in the formation of such hERG-dependent signaling complexes with β-integrins and other partners is an interesting one but has not been addressed yet. This hypothesis deserves further consideration *in vitro* and *in vivo* knowing that both Sig1R silencing and treatment with the sigma ligand igmesine decrease K562 cell adhesion capacity to fibronectin in a hERG-dependent manner (Crottes et al., 2011).

Channels of the EAG family are present in a number of tumor types. Stuhmer's group nicely demonstrated that CHO cells transfected with EAG exhibit a cancerous invasive phenotype *in vitro* and *in vivo* (Hemmerlein et al., 2006; Gomez-Varela et al., 2007; Pardo and Suhmer, 2008). Further signaling studies revealed that EAG-1 (Kv10.1) enhances cell resistance to hypoxia by increasing HIF-1 levels, thus stimulating VEGF secretion (Downie et al., 2008). The over expression of EAG-2 has been recently shown in human medulloblastoma (MB). In this cancer, EAG-2 promotes the progression of the MB tumor by regulating cell volume dynamics, in turn inhibiting the tumor suppressor p38 MAPK pathway (Huang et al., 2012). While the putative link between EAG channels and Sig1R has not been addressed so far, it is tempting to speculate such an interaction considering the molecular and structural proximity between EAG and hERG channels.

2-Voltage-gated Na^+ channels (VGNC)

The existence of VGNC has been first speculated by Hodgkin and Huxley to account for the fast depolarizing phase of the action potential of excitable cells (Hodgkin and Huxley, 1952). To date, the family of VGNC includes nine members mainly involved in the encoding of neuronal signaling, cardiac rhythm, muscle contraction and endocrine secretion (Hille, 1984; Harmar et al., 2009). Intriguingly, VGNC are expressed in metastatic cells of many cancers. In these cells, the sodium current driven by VGNC α subunits enhances the invasion and metastasis *in vivo* (Brisson et al., 2011, 2012; Yang et al., 2012). Expression of the cardiac Nav1.5 α subunit (SCN5A) is correlated with a poor prognosis in breast cancer specimens, suggesting that VGSCs may be used as prognosis marker in cancer progression (House et al., 2010; Yang et al., 2012). The mechanical link between Nav1.5 and cancer progression has been recently documented: in breast cancer cells, Nav1.5 associates with the Na^+/H^+ exchanger NHE1 in caveolae; Nav1.5 stimulates NHE1 activity, contributing to the acidification of the pericellular space. The low extracellular pH in turn potentiates the activity of different cathepsins involved in ECM degradation, a fundamental step for cancer cell invasion process (Gillet et al., 2009; Brisson et al., 2011, 2012). In recent studies, Jackson and Ruoho's groups have shown that sigma ligands reduce Nav1.5-dependent currents in cardiomyocytes of wild type mice. Interestingly, Nav1.5 current sensitivity to sigma ligands was lost in cardiomyocytes of knock-out mice for Sig1R (Fontanilla et al., 2009; Johannessen et al., 2011). The nature of the interaction occurring between Sig1R and Nav1.5 has been scrutinized in 2012 by atomic force microscopy (AFM) which revealed that Sig1R directly binds the channel with a four-fold symmetry in human embryonic kidney cell (HEK) heterologous expression system (Balasuriya et al., 2012). Because the Nav1.5 channel includes the four pore-forming α subunits within a single protein, this result suggests that Sig1R neither interacts with C- nor N-terminus, but rather with the transmembrane domains. This hypothesis is strengthened by the fact that deletions in the transmembrane domain of Kv1.3 subunits abolish their co-immunoprecipitation with Sig1R in *Xenopus* oocytes (Kinoshita et al., 2012). While the suppression of Sig1R expression in mice cardiomyocytes fails to alter any parameter of the native Nav1.5 current (Fontanilla et al., 2009), the Sig1R silencing in the highly aggressive MDA-MB-231 breast cancer cell line results in a strong reduction in current density, suggesting that Sig1R controls Nav1.5 trafficking in cancer cells but not in healthy cardiac cells (Balasuriya et al., 2012). From these observations, it can be hypothesized that Sig1R, by enhancing Nav1.5 membrane expression in breast cancer cells, modulates NHE1 activity, resulting in greater aggressiveness potency.

3-Voltage-gated calcium channels (VGCC)

VGCC are principally involved in fast synaptic transmission, cardiomyocyte and striated muscle contraction, as well as

stimulus-secretion coupling. The low threshold T-type channel (Cav-3) has however been involved in proliferation and differentiation in several cancer cell lines (Gackiere et al., 2008; Prevarskaya et al., 2010; Becchetti, 2011). While no data support an interaction between Sig1R and the T-type channel in the literature, it has been shown that Sig1R co-immunoprecipitate with high-threshold L-type channels in retinal ganglion and that the sigma ligand SKF 10.047 inhibits the corresponding current (Tchedre et al., 2008). These observations suggest that VGCC in cancer cells might be a client for Sig1R.

CALCIUM-ACTIVATED POTASSIUM CHANNELS (KCa)

KCa channels are involved in many physiological processes by regulating calcium entry through the control of the membranes'resting potential and Ca^{2+} driving force. A link between Sig1R and small-conductance KCa channels has been recently proposed in synaptic activity and plasticity in the hippocampus (Martina et al., 2007). In this report, the authors showed that Sig1R ligands potentiate the N-Methyl-D-Aspartate (NMDA) receptor responses and long-term potentiation (LTP) by inhibiting a small conductance Ca^{2+}-activated K^+ current (SK channel). Interestingly, SK3 channels play a predominant role in melanoma and breast cancer cell migration and are considered as potent targets for cancer therapy (Potier et al., 2006; Chantome et al., 2009; Girault et al., 2012). On the other hand, SK4 channels have been involved in the migration potency of glioblastoma stem cells (Ruggieri et al., 2012). The putative interaction between SK channels and Sig1R thus constitutes an interesting hypothesis that remains to be explored.

VOLUME-REGULATED CHLORIDE CHANNELS (VRCC)

VRCC, functionally coupled with K^+ channels, drive cell volume regulation by controlling chloride salt-associated water efflux (Hoffmann et al., 2009). Cell volume regulation and regulatory volume decrease (RVD) participate to at least three main aspects of cancer progression, i.e., cell cycle (G1/S and G2/M volume checkpoints) (Lang et al., 2000; Rouzaire-Dubois et al., 2000; Becchetti, 2011; Hoffmann, 2011; Huang et al., 2012), motility (control of cell shape dynamics through salt and water fluxes) (Cuddapah and Sontheimer, 2011) and apoptosis (Apoptosis Volume Decrease occurring at an early signaling step of programmed cell death) (Bortner and Cidlowski, 2011; Bortner et al., 2012). Our group has shown that sigma ligands strongly inhibit both VRCC and VGKC in T leukaemic and small cell lung carcinoma cells in a Sig1R-dependent manner. VRCC and VGKC inhibition lead to a strong reduction in RVD potency after a hypotonic shock. In isotonic conditions, cell treatment with sigma ligands lead to cell swelling, underlying an arrest of cell division at the late G_1 phase. These results indicate that the pharmacological alteration of Sig1R, by inhibiting channels involved in RVD, can block the cell division process (Renaudo et al., 2004, 2007). In these studies we also questioned the function of Sig1R in cancer cells in the absence of exogenous ligands. We observed that the over-expression of Sig1R in HEK cells was sufficient *per se* to significantly reduce the activation kinetics of VRCC upon hypotonic shock. We proposed that the presence of Sig1R induces a tonic reduction of VRCC activity, not sufficient to impede the cell cycle, but strong enough to protect cells from apoptosis by delaying AVD. This result was confirmed by showing that cells over-expressing Sig1R are less sensitive to staurospaurine-induced apoptosis than normal cells (Renaudo et al., 2007). Together with other reports, this study has unveiled the function of Sig1R as a protein involved in cell protection against environmental stress by modulating ion channels (Hayashi and Su, 2007; Renaudo et al., 2007).

CALCIUM SIGNALING AND ION CHANNELS AT THE MAM

Cell fate largely depends on calcium exchanges occurring between ER and mitochondria. These exchanges generally take place at specific membrane localization, the MAMs, which were originally described as sites for lipid synthesis and lipid transfer between ER and mitochondria membranes (Rusinol et al., 1994) for review: (Parys et al., 2012). Calcium fluxes between the two compartments involve various chaperones and signaling proteins as well as ion channels and transporters including IP3 receptors, voltage-dependent anion channels (VDAC) or the translocon. The regulation of calcium entry in the mitochondria participates in the control of the energy state and cell response to ER-mediated stress. Calcium homeostasis at MAM therefore constitutes a crossroad decision for cell engagement toward apoptosis, survival or autophagy (Tsai et al., 2009; Parys et al., 2012; Hammadi et al., 2013). Not surprisingly, MAM-associated transport machinery is disregulated in the context of environmental challenges in cancer such as hypoxia, low pH and dramatic nutrient deprivation (Moenner et al., 2007; Raturi and Simmen, 2013). As stated above, Hayashi and Su have demonstrated in CHO cells that the Sig1R chaperone plays a fundamental role in regulating the Ca^{2+} transport machinery within the MAMs, leading to a reinforced cell survival in response to environmental stress (Hayashi and Su, 2007). In particular, stress-activated Sig1R chaperones IP3 receptor and prevents its degradation. Moreover a recent report has shown that Sig1R is physically associated to VDAC2, a mitochondrial channel involved in cholesterol import into the mitochodria for metabolic regulation (Marriott et al., 2012). While no data is available on the function of MAM-associated Sig1R in tumors, it is conceivable that Sig1R contributes to the adaptation of cancer cells in restrictive environment.

CONCLUSION AND PERSPECTIVES

In summary, Sig1R is a stress-activated chaperone which controls, through different mechanisms, several families of ion channels at the plasma membrane and at the MAM. Studies realized in the retina, brain and heart strongly suggest that Sig1R participates in cell resistance to tissue injury, for instance infarction, stroke or ischemia (Kourrich et al., 2012). Several reports indicate that Sig1R exerts a role only in conditions of stress and remains generally "silent" in healthy organs or in steady-state conditions (Maurice and Su, 2009; Tsai et al., 2009). In good agreement with this idea, Sig1R KO mice present a normal development and behavior but are less resistant to experimental stroke (Ruscher et al., 2011). Moreover, the absence of side effects of Sig1R ligands in clinical trials in human suffering psychiatric disorders, improves the hypothesis of a dynamic and protective role of Sig1R in stressing conditions (Volz and Stoll, 2004; Banister

and Kassiou, 2012). Thus, it is tempting to speculate that tumor cells hijack the primary protective function of Sig1R to enhance their survival/growing/invasive potency in restrictive metabolic conditions encountered within the tumor tissue. As demonstrated by many authors, the aberrant expression of ion channels confers selective advantages for cancer cells to adapt their behavior and survival in the tumor environment. While research studies mainly focus on the function of one ion channel in a cancer type, it is important to consider that many ion channels are deregulated in the same cancer cell. Because a variety of ion channels are client proteins for Sig1R, we speculate that the Sig1R chaperone controls cancer cells'electrical plasticity by putatively "driving" ion channels to potentiate their function in proliferation, apoptosis resistance, migration and angiogenesis (**Figure 2**). At the time being, there is no real explanation on the process that controls the expression of all these ion channels in cancer cells and it is often postulated that this is due to the acquisition of an embryonic or developmental phenotype. The possibility that Sig1R expression might participate to this phenotype is an interesting hypothesis that has not been explored so far.

The literature strongly argues for a close interaction between Sig1R and ion channels that are already expressed in the cell. An alternative mechanism should however be considered: because

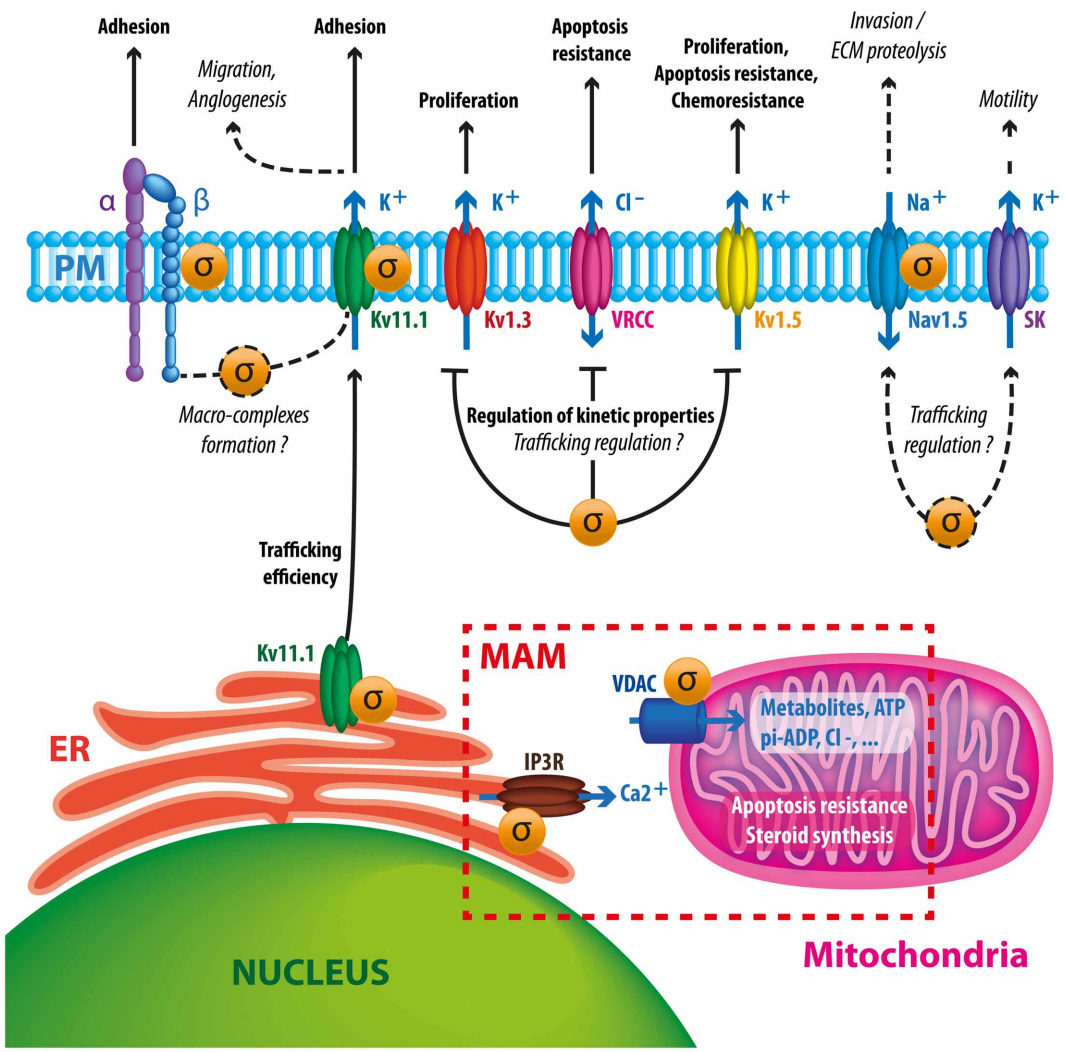

FIGURE 2 | Schematic diagram summarizing putative and documented interactions between Sig1R and ion channels in cancer cells. Sig1R promotes hERG maturation and membrane stability which may potentiate channel interaction with β1 integrin macrocomplexes, leading to enhanced migration and angiogenesis. Sig1R also directly binds to other VGIC such as Kv1.3, Kv1.5, and Nav1.5 controlling either current density or kinetic properties of channels. These interactions may have consequences on cell proliferation, apoptosis resistance, chemoresistance and invasive properties. The presence of Sig1R in cancer cells tonically reduces VRCC activity via a yet unknown mechanism, leading to a better resistance to apoptotic signals. Sigma receptors have also been associated to SK channels, suggesting that they may participate to cancer cell motility. At the MAM, Sig1R may enhance cell survival by regulating calcium fluxes between ER and mitochondria by chaperoning IP3 receptors and VDAC channels.

of the spatial dynamics of Sig1R within the cell, the protein could also behave as a transcriptional factor controlling either directly or indirectly a kit of genes encoding ion channels. While no data supports the presence of Sig1R in the nucleus, many reports have shown the involvement of Sig1R in a number of signaling pathways potentially targeting transcriptional activity (i.e., MAP kinases, PKA, PI3K/AKT, NFk-B, c-Fos, CREB) (for review: Hayashi et al., 2011).

It is noteworthy that ion channels expressed in cancer cells play important functions in healthy organs as well such as in the heart and brain. As a consequence, therapies based on toxins and drugs directly targeting ion channels present major drawbacks for cancer treatment. The unique properties of Sig1R may pave a new avenue to alter ion channels specifically within tumors. In this regard, many outstanding questions need to be addressed to unravel the importance of Sig1R in cancer such as the consequences of Sig1R silencing on the electrical signature of cancer cells and subsequent alteration of their behavior *in vitro* and *in vivo*. Promising anti-tumoral effects have been obtained *in vivo* with exogenous sigma ligands, but the innate function of Sig1R in cancer remains undetermined. Moreover, the molecular mechanisms of Sig1R ligands on Sig1R/ion channel complexes remain to be addressed.

Answers to these questions will open new strategies based on the targeting of Sig1R to target ion channels and associated cancer progression.

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Membrane potential and cancer progression

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Membrane potential (V_m), the voltage across the plasma membrane, arises because of the presence of different ion channels/transporters with specific ion selectivity and permeability. V_m is a key biophysical signal in non-excitable cells, modulating important cellular activities, such as proliferation and differentiation. Therefore, the multiplicities of various ion channels/transporters expressed on different cells are finely tuned in order to regulate the V_m. It is well-established that cancer cells possess distinct bioelectrical properties. Notably, electrophysiological analyses in many cancer cell types have revealed a depolarized V_m that favors cell proliferation. Ion channels/transporters control cell volume and migration, and emerging data also suggest that the level of V_m has functional roles in cancer cell migration. In addition, hyperpolarization is necessary for stem cell differentiation. For example, both osteogenesis and adipogenesis are hindered in human mesenchymal stem cells (hMSCs) under depolarizing conditions. Therefore, in the context of cancer, membrane depolarization might be important for the emergence and maintenance of cancer stem cells (CSCs), giving rise to sustained tumor growth. This review aims to provide a broad understanding of the V_m as a bioelectrical signal in cancer cells by examining several key types of ion channels that contribute to its regulation. The mechanisms by which V_m regulates cancer cell proliferation, migration, and differentiation will be discussed. In the long term, V_m might be a valuable clinical marker for tumor detection with prognostic value, and could even be artificially modified in order to inhibit tumor growth and metastasis.

Keywords: cancer, cell cycle, differentiation, ion channel, membrane potential, migration, proliferation, stem cell

INTRODUCTION

The presence of various ion channels and transporters at the plasma membrane provides different permeability to distinct ions, such as Na^+, K^+, Ca^{2+}, and Cl^-. Due to the unequal distribution of these ions, a voltage difference exists between the cytoplasm and the extracellular environment, which is known as the membrane potential (V_m). V_m is expressed relative to the extracellular environment. A cell is depolarized when the V_m is relatively less negative, whereas a hyperpolarized cell possesses a more negative V_m. V_m changes because of alterations in the conductance of one or more types of ion. The Goldman–Hodgkin–Katz equation shows that the V_m depends on the permeability (P) and both the intracellular and extracellular concentrations of major ions (Goldman, 1943; Hodgkin and Katz, 1949):

$$V_m = \frac{RT}{F}\ln\left(\frac{P_{Na^+}\left[Na^+\right]_o + P_{K^+}\left[K^+\right]_o + P_{Cl^-}\left[Cl^-\right]_o}{P_{Na^+}\left[Na^+\right]_i + P_{K^+}\left[K^+\right]_i + P_{Cl^-}\left[Cl^-\right]_i}\right)$$

where R is the ideal gas constant, T the temperature, and F the Faraday constant. In addition, intercellular communications (e.g., gap junction connections) are also able to influence V_m (Hulser and Lauterwasser, 1982; Levin, 2007a). In excitable cells, such as neurons and muscle fibers (Nakajima and Horn, 1967; Bean, 2007), changes in V_m underlie the action potential (AP) waveform. APs fire in response to a depolarization that exceeds a threshold value. Fine-tuning of APs is tightly regulated by the activities of several key ion channels and transporters, including voltage-gated Na^+ channels (VGSCs), voltage-gated K^+ channels (K_v), and the Na^+/K^+-ATPase (Caldwell and Keynes, 1957; Hille, 1992).

Emerging evidence suggests that the V_m also plays important functional roles in non-excitable cells. In the late 1960's, while studying mitotic activities in sarcoma cells, Clarence D. Cone Jr. reported that V_m underwent hyperpolarization before entering M phase, and suggested that the level of V_m correlated with cell cycle progression (Cone, 1969). He subsequently showed that membrane hyperpolarization reversibly blocked DNA synthesis and mitosis (Cone, 1970). He later generalized existing data at that time and postulated that the V_m level was correlated with the level of differentiation. For example, terminally differentiated cells (e.g., fibroblasts and epithelium) possess hyperpolarized V_m (Cone, 1971). Since then, changes in V_m, representing the long-term, slowly changing bioelectric gradient in non-excitable cells (Lobikin et al., 2012), have been shown to control critical cell functions including proliferation, migration, and differentiation (Binggeli and Weinstein, 1986; Schwab et al., 2007; Blackiston et al., 2009; Sundelacruz et al., 2009). Recently, studies have also demonstrated that V_m is able to, directly or indirectly, control wound healing (Nuccitelli, 2003a,b; McCaig et al., 2009), left-right patterning (Adams et al., 2006), development (Nuccitelli, 2003a; Adams, 2008), and regeneration (Levin, 2007b,

2009). Therefore, given the increasing evidence showing that ion channels/transporters functionally participate in cancer progression (Kunzelmann, 2005; Fiske et al., 2006; Stuhmer et al., 2006; Prevarskaya et al., 2010; Becchetti, 2011; Brackenbury, 2012), it is not surprising that V_m has been implicated in cancer development, since V_m is itself determined by the combined activities of ion channels/transporters at the cell membrane. This article aims to summarize current understanding of the V_m as a bioelectric regulator in cancer, and examines the therapeutic potential of V_m for tumor detection and treatment.

CANCER CELLS POSSESS DEPOLARIZED V_m

Cone's theory proposing the general correlation between proliferation and V_m (Cone, 1971) was supported by several previous studies which demonstrated significant V_m depolarization during malignant transformation of normal cells (Tokuoka and Morioka, 1957; Johnstone, 1959). Direct *in vitro* and *in vivo* comparisons of V_m levels between normal and cancerous breast cells (Marino et al., 1994), hepatocytes and hepatocellular carcinoma cells (Binggeli and Cameron, 1980; Stevenson et al., 1989), normal and neoplastic adrenocortical tissues (Lymangrover et al., 1975), normal embryonic fibroblasts and fibrosarcoma (Binggeli and Weinstein, 1985), benign and cancerous skin cells (Melczer and Kiss, 1957; Woodrough et al., 1975), and between normal and cancerous ovarian tissue (Redmann et al., 1972) showed that cancer cells tended to be more depolarized than their normal counterparts. In addition, the intracellular Na^+ level is markedly higher in tumors compared to non-cancerous tissues, whereas the K^+ level remains more stable (Smith et al., 1978; Cameron et al., 1980; Sparks et al., 1983). A similar scenario occurs in fast proliferating Chinese hamster ovary (CHO) and 3T3 cells (Cone and Tongier, 1973). Thus, an increased intracellular Na^+ concentration could be a determinant of a depolarized phenotype in rapidly cycling cancer cells.

Recordings from rodent and human tissues have revealed that proliferative cells, especially rapidly proliferating tumor cells, displayed depolarized V_m, whereas non-proliferating, terminally differentiated somatic cells, such as muscle cells and neurons, are characterized by their hyperpolarized V_m (**Figure 1**) [reviewed in Binggeli and Weinstein (1986)]. Given these findings, is V_m merely an epiphenomenon, which only indicates the outcome of the activities of various ion channels and transporters, or is it is actually a functional instructor that is capable of promoting tumorigenesis? A similar question had been posed 50 years ago soon after Cone revealed the relationship between mitotic activity and V_m level (Cone and Tongier, 1971). For example, depolarization can initiate mitosis in CHO cells and mouse spleen lymphocytes (Cone and Tongier, 1971; Kiefer et al., 1980). By contrast, hyperpolarized V_m immediately precedes mitotic arrest (Cone and Tongier, 1973). More recently, *in vivo* evidence shows that membrane depolarization itself, regardless of the types of ions and ion channel/transporter proteins, is able to bring cancerous transformation (i.e., increased proliferation, change in morphology and abnormal angiogenesis) in *Xenopus laevis* embryos (Lobikin et al., 2012).

Hanahan and Weinberg proposed 10 hallmarks of cancer, including sustaining proliferative signaling, activating invasion and metastasis, and angiogenesis (Hanahan and Weinberg, 2011). The following sections review the prevailing evidence that implicates V_m in several of these processes.

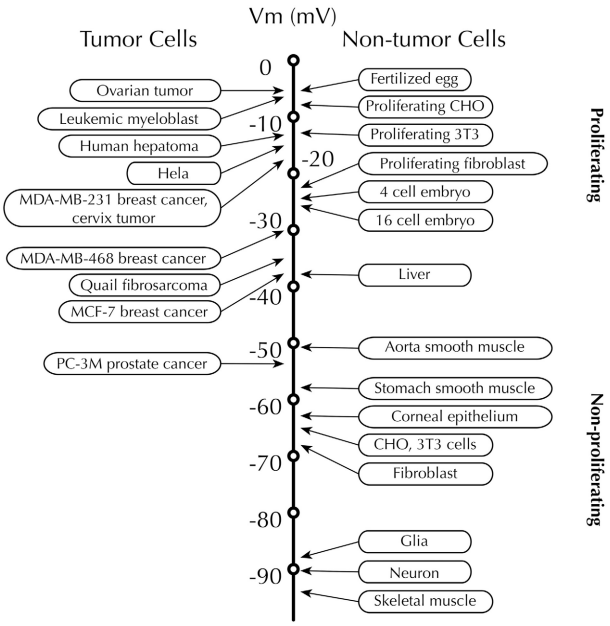

FIGURE 1 | Membrane potential (V_m) scale. Rapidly proliferating cancer cells possess depolarized V_m, while the V_m of quiescent cells is generally more negative. Proliferative somatic cells are also depolarized, suggesting that V_m is functionally instructive in cell development (Levin, 2007b). Scale adapted from Binggeli and Weinstein (1986), with additional data from Fraser et al. (2005); Mycielska et al. (2005); Yang et al. (2012).

V_m AND CANCER CELL PROLIFERATION

In general, in both highly proliferative tumor and non-tumor cells, depolarization is believed to serve as a signal that could initiate mitosis and DNA synthesis (Orr et al., 1972; Binggeli and Weinstein, 1986). Artificially altering V_m by modulating the extracellular ionic constitution or applying the Na^+/K^+-ATPase inhibitor ouabain revealed interesting results: First, hyperpolarizing CHO cells to −45 mV started to induce mitotic arrest and cell division was fully blocked at −75 mV. The cell cycle was resumed by depolarizing the cells to −10 mV (Cone, 1971). Secondly, quiescent (G_0) mature chick spinal cord neurons showed mitotic activity after depolarization (Cone and Cone, 1976) (**Figure 2**). Recently, artificial control of V_m was accomplished in *Xenopus laevis* embryos by expressing glycine-gated Cl^- channels and applying the activator ivermectin. Depolarization (caused by lowering the Cl^- concentration in the extracellular medium, which caused Cl^- efflux) was found to be directly responsible for malignant proliferation. This proliferation was ion and ion channel non-specific, because (1) the phenotype caused by depolarization could be rescued by expressing a hyperpolarizing channel gene, and (2) the malignant phenotype could be induced or suppressed simply by adjusting extracellular Cl^- concentration, as predicted by Goldman–Hodgkin–Katz equation (Lobikin et al., 2012). Therefore, the depolarized V_m frequently found in

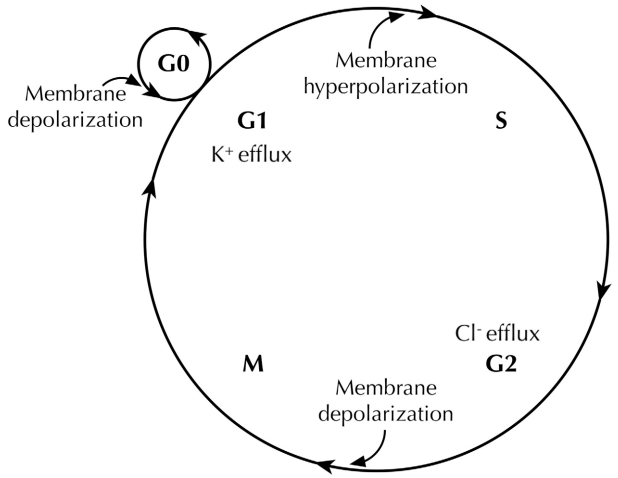

FIGURE 2 | Membrane potential (V_m) changes during the cell cycle. V_m undergoes hyperpolarization at G_1/S border, by virtue of K^+ efflux through various K^+ channels. Before cells enter M phase, increased Cl^- efflux accompanies V_m depolarization. Quiescent cells at G_0 stage show mitotic activities after V_m depolarization (Cone and Cone, 1976).

cancerous cell types could be regarded as a "sustaining proliferative signal" that instructs cells to rapidly advance in the cell cycle.

An additional layer of complexity in this model is that the V_m fluctuates during cell cycle progression, and follows a multi-step and rhythmic pattern (Wonderlin and Strobl, 1996; Blackiston et al., 2009) (**Figure 2**). A number of studies suggest that membrane hyperpolarization at the G_1/S checkpoint is generally required for S phase initiation. For example, depolarizing the cell membrane halts G_1/S progression in glia (Canady et al., 1990), Schwann cells (Wilson and Chiu, 1993), lymphocytes (Price et al., 1989; Freedman et al., 1992; Wang et al., 1992), V79 Chinese hamster lung cells (Sachs et al., 1974), C1300 mouse neuroblastoma cells (Boonstra et al., 1981), and MCF-7 human breast cancer cells (Wonderlin et al., 1995). The V_m then appears to remain relatively hyperpolarized through S phase in some cell types (Sachs et al., 1974; Boonstra et al., 1981; Strobl et al., 1995; Wonderlin et al., 1995), but is more depolarized in others (Arcangeli et al., 1995; Macfarlane and Sontheimer, 2000). The G_2/M transition exhibits a depolarized V_m (Sachs et al., 1974; Boonstra et al., 1981; Blackiston et al., 2009), although it is not known whether or not this depolarization is a prerequisite for progression. In fact, the exact V_m thresholds for driving progression appear to depend heavily on cell type, the state of differentiation, and the density of cell monolayer in culture (Cone and Tongier, 1973; Blackiston et al., 2009).

Importantly, the fluctuation of V_m levels across the cell cycle does not necessarily contradict the observation that depolarized V_m could be a hallmark of cancer cells. The mean V_m values in cancer cells are consistently depolarized relative to most normal somatic cell types (**Figure 1**). For example, MCF-7 cells arrested at G_1 phase have a V_m of -9 mV and hyperpolarize to ~ -30 mV in the S phase (Wonderlin et al., 1995). Both these values are more depolarized than normal breast cells, e.g., the mean V_m of unsynchronized MCF-10A cells is between -40 and -58 mV (Marino et al., 1994; Wonderlin et al., 1995; Fraser et al., 2005).

Evidence suggests that the fluctuation in K^+ concentration plays a significant contribution to changes in V_m during the cell cycle. For example, in neuroblastoma and Ehrlich ascites cells, there is a transient decrease in K^+ efflux before entering the G_2 phase, a relatively high level of K^+ efflux during the M phase (Mills and Tupper, 1976; Boonstra et al., 1981). Given the diversity of K^+ channel types (Hille, 1992; Miller, 2000; Wang, 2004), their relative contributions to the V_m and V_m-dependent cell cycle progression is probably context-dependent and highly complex. For example, inhibition of cell proliferation with K^+ channel inhibitors does not correlate with changes in the V_m in rat C6 glioma cells (Rouzaire-Dubois et al., 2000). In addition, the V_m is likely to be determined by the collective activities of a variety of ions/channels/transporters, which may exhibit reciprocal interactions and form a large and complex network responsible for V_m regulation and its downstream effects.

ION CHANNEL-DEPENDENT REGULATION OF PROLIFERATION AND V_m

Numerous studies have shown that pharmacological or genetic block of K_v channels reduces proliferation of cancer cells (e.g., Fraser et al., 2000; Ouadid-Ahidouch et al., 2000; Abdul and Hoosein, 2002; Chang et al., 2003; Menendez et al., 2010). Increasing evidence suggests that *Ether à go-go* (EAG) K^+ channels may serve as biomarkers for cancer (Ouadid-Ahidouch et al., 2001; Farias et al., 2004; Pardo et al., 2005; Hemmerlein et al., 2006; Ousingsawat et al., 2007; Ortiz et al., 2011; Rodriguez-Rasgado et al., 2012). Inhibition of EAG channel expression reduces proliferation in several cancer cell lines, whereas implantation of CHO cells over-expressing EAG channels in mice induces tumors (Pardo et al., 1999). In synchronized SH-SY5Y cells, human I_{EAG} is reduced to less than 5% in G_1 phase, compared to unsynchronized controls, suggesting that the activity of EAG channels is cell cycle-dependent (Meyer and Heinemann, 1998). Indeed, in MCF-7 cells, inhibiting EAG channels with astemizole increases the proportion of cells in G_1 phase and reduces the proportion in S phase (Borowiec et al., 2007). In contrast, activation of hEAG channels is responsible for hyperpolarization at late G_1 before the cells enter the S phase (Ouadid-Ahidouch et al., 2001). Interestingly, the hyperpolarization is accompanied by increased Ca^{2+}-activated K^+ (K_{Ca}) channel currents (Ouadid-Ahidouch et al., 2001), which might result from the elevated intracellular Ca^{2+} due to the increased electrochemical gradient (**Figure 3**) (Nilius and Wohlrab, 1992; Ouadid-Ahidouch and Ahidouch, 2008).

When K_{Ca} channels were found in Friend murine erythroleukemia cells, they were thought to be one of the main controllers of the V_m (Arcangeli et al., 1987). K_{Ca} channels have been found since in glioma (Liu et al., 2002), prostate cancer (Gessner et al., 2005), breast cancer (Haren et al., 2010), and the $CD133^+$ subpopulation of SH-SY5Y cells (Park et al., 2010). Inhibiting K_{Ca} channels with iberiotoxin arrests D54-MG glioma cells in S phase, and leads to apoptosis (Weaver et al., 2004).

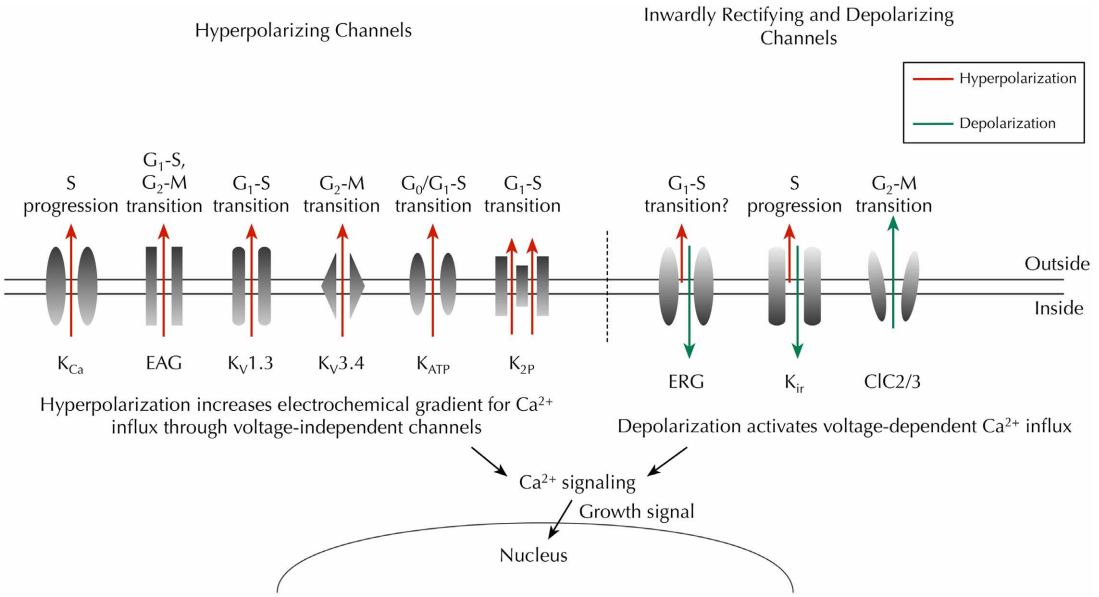

FIGURE 3 | Key ion channels that regulate V_m and cell cycle progression in cancer. Hyperpolarizing channels (outward I_K, red) would increase the driving force for Ca^{2+} influx through voltage-independent channels, whereas inwardly rectifying K^+ channels (predominantly inward I_K, green) and chloride channels (outward Cl^-, green) would depolarize the V_m, thus enabling activation of voltage-dependent Ca^{2+} influx (Schwab et al., 2012). Time- and domain-dependent Ca^{2+} signaling is then proposed to activate pathways that promote cell cycle progression and proliferation. Abbreviations: K_{Ca}, Ca^{2+}-activated K^+ channel; EAG, *ether à go-go* channel; K_V, voltage-gated K^+ channel; K_{ATP}, ATP-sensitive K^+ channel; K_{2P}, two-pore domain K^+ channel; ERG, EAG-related gene K^+ channel; K_{ir}, classic inward-rectifier K^+ channel; ClC2/3, chloride 2/3 channel.

Thus, the functional contribution of K_{Ca} channels to cell cycle regulation appears to be distinct from K_V channels. In addition, in MCF-7 cells, inhibition of ATP-sensitive K^+ (K_{ATP}) channels reversibly arrests cells in the G_0/G_1 phase (Woodfork et al., 1995). The two-pore domain K^+ channel, TREK1, increases proliferation of PC-3 and LNCaP prostate cancer cells (Voloshyna et al., 2008). In CHO cells, overexpression of TREK1 increases the number of cells in S phase, and reduces the number of cells at G_0/G_1 phase (Voloshyna et al., 2008).

Human EAG-related gene (HERG) K^+ channels are strongly inwardly rectifying and conduct K^+ influx when the voltage is more negative than the K^+ equilibrium potential (Trudeau et al., 1995; Smith et al., 1996). HERG channels are expressed at early developmental stages in the neural crest, central nervous system, dorsal root ganglion (DRG) and skeletal muscle, and are replaced by classic inward rectifier K^+ current (IK_{ir}) later in development (Arcangeli et al., 1997; Crociani et al., 2000). HERG channels are upregulated in a number of cancers (Arcangeli, 2005). Moreover, I_{HERG} increases tumor cell proliferation (Bianchi et al., 1998; Wang et al., 2002). The activity of I_{HERG} itself is cell cycle dependent (Arcangeli et al., 1995), suggesting a complex relationship between I_{HERG}, V_m, and proliferation. Additional inward rectifier K^+ (K_{ir}) channels have been reported in various cancer cell types, and are required for proliferation, including $K_{ir}2.2$ (Lee et al., 2010), $K_{ir}3.1$, and $K_{ir}3.4$ (Plummer et al., 2004; Takanami et al., 2004; Plummer et al., 2005; Wagner et al., 2010). In contrast, overexpression $K_{ir}4.1$ in glioma cells hyperpolarizes the V_m and increases the number of cells in quiescent G_0/G_1, reducing the proportion in G_2/M phase (Higashimori and Sontheimer, 2007). Thus, different K_{ir} channels may play opposing roles in regulation of V_m/proliferation, as a result of their heterogeneous voltage dependence (**Figure 3**). Cl^- conductance also appears to be linked to the cell cycle and regulate proliferation. For example, in D54-MG cells, Cl^- efflux through the outward rectifying ClC3 Cl^- channel is significantly increased during M phase (Habela et al., 2008). In addition, the ClC2 channel is expressed in M phase in transfected NRK-49F rat kidney fibroblast cells (Zheng et al., 2002).

The mechanisms underlying ion channel-dependent proliferation of cancer cells have been reviewed in detail elsewhere (Wang, 2004; Ouadid-Ahidouch and Ahidouch, 2008; Prevarskaya et al., 2010). These include possible non-conducting, direct interactions between ion channels and other pro-proliferative signaling mechanisms. For example, coexpression of HERG and tumor necrosis factor receptor 1 (TNFR1) has been found at the cell membrane of SKBR3 and SH-SY5Y cell lines, and HERG appears to recruit TNFR1 to the membrane, therefore enhancing TNF-α-induced cancer cell proliferation (Wang et al., 2002). Alternatively, ion channel-mediated V_m hyperpolarization would increase the electrochemical gradient for Ca^{2+} and therefore elevate the intracellular Ca^{2+} concentration through voltage-independent Ca^{2+} channels, such as transient receptor potential (TRP) channels (Nilius and Wohlrab, 1992; Wang, 2004; Ouadid-Ahidouch and Ahidouch, 2008). Ca^{2+} signaling is functional across the whole cell cycle (Santella et al., 2005). For example, Ca^{2+} is required for G_1 progression and G_1/S transition (Hazelton et al., 1979; Choi et al., 2006). In turn, intracellular Ca^{2+} and calmodulin (CaM) can regulate

K_{Ca} and EAG channels (Khanna et al., 1999; Ziechner et al., 2006; Ouadid-Ahidouch and Ahidouch, 2008). Thus, there may be a reciprocal, auto-regulatory relationship between ion channel activity, V_m, intracellular Ca^{2+} signaling, and proliferation.

In summary, a multiplicity of ion channels (predominantly K^+-conducting) participates in V_m regulation (both depolarization and hyperpolarization) in cancer cells. In turn, changes in V_m promote transition through cell cycle checkpoints. Changes in V_m are likely to trigger intracellular signaling messengers such as Ca^{2+} in order to drive sustained proliferation.

ROLE OF V_m IN CANCER CELL MIGRATION

Metastasis involves loss of adhesion at the primary site, increased migration and invasion, circulation through the vascular/lymphatic systems and growth of secondary tumors at distant sites (Gupta and Massague, 2006; Prevarskaya et al., 2010). Among the various steps in the metastatic cascade, it is well-established that cell migration is tightly controlled by the movement of ions and water [**Figure 4**; reviewed in depth in Schwab et al. (2007, 2012)]. V_m is regarded as an indirect factor that can affect cell migration, whose main regulatory role might be setting up the electrical driving force for Ca^{2+} (Prevarskaya et al., 2010; Schwab et al., 2012). A hyperpolarized V_m can increase intracellular Ca^{2+} via TRP channels, whereas membrane depolarization could activate voltage-gated Ca^{2+} channels (Schwab et al., 2012). Intracellular Ca^{2+} displays a concentration gradient in migrating cells, with lowest concentration at the leading edge (Brundage et al., 1991). During cell migration, oscillations in Ca^{2+} concentration are observed within microdomains, such that Ca^{2+} flickering is highest in the lamellipodia (Wei et al., 2009). These fluctuations play a role in regulating tractional forces (Lee et al., 1999; Ridley et al., 2003), direction sensing, and cytoskeleton reorganization (Pettit and Fay, 1998). V_m may also affect downstream intracellular signaling cascades that could contribute to cell migration in a Ca^{2+}-independent way (**Figure 4**). For example, in kidney epithelial cells, V_m depolarization induces diphosphorylation of myosin light chain (MLC) without inducing Ca^{2+} signaling, but instead by activating the Rho-Rho kinase (ROK) pathway (Szaszi et al., 2005). In addition, actin filaments undergo reorganization following V_m depolarization in bovine eye endothelial and epithelial cells (Chifflet et al., 2003, 2004), suggesting a functional role for V_m in cytoskeletal reorganization, although it is not clear whether or not Ca^{2+} is involved. Furthermore, applied electrical fields, which would impact on V_m, can enhance motility and galvanotaxis (Djamgoz et al., 2001; Levin, 2003, 2009; Schwab et al., 2012).

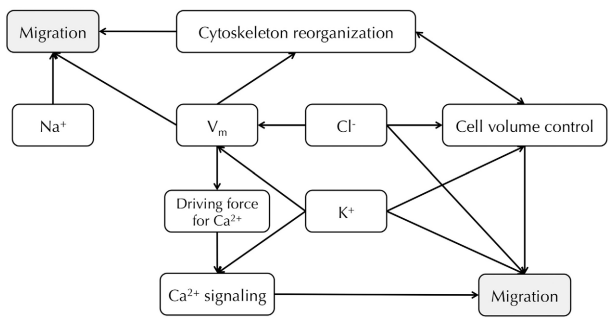

FIGURE 4 | Relationship between Na^+, K^+, Cl^- channels and V_m in cancer cell migration. V_m provides the driving force for Ca^{2+}, and downstream Ca^{2+} signaling leads to cell migration (Schwab et al., 2012). V_m also regulates cytoskeleton reorganization (Chifflet et al., 2003, 2004). Cl^- and K^+ channels both contribute to V_m regulation and cell volume control (Soroceanu et al., 1999; Sontheimer, 2008; Habela et al., 2009; Schwab et al., 2012). Inhibiting particular Na^+, K^+, and Cl^- channels can reduce cancer cell migration (Sontheimer, 2008; Brackenbury, 2012; Schwab et al., 2012).

A number of Na^+, K^+, and Cl^- channels, that potentially contribute to the V_m, are directly implicated in cancer cell migration. For example, functional VGSCs have been found in a number of cancer types [reviewed in Brackenbury (2012)], and suppressing VGSCs with siRNA or pharmacological agents inhibits migration and invasion (Roger et al., 2003; Fraser et al., 2005; Brackenbury et al., 2007; House et al., 2010; Yang et al., 2012). In several breast carcinoma/melanoma cell lines, $K_{Ca}2.3$, which is responsible for maintaining a hyperpolarized V_m, enhances migration, likely via promotion of intracellular Ca^{2+} signaling (Potier et al., 2006; Chantome et al., 2009). In addition, $K_{Ca}3.1$ activity causes a local shrinkage at the rear of migrating MDCK-F cells, therefore supporting retraction at this pole during movement (Schwab et al., 2006). In order to maintain electroneutrality, K^+ efflux must be accompanied by an anion, and Cl^- is the most likely candidate (Schwab et al., 2007, 2012). In agreement with this, Cl^- channels, which contribute to the depolarized V_m in glioma cells, enhance migration and invasion by permitting the release of K^+, Cl^-, and water at the leading edge, resulting in shrinkage and facilitating movement into tortuous extracellular spaces (Soroceanu et al., 1999; Sontheimer, 2008; Habela et al., 2009; Schwab et al., 2012).

In conclusion, a direct role for V_m in regulating cancer cell migration is much less clear than for proliferation. Given the great variety of ion channels and transporters that are involved in the process of cell migration, the concept of the "transportome" has been proposed (Schwab et al., 2012), which implies that rather than individual ion channels or transporters, it is a complex network of ion translocators that directs the migration and invasion of cells (**Figure 4**). Further work is required to establish to what extent V_m directly impacts on this network.

V_m AND THE DIFFERENTIATION OF CANCER STEM CELLS

Stem cells and cancer cells share similar properties, such as the ability to differentiate and self-renew, increased membrane transporter activity and the ability to migrate and metastasize (Wicha et al., 2006). The cancer stem cell (CSC) hypothesis contains two key concepts: (1) cancers arise from dysregulated transformation of normal tissue stem cells or progenitor cells, and (2) cellular components that display stem cell properties can lead to cancer progression (Wicha et al., 2006). In contrast to normal, regulated asymmetric division of stem cells during tissue homeostasis, where a stem cell produces one copy of itself and one cell that later differentiates into a mature cell, the dysregulation of transformed CSCs during tumorigenesis involves "symmetric division" in

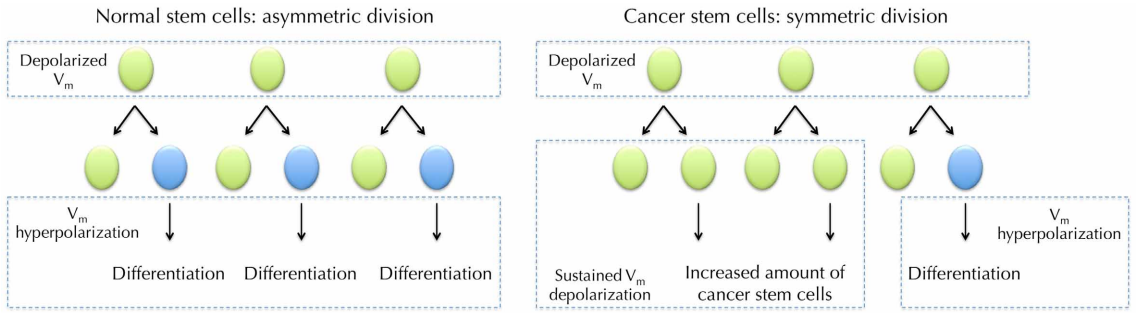

FIGURE 5 | V_m in normal stem cell (SC) differentiation and hypothesized role for V_m in cancer stem cells (CSCs). Depolarized V_m is needed during the maintenance of SCs. SC undergoes asymmetric division where it produces one copy of itself and one progeny that later differentiate into mature cells. The maturation requires V_m hyperpolarization (Sundelacruz et al., 2008). However, CSCs frequently undergo symmetric division, in which one CSC divides into two identical CSC progenies (Wicha et al., 2006). Sustained V_m depolarization may help to maintain the increasing CSCs in an undifferentiated state. Proliferation of CSCs then increases cancer malignancy.

which each malign CSC generates two identical daughter cells (giving rise to either proliferation or differentiation), which significantly expands the malign stem cell reservoir (**Figure 5**) (Liu et al., 2005).

A role for V_m in differentiation of normal stem cells has been previously reported. Studies in quail neural crest cells and a subpopulation of SH-SY5Y cells have demonstrated that stem cells exhibit distinct bioelectrical profiles during development (Arcangeli et al., 1997; Biagiotti et al., 2006; Sundelacruz et al., 2009). In particular, a hyperpolarized V_m is required during stem cell maturation (Sundelacruz et al., 2009). For example, K_{ir}-induced V_m hyperpolarization is required during human myoblast fusion (Liu et al., 1998). In a genome-wide microarray analysis of depolarization-regulated genes in postnatal mouse cerebellar granule neurons, among 87 depolarization-responsive genes, 22 are developmentally up-regulated and 26 are developmentally down-regulated (Sato et al., 2005). Remarkably, 18 of the 22 (82%) developmentally up-regulated genes coincide with depolarization down-regulated genes, and 20 of 26 (77%) developmentally down-regulated genes with depolarization up-regulated genes (Sato et al., 2005). V_m hyperpolarization is also a functional determinant of human mesenchymal stem cell (hMSC) differentiation. Pharmacologically-induced V_m depolarization suppresses adipogenic and osteogenic differentiation of hMSCs (Sundelacruz et al., 2008). In addition, depolarization reduces the differentiated phenotype of hMSC-derived cells and improves their ability to transdifferentiate, without fully restoring a stem cell-like genetic profile (Sundelacruz et al., 2013). Taken together, these data suggest that V_m depolarization may maintain cells in an undifferentiated stage at the gene expression level. Therefore, it is not unreasonable to postulate that depolarized V_m may also help maintain a population of undifferentiated CSCs (**Figure 5**). This possibility would raise additional, related questions: does a more depolarized V_m promote the proliferation of CSCs? Does V_m affect the pattern of symmetric vs. asymmetric division? Further work is required to investigate these possibilities.

CLINICAL IMPLICATIONS

Given that the fluctuation of V_m can functionally regulate tumorigenesis, differentiation, and promote cancer progression, it may serve as a potential marker for tumor detection and treatment, with prognostic value. For example, bioelectrical impedance analysis, which determines tissue electrical properties, has shown promise as a prognostic indicator to monitor cancer progression (Gupta et al., 2004a,b); , and recently, the development of non-invasive, voltage-sensitive optical probes provides a potential approach for *in vivo* V_m measurement (Adams and Levin, 2012; Chernet and Levin, 2013). Considering the vast array of therapeutic drugs that target ion channels (Sontheimer, 2008; Stuhmer and Pardo, 2010; D'amico et al., 2013; Djamgoz and Onkal, 2013), modulating the V_m of malign tissues by adjusting the activities of varies ion channels/transporters may provide a convenient clinical approach.

ACKNOWLEDGMENTS

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Ionizing radiation, ion transports, and radioresistance of cancer cells

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The standard treatment of many tumor entities comprises fractionated radiation therapy which applies ionizing radiation to the tumor-bearing target volume. Ionizing radiation causes double-strand breaks in the DNA backbone that result in cell death if the number of DNA double-strand breaks exceeds the DNA repair capacity of the tumor cell. Ionizing radiation reportedly does not only act on the DNA in the nucleus but also on the plasma membrane. In particular, ionizing radiation-induced modifications of ion channels and transporters have been reported. Importantly, these altered transports seem to contribute to the survival of the irradiated tumor cells. The present review article summarizes our current knowledge on the underlying mechanisms and introduces strategies to radiosensitize tumor cells by targeting plasma membrane ion transports.

Keywords: radiation therapy, cell cycle, DNA repair, ion channels

INTRODUCTION

Increasing pieces of evidence strongly indicate that ion transports across biological membranes fulfill functions beyond those described by classical physiology such as epithelial transports and neuronal or muscle excitability. More and more, it turns out that ion transports are involved in virtually all cell-biological processes. By modifying the chemistry, electricity and mechanics of cells, ion transports directly interact with cellular biochemistry and constitute signaling modules that are capable of altering protein function, gene expression (Tolon et al., 1996) and epigenetics (Lobikin et al., 2012). Moreover, ion transport-generating proteins such as ion channels have been identified to directly signal in macromolecular complexes with, e.g., surface receptors and downstream kinases (Arcangeli, 2011), or to directly bind to DNA as transcription factors (Gomez-Ospina et al., 2006).

Over the past two decades, ion transports came more and more in the focus of oncological research. Increasingly, data accumulate indicating tumor-suppressing as well as oncogenic functions of ion transport processes. In particular, ion transports have been identified as key regulators of neoplastic transformation, malignant progression, tissue invasion and metastasis (for review see Huber, 2013). Most recent data suggest that ion transports may also contribute to therapy resistance especially to radioresistance of tumor cells. The second chapter of this review article aims at giving an overview of those data. Since worldwide, only a handful of laboratories including ours are working in this research field only few data on ion transports in radioresistance are available and in most cases, the underlying molecular mechanisms of the observed phenomena remain ill-defined. Because tumor hypoxia is a major obstacle in radiotherapy, the second chapter also includes ion transports in the mitochondria that confer hypoxia resistance to normal tissue and probably also to tumor cells. At the end, this article provides some ideas how the acquired knowledge might be harnessed in the future for new strategies of anti-cancer therapy that combine ion transport-targeting and radiotherapy. To begin with, a brief introduction into radiotherapy and its radiobiological principles is given in the next paragraphs.

RADIOTHERAPY

According to the German Cancer Aid, 490,000 people in Germany are diagnosed with cancer every year (German-Cancer-Aid, 2013a) (data originating from February 2012), 218,000 die from their disease. About half of all cancer patients receive radiation treatment, half of all cures from cancer include radiotherapy (German-Cancer-Aid, 2013b). Radiotherapy is one of the main pillars of cancer treatment together with surgery and systemic therapy, mainly chemotherapy. Examples for curative radiotherapy without surgery are prostate (Eckert et al., 2013; Kotecha et al., 2013) and head and neck cancer (Glenny et al., 2010). Preoperative radiotherapy is applied in rectal cancer (Sauer et al., 2012), postoperative treatment in breast cancer (Darby et al.). Yet, also rare tumor entities like sarcoma and small cell carcinoma are treated with radiotherapy (Eckert et al., 2010a,b; Muller et al., 2012). Despite modern radiation techniques and advanced multimodal treatments local failures and distant metastases often limit the prognosis, especially due to limited salvage treatments (Muller et al., 2011; Zhao et al., 2012).

INTRINSIC AND HYPOXIC RADIORESISTANCE

Radiation therapy impairs the clonogenic survival of tumor cells mainly by causing double strand breaks in the DNA backbone. The number of double strand breaks increases linearly with the absorbed radiation dose (unit Gray, Gy). The intrinsic capacity

to repair these DNA damages by non-homologous end joining or homologous recombination determines how radio resistant a given tumor cell is. Irradiated tumor cells which leave residual DNA double strand breaks unrepaired lose their clonogenicity meaning that these cells cannot restore tumor mass. Ion transports may directly be involved in the cellular stress response to DNA damage by controlling cell cycle, metabolic adaptations or DNA repair and, thus, contribute to intrinsic radioresistance and the survival of the tumor cell.

Besides intrinsic factors, the microenvironment influences the radiosensitivity of a tumor. Hypoxic areas frequently occur in solid tumors. Hypoxic tumor cells, however, are somehow "protected" from radiotherapy [reviewed in Harada (2011)]. This is because ionizing radiation generates directly or indirectly radicals in the deoxyribose moiety of the DNA backbone. In a hypoxic atmosphere, thiols can react with those DNA radicals by hydrogen atom donation which results in chemical DNA repair. In the presence of oxygen, in contrast, oxygen fixes radicals of the deoxyribose moiety to strand break precursors (Cullis et al., 1987). This so called oxygen effect radiosensitizes tumor cells by a factor of two to three (oxygen enhancement ratio) as compared to the hypoxic situation (Langenbacher et al., 2013). Accordingly, patients with hypoxic tumors who undergo radiotherapy have a worse prognosis than those with normoxic tumors [e.g., cervical cancer (Fyles et al., 2002, 2006)]. Notably, ion transport processes have been identified as important players in the adaptation of tumor cells to a hypoxic microenvironment. Hence, ion transports via adaptation to hypoxia also indirectly contribute to the radioresistance of tumors.

In radiotherapy, fractionated treatment regimens have been established which may reoxygenate and thereby radiosensitize the irradiated tumor during therapy time. In addition, fractionated radiotherapy spaces out the single fractions in a way that allows DNA repair of normal tissue, that re-distribute cell cycle of the tumor cells in more sensitive phases and that minimize repopulation of the tumor during therapy. The next paragraphs will give an introduction to the underlying radiobiology.

FRACTIONATED RADIATION THERAPY. REPAIR, REOXYGENATION, REDISTRIBUTION, AND REPOPULATION

Early in historic development of radiotherapy fractionation was introduced as a means to limit side effects when giving therapeutic radiation doses (Bernier et al., 2004). Standard fractionation is defined as single doses of 1.8–2 Gy, once daily, 5 days per week.

The principal rationale for fractionation is based on the fact that recovery after radiation is better in normal tissue than in tumors, especially concerning late reacting tissues responsible for late side effects of radiotherapy (Jones et al., 2006) such as fibrosis, damage of spinal cord and brain, as well as most inner organs. Radiation with high single doses is only possible without increased side effects if the radiation field can be confined to the tumor (e.g., stereotactic radiotherapy of brain metastases [Rodrigues et al., 2013) and SBRT, stereotactic body radiation therapy (Grills et al., 2012)]. Yet, many situations in radiation oncology such as adjuvant treatment or irradiation of nodal regions require irradiation of significant volumes of normal tissue.

Alpha-beta ratios

Acute effects of ionizing irradiation on clonogenic cell survival *in vitro* as well as on late toxicity of the normal tissue in patients which underwent radiotherapy are described by the linear-quadratic model (Barendsen, 1982; Dale, 1985). The mathematical fit of the clonogenic survival (late toxicity) is calculated as follows: $N = N_0 \times E^{-(\alpha D - \beta D\hat{}2)}$ with N being the number of surviving cells (patients without late toxicity), N_0 being the initial number of cells (number of patients receiving radiotherapy), α [1/Gy] and β [1/Gy^2] being cell (tissue)-specific constants and D the delivered radiation dose. Low alpha-beta ratios (α/β) [Gy] as determined for many normal tissues indicate that dose fractionation in daily fractions of usually 2 Gy increases survival and decreases late toxicity as compared to a single equivalent dose. Tumors with high alpha-beta ratios, in contrast do not benefit from fractionation. For some tumors such as squamous cell carcinoma of the head and neck there is even a rationale for hyperfractionated radiotherapy with twice daily irradiation of 1.2–1.4 Gy per fraction [reviewed in Nguyen and Ang (2002)]. The theoretical advantage has been confirmed in clinical trials [e.g., EORTC trial 22791 in advanced head and neck cancer Horiot et al. (1992)]. Different fractionation schedules for distinct clinical situations are applied for example in whole-brain radiotherapy. In prophylactic radiation 2–2.5 Gy fractions are applied to limit neurocognitive deficits (Auperin et al., 1999; Le Pechoux et al., 2011; Eckert et al., 2012). For therapeutic radiation 3 Gy fractions or even 4 Gy fractions are preferred in a palliative setting and limited life expectancy to shorten the treatment time to 5 or 10 days (Lutz, 2007; Rades et al., 2007a,b).

Reoxygenation

As mentioned above, fractionated radiation may also lead to reoxygenation of the tumor during therapy (Withers, 1975; Pajonk et al., 2010). Blood vessels of tumors lack normal architecture and are prone to collapse whenever tissue pressure of the expanding tumor mass increases. This aggravates tumor malperfusion and accelerates intermittent or chronic tumor hypoxia. Being sublethal as related to the whole tumor, single radiation fractions in the range of 2 Gy kill a significant percentage of the tumor cells which give rise to tumor shrinkage. Shrinkage, in turn, is thought to increase blood and oxygen supply of the tumor by improving vessel perfusion and by increasing the ratio of vascularization and the residual tumor mass (Maftei et al., 2011; Narita et al., 2012). Increased oxygenation then reverses hypoxic radioresistance of the tumor and improves the therapeutic outcome of radiotherapy.

Redistribution and repopulation

The sensitivity to radiotherapy during cell cycle differs, being highest in M and lowest in late S phase of cell cycle (Pawlik and Keyomarsi, 2004). Often depending on p53 function, irradiated tumor cells accumulate in G_1 or G_2 phase of cell cycle to repair their DNA damages. In a radiation dose-dependent manner, irradiated cells are released from cell cycle arrest and re-enter cell

cycling and tumor repopulation. Importantly, repopulation after irradiation is often accelerated probably due to selection of more aggressive tumor cells (Marks and Dewhirst, 1991). Fractionated radiation regimes aim to re-distribute tumor cells in a more vulnerable phase of the cell cycle in the time intervals between two fractions and to impair repopulation (Pawlik and Keyomarsi, 2004).

CANCER STEM CELLS (CSCs)

Cancer stem cells (CSCs) may resist radiation therapy [for review see Pajonk et al. (2010)]. Mechanisms that might contribute to the relative resistance of CSCs as compared to the non-CSC cells of a given tumor include (i) higher oxidative defense and, therefore, lower radiation-induced insults, (ii) activated DNA checkpoints resulting in faster DNA repair, and (iii) an attenuated radiation-induced cell cycle redistribution. Fractionation regimes are designed that way that the macroscopically visible bulk of tumor cells (i.e., the non CSCs) and not the rare CSCs become redistributed into a more vulnerable phase of cell cycle between two consecutive fractions of radiotherapy. Finally, radiation therapy is thought to switch CSCs from an asymmetrical into a symmetrical mode of cell division; i.e., a CSC which normally divides into a daughter CSC and a lineage-committed progenitor cell is induced by the radiotherapy to divide symmetrically into two proliferative stem daughter cells. This is thought to accelerate repopulation of the tumor after end of radiotherapy (Pajonk et al., 2010).

In summary, fractionated radiotherapy may radio sensitize tumor cells by reoxygenation of the tumor and redistribution of the tumor cells in more vulnerable phases of cell cycle while protecting at the same time normal tissue if the alpha-beta ratio of the tumor exceeds that of the normal tissue. On the other hand, the applied fractionation protocols might spare CSCs due to their radiobiology that differs from that of the bulk of non-CSCs. Furthermore, single radiation fractions apply sublethal doses of ionizing radiation. Data from *in vitro* and animal studies suggest that sublethal doses of ionizing radiation may stimulate migration and tissue invasion of the tumor cells. Translated into the *in vivo* situation, this might imply that cells at the edge of solid tumors might be stimulated by the first radiation fractions to migrate out of the target volume of radiation resulting in survival of the evaded cells and tumor relapse. Moreover, if radiation fractions further induce tumor cell invasion into blood or lymph vessels, fractionated radiotherapy regimes might also boost metastases. As described in the next paragraphs, ion transports fulfill pivotal functions in cell migration especially in radiation-induced migration.

ION TRANSPORTS AND RADIORESISTANCE

Ion transports can be assessed by tracer-flux measurements, fluorescence microscopy/photometry using ion species-specific fluorescence dyes such as the Ca^{2+}-specific fluorochrome fura-2, as well as by electrophysiological means. The latter can be applied if ion transports are electrogenic. Measurements of ion transports during treatment with ionizing radiation are hardly feasible. Reported electrophysiological *in vitro* data on irradiated tumor cells indicate that radiation-induced transport modifications may occur instantaneously and may last up to 24 h post irradiation (Kuo et al., 1993). They further suggest that these modifications may be induced by doses used for single fractions in the clinic (Steinle et al., 2011). The following paragraphs summarize radiation-induced transport modifications as observed in *in vitro* studies on tumor cell lines and their putative contribution to the radioresistance of tumor cells. Whether these processes may indeed underlie therapy failure in tumor patients can only be answered if more data from tumor mouse models and clinical trials become available.

Tumor cells have been proposed to adapt either a "Grow" or a "Go" phenotype in dependence on changes in their microenvironment. When developing a certain mass, growing solid tumors are prone to become malperfused because of the insufficient tumor vasculature. As a consequence of malperfusion, microenvironmental stress by hypoxia, interstitial nutrient depletion, and low pH increases (Stock and Schwab, 2009; Hatzikirou et al., 2012) which is thought to trigger at a certain point the induction of the "Go" phenotype. By migration and tissue invasion "Go" tumor cells may evade the locally reined stress burden and resettle in distant and less hostile regions. Once re-settled, tumor cells may readapt the "Grow" phenotype by reentering cell cycling and may establish tumor satellites in more or less close vicinity of the primary focus. Moreover, this stress evasion may lead to metastases if the "Go" cells invade into blood or lymph vessels.

Migration and tissue invasion are directed by extracellular hapto- and chemotactic signals which trigger preset "Go" programs (Schwab et al., 2007, 2012). The latter comprise intracellular signaling, cellular motor functions including cell volume changes and cytoskeletal dynamics, as well as extracellular matrix digestion and reorganization. Ion transports have been suggested to contribute to all of these processes (Schwab et al., 2007, 2012). As a matter of fact, highly invasive and metastatic phenotypes of tumor cells often show aberrant activity of certain ion transports. The following paragraphs describe the role of these ion transports in particular of those across the plasma membrane using the example of glioblastoma cells.

MOTOR FUNCTION

Glioblastoma cells exhibit a highly migrative phenotype and "travel" long distances throughout the brain (Johnson et al., 2009). Primary foci of glioblastoma show, therefore, even at early stages of diagnosis a characteristic diffuse and net-like brain infiltration (Niyazi et al., 2011). Tumor margins are often not definable and complete surgical tumor resection as well as capture of all residual tumor cells by the radiation target volume is hardly possible (Weber et al., 2009). This results in therapy failure accompanied by very bad prognosis for the survival of the patient in almost all cases of glioblastoma (Niyazi et al., 2011). Glioblastoma cells typically migrate into the surrounding brain parenchyma primarily by using nerve bundles and the vasculature as tracks. The close vicinity to the vasculature has the advantage for the migrating glioblastoma cell of a continuous and sufficient supply of oxygen, nutrients, growth factors, chemokines, and cytokines (Montana and Sontheimer, 2011). Glioblastoma cells have to squeeze through very narrow interstitial spaces during their brain invasion along those tracks. This

requires highly effective local cell volume decrease and re-increase procedures. Notably, glioblastoma cells are capable to lose all unbound cell water leading to maximal cell shrinkage (Watkins and Sontheimer, 2011). Unusually high cytosolic Cl^- concentrations (100 mM) provide the electrochemical driving force for this tremendous cell volume decrease. The cytosolic Cl^- concentration is built up highly above its electrochemical equilibrium concentration by the Na/K/2Cl cotransporter NKCC1 (Haas and Sontheimer, 2010; Haas et al., 2011) allowing glioblastoma cells to utilize Cl^- as an osmolyte.

Local regulatory volume increase and decrease have been proposed to drive migration mechanics. The latter is generated by the loss of Cl^- and K^+ ions along their electrochemical gradients followed by osmotically obliged water fluxes. Involved transporters probably are ClC-3 Cl^- channels (Olsen et al., 2003; Cuddapah and Sontheimer, 2010; Lui et al., 2010), Ca^{2+}-activated high conductance BK (Ransom and Sontheimer, 2001; Ransom et al., 2002; Sontheimer, 2008) as well as intermediate conductance IK K^+ channels (Catacuzzeno et al., 2010; Sciaccaluga et al., 2010; Ruggieri et al., 2012) and AQP-1 water channels (Mccoy and Sontheimer, 2007; Mccoy et al., 2010). To a lower extent, K^+ and Cl^- efflux is probably also mediated by KCC1-generated cotransport (Ernest et al., 2005). These transports are crucial for glioblastoma migration since either transport blockade inhibits glioblastoma cell migration and invasion (Ernest et al., 2005; Mcferrin and Sontheimer, 2006; Catacuzzeno et al., 2010; Haas and Sontheimer, 2010; Lui et al., 2010; Sciaccaluga et al., 2010).

Notably, Ca^{2+}-activated BK (Ransom and Sontheimer, 2001; Liu et al., 2002; Ransom et al., 2002; Weaver et al., 2006) and IK K^+ channels (Ruggieri et al., 2012) are ontogenetically down-regulated or absent in mature glial cells but up-regulated with neoplastic transformation and malignant tumor progression as shown in expression studies in human glioma tissue. Moreover, glioblastoma cells up-regulate a unique splice variant of the BK channel (Liu et al., 2002) which exhibits a higher Ca^{2+} sensitivity than the other isoforms (Ransom et al., 2002) and is indispensable for glioblastoma proliferation *in vitro*. Similarly, ClC-3 Cl^- channels are mal-expressed in glioblastoma tissue where they traffic, in contrast to normal tissue, to the plasma membrane (Olsen et al., 2003). The predominant (surface) expression of ClC-3 and the BK splice variant by glioblastoma cells renders both channel types putative glioblastoma-specific therapeutic targets.

EVASION FROM RADIATION STRESS

External beam radiation may induce the "Go" phenotype in tumor cells similarly to the situation described for stress arising from an adverse tumor microenvironment (**Figure 1**). Ionizing radiation at doses used in single fractions during fractionated radiotherapy has been demonstrated *in vitro* and by a mouse study (Wild-Bode et al., 2001) to induce migration, invasion and spreading of head and neck squamous carcinoma (Pickhard et al., 2011), lung adenocarinoma (Jung et al., 2007; Zhou et al., 2011), meningioma (Kargiotis et al., 2008), medulloblastoma (Asuthkar et al., 2011), and glioblastoma cells (Wild-Bode et al., 2001; Wick et al., 2002; Badiga et al., 2011; Canazza et al., 2011; Rieken et al., 2011; Steinle et al., 2011; Kil et al., 2012; Vanan et al., 2012). The phenomenon of radiation-stimulated migration might be particularly relevant for highly migrating and brain-infiltrating glioblastoma cells.

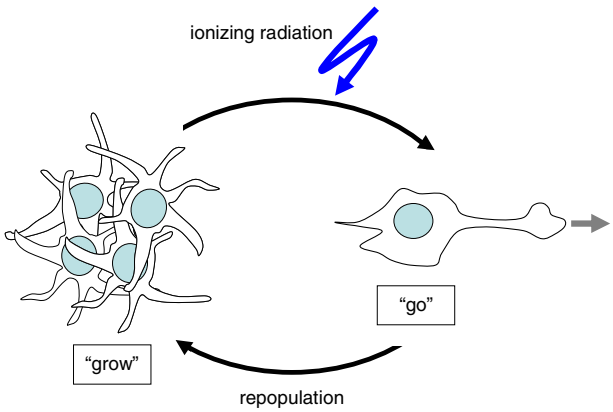

FIGURE 1 | Cellular stress such as ionizing radiation induces the switch from "Grow" to "Go" phenotype.

After macroscopic complete resection glioblastoma is usually treated by adjuvant radiotherapy of the tumor bed applying 54–60 Gy in daily fractions of 1.8–2 Gy combined with temozolomide (Stupp et al., 2005). The median progression-free survival after therapy ranges between 5 and 7 months (Stupp et al., 2005). The recurrence of glioblastoma is typically observed within the former target volume of the adjuvant fractionated radiotherapy. This might be due either to a high intrinsic radioresistance of the glioblastoma cells or to re-invasion of tumor cells into the area of the irradiated primary. One might speculate that this necrotic area, meanwhile cleared by phagocytes, offers optimal growth conditions for such re-invading tumor cells. In this latter scenario, re-invading cells might be recruited from glioblastoma (stem) cells pre-spreaded prior to radiotherapy onset in areas outside the target volume, or from cells that successfully evaded during radiation therapy.

Radiation-induced up-regulation of integrin- (Wild-Bode et al., 2001; Nalla et al., 2010; Canazza et al., 2011; Rieken et al., 2011), VEGF- (Sofia Vala et al., 2010; Badiga et al., 2011; Kil et al., 2012), EGF- (Kargiotis et al., 2008; Pickhard et al., 2011) or/and TGFbeta signaling (Canazza et al., 2011; Zhou et al., 2011) has been proposed to promote tumor cell migration. Downstream ion transport processes have been reported for glioblastoma cells (Steinle et al., 2011). In this study, BK K^+ channel activation and subsequent BK-dependent activation of the CaMKII kinase were identified as key triggers of radiation-induced migration (Steinle et al., 2011). Additionally, ClC-3 anion channels were identified as downstream targets of radiation-induced CaMKII activity (Huber, 2013). This suggests on the one hand motor function (i.e., volume decrease) of radiation-induced BK and ClC-3 currents, on the other hand, it points to a signaling function of BK channels in the programming of radiation-stimulated glioblastoma migration (**Figure 2**). Similar to the situation in migrating glioblastoma cells, radiation-induced plasma membrane K^+ currents and downstream CaMKII activation have been defined as key signaling events in cell cycle control of irradiated leukemia cells as introduced in the following paragraphs.

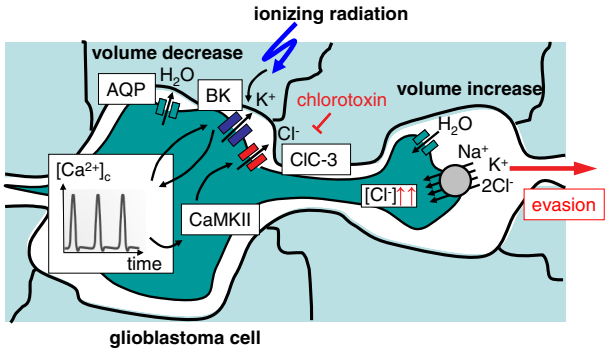

FIGURE 2 | Ionizing radiation (IR)-induced activation of BK K^+ channels results in evasion of glioblastoma cells from radiation stress. Activation of BK channels occurs upstream of CaMKII and ClC-3 Cl^- channels. The $Na^+/K^+/2Cl^-$ cotransporter accumulates Cl^- above its electrochemical equilibrium. Cl^- is used as osmolyte for volume decrease.

DNA REPAIR

Survival of irradiated tumor cells critically depends on DNA repair. This involves cell cycle arrest, elevated energy consumption, chromatin relaxation, and formation of repair complexes at the site of DNA damage. Recent *in vitro* observations suggest that radiation-induced ion transports may contribute to these processes in an indirect manner.

Cell cycle control

Survival of irradiated human leukemia cells depends on Ca^{2+} signaling. Radiation reportedly stimulates Ca^{2+} entry through TRPV5/6-like channels and subsequently activates CaMKII, which in turn fosters G_1/S transition, S progression and accumulation in G_2 phase of the cell cycle (Heise et al., 2010). Moreover, Ca^{2+} signaling in human leukemia cells has been demonstrated to be tightly regulated by voltage-gated $K_v3.4$ K^+ channels and translates into G_2/M cell cycle arrest by CaMKII-mediated inhibitory phosphorylation of the phosphatase cdc25B resulting in inactivation of the mitosis promoting factor and G_2/M arrest. Radiation activates Kv3.4 currents without changing the surface expression of the channel protein. Most importantly, inhibition of $K_v3.4$ by tetraethylammonium and blood-depressing substance-1 and substance-2 or silencing of the $K_v3.4$ channels by RNA interference prevents TRPV5/6-mediated Ca^{2+} entry, CaMKII activation, as well as cdc25B inactivation which results in release from radiation-induced G_2/M arrest, increased apoptosis, and decreased clonogenic survival. Thus, targeting of $K_v3.4$ radiosensitizes the leukemia cells demonstrating the pivotal role of this channel in cell cycle arrest required for DNA repair (Palme et al., 2013). Similar results have been obtained in prostate cancer cells, where TRPV6 inhibition by capsaicin resulted in radiosensitization (Klotz et al., 2011).

Glucose fueling and chromatin relaxation

In addition to cell cycle control, radiation-induced ion transports are proposed to improve glucose fueling of irradiated tumor cells. Fast proliferating tumor cells have a high metabolism at low external glucose and oxygen concentration in the usually chronically under-perfused growing tumor tissue. At the same time, many tumor cells cover their high energy requirements by anaerobic glycolysis with low ATP yield per metabolized glucose even under normoxic conditions. To sustain sufficient glucose fueling, tumor cells may up-regulate the Na^+/glucose co-transporter (SGLT). SGLTs are capable to take up glucose into the tumor cell even against a high chemical gradient (Ganapathy et al., 2009). Several tumor entities such as colorectal, pancreatic, lung, head and neck, prostate, kidney, cervical, mammary, and bladder cancer as well as chondrosarcomas and leukemia have indeed been shown to up-regulate SGLTs (Nelson and Falk, 1993; Ishikawa et al., 2001; Helmke et al., 2004; Casneuf et al., 2008; Weihua et al., 2008; Yu et al., 2008; Leiprecht et al., 2011; Wright et al., 2011). The inwardly directed Na^+ gradient and the voltage across the plasma membrane drive the electrogenic SGLT-generated glucose transport into the cell. The membrane voltage is tightly regulated by the activity of voltage gated K^+ channels which counteract SGLT-mediated depolarization.

Ionizing radiation has been demonstrated to activate EGF receptors (Dittmann et al., 2009). In addition, SGLT1 reportedly is in complex with and under the direct control of the EGF receptor (Weihua et al., 2008) suggesting radiation-induced SGLT1 modifications. As a matter of fact, ionizing radiation stimulates a long lasting EGFR-dependent and SGLT-mediated glucose uptake in A549 lung adenocarcinoma and head and neck squamous carcinoma cell lines (but not in non-transformed fibroblasts) as shown by ^{3}H-glucose uptake and patch-clamp, current clamp recordings (Huber et al., 2012). In the latter experiments, radiation-induced and SGLT-mediated depolarization of membrane potential was preceded by a transient hyperpolarization of the plasma membrane indicative of radiation-induced K^+ channel activation (Huber et al., 2012). Such radiation-induced increase in K^+ channel activity has been reported for several tumor cell lines including A549 lung adenocarcinoma cells (Kuo et al., 1993). In this cell line, radiation at doses between 0.1 and 6 Gy stimulates the activity of voltage gated K^+ channels within 5 min, which gradually declines thereafter. It is tempting to speculate that this radiation-stimulated K^+ channel activity counteracts the depolarization of the membrane potential caused by the SGLT activity shortly after radiation and sustains the driving force for Na^+-coupled glucose uptake (Huber et al., 2012).

Ionizing radiation may lead to necrotic as well as apoptotic cell death depending on cell type, dose, and fractionation (Verheij, 2008). In particular, necrotic cell death may be associated with ATP depletion (Dorn, 2013). Increased SGLT activity in irradiated tumor cells might contribute to ATP replenishment counteracting necrotic cell death. Such function has been suggested in irradiated A549 cells by experiments analyzing cellular ATP concentrations, chromatin remodeling, residual DNA damage, and clonogenic survival of irradiated tumor cells (Dittmann et al., 2013). The data demonstrate that radiation of A549 lung adenocarcinoma cells leads to a transient intracellular ATP depletion and to histone H3 modifications crucial

for both chromatin remodeling and DNA repair in response to irradiation.

Importantly, recovery from radiation-induced ATP crisis was EGFR/SGLT-dependent and associated with improved DNA-repair and increased clonogenic cell survival. The blockade of either EGFR or SGLT inhibited ATP level recovery and histone H3 modifications. *Vice versa*, inhibition of the acetyltransferase TIP60, which is essential for histone H3 modification, prevented chromatin remodeling as well as ATP crisis (Dittmann et al., 2013). Together, these data suggest that radiation-associated interactions between SGLT1 and EGFR result in increased glucose uptake, which counteracts the ATP crisis in tumor cells caused by chromatin remodeling. Importantly, the blockade of recovery from ATP crisis by SGLT1 inhibition may radio-sensitize tumor cells as demonstrated in lung adenocarcinoma and head and neck squamous carcinoma cell lines (Huber et al., 2012; Dittmann et al., 2013).

Formation of repair complexes

In addition to SGLT-generated glucose uptake, radiation-induced electrosignaling via transient receptor potential melastatin 2 (TRPM2) and vanilloid 1 (TRPV1) cation channels, has been shown to stimulate Ataxia telangiectasia mutated (ATM) kinase activation, histone 2AX (H2AX) phosphorylation, and γH2AX focus formation in A549 lung adenocarcinoma cells, processes required to recruit further repair proteins to the DNA double strand break (Masumoto et al., 2013). Furthermore, radiation-induced TRPM2 induces ATP release and P2Y signaling in A549 cells (Masumoto et al., 2013). Radiation-stimulated and $P2X_7$ receptor- and gap junction hemichannel connexin43-mediated ATP release has been suggested to signal in a paracrine manner to unirradiated bystander cells in the B16 melanoma model (Ohshima et al., 2012).

Combined, these recent data indicate that ion transports may regulate processes that mediate intrinsic radioresistance. The investigation of ion transports in radiobiology is at its very beginning and the few data available are mostly phenomenological in nature. The molecular mechanisms that underlie, e.g., regulation of DNA repair by ion transports are ill-defined. Nevertheless, the data prove functional significance of ion transports and electrosignaling for the survival of irradiated tumor cells and might have translational implications for radiotherapy in the future.

Similar to intrinsic radioresistance, the function of ion transports in hypoxia resistance and associated hypoxic radioresistance of tumor cells is not well-defined. The following paragraphs give a summary of what is known about mitochondrial transports and hypoxia resistance of normal tissue and how these findings might also apply for tumor cells.

MITOCHONDRIAL UNCOUPLING AND RESISTANCE TO HYPOXIA, CHEMO-, AND RADIOTHERAPY

Intermittent hypoxia is a common feature of vascularized solid tumors. The pathophysiologial aspects of hypoxia and reoxygenation are well-known from ischemia-reperfusion injuries observed in normal tissue. Reoxygenation-associated production of reactive oxygen species (ROS) is a major cause of the hypoxia/reoxygenation injury after myocardial, hepatic, intestinal, cerebral, renal and other ischemia and mitochondria have been identified as one of the main sources of ROS formation herein (Li and Jackson, 2002). Mitochondrial ROS formation mutually interacts with hypoxia/reoxygenation-associated cellular Ca^{2+} overload. Brief hypoxic periods induce an adaptation to hypoxia in several tissues which lowers ischemia-reperfusion injuries of subsequent ischemic insults (so-called ischemic preconditioning). Similar adaptations which involve alterations in mitochondrial ion transport have been proposed to confer hypoxia resistance of tumor cells.

Mitochondrial ROS formation

Activity and efficacy of the mitochondrial respiration chain are fine-tuned by the dependence of the ATP synthase (complex V) on the membrane potential $\Delta\Psi_m$, by the ATP/ADP ratio, as well as by reversible phosphorylation of the complexes I and IV (**Figure 3**) (Kadenbach, 2003). It is suggested that under physiological conditions (high ATP/ADP ratios), the membrane potential $\Delta\Psi_m$ is kept low [around −100 to −150 mV (Kadenbach, 2003)]. The efficacy of the respiratory chain at low $\Delta\Psi_m$ is high. At higher ATP demand or decreasing cellular ATP levels, cytochrome c oxidase (complex IV) is relieved from ATP blockade and $\Delta\Psi_m$ increases. High $\Delta\Psi_m$ values (up to −180 mV), however, lower the efficacy of cytochrome c oxidase (Kadenbach, 2003) and increase the probability of single electron leakage at complex I and III to molecular oxygen resulting in an increased $O_2\cdot^-$ production (**Figure 3**) (Korshunov et al., 1997; Skulachev, 1998; Kadenbach, 2003).

The respiratory chain is also regulated by the cytosolic ($[Ca^{2+}]_i$) and mitochondrial matrix free Ca^{2+} concentration ($[Ca^{2+}]_m$) in a complex manner (for review see Pizzo et al., 2012). The phosphatases that dephosphorylate (and thereby switch-off) the NADH oxidase and that relieve the ATP blockade of complex IV are inhibited by $[Ca^{2+}]_m$ and activated by $[Ca^{2+}]_i$, respectively (**Figure 3**). As a consequence, increase in $[Ca^{2+}]_m$ and $[Ca^{2+}]_i$ results in a higher $\Delta\Psi_m$ and a concurrently increased production of reactive oxygen species (Kadenbach, 2003).

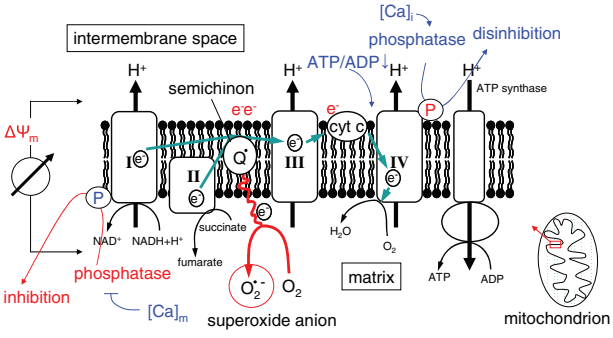

FIGURE 3 | Mitochondrial ROS formation in dependence on $\Delta\Psi_m$ (I, II, III, and IV, electron transport complexes I, II, III, and IV; Q: semiquinone radical; cyt C: cytochrome C). Ca^{2+} and the ATP/ADP ratio regulate the electron chain at complexes I and IV.

Hypoxia decreases the activity of the mitochondrial manganese superoxide dismutase (Mn-SOD) and of the cytochrome c oxidase. Depletion of the final electron acceptor, however, increases the formation of $O_2{\cdot}^-$ during reoxygenation by the enhanced leakage of single electrons from more proximal complexes of the respiration chain (for review see Li and Jackson, 2002; Sack, 2006). Lowered $O_2{\cdot}^-$-detoxifying capability combined with simultaneous elevated $O_2{\cdot}^-$ production results in a highly elevated $O_2{\cdot}^-$ concentration which, e.g., in hepatocytes increases 15-fold within 15 min of reoxygenation (Caraceni et al., 1995).

Hypoxia/reoxygenation-associated Ca^{2+} overload

Hypoxia-associated energy depletion and the concomitant impairment of plasma membrane Na^+ and Ca^{2+} pump activity lead to a decline of the chemical Na^+, Ca^{2+} and K^+ gradients across the plasma membrane and to the depolarization of the plasma membrane potential. In parallel, increased lactic acid fermentation during hypoxia increases the cytosolic proton concentration and lowers the intracellular pH. The proton extrusion machinery that is already active during hypoxia becomes massively activated during reoxygenation and restores a physiological pH by wash-out of lactic acid and activation of the sodium/hydrogen exchanger and sodium/bicarbonate symporter. The latter, Na^+-coupled transports, in turn, further increase the cytosolic Na^+ concentration to a level, where the low affinity high capacity sodium/calcium exchanger in the plasma membrane starts to operate in the reverse mode (i.e., to extrude Na^+ at the expense of Ca^{2+} uptake). At that time, reoxygenation-mediated oxidative stress (see above) stimulates further Ca^{2+} entry through Ca^{2+}-permeable channels in the plasma membrane and the release of Ca^{2+} from the endoplasmic reticulum resulting in an abrupt rise in $[Ca^{2+}]_i$ during the first minutes of reoxygenation. Cytosolic Ca^{2+} is buffered by $\Delta\Psi_m$-driven and uniporter-mediated Ca^{2+} uptake into the mitochondrial matrix which increases $[Ca^{2+}]_m$. Elevated $[Ca^{2+}]_m$ and $[Ca^{2+}]_i$ values, in turn, signal back to the respiratory chain by further increasing $\Delta\Psi_m$ (see above). Exceeding the Ca^{2+} threshold concentration in the matrix, $[Ca^{2+}]_m$ activates the permeability transition pore which leads to breakdown of $\Delta\Psi_m$, swelling of the mitochondrial matrix and eventually release of cytochrome c from the intermembrane space into the cytosol (Crompton, 1999; Rasola and Bernardi, 2011). By reversing the ATP synthase activity, into the ATPase proton pump mode, the F_0/F_1 complex in the inner mitochondrial delays the break-down of $\Delta\Psi_m$ at the expense of ATP hydrolysis. In addition to this ATP depletion, the loss of cytochrome c and the concurrent decline of the final electron acceptor (cytochrome c oxidase of complex IV) further increases the formation of $O_2{\cdot}^-$ by more proximal complexes. The pivotal role of membrane transports in this process is illustrated by the fact that inhibition of the sodium/hydrogen antiporter in the plasma membrane, the Ca^{2+} uniporter in the inner mitochondrial membrane, or Ca^{2+} channels in the endoplasmic reticulum (ER) decreases the hypoxia/reoxygenation injury *in vitro* (for review see Crompton, 1999; Li and Jackson, 2002; Sack, 2006; Yellon and Hausenloy, 2007).

Ischemic pre-conditioning

Cells can also adapt to repetitive periods of hypoxia. This so-called ischemic preconditioning has been demonstrated in the myocardium where it reduces ischemia-caused infarct size, myocardial stunning, and incidence of cardiac arrhythmias (Gross and Peart, 2003). Since mitochondrial ROS formation increases with increasing $\Delta\Psi_m$ (Korshunov et al., 1997; Skulachev, 1998; Kadenbach, 2003) lowering of the mitochondrial $\Delta\Psi_m$ is proposed to be a key adaptation event in ischemic preconditioning (Sack, 2006). Lowering of $\Delta\Psi_m$ reduces not only mitochondrial $O_2{\cdot}^-$ production but also the mitochondrial Ca^{2+} overload during reoxygenation (Gross and Peart, 2003; Prasad et al., 2009). The hypoxic preconditioning-associated reduction of $\Delta\Psi_m$ is in part achieved by up-regulation of ATP-sensitive (mitoKATP) and Ca^{2+}-activated (mitoKCa) K^+ channels in the inner mitochondrial membrane which short-circuit $\Delta\Psi_m$ (Murata et al., 2001; Gross and Peart, 2003; Prasad et al., 2009; Singh et al., 2012; Szabo et al., 2012). The uncoupling proteins-2 and -3 (UCP-2, -3) constitute two further proteins that have been suggested to play a role in counteracting cardiac hypoxia/reoxygenation injury and in hypoxic preconditioning in heart and brain (Mcleod et al., 2005; Sack, 2006; Ozcan et al., 2013). Activation of these proteins results in a modest depolarization of $\Delta\Psi_m$ by maximally 15 mV (Fink et al., 2002). High expression of UCP-3 has also been demonstrated in skeletal muscle where it suppresses mitochondrial oxidant emission during fatty acid-supported respiration (Anderson et al., 2007). Accordingly, overexpression of UCP-3 in cultured human muscle cells lowers $\Delta\Psi_m$, raises the ATP/ADP ratio, and favors fatty acid vs. glucose oxidation (Garcia-Martinez et al., 2001). Conversely, knockdown of UCP-3 increased the coupling between electron and proton transfer across the inner mitochondrial membrane and ROS production (Vidal-Puig et al., 2000; Talbot and Brand, 2005). UCP-3 protein is robustly up-regulated in chondrocytes (Watanabe et al., 2008) and skeletal muscle during hypoxia and the absence of UCP-3 exacerbates hypoxia-induced ROS (Lu and Sack, 2008). UCP-3 is not constitutively active. $O_2{\cdot}^-$ has been demonstrated to stimulate the activity of UCP-3 in skeletal muscle suggesting that UCP-3 is the effector of a feed back loop which restricts overshooting ROS production (Echtay et al., 2002).

Mitochondrial uncoupling in tumor cells

Recent studies suggest that UCPs are upregulated in a number of aggressive human cancers. In particular, over-expression of UCP2 has been reported in leukemia as well as in breast, colorectal, ovarian, bladder, esophagus, testicular, kidney, pancreatic, lung, and prostate cancer (Ayyasamy et al., 2011; Su et al., 2012). In human colon cancer, UCP2 mRNA and protein expression reportedly is increased by factor of 3–4 as compared to peritumoral normal epithelium. In addition, UCP2 expression gradually increases during the colon adenoma-carcinoma sequence (Horimoto et al., 2004) and is higher in clinical stages III and IV colon cancer than in stage I and II (Kuai et al., 2010). Similarly, UCP4 expression has been shown to correlate with lymph node metastases in breast cancer (Gonidi et al., 2011) and UCP1 expression in prostate cancer with disease progression from primary to bone metastatic cancers (Zhau et al., 2011). Moreover, postmenopausal breast

tumors with low estrogen receptor (ER) alpha to ER beta ratios that associate with higher UCP5 expression and higher oxidative defense have a poor prognosis (Sastre-Serra et al., 2013). Finally, ectopic expression of UCP2 in MCF7 breast cancer cells has been demonstrated to enhance proliferation, migration and matrigel invasion *in vitro* and to promote tumor growth *in viv*o (Ayyasamy et al., 2011). Together, these observations suggest that UCPs may contribute to the malignant progression of tumor cells.

In addition to malignant progression, UCPs may alter the therapy sensitivity of tumor cells. In specimens of human ovarian cancers carboplatin/paclitaxel-resistant cancers showed decreased UCP2 protein abundances as compared to the sensitive ones (Pons et al., 2012). Likewise, progression-free and overall survival of patients with inoperable lung cancer who received cisplatin-based chemotherapy was higher when tumors expressed high levels of UCP2 as compared to tumors with low UCP2 levels (Su et al., 2012). A possible explanation of the latter observation is that especially in lung tumors with mutated p53, cisplatin elicits oxidative stress that induces pro-survival signaling. High UCP2 expression, however, diminishes cisplatin-evoked oxidative stress and, in turn, decreases the pro-survival signals (Su et al., 2012).

In lung cancer cell lines with wildtype p53, in contrast, down-regulation of UCP2 results in significantly increased paclitaxel-induced cell death (Su et al., 2012). Similarly, overexpression of UCP2 in a human colon cancer cell line has been shown to blunt topoisomerase I inhibitor CPT-11-induced accumulation of reactive oxygen species and apoptosis *in vitro* and to confer CPT-11 resistance of tumor *xeno*grafts (Derdak et al., 2008). In addition, in pancreatic adenocarcinoma, non-small cell lung adenocarcinoma, and bladder carcinoma cell lines IC_{50} values of the anticancer drug gemcitabine increase with intrinsic UCP2 mRNA abundance. Furthermore, UCP2 overexpression strongly decreases gemcitabine-induced mitochondrial superoxide formation and protects cancer cells from apoptosis (Dalla Pozza et al., 2012). Finally, metabolic changes including UCP2 up-regulation and UCP2-mediated uncoupling of oxidative phosphorylation have been demonstrated in multidrug-resistant subclones of various tumor cell lines (Harper et al., 2002). Similarly, in acute myeloid leukemia cells, UCP2 up-regulation has been shown to foster the Warburg effect (i.e., anaerobic glycolysis in the absence of respiratory impairment) (Samudio et al., 2008).

UCP2 expression is stimulated by co-culturing of these leukemia cells with bone marrow-derived mesenchymal stromal cells (Samudio et al., 2008). Other stimuli of UCP expression/activity are hydrogen peroxide as shown for UCP5 in colon cancer cells (Santandreu et al., 2009) and gemcitabine chemotherapy as reported for UCP2 in pancreatic, lung and bladder cancer cell lines (Dalla Pozza et al., 2012). Collectively, these data suggest that tumor cells may acquire resistance to chemotherapy by up-regulation of UCPs and lowering of the therapy-evoked mitochondrial formation of reactive oxygen species (Robbins and Zhao, 2011).

Accordingly, experimental targeting of UCPs has been demonstrated to sensitize tumor cells to chemotherapy *in vitro*. For instance, genipin-induced inhibition or glutathionylation of UCP2 sensitizes drug-resistant leukemia subclones to chemotherapy with menadione, doxorubicin, or epirubicin (Mailloux et al., 2010; Pfefferle et al., 2012). Likewise, UCP2 inhibition by genipin or UCP2 mRNA silencing strongly enhances gemcitabine-induced mitochondrial superoxide generation and apoptotic cell death of pancreatic, lung and bladder cancer cell lines (Dalla Pozza et al., 2012). Moreover, UCP2 inhibition has been reported to trigger reactive oxygen species-dependent nuclear translocation of GAPDH and autophagic cell death in pancreatic adenocarcinoma cells (Dando et al., 2013) Together, this suggests that targeting UCPs might be a promising strategy to overcome resistance to anti-cancer therapies in the clinic. Notably, in an acute myeloid leukemia cell line, the cytotoxicity of cisplatin has been proposed to be in part mediated by cisplatin-dependent down-regulation of UCPs (Samudio et al., 2008) suggesting that established chemotherapy regimes already may co-target UCPs.

It is tempting to speculate that UCPs may also confer resistance to radiotherapy. One could hypothesize that UCPs adapt the tumor cells to a "relatively radioprotected" hypoxic microenvironment by decreasing hypoxia-associated mitochondrial formation of reactive oxygen species. Such UCP function in hypoxia resistance has been demonstrated for a lung adenocarcinoma cell line (Deng et al., 2012). Notably, radiation induces up-regulation of UCP2 expression as shown in colon carcinoma cells (Sreekumar et al., 2001) and in a radiosensitive subclone of B cell lymphoma (Voehringer et al., 2000). On the one hand, this UCP2 up-regulation might facilitate radiation-induced apoptosis induction by accelerating the break-down of $\Delta\Psi_m$ as proposed by the authors of these studies. On the other hand, radiation-induced UCP2 upregulation might be radioprotective by lowering the radiation-induced burden of reactive oxygen species. As a matter of fact, multi-resistant subclones of leukemia cells show higher UCP2 protein expression, lower $\Delta\Psi_m$, lower radiation induced formation of reactive oxygen species and decreased DNA damage as compared to their parental sensitive cells (Harper et al., 2002).

In summary, UCPs suppress the formation of $O_2{\cdot}^-$, a byproduct of the mitochondrial respiration chain and a major source of oxidative stress. In some cancers UCPs in particular UCP2 are highly upregulated and may contribute to the reprogramming of the cell metabolism that results in chemoresistance (for review see Baffy, 2010; Baffy et al., 2011) or even radioresistance. Moreover, recent studies imply that UCP2 may repress p53-mediated apoptosis providing a potential new mechanism of how UCP2 contributes to cancer development (Robbins and Zhao, 2011).

Together, these observations suggest that ion transport processes are critically involved in evasion from radiation stress, and intrinsic or hypoxic radioresistance. Since ion transport-mediated radioresistance might underlie failure of radiotherapy, concepts which combine ion transport targeting with radiotherapy hold promise for new therapy strategies in the future. A summary of how ion transport can be harnessed for anticancer therapy and how these therapy strategies might be combined with radiotherapy is given in the next paragraphs.

TARGETING ION TRANSPORTS IN RADIOTHERAPY

An important reason for the study of ion transports in the context of radiotherapy is the possible translation of the acquired knowledge into anti-cancer therapy. Many pharmacological modulators

of ion transports are already in clinical use or currently tested in clinical trials (Wulff and Castle, 2010). Moreover, tumors often over-express certain types of transport proteins.

These proteins such as the transient receptor melastatin 8 (TRPM8) non-selective cation channel in prostate cancer have been used in clinical trials as tumor-associated antigen for anti-tumor vaccination (Fuessel et al., 2006). Tumor promoting inflammation and anti-tumor immune effects are evolving fields of preclinical and clinical research (Hanahan and Weinberg, 2011). Preclinical evidence supports the thesis that tumors have to develop immune-evading capacities in order to grow into macroscopic, clinically detectable lesions (Koebel et al., 2007; Teng et al., 2008). Possible mechanisms are the secretion of cytokines and chemokines by cancer and tumor stroma cells (Vianello et al., 2006; Shields et al., 2010), the priming of infiltrating T-lymphocytes toward immunosuppressive regulatory T-cells and the recruitment of myeloid-derived suppressor cells and tumor-associated macrophages (Tanchot et al., 2013; Oleinika et al., 2013). Irradiation of tumors has been shown to impair on the one hand the immunosuppressive action of the tumor and on the other to induce so-called "immunogenic" cell death within the tumor with translocation of calreticulin to the plasma membrane, release of HMGB1 or ATP (Formenti and Demaria, 2013). Preclinical studies showed a synergistic effect of irradiation and several immunotherapeutic approaches such as dendritic cell injection (Finkelstein et al., 2012), anti-CTLA-4-antibody (Grosso and Jure-Kunkel, 2013), and vaccines (Chakraborty et al., 2004). Interestingly, for combination with anti-CTLA-4 antibody a synergistic effect could only be demonstrated for fractionated but not for single-dose irradiation (Demaria and Formenti, 2012).

In addition, over-expressed transport proteins in tumors can be harnessed to target drugs, cytokines, or radioactivity to the tumor cells (Hartung et al., 2011). One example is the specific surface expression of ClC-3 Cl^- channels by glioblastoma (and other tumor entities) which suggests ClC-3 as an excellent and highly specific target for anti-glioblastoma therapy. Chlorotoxin which is a 36 amino acid-long peptide from the venom of the scorpion *Leiurus quinquestriatus* has been found to inhibit ClC-3 and to preferentially bind to the cell surface of a variety of human malignancies. This specificity probably comes from the highly affine binding of chlorotoxin to a lipid raft-anchored complex of matrix metalloproteinase-2, membrane type-I MMP, and transmembrane inhibitor of metalloproteinase-2, as well as ClC-3 (Veiseh et al., 2007). Ongoing clinical trials successfully used ^{131}I-labeled chlorotoxin as glioblastoma-specific PET-tracer (Hockaday et al., 2005) and for targeted radiation of glioblastoma cells (Mamelak and Jacoby, 2007). Due to the low surface expression of ClC-3 in normal tissue, chlorotoxin exhibits little or no affinity to normal cells (Lyons et al., 2002). If the *in vitro* and mouse data on radiation-stimulated glioblastoma migration reflect indeed the *in vivo* situation in glioblastoma patients, a clinical setting might be envisaged in which radiation-induced glioblastoma spreading is prevented by combining radiotherapy with chlorotoxin blockade of ClC-3 channels.

CONCLUDING REMARKS

Interdisciplinary approaches linking radiobiology with physiology brought about the first peaces of evidence suggesting a functional significance of ion transport processes for the survival of irradiated tumor cells. The few reports published up to now on this topic are confined to phenomena occurring in the plasma membrane due to the methodological restrictions of studying these processes in the membranes of mitochondria, endoplasmic reticulum, or nuclear envelope. Intracellular membrane transports, however, might similarly impact tumor cell radiosensitivity. This is suggested by the notion that intracellular Cl^- channel CLIC1 protein expression regulates radiosensitivity in laryngeal cancer cells (Kim et al., 2010). However, the molecular mechanisms underlying, e.g., radiation-induced transport modifications, or downstream signaling events are far from being understood. Despite all these limitations, our current knowledge already clearly indicates that the observed transport processes may be crucial for the survival of the tumor and, thus, are worthwhile to spend further and more effort in this field which might lead to new strategies for cancer treatment in the future.

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PIST (GOPC) modulates the oncogenic voltage-gated potassium channel $K_V10.1$

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Although crucial for their correct function, the mechanisms controlling surface expression of ion channels are poorly understood. In the case of the voltage-gated potassium channel $K_V10.1$, this is determinant not only for its physiological function in brain, but also for its pathophysiology in tumors and possible use as a therapeutic target. The Golgi resident protein PIST binds several membrane proteins, thereby modulating their expression. Here we describe a PDZ domain-mediated interaction of $K_V10.1$ and PIST, which enhances surface levels of $K_V10.1$. The functional, but not the physical interaction of both proteins is dependent on the coiled-coil and PDZ domains of PIST; insertion of eight amino acids in the coiled-coil domain to render the neural form of PIST (nPIST) and the corresponding short isoform in an as-of-yet unknown form abolishes the effect. In addition, two new isoforms of PIST (sPIST and nsPIST) lacking nearly the complete PDZ domain were cloned and shown to be ubiquitously expressed. PIST and $K_V10.1$ co-precipitate from native and expression systems. nPIST also showed interaction, but did not alter the functional expression of the channel. We could not document physical interaction between $K_V10.1$ and sPIST, but it reduced $K_V10.1$ functional expression in a dominant-negative manner. nsPIST showed weak physical interaction and no functional effect on $K_V10.1$. We propose these isoforms to work as modulators of PIST function via regulating the binding on interaction partners.

Keywords: potassium channels, $K_V10.1$, KCNH1, PIST, FIG, GOPC, trafficking

INTRODUCTION

The voltage gated potassium channel $K_V10.1$ is the founding member of the family of *ether à go-go channels* (Warmke and Ganetzki, 1994). The function of this ion channel in the central nervous system, where it is normally exclusively expressed, still remains elusive (Occhiodoro et al., 1998; Pardo et al., 1999). Ectopically expressed $K_V10.1$ induces a transformed phenotype, and the channel is expressed in 70% extra-cranial tumors (Hemmerlein et al., 2006; Mello De Queiroz et al., 2006; Ding et al., 2007a,b, 2008; Ousingsawat et al., 2007; Wadhwa et al., 2009; Agarwal et al., 2010; Asher et al., 2010). Although there is evidence pointing to an additional non-canonical, permeation-independent role of the channel in cancer initiation and growth (Downie et al., 2008; Chen et al., 2011), inhibition of channel function exclusively at the plasma membrane by a monoclonal antibody (Gomez-Varela et al., 2007) reduces tumor progression *in vivo*, indicating that the membrane population of the channel is very important for its oncogenic properties. However, the mechanisms regulating the expression of $K_V10.1$ at the surface are still unclear, although it is known that the membrane residence time of the channel is relatively short, because it is internalized at a rate of 2% per minute (Kohl et al., 2011), and that both rabaptin 5 and cortactin are important to determine the abundance of $K_V10.1$ at the plasma membrane at a given time(Herrmann et al., 2012; Ninkovic et al., 2012).

PIST (PDZ domain protein interacting specifically with TC10), also called GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein), CAL (CFTR-associated ligand) or FIG (fused in glioblastoma) was first described in 2001 as a coiled-coil and PDZ domain-containing protein that specifically interacts with the GTPase TC10 (Neudauer et al., 2001). Depending on one of its coiled-coil regions, PIST mainly resides in the Golgi apparatus, but can also co-localize with its binding partners at the plasma membrane (Charest et al., 2001; Yao et al., 2001; Wente et al., 2005b). Many studies have addressed the possible interaction partners of PIST, in order to unravel the function of this protein. Proteins known to be located at the *trans*-Golgi network (TGN) like Syntaxin 6 or Golgin160 are able to bind the coiled-coil region of PIST, pointing to an involvement of this protein in processes like TGN sorting and vesicle trafficking (Charest et al., 2001; Hicks and Machamer, 2005). A crystal structure of this domain of PIST is available (Shin et al., 2013). The ability of PIST to bind activated small GTPases like TC10 and Rab6a may serve as a mechanism controlling PIST function in response to signaling pathways, as shown for cystic fibrosis transmembrane conductance regulator (CFTR) (Neudauer et al., 2001; Cheng et al., 2005; Bergbrede et al., 2009). Most other interaction partners of PIST are reported to bind to its PDZ domain, among them CFTR itself (Cheng et al., 2002), the G-protein-coupled receptors *frizzled* 5 and 8 (Yao et al., 2001), the chloride channel CIC-3B (Gentzsch

et al., 2003), the β1-adrenergic receptor (He et al., 2004), the somatostatin receptor subtype 5 [SSTR5 (Wente et al., 2005b)], a metabotropic glutamate receptor [mGluR1a, (Zhang et al., 2008)] and Cadherin 23 (Xu et al., 2010). Several studies discuss that overexpression of PIST might lead to different surface expression patterns of some of these membrane proteins by holding them back in the Golgi (He et al., 2004; Wente et al., 2005b; Xu et al., 2010). A different and more detailed process is postulated for CFTR, where PIST enhances degradation by facilitating its targeting to lysosomes (Cheng et al., 2005). A fusion between PIST and the proto-oncogene ROS1 has been detected, initially in glioblastoma and subsequently in other cancer types (Charest et al., 2003; Birch et al., 2011; Gu et al., 2011; Suehara et al., 2012).

Relevant functions of PIST in the brain appear to be mediated by an alternatively spliced isoform (nPIST) that contains an eight amino acid insertion in the second coiled-coil region (Yue et al., 2002). This isoform is able to interact with Beclin1 over its coiled-coil domain and therefore was linked to autophagy in neurons of lurcher mice (Yue et al., 2002), although constitutive ion fluxes were able to induce cell death regardless of the nPIST-Beclin1 interaction (Nishiyama et al., 2010). Additionally, glutamate receptors are able to bind to the PDZ domain of nPIST over their extreme C-terminus (Yue et al., 2002; Cuadra et al., 2004). This interaction in concert with Stargazin has been shown to enhance synaptic clustering of AMPA receptors in hippocampal neurons (Cuadra et al., 2004).

In this study, we identified PIST as a new interaction partner of K_V10.1. We show physical as well as functional interaction between PIST and K_V10.1. We were also able to identify and clone two new isoforms of PIST, and provide evidence that the four isoforms of PIST differ in terms of binding to and regulation of K_V10.1.

MATERIALS AND METHODS

YEAST TWO-HYBRID

The yeast reporter strain L40 (Vojtek et al., 1993) (*MATa, trp1, leu2, his3, LYS::lexA-HIS3, URA3::lexA-lacZ*) was transformed with pLexN-hEag1 by the lithium acetate method and grown on synthetic medium lacking tryptophan. After additional transformation with plasmid pVP16-3-cDNA [postnatal 8 rat brain cDNA library, (Okamoto and Sudhof, 1997)], double transformants were plated on synthetic medium lacking histidine, leucine, uracil, lysine, tryptophan and in the presence of the competitive inhibitor of the HIS3 protein 3-amino-1,2,4-triazole (3-AT). Positive colonies were picked after 4–6 days and tested for ß-galactosidase activity using plate assay. Plasmids from positive clones were rescued and transformed in *E. coli* strain HB101. *E. coli* cells were plated on leucine-lacking medium. Positive clones were further analyzed by yeast retransformation and DNA sequencing.

RNA PURIFICATION, cRNA SYNTHESIS AND RT-PCR

HEK 293 cells were washed 3 times with ice cold PBS, harvested and directly used for RNA purification using RNAeasy Mini Kit (Qiagen). First strand cDNA was produced using SuperScript (Invitrogen) with oligo-dT. For cloning, PIST was amplified in a standard PCR reaction using Taq DNA polymerase (NEB) and sense and antisense primers (see below). PCR products were analyzed by electropohoresis, fragments excised out of gel and purified using NucleoSpin Extract (Macherey-Nagel) and subcloned in pGEMT-easy (Promega) following manufacturer's instructions.

Mouse first strand cDNA was a generous gift from Dr. R. Ufartes (Ufartes et al., 2013).

To distinguish between isoforms, PCR products were obtained in a standard PCR procedure using 100 ng cDNA mouse first strand cDNA, and a set of 2 or 3 specific primers in the appropriate combinations to distinguish between isoforms (see below) and Biotherme Polymerase (Genecraft).

Primers for human PIST (accession numbers NM_001017408.2—PIST- and NM_020399.3—sPIST-) were:

5′ ATGTCGGCGGGCGGTCCATGC3′ and

5′TTAATAAGATTTTTTATGATACAGAG3′

For mouse PIST (accession numbers NM_001199272.1 and NM_053187.3):

5′GCAGAGGGCGCAACGACTT3′ and

5′ATTCTCATGCGCATCCCTCACTG3′

For selective amplification of the previously described transcript variants, we used different forward primers (nPIST; NM_020399.3: 5′ CAAGGCAAAATTGTCTGTCCAC 3′; PIST, NM_001017408.2: 5′ CAAGGCAAAATTGGAAAGAGAAC 3′), and to distinguish between short and long forms, the following antisense primers were used:

Short 5′ CTCTCTGCTGGGAGAGTATAG 3′

Long 5′ CTCTCCTCTCTGTGTGATAGA 3′

CELL CULTURE AND TRANSFECTION

HeLa cells were cultured in MEM+GlutaMax (Invitrogen) supplemented with 10% FCS (PAA), HEK293 cells in DMEM/F12+GlutaMax (Invitrogen) supplemented with 10% FCS and for stable cell line HEK293-K_V10.1-BBS [HEK-BBS, (Kohl et al., 2011)] with Zeocin (Invitrogen) at 5% CO_2 and 37°C.

Transfection was performed using Lipofectamine 2000 or Lipofectamine (Invitrogen) according to the manufacturer's instructions. K_V10.x-BBS Venus and CFP-PIST were generated by cloning into pcDNA3 or pECFP-N1 vectors. Empty vectors were used as controls.

FRACTIONAL LABELING, QUANTIFICATION AND PURIFICATION OF K_V10.1-BBS

Labeling of whole-cell K_V10.1-BBS was performed in cell lysates in buffer LP [20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% Nonidet P40, protease inhibitors (Roche)] with α-bungarotoxin (BTX)-biotin conjugate (Invitrogen) in a final concentration of 0.2 μg/ml for 30 min on ice. To detect membrane and/or internalized K_V10.1-BBS, transfected HEK-BBS cells were incubated in media supplemented with α-BTX-biotin conjugate (Invitrogen) in a final concentration of 2.5 μg/ml and kept at room temperature for 10 min (membrane) or at 37°C for one hour (internalized). For internalized K_V10.1-BBS, cells were washed with ice-cold acid wash buffer (150 mM NaCl, pH 3.0) for 3 min to remove membrane labeling of K_V10.1-BBS. Twice washing with cold PBS

removed residual α-BTX-biotin conjugate. Cells were then harvested and lysed with LP buffer for 20 min on ice. Insoluble fraction was removed by centrifugation at 18,000 × *g* at 4°C, and the supernatant used for ELISA or pull down experiments.

For pull down approaches, labeled K_V10.1-BBS was bound on streptavidin-coated magnetic beads (T1, Invitrogen) for 2 h at 4°C. Unbound protein was removed by washing 5 times with buffer LP supplemented with protease inhibitors (Roche). Bound protein was eluted at 70°C for 10 min using LDS sample buffer containing reducing agent (Invitrogen) and analyzed by SDS PAGE (Invitrogen) and western blotting.

Quantification of the amount of labeled K_V10.1-BBS was performed by ELISA. After labeling, total cell lysates (30 and 150 μg protein), were immobilized on streptavidin-coated plates (Pierce) and detected using a C-terminal monoclonal anti-K_V10.1 antibody [Ab33, 5 μg/ml (Hemmerlein et al., 2006)] and a polyclonal anti-mouse secondary antibody (Pierce, 1:500) coupled to peroxidase. ABTS (Invitrogen) was used as a substrate for development and detected in a Wallac Victor2 reader at 405 nm (reference 490 nm). Experiments were performed in duplicates.

PULL DOWN EXPERIMENTS

For immunoprecipitation, rat brain lysates (800 μg total protein) were incubated overnight at 4°C with 2–5 μg of antibody [anti-K_V10.1 33/62 (Hemmerlein et al., 2006); anti-PIST (Millipore); non-specific mouse IgGκ2b) in buffer A (0,5% Triton X-100, 25 mM TrisHCl pH7.5, 75 mM NaCl, 2.5 mM EDTA and protease inhibitors (Roche)]. Pull down was performed by adding 20 μl of Protein G/A-coated Sepharose beads (Calbiochem) for 2 h at 4°C under rotation. After washing five times with buffer A, bound protein was eluted at 70°C for 10 min using LDS sample buffer containing reducing agent (Invitrogen) and analyzed by SDS PAGE (Invitrogen) and western blotting.

ELECTROPHYSIOLOGY

Recordings on *Xenopus* oocytes were performed as described by Stuhmer (1998) using a Turbo TEC-10CD amplifier (NPI electronics). cRNA was synthesized using the mMessage mMachine kit (Ambion) according to the manufacturer's instructions. 0.1–1 ng cRNA per oocyte were microinjected 1–3 days prior to recording. Cells were kept at 18°C in ND 96 solution (96 mM NaCl, 2 mM KCl, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Hepes/NaOH, pH 7.5) supplemented with theophylline (0.5 mM) to avoid maturation of the oocytes. For voltage clamp recordings, pipettes had resistances ranging from 0.5 to 1.2 MΩ when filled with 2 M KCl. External solution (NFR) contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM Hepes/NaOH, pH 7.2. Current was digitized at 5 kHz and filtered at 1 kHz. Currents were elicited by 1 s depolarizations from a holding potential of −100 mV to values ranging from +80 to −100 mV. Current amplitude was determined as mean value in the last 200 ms of the depolarization. To fit current-voltage relationships, we used an equation of the form:

$$I(V) = \Gamma \cdot \frac{(V - V_{rev})}{\left(1 + e^{\frac{V_{50}-V}{k}}\right)^4}$$

where Γ is the total conductance, V_{rev} the reversal potential, V_{50} the potential of half activation per subunit and *k* the slope factor.

Data acquisition and analysis was performed with PulsePulseFit (HEKA Electronics) and IgorPro (WaveMetrics).

MICROSCOPY

For confocal microscopy, HeLa cells were grown on glass coverslips and transfected with Venus-tagged K_V10.1 and CFP-tagged isoforms of PIST. Empty vectors served as control. 24 h after transfection, the Golgi marker BODIPY FL C_5 Ceramide (Invitrogen) was used according to manufacturer's instructions to visualize Golgi apparatus. Cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, washed thoroughly with HBBS and mounted with ProLong Gold Antifade Reagent (Invitrogen). Image acquisition was performed using a Zeiss LSM 510 Meta confocal microscope with a Zeiss 40x 1.2 Korr water immersion objective. Single channel gray scale images are presented using color tables according to the fluorophore used. Merged images were generated by combination of the channel images into a single RGB file. Only linear contrast enhancement and Gaussian blur were applied off-line to the images; analysis was carried out with Fiji (Schindelin et al., 2012).

RESULTS

PIST INTERACTS WITH THE VOLTAGE-GATED ION CHANNEL K_V10.1

Finding new interaction partners of K_V10.1is crucial to unravel the role of this ion channel both in the healthy brain and in tumors. To identify candidate interaction partners of K_V10.1, we performed yeast two-hybrid screens using a rat brain expression library. The C-terminus of K_V10.1 in fusion to the DNA binding domain of LexA transcription factor was used as a bait to screen a rat brain cDNA library fused to the activation domain of VP16 transcription factor. As previously reported (Ninkovic et al., 2012), out of 2 million screened clones, eight were positive for the HIS3 marker. One of them encoded most of the open reading frame of the murine PIST, including its PDZ domain (Neudauer et al., 2001).

We then performed co-immunoprecipitation studies to confirm the interaction. Precipitation of PIST using a specific antibody was able to pull down K_V10.1 out of rat brain lysates (**Figure 1A**). In reverse co-IP experiments, PIST co-precipitated K_V10.1 (**Figure 1B**). Non-specific isotype antibodies did not precipitate either of the two proteins.

In a second pull-down approach we used HEK293 cells stably expressing a modified K_V10.1 bearing an α-bungarotoxin (BTX) binding site [K_V10.1_BBS, (Kohl et al., 2011)]. Specific binding of a BTX-biotin conjugate to its binding site allows precipitation of K_V10.1_BBS from cell lysates. After transfection with CFP-labeled PIST, pull down of K_V10.1_BBS with BTX-biotin and immunoblotting using anti-PIST antibodies revealed two bands compatible with endogenous PIST as well as CFP-labeled PIST (**Figure 1C**, lane +Bb); no bands were detected if BTX-biotin was omitted (**Figure 1C**, lane −Bb). The fact that both endogenous and CFP-tagged PIST are detected argues in favor of the specificity

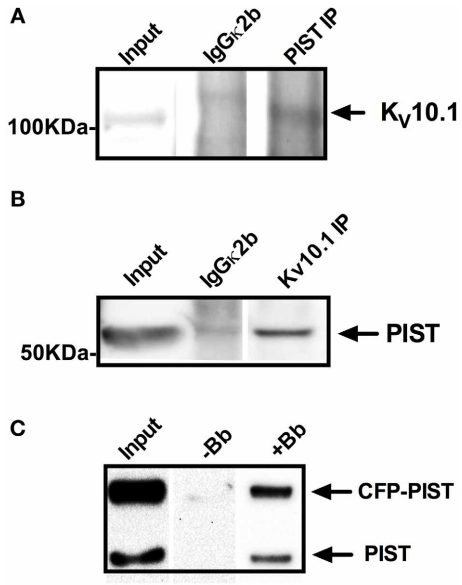

FIGURE 1 | Co-precipitation of K_V10.1 and PIST out of (A,B) rat brain lysates and (C) HEK293_K_V10.1_BBS cells. Specific antibodies against PIST **(A)** or K_V10.1 **(B)** were used for precipitation out of rat brain lysates using Protein A/G coupled sepharose beads. Isotype control antibodies served as negative controls. Co-precipitation of Kv10.1 by anti PIST immunoprecipitation **(A)** as well as precipitation of PIST by anti K_V10.1 immunoprecipitation **(B)** were detected by western blot. **(C)** K_V10.1_BBS was labeled with an α-bungarotoxin-biotin conjugate after lysis of HEK293_K_V10.1_BBS cells and pulled down with streptavidin-coated beads. Detection on a western blot with and anti-PIST antibody shows bands compatible with precipitation of both CFP-tagged and endogenous PIST.

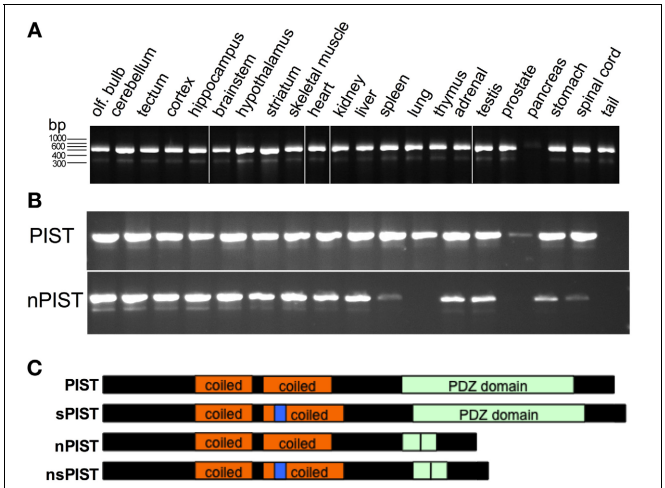

FIGURE 2 | New PIST isoforms. (A) PCR on cDNA from different mouse tissues show amplification of short (205 bp) and a long (408 bp) fragments corresponding to sPIST and PIST. **(B)** A PCR designed to differentiate neuronal and non-neuronal forms of PIST detect both short and long bands revealing the widely extended expression of both forms. **(C)** Schematic representation of the domains of all four PIST isoforms. Coiled-coil regions are depicted in orange, the insertion in n-PISTs in the second coiled-coil is represented in blue. The PDZ domain is represented in green, and a vertical line represents the site of deletion of exon 6.

of PIST detection. As a control, the same experiments using unlabeled K_V10.1_BBS did not precipitate PIST, also indicating specificity of the co-IP. Taken together, these results strongly indicate that PIST and K_V10.1 interact physically both *in vitro* and *in vivo*.

TWO NEW SHORT PIST ISOFORMS LACKING THE PDZ DOMAIN ARE EXPRESSED IN ALL TISSUES TESTED

For further analysis we amplified and cloned the human PIST out of cDNA obtained from untransfected HEK293 cells, using 5′ and 3′ flanking primers. Interestingly, during the cloning process we systematically observed two bands, one of them at the expected size of full length PIST and a shorter one. Cloning and subsequent sequencing of the shorter band revealed that it corresponds to a PIST variant lacking the complete exon 6, which is the region coding for the majority of the PDZ domain indicating that this clone corresponds to a new splice variant of PIST. To further proof the existence of the new isoform, we designed primers to generate an amplicon containing the skipped exon, in order to discriminate between the longer and the shorter form of PIST. We were able to amplify fragments with the expected sizes out of cDNA from HEK293 cells (data not shown). To evaluate the tissue distribution of the splice variant we used mouse cRNA from different tissues. The variant (lacking in the mouse exon 5) was detected in all tissues tested except pancreas, where amplification of full-length PIST was also much weaker (**Figure 2A**). The signals for the short form were consistently weaker than those for full-length PIST.

In 2002, a second isoform of PIST was cloned and termed "neuronal PIST" (nPIST) because it is believed to be the main form in the brain. It differs from PIST by an 8 amino acid insertion in the coiled-coil region (Yue et al., 2002). The sequence we identified here codes for a protein that lacks the main part of the PDZ domain but bears the 8 amino acids of the neuronal form. We therefore asked if there is a fourth isoform, lacking the PDZ as well as the 8 amino acid insertion. PCR with primer pairs distinguishing between nPIST and PIST and producing different fragment length depending on the presence of the alternatively skipped exon was used to answer this question. Amplification of the expected fragments out of cDNA from HEK293 cells as well as from different murine tissues indicated that all four isoforms are expressed in nearly every tissue (**Figure 2B**). Weak or absent expression of nPIST in liver and pancreas correlated with no detection of the short form (nsPIST) of it, indicating that expression of the shorter form depends on the expression of the corresponding longer one (**Figure 2B**). The cartoon in **Figure 2C** depicts the domain structure of the four PIST isoforms. Taken together, our data suggests that two new short forms of PIST (from here on termed sPIST and nsPIST) exist and are expressed in nearly every tissue. According to our semi-quantitative approach, both seem to be less abundant than their corresponding longer form. sPIST and nsPIST sequences have been deposited under accession numbers KF420122 and KF420123, respectively.

SHORT AND LONG ISOFORMS OF PIST HAVE DIFFERENT CAPABILITIES FOR BINDING TO K_V10.1

PIST is known to form homodimers depending on the second coiled-coil motif, which bears also a leucine zipper (Neudauer et al., 2001). As this region is intact in every identified isoform, we tested if the shorter variants can form heterodimers with the longer ones. To this end, we transfected CFP-labeled PIST constructs and used a GFP-specific antibody to immunoprecipitate the extracts that were subsequently blotted using a PIST-antibody. Precipitation of CFP-tagged PIST and nPIST co-precipitated a band compatible with endogenous PIST (or nPIST), strongly suggesting that dimerization of PIST occurs in our system (**Figure 3A**). The major band that co-immunoprecipitated with CFP-tagged nsPIST was smaller than the one pulled by full-length forms and compatible with the size of an endogenous short isoform, suggesting that the short isoforms (or at least nsPIST) do occur at the protein level *in vivo*. Expression of sPIST-CFP was not clearly detectable even after immunoprecipitation, rendering interpretation of the results difficult with this isoform, even though an endogenous band was detected. We therefore excluded sPIST from transfection-based experiments and concentrated on the other three variants.

We cannot exclude formation of heterodimers between nPIST and PIST isoforms, because HEK293 cells express all four isoforms of PIST and in this approach we cannot resolve the eight residues difference between PISTs and nPISTs.

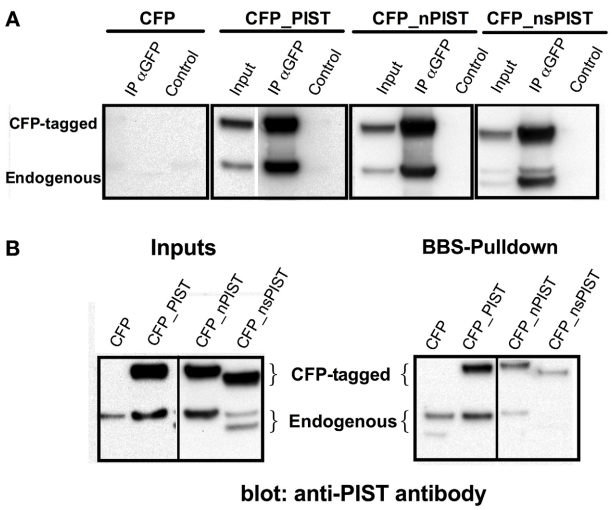

FIGURE 3 | (A) Heteromerization of long and short forms of PIST. **(A)** GFP antibody was used to precipitate CFP-tagged PIST isoforms out of lysates of transfected HEK293 cells. PIST isoforms were detected using a polyclonal antibody against PIST. Bands compatible with both the GFP-tagged forms and with endogenous PIST were detected, indicating association between the transfected CFP-PIST and the endogenous one. **(B)** Co-precipitation of PIST isoforms with K_V10.1_BBS. Lysates of KV10.1_BBS-expressing cells were labeled with an α-BTX-biotin conjugate and pulled down with streptavidin-coated beads (see **Figure 1**). Anti-PIST specific antibodies detected co-immunoprecipitation of bands compatible with the corresponding CFP-tagged form, together with an endogenous PIST band.

We next tested the different isoforms for their capability to bind K_V10.1. Precipitation of K_V10.1_BBS out of stably expressing HEK293 cells was able to co-precipitate over-expressed CFP-tagged PIST and nPIST (**Figure 3B**). In comparison to the long forms, co-precipitation of the tagged short isoform CFP_nsPIST was much less evident in this assay (**Figure 3B**). Since the short forms lack most of the PDZ domain, these data suggest an involvement of the PDZ domain of PIST in K_V10.1 binding. Interestingly, overexpression of the long isoforms resulted in higher intensity in bands compatible with the endogenous full-length PIST, while the shorter isoform rendered more intense bands corresponding to short isoforms (**Figure 3B**).

PIST INFLUENCES CURRENTS MEDIATED BY K_V10.1, BUT NOT BY K_V10.2

We next tested if there are functional consequences of the interaction between PIST and K_V10.1. As a model we used voltage clamp recordings in the *Xenopus* oocyte expression system and checked if the overexpression of any PIST isoform can influence K_V10.1-mediated current. Regardless of the concomitant overexpression of human PIST isoforms, macroscopic K_V10.1 currents showed very similar voltage dependence (**Figure 4**); in fact, the current-voltage relationship could be fitted using the same parameters used for K_V10.1 alone (**Figure 4B**). We did not appreciate differences in kinetics, and the main properties of K_V10.1 were conserved in the presence of PIST isoforms (not shown).

The total current amplitude, however, was influenced by PIST expression (**Figures 4A,C**). Over-expression of PIST was able to nearly double the amplitude of K_V10.1 mediated current ($189 \pm 33\%$ of control levels). The difference was more significant the more depolarized the stimulus (reaching $p < 0.0001$ at $+80$ mV and above). Injection of nPIST and nsPIST did not affect the amplitude. These observations are compatible with an influence of PIST on the membrane expression of K_V10.1, similar to the one described for CFTR (Cheng et al., 2005). In the presence of PDZ and coiled-coil domain, the amplitude of K_V10.1 currents was increased. We observed no functional interaction with the insertion in the coiled-coil of "n" forms.

We previously stated that very poor and irregular expression of sPIST did not allow us to document physical interaction between this isoform and K_V10.1. However, since experiments in oocytes do not depend on transfection or transcription, which is performed *in vitro* previous to oocyte injection, we also tested if injection of synthetic cRNA encoding for sPIST affects the amplitude of the K_V10.1-mediated current. Surprisingly, sPIST coinjection induced a reduction in current amplitude to $55 \pm 4\%$ of control levels. Although they had opposite effects, coinjection of a mixture of PIST and sPIST together with K_V10.1, did not results on compensation of the effects, but rather sPIST showed a dominant-negative effect, in such a way that the current was reduced to the same extent as when sPIST was injected alone (**Figure 4D**).

A Golgi-resident protein like PIST might have a general effect on the expression level of ion channels in the oocyte expression system, enhancing transport mechanism toward the membrane. As a measure for the specificity of our observations, we tested the effect of PIST overexpression on K_V10.2, the closest relative of

K_V10.1. PIST overexpression did not change voltage-dependence or kinetic parameters of K_V10.2. In contrast to K_V10.1, there was also no change in current amplitudes of K_V10.2-mediated currents (**Figure 4E**), pointing toward a highly specific effect of PIST expression on K_V10.1, but not K_V10.2 current amplitude.

CELLULAR DISTRIBUTION OF K_V10.1 CHANGES IN RESPONSE TO PIST OVEREXPRESSION

Having shown that PIST is able to interact physically and functionally with K_V10.1, we next asked if both proteins co-localize *in vivo*. PIST is reported to reside mainly in the Golgi apparatus, depending on at least one coiled-coil region, but can also be pulled to the membrane in co-expression with its interaction partners (Charest et al., 2001; Yao et al., 2001; Wente et al., 2005b). To perform subcellular localization studies, we used CFP-tagged forms of PIST. As described in the literature, the longer forms of PIST were mainly located in the Golgi, the neuronal form being slightly more diffuse (**Figures 5**, **6A**). nsPIST localized to discrete areas of the Golgi.

Co-expression of a Venus-tagged K_V10.1 led to a slight change in the localization of PIST only, a fraction of which showed expression at the membrane; this also resulted in a clearer membrane localization of K_V10.1 itself. Expressed alone, K_V10.1_Venus showed a typical distribution of this ion channel in the cell, where most K_V10.1 stays intracellular and only weak expression can be seen at the membrane (**Figure 6B**). PIST coexpression led to peripheral venus signal compatible with increased channel localization at the plasma membrane (**Figures 6C,D**). Overexpression of nPIST as well as its short form nsPIST did not change K_V10.1 localization in the cell (not shown). This data further supports the idea that a trafficking effect of PIST might be involved in the regulation of macroscopic current amplitude.

PIST CHANGES MEMBRANE EXPRESSION OF K_V10.1

To get more quantitative data we made use of another system recently established in our lab, which is based on K_V10.1 bearing an α-bungarotoxin (BTX) binding site between transmembrane segments 3 and 4 (K_V10.1_BBS). Different labeling techniques with an α-BTX-biotin conjugate allow to distinguish between whole cell, membrane bound and internalized K_V10.1 out of stably expressing cells and to quantify this different fractions via ELISA (Kohl et al., 2011). Specific labeling of membrane K_V10.1_BBS is possible by incubating intact cells stably expressing K_V10.1_BBS at low temperature (to avoid internalization) with an α-BTX-biotin conjugate. Quantification of

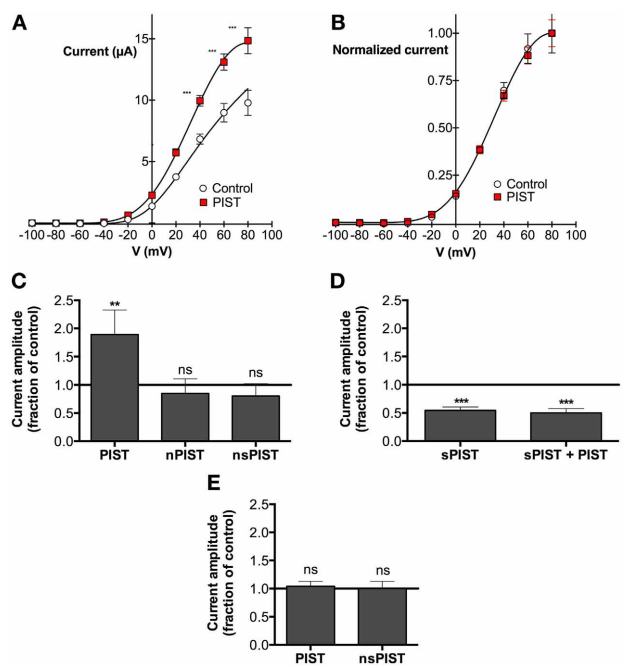

FIGURE 4 | PIST influences K_V10.1 function. (A) Current-voltage relationship of K_V10.1 expressed in *Xenopus* oocytes with or without PIST. Current amplitudes were significantly larger in oocytes coinjected with PIST ($N = 4$; $^{**}p < 0.01$; $^{***}p < 0.001$). **(B)** After normalization to the current value at +100 mV, the current-voltage plots overlap, indicating that the voltage-dependence of the channel is not altered by PIST. **(C)** Relative current amplitude (at + 80 mV) in oocytes co-expressing PIST isoforms with respect to K_V10.1 alone ($N = 19$). PIST ($N = 18$) induced a significant increase ($p < 0.05$), while nPIST ($N = 10$) and nsPIST ($n = 14$) did not alter current amplitude. **(D)** Co-injection of sPIST reduced the current amplitude both in the absence ($N = 8$) and in the presence ($N = 8$) of PIST. **(E)** PIST expression did not affect K_V10.2 current amplitude.

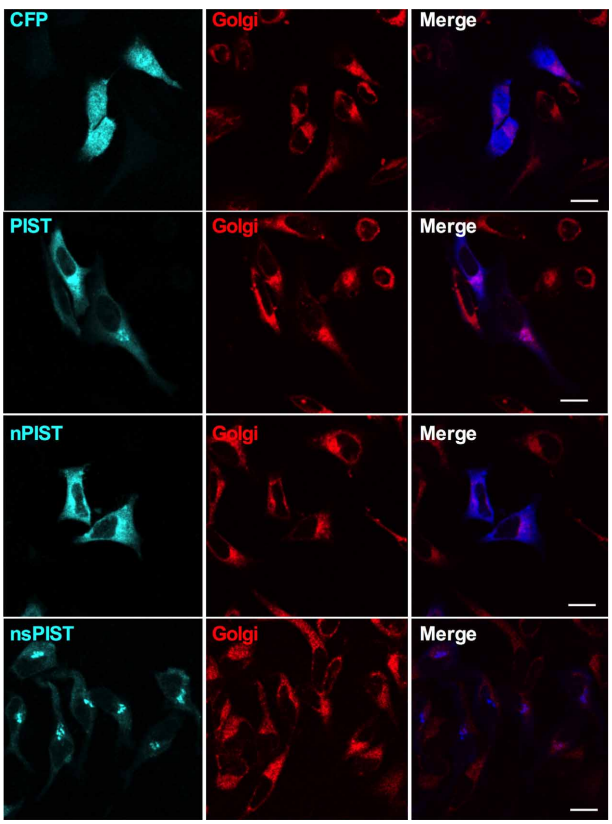

FIGURE 5 | Single confocal slices of cells expressing CFP-tagged PIST isoforms (blue). The Golgi apparatus was stained with BODIPY-C5 ceramide (red). Scale bar, 10 μm.

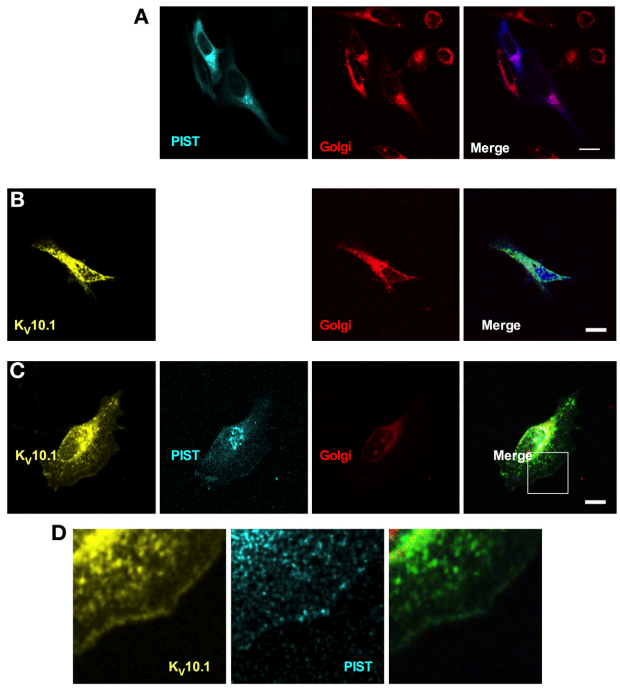

FIGURE 6 | Cellular distribution of K_V10.1_Venus and CFP-tagged PIST. CFP-PIST, when expressed alone **(A)**, reproduced from **Figure 5**, gave a diffuse distribution with preferential location to the Golgi (stained in red). Overexpressed K_V10.1 showed a preferentially intracellular distribution **(B)**. When co-expressed **(C)**, both CFP-PIST and K_V10.1 were detectable at the cell periphery, compatible with an increase of membrane localization of both proteins. **(D)** represents a larger magnification of the area represented by a white square in the "merged" panel in **(C)**, Scale bar: 10 μm.

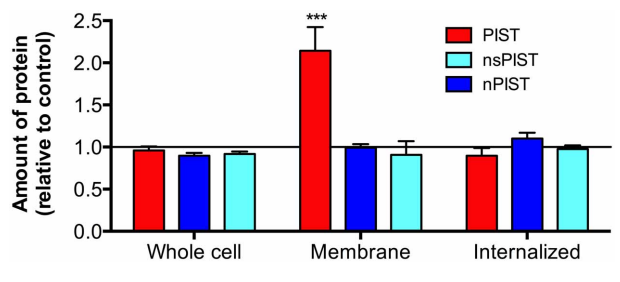

FIGURE 7 | Over-expression of PIST isoforms differently influences cellular fractions of K_V10.1_BBS. Different labeling protocols allowed distinguishing between whole cell, membrane bound and internalized K_V10.1_BBS by ELISA (see Materials and Methods). The fraction of membrane bound K_V10.1_BBS, labeled on living cells, was clearly enriched by expression of PIST but not of the other isoforms ($N = 3$; $^{***}p < 0.001$).

this fraction of membrane bound K_V10.1_BBS revealed that the overexpression of CFP-tagged PIST can indeed increase surface K_V10.1_BBS to 214 ± 28% of control levels (**Figure 7**). In agreement with findings in the oocyte expression system, the neuronal forms of PIST (nPIST and nsPIST) did not have any effect on membrane K_V10.1_BBS, as values were not different (94 ± 15% nPIST, 91 ± 16% nsPIST) from control levels.

Both increased expression levels and increased stability of plasma membrane K_V10.1_BBS could explain this observation. To distinguish between both possibilities, we examined the effect of overexpressing PIST isoforms on whole cell K_V10.1_BBS, labeled after lysis of the cells. Neither isoform of PIST had an effect on the amount of total K_V10.1_BBS (**Figure 7**), giving values of 96 ± 5% (PIST), 92 ± 3% (nsPIST) or 102 ± 9% (nPIST) in comparison to control levels, indicating that neither isoform of PIST affect total expression levels of K_V10.1_BBS. Therefore, they may be involved in the transport of this voltage gated ion channel to and from the plasma membrane.

The K_V10.1_BBS system easily allows for the quantification of internalized K_V10.1_BBS within a given time frame (Kohl et al., 2011). For this, we incubated living cells with α-BTX-biotin conjugate at 37°C and allowed internalization for one hour. Removal of the label of membrane-bound K_V10.1_BBS by acid wash afterwards leaves only the internalized labeled K_V10.1_BBS for quantification. Interestingly, neither isoform had an effect on the total amount of internalized K_V10.1_BBS (PIST 90 ± 9%, nsPIST 92 ± 13%, nPIST 98 ± 8%). As membrane expression of K_V10.1_BBS is clearly increased in response to PIST overexpression (**Figure 7**), one would expect more channels internalized if the rate of internalization is not affected by this protein.

DISCUSSION

In this study we identified PIST as a binding partner of K_V10.1 and cloned two new isoforms of PIST. The novel variants lack a significant fraction of the PDZ domain and are expressed in all tissues examined, although with lower abundance than their respective longer forms. The shorter form was also detected at the protein level in HEK293 cells (**Figure 3**). The new isoforms correspond to skipping of exon 6 in the human gene, encoding for most of the PDZ domain. Interaction of K_V10.1 and PIST appears to require the PDZ domain of the latter, since deletion of this domain reduced binding in pull-down assays. On the channel side, the C-terminus of the protein was the part that showed interaction with PIST in a yeast two-hybrid screen, and therefore is a probable mediator of the interaction. However, the interaction might need an additional binding partners, because pull down experiments using recombinant purified proteins (K_V10.1 C-terminus and PIST) were unsuccessful (not shown).

We found K_V10.1-mediated current to be influenced by the overexpression of PIST in *Xenopus* oocytes. This effect is apparently not due to changes in the electrophysiological properties of K_V10.1 but rather due to a change in the surface expression of the channel, which was clearly enhanced by PIST as indicated by surface-labeling experiments. Binding of endogenous PIST was enhanced by overexpression of CFP-tagged PIST, leading to the idea that binding gives a positive feedback that stabilizes or improves association of both proteins *in vivo*.

Forced expression of the short isoform of PIST (sPIST) acts as a dominant negative in *Xenopus* oocytes, in the sense that leads to reduction of functional expression of K_V10.1 (**Figure 4D**). These data are in good agreement with previous observations regarding the interaction of PIST with AMPA receptors (Cuadra

et al., 2004). Although binding of several proteins to the coiled-coil region of PIST is reported, functional consequences have only been described through interaction with the PDZ domain (Cuadra et al., 2004; He et al., 2004; Cheng et al., 2005; Wente et al., 2005a; Xu et al., 2010). Deletion of the PDZ domain results in a dominant negative effect in AMPA receptor clustering (Cuadra et al., 2004). This led to the hypothesis that the short forms of PIST might serve as negative regulators of PIST function, in good agreement with our observations in oocytes.

nPIST, which differs from PIST by an insertion of eight amino acids, was not able to regulate $K_V10.1$ in our model systems. Since the insertion occurs in the coiled-coil region, the lack of regulation by nPIST indicates that, although the physical interaction with $K_V10.1$ depends on the PDZ domain, the functional effect requires additionally the coiled-coil region of PIST. This raises the question if and how the surface expression of $K_V10.1$, which is normally expressed exclusively in the central nervous system (Pardo et al., 1999), is controlled by nPIST and PIST *in vivo*. We cannot exclude from our data that nPIST might also control surface levels of $K_V10.1$ *in vivo* because, as already mentioned, it is likely that additional partners are required the functional interaction of nPIST and $K_V10.1$. These additional factor(s) could be different for nPIST and PIST, and its (their) absence might preclude an nPIST-mediated effect in our systems. Our results point to an at least much more efficient influence of PIST compared to nPIST in positively affecting $K_V10.1$ surface expression. This implies that, in tumors outside the brain, the surface expression of ectopically expressed $K_V10.1$ would be additionally boosted by the expression of PIST.

From our data, we can only speculate about the mechanism by which PIST can influence the surface expression of $K_V10.1$. However, we can exclude mechanisms involving an overall increased amount of $K_V10.1$, induced either by enhanced synthesis or decreased degradation of the ion channel, since we observed no changes in total $K_V10.1$ in cells overexpressing PIST.

Although there are apparently more active channels at the membrane, the amount of internalized $K_V10.1$ is not altered by overexpression of PIST, indicating that PIST induces a more efficient transport of $K_V10.1$ to the plasma membrane. However, this interpretation might be an oversimplification, because it would require that the amount of $K_V10.1$ channels at the plasma membrane does not influence the rate of internalization, which is counterintuitive. Trafficking of $K_V10.1$ is not yet completely understood. We have recently described Rabaptin 5 (RABEP1) as an interaction partner of $K_V10.1$, linking this ion channel to early endosomes (Ninkovic et al., 2012). As an early event in the maturation and function of early endosomes Rabaptin 5 is able to bind and stabilize the GTP form Rab5, which then recruits effector proteins (Stenmark et al., 1995; Lippe et al., 2001). PIST interacts with Syntaxin 6, a member of the Syntaxin family of SNAREs that is also a Rab5 effector (Charest et al., 2001). Syntaxin 6, together with Syntaxin 13, VTI1a (Vps10p tail interactor 1) and VAMP4 were identified as the SNARE machinery involved in homotypic fusion of early endosomes to where it is recruited by direct binding of early endosomal antigen-1 (Simonsen et al., 1999; Brandhorst et al., 2006; Zwilling et al., 2007). PIST could therefore interfere with this machinery to block $K_V10.1$ internalization. Inhibition of internalization processes by overexpression of a dominant-negative point mutant of Rab5(S34N), shown to block endocytosis (Li and Stahl, 1993), drastically reduced the amount of internalized $K_V10.1$, but was not able to increase its surface expression significantly (data not shown). If PIST only impairs endocytosis of $K_V10.1$, we therefore would not expect such a high increase in its surface expression as seen here.

PIST is also involved in sorting mechanisms, facilitating the trafficking of CFTR to lysosomes when over-expressed (Cheng et al., 2002, 2004, 2005; Gentzsch et al., 2003). Moreover, the Rho GTPase TC10 can bind to PIST and modulate this effect (Neudauer et al., 2001; Cheng et al., 2005). Another example of PIST function is the enhancement of synaptic clustering of AMPA receptors in hippocampal neurons in response to overexpression of the neuronal form of PIST (Cuadra et al., 2004). Deletion of the PDZ domain served as dominant negative form of nPIST clearly reducing AMPA clustering at the membrane (Cuadra et al., 2004). Endosomal sorting of AMPA receptors beginning in early endosomes towards either recycling or late endosomes is well documented to be a main process in long term synaptic plasticity in hippocampal neurons [reviewed in Hanley (2010)]. Therefore nPIST, like PIST for CFTR, might be involved in sorting processes for AMPA receptors, favoring recycling.

A similar effect might be postulated for the $K_V10.1$-PIST interaction. PIST may serve as a molecular switch to control the fate of the channel after internalization in early endosomes by enhancing its recycling. This would lead to an increased membrane expression of the channel, while the amount of internalized $K_V10.1$ would be reduced, leading in our case to equal amounts of internalized $K_V10.1$ despite drastic changes in the membrane expression. Another interaction partner of PIST might regulate this process by binding to the coiled-coil region of PIST, like TC10 does for CFTR (Cheng et al., 2005). Candidate proteins for this interaction are small Rho GTPases like TC10 or Rab proteins, as Rab6a was shown to bind PIST (Bergbrede et al., 2009). The insertion of eight amino acids in the neuronal form of PIST lies in the region putatively responsible for this interaction, leading to the hypothesis that the isoforms can be regulated by different binding partners. These binding partners might decide if a protein bound to the PDZ domain of PIST is sorted to lysosomes or to a recycling process. The lack of the right binding partner for nPIST in our systems would explain why we observed a different behavior of PIST and nPIST although both are able to bind $K_V10.1$ in pull down assays. The shorter forms of PIST may serve as a dominant negative variant, whose expression controls PIST function *in vivo* by trapping the binding partners of the coiled-coil region without linking them to effected proteins like $K_V10.1$.

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K^+ channels and cell cycle progression in tumor cells

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K^+ ions play a major role in many cellular processes. The deregulation of K^+ signaling is associated with a variety of diseases such as hypertension, atherosclerosis, or diabetes. K^+ ions are important for setting the membrane potential, the driving force for Ca^{2+} influx, and regulate volume of growing cells. Moreover, it is increasingly recognized that K^+ channels control cell proliferation through a novel signaling mechanisms triggered and modulated independently of ion fluxes. In cancer, aberrant expression, regulation and/or sublocalization of K^+ channels can alter the downstream signals that converge on the cell cycle machinery. Various K^+ channels are involved in cell cycle progression and are needed only at particular stages of the cell cycle. Consistent with this idea, the expression of Eag1 and HERG channels fluctuate along the cell cycle. Despite of acquired knowledge, our understanding of K^+ channels functioning in cancer cells requires further studies. These include identifying the molecular mechanisms controlling the cell cycle machinery. By understanding how K^+ channels regulate cell cycle progression in cancer cells, we will gain insights into how cancer cells subvert the need for K^+ signal and its downstream targets to proliferate.

Keywords: K^+ channels, cell cycle, tumor cell, cyclins, CDK, membrane potential, calcium, volume control

INTRODUCTION

Cell homeostasis requires a delicate balance between formation of new cells by cell proliferation and their elimination by apoptosis. Cell proliferation involves, at some point, activation of Cl^-, K^+, aquaporin, and Ca^{2+} channels (Pardo, 2004; Lang et al., 2007; Lehen'kyi et al., 2007). As the respective channel inhibitors have been reported to interfere with cell proliferation, the channels appear to play an active role in the machinery triggering cell entry and progression through the cell cycle (Wonderlin and Strobl, 1996; Ouadid-Ahidouch and Ahidouch, 2008; Becchetti, 2011).

Perturbations in the cell cycle results in unlimited proliferation and confers the apoptosis resistance leading to cancer development (Malumbres and Barbacid, 2001, 2009). The progression of cells through the cell cycle is regulated by different cyclin/Cyclin-Dependent Kinase (CDKs) complexes. The cyclin-CDK complexes govern a linear progression of the events that lead cells from a resting state (G0), to growth phase (G1), DNA replication phase (S), and finally to cell division (M). Cyclins D (D1, D2, and D3) and their associated CDKs (4/6) are important during the G1 phase (Caldon et al., 2006). Cyclins E and A, along with their partner, CDK2, are important for the G1/S phase transition and S-phase progression, respectively, and cyclins A and B (with Cdc2) are important for entry of the cells into the M phase (Schwartz and Shah, 2005). The activity of the cyclin/CDK complex can be inhibited by Cyclin-Dependent Kinase inhibitors (CDKi) such as $p21^{WAF1/Cip1}$ and $p27^{kip1}$ (Besson et al., 2008).

Over the past two decades, ion channels have been identified as important contributors to cell proliferation of normal and cancerous cells (for review see Wonderlin and Strobl, 1996; Kunzelmann, 2005). Otherwise, K^+ channels are thought to be important for setting the membrane potential and the driving force for Ca^{2+} influx and both mechanisms are considered crucial for volume regulation of growing cells (Nilius and Wohlrab, 1992; Kunzelmann, 2005; Lang et al., 2007; Ouadid-Ahidouch and Ahidouch, 2008). Moreover, it is increasingly recognized that K^+ channels control cell proliferation through a novel signaling mechanisms triggered and modulated independently from ion fluxes (Hegle et al., 2006; Kaczmarek, 2006; Glassmeier et al., 2012).

Although the K^+ channels contribute to regulation of different cell cycle checkpoints in cancer cells, no direct evidence about the mechanism at issue has been provided concerning how they control the cell cycle machinery, particularly in regard to CDKi and/or cyclin expression (Evan and Vousden, 2001). This review focuses on the regulation of proliferation by K^+ channel-mediated signals and discusses how these signals may influence the cell cycle progression in tumor cells.

K^+ CHANNELS AND CELL PROLIFERATION

K^+ channels belong to a large and heterogeneous group of ion channels. The first K^+ currents were described by Hodgkin and Huxley in the early 50s during their work on the identification of ionic species responsible for the propagation of the action potential in the squid giant axon. Today, several types of K^+ channels have been identified by molecular cloning. They are responsible for the passive diffusion of K^+ ion across the plasma membrane and are detectable in both excitable and non-excitable cells. K^+ channels play a major role in several physiological functions such as synaptic transmission, muscle contraction, release of hormones, such as insulin (for review see Sandhiya and Dkhar, 2009), or in proliferation, apoptosis, migration, invasion, and angiogenesis (Pardo, 2004; Cherubini et al., 2005; Ouadid-Ahidouch and

Ahidouch, 2008; Schwab et al., 2008; Becchetti, 2011; Cuddapah and Sontheimer, 2011; Jehle et al., 2011; Girault et al., 2012). The physiological importance of K^+ channels is undoubtedly underscored considering the increasing number of genetic diseases associated with them. Indeed, Kv gene mutations trigger episodic ataxia, long QT syndrome, pancreatic diseases, and epilepsy (Ashcroft and Gribble, 2000; Goonetilleke and Quayle, 2012).

The early studies that have linked K^+ channels to the proliferative processes were carried on T lymphocytes (DeCoursey et al., 1984; Matteson and Deutsch, 1984). In cancer, the first works were performed on melanoma cells, neuroblastoma, breast, lung, and bladder cancer cells (Rouzaire-Dubois and Dubois, 1990; Lepple-Wienhues et al., 1996; Wonderlin and Strobl, 1996; Ouadid-Ahidouch and Ahidouch, 2008). Since then, a wide variety of K^+ channels has been shown to be implicated in the regulation of proliferation of myeloid leukemia, prostate, colon, neck, and head cancer, lymphoma and hepatocarcinoma cells (Zhou et al., 2003; Conti, 2004; Pardo, 2004; Wang, 2004; Kunzelmann, 2005; Wang et al., 2007a; Arcangeli et al., 2009; Jehle et al., 2011; Lehen'kyi et al., 2011). These data are based on the fact that, *in vitro*, genetic or pharmacological inhibition of K^+ channels inhibits the growth of cancer cells. Moreover, K^+ channel openers can also be responsible for an increase in proliferation rate. Indeed, minoxidil or cromakalim increased activity of K_{ATP} channels inducing an increase in DNA synthesis (Malhi et al., 2000). KCa3.1 channels activator, 1-EBIO, increased prostate cancer cell proliferation (Parihar et al., 2003), but diminished the proliferative effect on keratinocytes (Koegel et al., 2003).

Several hypotheses argue in favor of a mechanism of K^+ influence on the cell cycle involving Ca^{2+} signaling, membrane potential, and cell volume (Nilius and Wohlrab, 1992; Lepple-Wienhues et al., 1996; Wonderlin and Strobl, 1996; Kunzelmann, 2005; Higashimori and Sontheimer, 2007; Zhanping et al., 2007; Ouadid-Ahidouch and Ahidouch, 2008; Blackiston et al., 2009; Becchetti, 2011).

One of the most accredited models is based on the involvement of K^+ channels in the regulation of membrane potential, which drives cancer cells into certain phases of the cell cycle. In cancer cells, it has been shown that cells in the early G_1 phase are depolarized; however, they are hyperpolarized during the progression through G_1 and into the S phase (Wang et al., 1998). Inhibition of K^+ channels or blocking cells in G1 phase by serum starvation is accompanied by membrane depolarization (Nilius and Wohlrab, 1992; Wonderlin et al., 1995; Wang et al., 1998; Ouadid-Ahidouch et al., 2001). Similarly, membrane depolarization caused by an increase in the concentration of extracellular K^+ mimics the effects of K^+ channel blockers (Freedman et al., 1992).

Many K^+ channels have been implicated in the regulation of the membrane potential, (for review see: Wonderlin and Strobl, 1996; Pardo, 2004; Kunzelmann, 2005; Ouadid-Ahidouch and Ahidouch, 2008; Becchetti, 2011). In some cases, the membrane potential of cycling cells is regulated by the ratio of different K^+ channel isoforms. For example, in neuroblastoma, the membrane potential can be controlled by Kv11.1 and blocking this channel inhibits mitosis (Arcangeli et al., 1995; Crociani et al., 2003; Becchetti, 2011). In these cells, the membrane potential tends to depolarize during the S phase. The effect is caused by oscillation of the expression balance of the full-length Kv11.1a isoform and the N-deleted Kv11.1b (Crociani et al., 2003). Indeed, the truncated Kv11.1b form is up-regulated during the S phase, while the full-length Kv11.1a protein increases its expression on the plasma membrane during the G1 phase (Arcangeli et al., 1995). The increase of the ratio between Kv11.1b and Kv11.1 leads to the depolarization of the membrane potential occurring during S phase progression (Arcangeli et al., 1995).

Ca^{2+} signaling appears to be required for progression through G1, the G1/S transition, and G2/M in several cell types (Kahl and Means, 2003; Roderick and Cook, 2008; Ding et al., 2010). The relationship between K^+ channels, membrane potential, and Ca^{2+} influx has been first reported in T cells by Cahalan's group. Indeed, the authors proposed that the membrane hyperpolarization generated by the activation of K^+ channels increases the driving force for Ca^{2+} influx (Hess et al., 1993; Lewis and Cahalan, 1995), which in turn, activates Ca^{2+}-dependent transcriptional factors leading to expression of cell cycle regulatory proteins, such as cyclins, CDKs, and the inhibition of CDKi expression (Nilius and Wohlrab, 1992; Yao and Kwan, 1999; Ouadid-Ahidouch et al., 2004; Zhanping et al., 2007; Borowiec et al., 2011). The inhibitors of voltage-gated K^+ channels block the Ca^{2+} influx and the cell proliferation (Yao and Kwan, 1999; Ouadid-Ahidouch et al., 2004; Zhanping et al., 2007).

In agreement with these general considerations, in prostate LNCaP cells, a member of Transient Receptor Potential (TRP) channel family, TRPV6 was identified as a major provider of passive calcium influx in response to hyperpolarization associated with activation of the intermediate-conductance Ca^{2+}-activated K^+ channel (KCa3.1) (Lallet-Daher et al., 2009). The same team showed that calcium entry through TRPV6 channel in these cells induced a subsequent downstream activation of Nuclear Factor Activated T cell (NFAT) leading to cell proliferation (Lehen'kyi et al., 2007). In breast cancer cells, it was demonstrated that Eag1 (Kv10.1) K^+ channel function is required for controlling the Ca^{2+} entry trough Orai1 channels (Hammadi et al., 2012).

K^+ channels also control cellular proliferation by affecting cell volume (Rouzaire-Dubois et al., 2000). Alterations of cell volume require participation of ion transport across cell membrane and accordingly, Cl^- and K^+ channels (Lang et al., 2007). Recently, aquaporin channels have emerged as regulators of cell cycle progression (Di Giusto et al., 2012). Cell volume regulation may favor maintenance of appropriate levels of critical cell cycle regulatory proteins that are necessary for controlling cell cycle progression through G1 to S or G2/M phases (Tao et al., 2008; Huang et al., 2012).

In addition to their ability to permit ions to cross the membrane, ion channels can also have non-conducting functions that enable them to interact with cell signaling pathways to directly regulate biochemical events (Kaczmarek, 2006). It has been demonstrated that expression of a mutant form of Eag1, that cannot conduct current, promotes proliferation of NIH3T3 fibroblasts and C2C12 myoblasts cells (Hegle et al., 2006). In contrast to the wild type Eag1, the no-conducting Eag1 is independent of serum and is unaffected by changes in extracellular

Ca^{2+}. Moreover, proliferation induced by Eag1 is unaffected by changes in extracellular Ca^{2+}, suggesting that increased Ca^{2+} influx is not an essential downstream component of Eag1-induced signaling. The same authors reported that Eag1 is able to regulate cell proliferation in fibroblasts via activation of the p38 Mitogen-Activated Protein (MAP) kinase pathway but not extracellular signal-regulated kinase 1/2 (ERK1/2). Ectopic expression of pore-dead mutant Eag1, in CHO cells, promotes cell growth *in vitro* and *in vivo* (xenografts) (Downie et al., 2008). Recently, the ion-conducting function of HERG1 has also proved to be important for cell growth of human small cell lung cancer cells (Glassmeier et al., 2012). Indeed, the knockdown of HERG1 inhibits cell proliferation, while its pharmacological inhibition by E4031 fails to affect cell proliferation (Glassmeier et al., 2012). Additionally, the expression of non-conducting mutant KCa3.1 induced HEK293 cells proliferation not by enhancing Ca^{2+} entry but via a direct interaction with ERK1/2 and c-jun N-terminal kinase (JNK) signaling (Millership et al., 2011). Increasing number of reports show that certain K^+ channels interact with signaling molecules directly to regulate cellular signaling. Indeed, the N- and C-terminal domains of hEag1 interacts with calmodulin (Schönherr et al., 2000; Ziechner et al., 2006; Gonçalves and Stühmer, 2010), cortactin (Herrmann et al., 2012); KCa3.1 channels interact with ERK1/2 (Millership et al., 2011), and HERG1 channels with the adaptor protein 14-3-3 (Kagan et al., 2002), Src tyrosine kinase (Cayabyab and Schlichter, 2002), and the TNF-α receptor (Wang et al., 2002). In addition, HERG channel proteins have been shown to interact with integrins, thereby regulating cell survival, adhesion and migration (Arcangeli and Becchetti, 2006; Arcangeli, 2011).

The mechanisms that allow K^+ channels to regulate cell growth in cancer cell lines appear to be different from the ones in normal cells. It has been reported that cancer cells express several K^+ channel isoforms that may be physiologically different as compared to a wild type channel. For example, proliferation of neuroblastoma cells is regulated by an oscillation balance of expression of the full-length HERG 1a (Kv11.1a) isoform and the N-deleted HERG 1b (Kv11.1b) (Crociani et al., 2003). It should be also noted that the effects of K^+ channels on cell proliferation can involve their trafficking to the micro-domains within the cell.

It follows from the foregoing that the K^+ channels influence cell proliferation through as many mechanisms as the number of their families and correspondent isoforms. That is undoubtedly dependent on intrinsic features of each cell type and the isoforms expressed.

K^+ CHANNEL ACTIVITY DURING THE CELL CYCLE

A direct link between channel activity and particular stages of the cell cycle has been reported. For example, in HeLa cells, the K^+ current-density increases during M and G1 phases (Takahashi et al., 1993), in unfertilized mouse oocytes, a large-conductance, voltage-activated K^+ channel (BKCa, 240 pS), is active throughout M and G1 phases, and switches off during the G1-to-S transition (Day et al., 1993). In Xenopus oocytes, the expressed rat Eag (rEag1) rEag1 displays decrease in current-density in meiotic phase induced by progesterone or by Mitosis promoting factor (Brüggemann et al., 1997). Furthermore, the partial synchronization of cells in G1 or M phases greatly increases the current blockade by intracellular Na^+ and Cs^+ (Pardo et al., 1998). In cancer cells, the K^+ channels activity has been also found to be cell cycle-dependant. For example, the activity of Eag1 is at high rate in G1 phase and decreases when cells enter S phase (Ouadid-Ahidouch et al., 2004), and in neuroblastoma cells, HERG current activity has been shown to be cell cycle-dependent (Arcangeli et al., 1995).

The mechanisms linking the activity of each of these channels to the cell cycle appear to be different and include regulation by cytoskeletal elements (Camacho et al., 2000), the activation of cyclin-dependent kinase 1 (CDK1) cyclin B (Brüggemann et al., 1997), a cytoplasmic cell cycle that can run independently of the nuclear cell cycle (Day et al., 1998), or channel trafficking.

Studies on K^+ channels trafficking are increasingly emergent. It has been suggested that Eag1 turns over rapidly (8–12 h) at the cell surface (Weber et al., 2006). This process involves surface expression followed by constitutive internalization and degradation in lysosomes (Kohl et al., 2011). Indeed, the Eag1 trafficking has been reported to be regulated by several proteins including cortactin, rabaptin-5 and epsin. Depletion of cortactin decreases the number of functional Eag1 channels without altering their open probability or conductance (Herrmann et al., 2012). These authors suggest that cortactin connects Eag1 to the cytoskeleton. Moreover, knockdown of rabaptin-5 reduces recycling rates of Eag1 and leads to a reduction of Eag1 current-density (Ninkovic et al., 2012). Eag1 also interacts with epsin that modulates its gating in rat brain (Piros et al., 1999). In view of these studies, the expression of K^+ channels during the cell could likely due to their trafficking, although the involvement of this particular pathway in cell proliferation is still not proven.

MOLECULAR MECHANISMS UNDERNEATH CELL CYCLE REGULATION BY K^+ CHANNELS (SEE TABLE 1 AND FIGURE 1)

Aberrantly expressed cell cycle-related cyclins are highly associated with several cancer types including breast cancer (for review Williams and Stoeber, 1996). As such, cyclin D1 is overexpressed in ∼50% of breast cancers (Alle et al., 1998). Cyclin E is overexpressed in 30% of breast tumors (Wang and Shao, 2006), and the elevation of cyclin E is correlated with low levels of $p27^{Kip1}$. A decrease in the expression of $p27^{Kip1}$ is strongly detected in breast cancer tissues (for review, Alkarain et al., 2004). Further genetic abnormalities also affect other CDKi, such as $p16^{INK4a}$, $p15^{INK4b}$, and $p57^{Kip2}$ (for review, Williams and Stoeber, 1996). These observations led to consider the cell cycle regulators as potential targets for selective inhibition in the treatment of cancer (Stone et al., 2012). The studies conducted so far show that the modulation of these key proteins of the cell cycle correlates with tumor development. Thus, it is clear that mitogenic factors capable of modulating their expression positively can intensify the process of carcinogenesis.

Despite K^+ channel blockers modulate several proliferative signaling pathways evidence for a direct mechanistic link between K^+ channels and cell cycle proteins remains sparse to date. Two cell-cycle blocks are often observed when K^+ channels are inhibited: at G1 (early G1, late G1, G1/S transition) and at G2/M block

Table 1 | K^+ channels and cell cycle protein actors in cancers.

Cell line type	K^+ channel family	Cell cycle phase	Main actors	References
MCF-7 (Breast cell line)	hEag1 (Kv10.1; KCNH1)	G1 and G1/S	Cyclin D1 Cyclin E P-Rb No/CDK4 No/CDK2 No/$p21^{waf1/cip1}$ No/$p27^{kip1}$	Borowiec et al., 2011
LoVo (Human colon cell line)	KCa3.1 (IK1; IKCa1; KCNN4)	G2/M	p-Cdc2	Lai et al., 2011
549 (Human lung cell line)	Kv1.3 (KCNA3)	G1	$p21^{Waf1/Cip1}$ CDK4 Cyclin D3	Jang et al., 2011
U87 and U251 (Human glioma cell lines)	Kir6.2 (KCNJ11) ATP-sensitive	G1	p-ERK	Huang et al., 2009
LNCaP PC-3 DU-145 (Prostate cancer cell lines)	KCa3.1 (IK1; IKCa1; KCNN4)	Prolif (G1/S)	$p2^{Cip1}$ No/$p27^{Kip1}$	Lallet-Daher et al., 2009
MB (Medulloblastoma) CNS tumor	EAG2 (Kv10.2; KCNH5)	G2	Cyclin B1 p38 MAPK	Huang et al., 2012
HL-60 (leukemia cells)	HERG1 (Kv11.1)	G1	β-catenin, cyclin-D1 c-myc	Zheng et al., 2011

with or without changes in the proportion of the cells entering S-phase. The G1 block is associated with a reduction of the expression of cyclins (D and E), CDKs (4 and 2), pRb activation, and overexpression of CDKi ($p21^{Waf1/Cip1}$ and $p27^{kip1}$), while the block in G2/M is associated with a reduction of cyclin B1 expression or changes in Cdc2 (CDK1) phosphorylation.

The first studies used K^+ channels pharmacological inhibitors such as TetraEthyl Ammonium (TEA) and 4-Amino-Pyridine (4-AP) to demonstrate that inhibition of the two K^+ channels (Kv1.3 and Kv1.5) resulted in cell cycle arrest at G1 in rat oligodendrocyte precursors (Ghiani et al., 1999; Chittajallu et al., 2002). The authors proposed that changes in the membrane potential (depolarization) could activate a signaling pathway involving the $p27^{kip1}$ and $p21^{Waf1/Cip1}$ proteins expression. Similar results were obtained when the membrane potential was depolarized by increasing extracellular K^+ (Renaudo et al., 2004).

In small cell lung cancer (SCLC, NCI-H209, and NCI-H146) and leukemic (Jurkat) cell lines, inhibition of K^+ channels has also been implicated in upregulation of the $p27^{kip1}$ protein and a reduction in cyclin. A expression resulting in cell arrest in G1/S phase transition (Renaudo et al., 2004). Athough the mechanisms, by which K^+ channels regulate cell cycle in these cells remain undetermined, the authors suggested involvement of cytoskeleton rearrangements due to cell volume changes.

A detailed mechanism underlying the role of Eag1 in the cell cycle was studied in MCF-7 breast cancer cells. In this work, mitogenic stimulation [using serum or Insulin Growth Factor 1, (IGF-1)] up-regulates Eag1 expression and activity (Borowiec et al., 2007). IGF-1 triggers cell cycle progression by increasing expression of cyclin D1, cyclin E, CDK4, CDK2, and phosphorylation of pRb (Dufourny et al., 1997; Borowiec et al., 2011). Inhibition of Eag1 by astemizole or by siRNA induced a decrease in cyclin D1 expression along with a strong decrease of pRb phosphorylation and an arrest of the cells in G1 phase of cell cycle. The effect of Eag1 inhibition was accompanied by a decrease of cyclin E expression, the key regulator of the G1/S transition necessary for cell entry into S phase (Skelding et al., 2011). However, inhibition of Eag1 failed to modify the $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and CDK4/2 expression.

Interestingly, when Eag1 is inhibited, the cyclin D1 level is below its level in serum-deprived cells, the condition known to synchronize cells in G1 phase (Borowiec et al., 2011). It was thus suggested that Eag1 control of the cell cycle could be upstream of the G1 phase synchronization by serum deprivation. Indeed, the cell cycle arrest site under Eag1 inhibition is both upstream of that obtained by serum-deprivation, and downstream of that induced by lovastatin, which synchronizes the cells in the early G1 phase (Borowiec et al., 2011). Altogether, these results demonstrate that Eag1 activation is positioned upstream of the upregulation of both cyclins D1 and E expression.

It has been reported recently that the isoform 2 of Eag (Eag2) is essential for mitotic entry of medulloblastoma (MB) cells (Huang et al., 2012). Indeed, the authors observed a correlation between

temporal Eag2 translocation to plasma membrane and mitotic entry from the late G2 phase concurrent with an increase of outward K^+ current during mitosis. Moreover, Eag2 knockdown accumulates cells in G2/M in association with a strong decrease of cyclin B1 (essential for G2/M transition), but fails to affect the expression of cyclin A, cyclin D1, cyclin E, and CDK1 (Huang et al., 2012). The G2/M arrest is due to an alteration in cell volume control that is linked to hyperactivation of p38 MAPK pathway without any effect on p53 expression. It has been then hypothesized that Eag2 regulates cell volume during the MB cell cycle progression (Habela and Sontheimer, 2007; Boucrot and Kirchhausen, 2008).

Ca^{2+}-activated K^+ channels (KCa) also regulate cell cycle progression. KCa3.1 is one of the KCa channels that regulate proliferation and migration of cancer cells (Ouadid-Ahidouch et al., 2004; Faouzi et al., 2010; Catacuzzeno et al., 2012). KCa3.1 controls G1 (mainly late G1) phase, G1/S transition, and G2/M phase. In breast, prostate and endometrial cancers, KCa3.1 regulates G1 and G1/S transition (Ouadid-Ahidouch et al., 2004; Wang et al., 2007b; Lallet-Daher et al., 2009). Pharmacological or genetic blockade of KCa3.1 increases $p21^{Waf1/Cip1}$ expression and decreases the expression of cyclin E. According to the "potential membrane model," activation of K^+ channels amplifies the Ca^{2+} signals by hyperpolarizing the membrane potential, thus increasing driving force for Ca^{2+} influx, which, in turn, activates Ca^{2+}-dependent transcriptional factors leading to expression of G1 cyclins and CDK proteins (Roderick and Cook, 2008). Consistent with this notion, it has been shown that TRPV6 may be the major provider of passive Ca^{2+} influx in response to the hyperpolarization associated with KCa3.1 channels activation in prostate cancer cell line (Lallet-Daher et al., 2009). In breast cancer cells, it has been demonstrated that Eag1, by regulating membrane potential, controls Ca^{2+} entry through Orai1 channels (Hammadi et al., 2012).

KCa3.1 channels also control the cell volume and regulate G1/S transition and G2/M phases (Tao et al., 2008; Lai et al., 2011). In mouse mesenchymal stem cells, KCa3.1 channels associated with Cl^- -volume channels regulate cell cycle progression by modulating cyclin D1 and cyclin E expression (Tao et al., 2008). However, in human colon cancer cells (LoVo), their inhibition accumulates cells in G2/M phase and increases the phosphorylation of Cdc2 (Lai et al., 2011).

In summary, several K^+ channels have been implicated in proliferation of various types of cancers cells including Kv (Menéndez et al., 2010; Asher et al., 2011; Jang et al., 2011; Jeon et al., 2012), KCa (Jäger et al., 2004; Faouzi et al., 2010; Oeggerli et al., 2012), Kir (Huang et al., 2009), K2P (Bayliss and Barrett, 2008; Innamaa et al., 2013). Higher expression or activity of these channels in cancer cells appears to correlates with deregulation of cell cycle protein expression and/or function leading to enhanced cell proliferation.

CONCLUSIONS

In summary, K^+ channels are often overexpressed in tumor cells and regulate proliferation. They are needed to control specific checkpoints in the cell cycle progression (early G1, the G1/S and G2/M transitions). Accordingly, several K^+ channels exhibit a cell-cycle-dependent expression and activity. In addition, cell cycle progression is often accompanied by oscillations of Cl^- channel expression and Ca^{2+} signals that regulate cell proliferation. Recent evidence shows the involvement of aquaporin in cell proliferation. The links between K^+ channels and cell-cycle machinery (cyclins, CDKs, and CDKi) start to emerge (**Figure 1**). However, we are still far from full understanding the complex mechanistic link between channel expression/activity

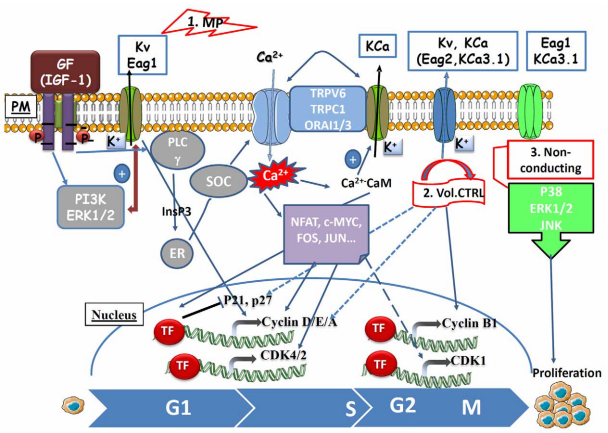

FIGURE 1 | Schematic illustration of the role of K^+ channels during cell cycle progression and proliferation. (1) Membrane potential model: during progression into G1 phase, the membrane potential becomes hyperpolarized relative to the normal resting potential. The hyperpolarization relates to Kv channels activation (for example Kv1.3, Eag1) in early G1 and to Ca^{2+}−activated K^+ channels (for example KCa3.1) activation in late G1 and/or G1/S transition. Multiple growth factors in serum have been well-described to play an important role in initiating G1 progression to the S phase of the cell cycle, in which Ca^{2+} influx is a major determinant in serum-induced DNA synthesis. Growth factors (for example IGF-1) binding to the receptor tyrosine kinases [RTKs; for example, Insulin growth factor receptor (IGF-1R)] can activate effectors [Extracellular signal-Regulated Kinase 1/2 (ERK1/2), phosphatidylinositol 3-kinase (PI3K)] that increase the expression and the activity of K^+ channels inducing a hyperpolarization of the membrane potential (MP). Moreover, RTKs also activate phospholipase C (PLCγ) to promote the generation of inositol-1,4,5-trisphosphate (InsP3) and the release of Ca^{2+} from the endoplasmic reticulum (ER) into the cytosol. Ca^{2+} enters across plasma membrane by store-operated capacitive Ca^{2+} entry (SOCE) through the ORAI1 (Hammadi et al., 2012) or ORAI3 (Faouzi et al., 2011) channels, through TRP Ca^{2+} channels (for example TRPC1, El Hiani et al., 2009), or via the constitutively active TRPV6. The Ca^{2+} entry, in turn, regulates the activity and/or the expression of Ca^{2+}-activated K^+ channels (for example KCa3.1), which maintains the hyperpolarization promoting a significant Ca^{2+}-entry. The increase in $[Ca^{2+}]i$ triggers the activation of Ca^{2+}-dependent signaling enzymes that may act by regulating the expression or activity of the transcription factors (TF), such as FOS, JUN, NFAT, C-MYC leading to the expression of cyclins and CDKs and the inhibition of the CDK inhibitor proteins ($p27^{KIP1}$ and $p21^{waf1/cip1}$). (2) Volume control: K^+ channels in association with chloride channels regulate cell cycle progression by controlling the cell volume. For example, Eag2 channels control M phase by regulating the expression of cyclin B1 through the p38 MAP Kinase pathway. (3) Non-conducting roles: K^+ channels may also promote cell proliferation independently of their ion permeation function. For example: Eag1 and KCa3.1 induce cell proliferation by (direct or indirect) interaction with MAP kinase signaling pathways (p38 for Eag1, ERK1/2, and JNK for KCa3.1). MP: membrane potential, (vol. CTRL): volume control, (PM): plasma membrane.

and signaling in proliferating cells. The detailed understanding of the role of K^+ channels and their connection with Ca^{2+} signals in regulation of cell cycle proteins and/or transcription factors will offer significant opportunities to develop more specific tools for cancer therapy.

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Cell volume regulation in epithelial physiology and cancer

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The physiological function of epithelia is transport of ions, nutrients, and fluid either in secretory or absorptive direction. All of these processes are closely related to cell volume changes, which are thus an integrated part of epithelial function. Transepithelial transport and cell volume regulation both rely on the spatially and temporally coordinated function of ion channels and transporters. In healthy epithelia, specific ion channels/transporters localize to the luminal and basolateral membranes, contributing to functional epithelial polarity. In pathophysiological processes such as cancer, transepithelial and cell volume regulatory ion transport are dys-regulated. Furthermore, epithelial architecture and coordinated ion transport function are lost, cell survival/death balance is altered, and new interactions with the stroma arise, all contributing to drug resistance. Since altered expression of ion transporters and channels is now recognized as one of the hallmarks of cancer, it is timely to consider this especially for epithelia. Epithelial cells are highly proliferative and epithelial cancers, carcinomas, account for about 90% of all cancers. In this review we will focus on ion transporters and channels with key physiological functions in epithelia and known roles in the development of cancer in these tissues. Their roles in cell survival, cell cycle progression, and development of drug resistance in epithelial cancers will be discussed.

Keywords: K^+ channels, Cl^- channels, tumour microenvironment, drug resistance, pancreatic cancer, breast cancer, stroma, secretion

INTRODUCTION

Broadly speaking, epithelia are organized into sheets, tubes, or glandular structures, and perform complex tasks of transporting ions, organic molecules, and water for which specific ion channels/transporters are required. The majority of cancers are of epithelial origin, and the altered ion channel/transporter expression, which is emerging as one of the hallmarks of cancer in general (Prevarskaya et al., 2010; Lehen'kyi et al., 2011), is also a marked characteristic of epithelial cancers. In this review we will first outline the ion transport mechanisms operating in epithelia under physiological conditions of ion/fluid transport and cell volume regulation. Next, we will review and critically discuss how dys-regulation of cell volume or given ion transporters can lead to loss of epithelial architecture, altered cell survival, tumor progression, and drug resistance. The focus will be on cancers of secretory epithelia, primarily pancreatic ductal adenocarcinoma (PDAC) and mammary cancer.

Abbreviations: ABC, ATP-binding cassette; AVD, apoptotic volume decrease; α-SMA, α-smooth muscle actin; BK, big conductance K^+ channel, also named KCa1.1 and maxi-K^+; CA, carbonic anhydrase; CAFs, cancer associated fibroblasts; $[Ca^{2+}]_I$, intracellular Ca^{2+} activity; CFTR, the cystic fibrosis transmembrane conductace regulator; EATC's, Ehrlich ascites tumour cells; ECM, extracellular matrix; EGF, epidermal growth factor; ELA, Ehrlich Lettre ascites carcinoma; EMT, epithelial-to-mesenchymal transition; ERK1/2, extracellular signal regulated kinase; HICCS, hypertonicity-induced cation channels; HIF1α, hypoxia-inducible factor-1α; IK, intermediate conductance K^+ channel, also named KCa3.1; MAPK, mitogen-activated protein kinase; MCT, monocarboxylate transporters; MDR, multi drug resistance; NBC, Na^+-HCO_3^- transporter; NHE, Na^+/H^+ exchanger; NKCC1, Na^+-K^+-$2Cl^-$ cotransporter; VRAC, volume regulated Cl^- channel; OSR1, oxidative stress responsive kinase; pH_i, intracellular pH; pH_e, extracellular pH; PCD, programmed cell death; PSCs, pancreatic stellate cells; PDAC, pancreatic ductal adenocarcinoma; RVI, regulatory volume increase; RTK, receptor tyrosine kinase; RVD, regulatory volume decrease; SOCE, store-operated calcium entry; SPAK, SPS-related proline/alanine-rich kinase; TME, tumor microenvironment; VDAC-1, mitochondrial voltage-dependent anion channel; TRP, transient receptor potential channels; VEGF, vascular endothelial growth factor; WNK, with no lysine kinase; ZO-1, tight junction protein.

PHYSIOLOGY OF EPITHELIAL TRANSPORT AND ROLE OF CELL VOLUME

Animal cells are subjected to transmembrane osmotic gradients in a number of physiologically relevant conditions, including: (i) ion/nutrient transport followed by osmotically obliged water movement; (ii) metabolic activity generating or requiring osmotically active substances; or (iii) altered extracellular osmolarity of the environment [see Hoffmann et al. (2009)]. Epithelial cells are of special interest because they carry out net transport of electrolytes, nutrients, and water in the secretory or reabsorptive direction, conditions in which cell volume regulation is a particular challenge. A question that has raised substantial interest in the field is how well cell volume regulation is achieved under these conditions, and to what extent cell volume changes contribute to the regulation of secretion/absorption. Furthermore, little is known about what happens to cell volume regulation if the normal vectorial epithelial transport is prevented or dys-regulated. It is well documented that several pathophysiological conditions, including altered Na^+/K^+ balance and acid/base disturbances caused by renal disease, or cardiac or brain ischemia, are associated with dys-regulation of cell volume regulatory transporters, and that the associated cell volume disturbance

contributes importantly to the pathology of these conditions (for reviews, see Lang, 2007; Hoffmann et al., 2009; Pedersen et al., 2011).

In *absorptive* epithelia such as the renal tubules, small intestine, gallbladder, and skin, the most common mechanism of transepithelial transport involves luminal channels and transporters that utilize the plasma membrane Na^+ gradient for salt and nutrient transport, which would tend to swell the cells. Isosmotic transport and recovery of cell volume under these conditions is likely achieved through activation of basolateral stretch-activated K^+ channels, volume regulated Cl^- channels (VRAC), and increased activity of the Na^+/K^+ pump, followed by exit of ions/nutrients and osmotically obliged water across the basolateral membrane (Lang et al., 1998; Vanoye and Reuss, 1999; Schultz and Dubinsky, 2001; Hoffmann et al., 2009; Bachmann et al., 2011).

Here, we will focus on *secretory* epithelia such as pancreas, salivary glands, colorectum, stomach, mammary glands, and prostate, which, as will be discussed below, might not fully regulate their cell volume during stimulated secretion. Notably, several of these epithelia are among the tissues in the body that are most commonly afflicted by cancer (Siegel et al., 2013). One of the most common mechanisms for initiating fluid secretion by agonists or hormones is opening of luminal Cl^- channels and luminal and basolateral K^+ channels, and this also leads to a cell volume decrease. A number of transport mechanisms on the basolateral membrane are activated to provide ions for luminal exit and thus secretion, and this will potentially lead to regain of cell volume. Concurrently, the cells need to regulate their intracellular pH (pH_i), and for cells exhibiting net secretion of H^+ or HCO_3^- (stomach, pancreatic ducts), this is a particular challenge. **Figure 1A** shows the basic model for ion transport across secretory cells such as pancreatic duct cell. As seen, this model includes a toolbox of ion channels and transporters (Novak et al., 2011; Frizzell and Hanrahan, 2012; Wilschanski and Novak, 2013), some of which are dys-regulated in cancer, as will be described below. The ion channels include: the cystic fibrosis transmembrane conductace regulator (CFTR) and Ca^{2+}-activated Cl^- channels (ANO1/TMEM16A), intermediate and large conductance K^+ channels (IK—KCa3.1; BK—KCa1.1), volume sensitive KCNQ1 channels, and possibly voltage-regulated channels (HERG—Kv11.1; EAG2—Kv10.2) (Hayashi et al., 2012; Wang et al., 2013). The ion transporters include Na^+-K^+-$2Cl^-$ cotransporters (NKCC1), Na^+/H^+ exchangers (NHEs), Cl^-/HCO_3^- exchangers (SLC26A3,6 and SCL4A family), Na^+-HCO_3^- transporters (NBCs) and H^+/K^+-pumps. Another mechanism of achieving secretion, which is beyond the scope of this review, is that driven at least in part by exocytosis, such as in mammary epithelial cells secreting milk, or, for example, parietal cell secreting hydrochloric acid following exocytotic recruitment of the H^+/K^+ pump from tubulovesicles to the apical membrane (Forte and Zhu, 2010).

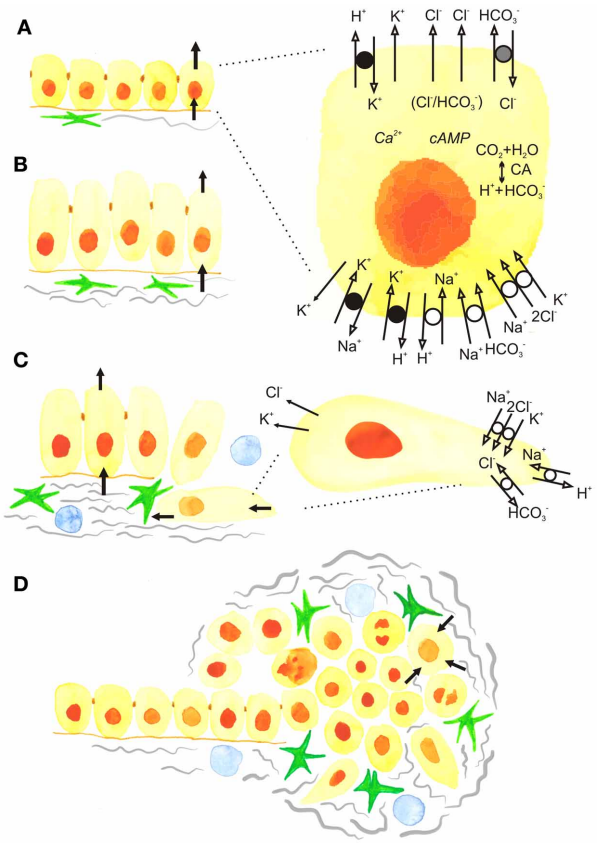

FIGURE 1 | The development of epithelial cancer and roles of ion transport and cell volume. (A) Normal secreting epithelium showing net movements of ions and fluid across the basolateral and luminal membranes. Black arrows show the movement of ions and fluid across cell membranes. The insert shows the detailed model of a cell with basic ion channels and transporters that operate, for example, in pancreatic ducts, but are also applicable to other secreting epithelia. The luminal Cl^- channels include CFTR and TMEM16A/ANO1, as well as a SLC26 family Cl^-/HCO_3^- exchanger. The basolateral membrane contains the $Na^+/K^+/2Cl^-$ transporter NKCC1, SLC7 family Na^+-HCO_3^- cotransporters (NBCs), and the Na^+/H^+ exchanger NHE1. The epithelium also expresses H^+/K^+ pumps, as well as several types of K^+ channels such as IK-KCa3.1, BK-KCa1.1, KCNQ1 and voltage-activated K^+ channels, some of which may be expressed on both luminal and basolateral membranes. The major Ca^{2+} and cAMP signalling pathways are not elaborated and for simplicity, Ca^{2+} -channels/transporters and aquaporins are not included. **(B)** Carcinogenesis and postulated dysregulation of cells volume and secretion. Increased activity of fibroblastic cells, such as cancer associated fibroblasts (CAFs) and pancreatic stellate cells (PSCs) (green). **(C)** The epithelial-to-mesenchymal (EMT) transition showing cells that loose apico-basal polarity and the appearance of some ion transporters from the lumimal membrane in the rear of the cells and some from the basolateral membrane in the leading edge, contributing to driving cell migration. **(D)** Progression to cancer, showing tumor with extensive fibrosis (gray), fibrogenic cells (green) and immune cells (blue). (Blood vessels are not shown). In the center of the tumor cells may die, while surrounding cells are proliferating and cell volume and corresponding ion transport is up-regulated (see the text).

In terms of cell volume, the crucial question is how ion/fluid transport on the two opposing membranes is coordinated. The main driving force for all these secondary- or tertiary- active processes is provided by the Na^+/K^+-ATPase. For secretory epithelia, the classical view is that basolateral transporters are activated secondarily to ion movements across the apical membrane due to alterations in electrochemical gradients or cell volume changes. Regarding the cell volume, known shrinkage-activated

proteins are NHE1, NKCC1, and some Transient receptor potential vanniloid (TRPV) channels; and swelling activated proteins are volume regulated anion channels (VRAC), KCNQ1, two-pore K^+ channels and Ca^{2+}-activated K^+ channels (Hoffmann et al., 2009). In addition to these transporters and channels, other plasma membrane transporters are regulated by volume-sensitive signaling pathways, including intracellular messengers, phosphorylation, and complex interactions involving cytoskeletal reorganization, Ca^{2+}-signaling, and signaling via integrins and receptor tyrosine kinases (RTKs). For overview of these topics the reader is referred to the recent review (Pedersen et al., 2011). Here we just point out that recently discovered cell signaling pathways involving volume- and low Cl^--sensitive With No Lysine kinases (WNK), acting via Ste20-like kinases, SPS-related proline/alanine-rich kinase (SPAK) and oxidative stress responsive kinase (OSR1), may be key factors in secretory epithelia as they regulate NKCC1 and other transporters (Kahle et al., 2006; Hoffmann et al., 2009; McCormick and Ellison, 2011; Park et al., 2012). Similarly, autocrine and paracrine signaling via volume-sensitive ATP release and purinergic receptors may be important regulators of key short- and long-term cell volume and ion transport in epithelia and tumor models (Hug et al., 1994; Pedersen et al., 1999; Sørensen and Novak, 2001; Koltsova et al., 2011; see Novak, 2011). A number of ATP release mechanism have been proposed, including ion channels and transporters, and they utilize favorable electrochemical gradient (see Novak, 2011).

Nevertheless, in the acute/secretory state, the cell volume of many native epithelial cells recovers only partially or does not recover until the stimulus is withdrawn (Manabe et al., 2004; Bachmann et al., 2007). For example, some secretory cells shrink by more than 20% during stimulation and remain shrunken until the stimulus is withdrawn (Dissing et al., 1990; Foskett, 1990; Nakahari et al., 1990, 1991; Lee and Foskett, 2010) (**Figure 1A**). The chronic events of altered volume regulation and/or ion transporter expression might lead to pathological developments associated with cancer.

LOSS OF EPITHELIAL POLARITY—IMPLICATIONS FOR ION TRANSPORT

The polarized organization of ion transport proteins is essential for the normal function of epithelia, and appears to involve the interplay between the targeted delivery of transporters, restriction by cell-cell junctions, and the fact that the transporters reside in large protein-protein complexes linking them to the actin- and spectrin-based cytoskeleton (Nelson, 2009). During early stages of cancer development, the epithelial layer becomes disorganized, loses its cell-cell adhesions, and undergoes a dramatic change from apical-basal polarity to a mesenchymal cell type organization with a front-rear polarity (**Figures 1B,C**). This process is known as epithelial-to-mesenchymal transition (EMT), and has been well studied both for breast and pancreatic adenocarcinomas (Foroni et al., 2012; Rhim et al., 2012). Although the signaling mechanisms involved in EMT are far from fully elucidated and are partially context- and cell-type dependent, several central themes have been established. Upstream EMT features include up-regulation of transcription factors such as Slug, Snail, and Twist. Markers of the full-blown EMT include up-regulation of α-smooth muscle actin (α-SMA), vimentin, and fibronectin, and down-regulation of epithelial markers such as E-cadherin, cytokeratins, and the tight junction protein ZO-1 (Kalluri and Weinberg, 2009; Nelson, 2009; De Craene and Berx, 2013). Notably, although a number of factors involved in polarity switching are described (Nelson, 2009; Godde et al., 2010), essentially nothing is known regarding the roles and regulation of polarized transport proteins during EMT. Thus, it is an open question how the tightly compartmentalized localization of transport proteins gets "reinstructed" upon transition from apical-basal to a front-rear polarity (**Figure 1C**). The net result, however, is that at least some apical ion channels and transporters relocalize to the rear end, while several that are basolaterally located in epithelia move to the leading edge of the cell (compare **Figures 1A,C**). This specific reorganization of ion channels and transporters contributes importantly to cell migration (Schwab et al., 2012). Given the known roles of many of these transport proteins in cytoskeletal organization, signaling, and motility, we speculate that contributions to EMT might be added to the list of roles for dys-regulation of transport proteins in epithelial cancers.

THE TUMOR MICROENVIRONMENT (TME)

Tumors are highly complex tissues in which the cancer cells themselves are often the minority and co-exist with numerous other cell types in a physical/chemical microenvironment which differs dramatically from that of the normal tissue (**Figure 1D**). The tumor microenvironment (TME) undergoes extensive reciprocal interactions with the cancer cells and provides oncogenic signals that exacerbate cancer progression. The detailed properties of the TME have been excellently reviewed elsewhere (Mueller and Fusenig, 2004; Kalluri and Zeisberg, 2006; Pandol et al., 2009; Hanahan and Weinberg, 2011; Feig et al., 2012; Hanahan and Coussens, 2012). In the following, we set the stage for discussing the interrelationship of the TME with dys-regulated ion transport, focusing on PDAC and mammary adenocarcinoma.

THE CELLULAR COMPONENT OF THE TME

The predominant stromal cell type in many carcinomas, including breast cancers, is *cancer associated fibroblasts (CAFs)* (Kalluri and Zeisberg, 2006; Hanahan and Coussens, 2012). CAFs secrete extracellular matrix (ECM) components and matrix-degrading enzymes, and, being contractile, mechanically pull at the ECM, increasing its stiffness (Kalluri and Zeisberg, 2006; Hanahan and Coussens, 2012). CAFs also secrete numerous growth factors, cytokines and vascular endothelial growth factor (VEGF), stimulating tumor growth and, in general, angiogenesis (Kalluri and Zeisberg, 2006; Hanahan and Coussens, 2012), though paradoxically solid tumors show poor vascularization (see below). In PDAC, *pancreatic stellate cells* (*PSCs*) play a role similar to that of CAFs in breast cancer (Pandol et al., 2009; Feig et al., 2012), although CAFs *per se* are also present in PDAC (Scarlett, 2013). Quiescent PSCs are present in low numbers in the normal exocrine pancreas. PSCs become activated by exposure to factors secreted by the cancer cells, rendering them myofibroblast-like,

highly proliferative, and motile (Pandol et al., 2009; Feig et al., 2012; Li et al., 2012). Excessive ECM deposition by PSCs is the main source of the marked desmoplasia in PDAC (**Figure 1D**). The PSCs also secrete growth factors, cytokines and chemokines, stimulating immune cell infiltration, angiogenesis, and cancer cell proliferation and motility (Pandol et al., 2009; Feig et al., 2012; Li et al., 2012). Infiltrating immune cells are of major importance in both mammary and pancreatic adenocarcinomas (Clark et al., 2007). Recruited *tumor-associated macrophages* release growth factors, chemokines, cytokines, and matrix-degrading enzymes, stimulating angiogenesis, cancer cell growth and invasiveness and further recruitment of pro-tumorigenic immune cells, while blocking activation of anti-tumorigenic T cells (Kalluri and Zeisberg, 2006; Pandol et al., 2009; Hanahan and Weinberg, 2011; Kees and Egeblad, 2011). Other central cellular stromal components are *endothelial cells* and *pericytes* (smooth-muscle-derived cells surrounding the endothelium). Finally, *cancer stem cells* or tumor-initiating cells have been found in the TME in both mammary and pancreatic cancer (Hermann et al., 2007; Iqbal et al., 2013).

CHEMICAL/PHYSICAL PROPERTIES OF THE TME

In addition to the wealth of cell types and secreted signaling factors mentioned above that sets the TME apart from the normal tissue, the TME also differs markedly from the normal tissue in its physical/chemical properties (see Harris, 2002; Heldin et al., 2004; Vaupel, 2004; Egeblad et al., 2010; Provenzano and Hingorani, 2013). Similar to the cellular component, the physical/chemical microenvironment exhibits distinct spatial heterogeneity throughout the tumor and develops dynamically as the cancer progresses. Because of the generally insufficient or collapsed tumor vasculature in many solid tumors, many areas of the TME are hypoxic or even anoxic (Harris, 2002). This has been shown directly for breast cancer (Vaupel, 2004), whereas evidence is more sparse for PDAC (see Feig et al., 2012). In conjunction with cancer-associated metabolic changes and high demand for energy and building blocks for anabolic reactions, this results in glucose deprivation, elevated lactate levels, and acidic extracellular pH (pH_e) (Heldin et al., 2004; Vaupel, 2004). Another consequence of the inefficient tumor vasculature and lymph outflow is elevated interstitial fluid pressure. A third physical characteristic of mammary and especially pancreatic cancers is that of desmoplasia—excessive accumulation and crosslinking of fibrillar collagens. This stiffens the ECM, in turn favoring cancer progression through effects on cell motility, differentiation, proliferation, and treatment response (Egeblad et al., 2010). In PDAC tumors, which are highly fibrotic and hypovascular, it is difficult for therapeutic agents to reach the tumor cells (Feig et al., 2012; Provenzano and Hingorani, 2013). Recent studies show that enzymatic targeting of stroma, ablation of the physical barrier improves vasculature and promotes drug delivery (Provenzano et al., 2012).

It seems likely that, in addition to selecting for hypoxia resistance and increased acid extrusion capacity (section Functional interactions between the TME and ion transport dys-regulation), the physically restricted TME with elevated interstitial pressure will tend to select for increased cell volume regulatory capacity due to the increased osmotic stress exposure. However, to our knowledge, this has never been directly studied. In addition, one might expect that physical constraints, hypoxia and necrosis will influence the concentration profiles of extracellular nucleosides/-tides within the tumor, in turn affecting a spectrum of tumor resident cells via purinergic signalling (Di Virgilio, 2012) (**Figure 1D**).

FUNCTIONAL INTERACTIONS BETWEEN THE TME AND ION TRANSPORT dys-REGULATION

While this has still been relatively little studied, it is clear that dys-regulation of ion transport in cancer is involved in important functional interactions with the TME. Firstly, the metabolic switch induced (in part) by hypoxia increases acid production in the cancer cells. This, in conjunction with hypoxia-induced elevation of hypoxia-inducible factor-1α (HIF1α) levels increases the expression and/or activity of acid-extruding ion transport proteins and carbonic anhydrases (CAs). In breast cancer, these include the Na^+/H^+ exchanger NHE1, the Na^+-HCO_3^- cotransporter NBCn1, monocarboxylate transporters MCT1 and MCT4, and CAIX (Bartosova et al., 2002; Lauritzen et al., 2010, 2012; Pinheiro et al., 2010; Boedtkjer et al., 2013; see Cardone et al., 2005) (**Figure 2**). In PDAC, evidence is much sparser, although neurotensin-induced NHE1 activation in PDAC cell lines is reported (Olszewski et al., 2010). Cytokines and growth factors secreted by the cancer cells and stromal cells likely also contribute to the up-regulation of ion transport. For instance, ErbB2

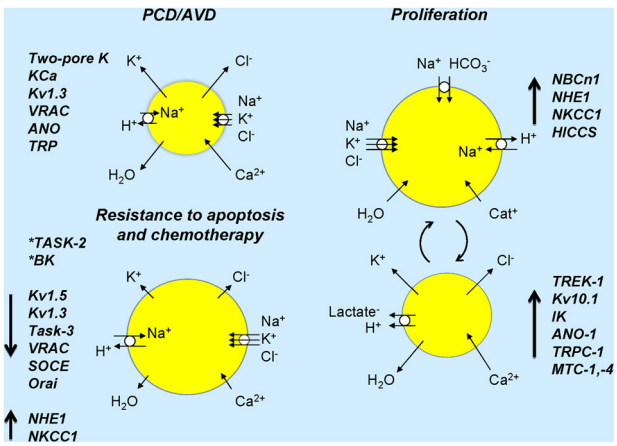

FIGURE 2 | Ion channels and transporters and cell volume changes associated in normal and cancer cells. Cell sizes refer to expected cell volume changes and lengths of arrows on cells indicates up- or down-regulation or ion transporters/channels. Resistance to apoptosis is associated with down-regulation of several channels and inhibition of some channels (asterisks) induces resistance to apoptosis. In proliferation, several transporters and channels are up-regulated and over-expressed in cancer (see text). The right part of the figure shows ion transporters and channels that would lead to cell volume increase and those in the lower part indicate those that would lead to cell volume decrease. Large arrows next to named ion channels/transporters indicate their up- or down-regulation in cancer. *Chronic activation* of ion transport may lead to cell death. *Dynamic activation* or suppression of ion transport/cell volume with specific signals, in time or in given cells may lead to cancer development and progression.

signaling increases NBCn1 expression and post-translationally activates NHE1 by phosphorylation in its C-terminal cytoplasmic domain (Lauritzen et al., 2010, 2012). In turn, ion transporters play major roles in creating the TME. Increased acid extrusion from the cancer cells can cause extracellular pH (pH_e) to become as low as 6.0 in some tumor regions (Vaupel, 2004). This favors further cancer development, e.g., through facilitating ECM degradation and cell motility, resistance to chemotherapy, and compromised anti-tumor function of cytotoxic T-cells and natural killer cells (Ward et al., 2013), while their role in maintaining pH_i at or above the normal pH 7.0–7.4 favors metabolic, migratory, and proliferative activity and counteracts apoptotic death (Parks et al., 2011; Webb et al., 2011; Boedtkjer et al., 2012). Finally, it has been suggested that NHE1 may directly regulate ECM deposition by fibroblasts (Karydis et al., 2009).

ROLES OF CELL VOLUME REGULATION IN CELL PROLIFERATION AND PROGRAMMED CELL DEATH (PCD)

Importantly, cells do not have one preferred volume. Rather, the volume *set point* depends on the functional state of the cell and changes in cell volume serve as key physiological signals initiating downstream responses, such as transepithelial transport (see above), proliferation, migration and cell death (**Figure 2**) (see Hoffmann et al., 2009). Consequently, dysfunction of volume-sensitive membrane transport proteins is associated with pathophysiological conditions related to control of these processes, including cancer.

CELL PROLIFERATION

Cell volume is a major factor in the regulation of cell cycle progression, with cell proliferation generally being inhibited by cell shrinkage and stimulated by cell swelling, respectively (Anbari and Schultz, 1993; Dubois and Rouzaire-Dubois, 2004; Rouzaire-Dubois et al., 2005). Cell cycle progression depends on an increase in cell volume, and the capacity for regulatory volume decrease (RVD) changes during the cell cycle (see e.g., Hoffmann et al., 2009). Accordingly, cell volume was found to be greatest in the M phase and smallest in the G1 phase in CNE-2Z cells and to increase in parallel to the G1-S transition in fibroblasts (see Hoffmann et al., 2009) In Ehrlich Lettre ascites carcinoma (ELA) cells, significant water uptake and cell swelling occur in S phase (Klausen et al., 2010). The direct effects of changes in cell volume on the cell cycle control are still not clear, but it seems that RTKs and mitogen-activated protein kinases (MAPKs) play important roles. Accordingly, cell swelling induced by hyposmotic stress in general stimulates extracellular signal regulated kinase (ERK1/2), a major player in control of cell cycle progression (see e.g., Meloche and Pouyssegur, 2007; Hoffmann et al., 2009) and multiple Src family kinases are activated in response to cell swelling (Cohen, 2005). An interesting example, somewhat in contrast to the general picture given above, is described in glioma cells, where a marked premitotic cell shrinkage is necessary for the following cell division (Habela and Sontheimer, 2007).

Several types of ion channels have been implicated in the dysregulated control of cell cycle progression in cancer (**Figure 2**).

TRP channels. The resting level of $[Ca^{2+}]_i$ varies through the cell cycle (Schreiber, 2005). Thus, transient changes in $[Ca^{2+}]_i$ occur at the exit from quiescence in early G1, at the G1/S phase transition and at the exit from M phase (Munaron, 2002; Munaron et al., 2004). In some cell types, TRPC1 is proposed to be involved in Ca^{2+} influx, RVD and cell cycle progression (Golovina et al., 2001; Salido et al., 2011; Madsen et al., 2012). A variety of *K^+ channels* have been implicated in the regulation of proliferation (Takahashi et al., 1993; Pei et al., 2003; Wang, 2004; Voloshyna et al., 2008) and cell cycle progression (Wang et al., 1998; Felipe et al., 2006). Accordingly, epithelial carcinomas often show high K^+ channel activity (Patel and Lazdunski, 2004; Wang, 2004; Felipe et al., 2006). Thus increased TREK-1 channel expression is associated with abnormal cell proliferation in prostate cancer cell lines and TREK-1 may be a novel molecular target in prostate cancer (Voloshyna et al., 2008). The $K_v10.1$ (KCNH1) channel, which is widely studied in cancer, is important for cell cycle progression and is regulated through the cell cycle (Pardo et al., 2012). Thus, developing specific blockers for these channels in the treatment of cancer is a promising field (Felipe et al., 2006; Li and Xiong, 2011; Pardo et al., 2012). In PDAC, in addition to $K_v10.1$ (Gomez-Varela et al., 2007), expression of IK (KCa3.1) is up-regulated in cancer tissue and some PDAC cell lines in which it contributes to stimulation of cell proliferation (Jager et al., 2004). *Cl^- channels* are also involved in control of cell proliferation, and Cl^- channel blockers inhibit cell proliferation (Voets et al., 1995; Pappas and Ritchie, 1998; Rouzaire-Dubois et al., 2000; Shen et al., 2000; Wondergem et al., 2001; Chen et al., 2007; Klausen et al., 2010) Several studies have found that VRAC currents differ in magnitude during the cell cycle (Shen et al., 2000; Doroshenko et al., 2001; Klausen et al., 2007, 2010). In nasopharyngeal carcinoma cells, VRAC activity was found to be central in control of passage through the G1 restriction point (Chen et al., 2007). The Ca^{2+}-activated Cl^- channel TMEM16A (ANO-1) is overexpressed in many carcinomas, including human prostate carcinoma (Liu et al., 2012) and head and neck squamous cell carcinomas, where it induces stimulation of ERK1/2 and contributes to cell proliferation (Duvvuri et al., 2012). In mammary cancer, where TMEM16A (ANO-1) is also over-expressed and supports proliferation, it is linked to EGF receptor and calmodulin-dependent kinase II signaling (Britschgi et al., 2013). Thus, specific blockers of Cl^- channels are also a potentially interesting field in the treatment of cancer (Duvvuri et al., 2012; Mazzone et al., 2012). Also several volume-regulatory transporters, including NHE1 (Putney and Barber, 2003) and NKCC1 (Panet et al., 2000) have been shown to exhibit cell-cycle dependent regulation and/or roles in regulation of cell proliferation, although the specific mechanisms are not fully elucidated and for NHE1 likely include effects both on pH_i and cell volume.

In conclusion, ion channels and transporters have been implicated in the control of cell cycle checkpoints in normal as well as cancer cells, and specific types of ion channels seem to play an important role in tumor cell proliferation. However, a comprehensive mechanistic picture of the functional relation between ion channels and cell proliferation is yet not available (Becchetti, 2011).

PROGRAMMED CELL DEATH (PCD)

A hallmark of PCD (or its more restrictive term, apoptosis) is a marked cell shrinkage (Kerr et al., 1972), which is entitled *Apoptotic volume decrease*, or AVD (Maeno et al., 2000) (**Figure 2**). AVD is an early event required for triggering of full-blown apoptosis (Maeno et al., 2000; Poulsen et al., 2010), and there is strong evidence that preventing cell volume regulation after shrinkage is associated with induction of apoptosis (Lang and Hoffmann, 2012). AVD results from a loss of KCl via K^+ and Cl^- channels, and concomitant loss of water (Bortner and Cidlowski, 1998; Okada and Maeno, 2001; Okada et al., 2001; Okada, 2004; Lang et al., 2007; Poulsen et al., 2010). Apoptosis thus depends on K^+, Cl^- and Ca^{2+} (to activate Ca^{2+} activated K^+ and Cl^- channels) channels, such as, e.g., various voltage-dependent K^+ channels, two-pore K^+ channels, Ca^{2+} activated K^+-channels, VRAC, some Ca^{2+} -activated Cl^- channels of the ANO family and some Ca^{2+} permeable TRP channels (see Lehen'kyi et al., 2011; Lang and Hoffmann, 2012). Enhanced expression of these ion channels in cancer cells will, as described above, typically stimulate proliferation and migration, but it will in general also be expected to be pro-apoptotic. It seems to be a paradox that cancer cells manage to up-regulate channels mainly involved in proliferation and migration, while at the same time avoiding the expected pro-apoptotic effect of these channels. We favor the interpretation that proliferation /cell cycle progression is dependent on specific windows of temporal-/spatial-/signal-specific modulation of Cl^- and K^+-channel activity, whereas apoptosis may be the result of a longer-term activation of Cl^- and K^+-channels (**Figure 2**). However, elucidation of this important question will require complete characterization of the cell-cycle dependent expression- and activity pattern of the specific channels involved and mapping of their precise subcellular localization.

Proapoptotic effects of enhanced K^+ channel expression include: (i) hyperpolarization and associated Ca^{2+} overload; (ii) AVD; and (iii) increased proteolytic cleavage of pro-caspase 3 secondary to the decrease in intracellular K^+ (Lehen'kyi et al., 2011). The proapoptotic effect of VRAC expression is predominantly on AVD (see e.g., Poulsen et al., 2010). The TRP channels are particularly involved in the control of Ca^{2+} influx participating in the PCD process (Lehen'kyi et al., 2011). Collectively, these findings strongly indicate that ion channel dys-regulation can underlie cancer cell resistance to apoptosis (see below). This is also the case for several ion transporters. Thus, during AVD, cells lose the capacity for counteracting cell shrinkage by triggering a regulatory volume increase (RVI) response (Maeno et al., 2006), which would be normally operating in a healthy cell. In fact, in HeLa cells undergoing apoptosis, the RVI mechanism seems to be weakened (Numata et al., 2008). The transporters involved in RVI thus tend to counteracts apoptosis. As the most important transport systems in RVI are NKCC1, NHE1, the Na^+/K^+ ATPase, and in some cells also ENaC type cation channels (Hoffmann et al., 2009), it seems likely that increased expression or function of these in epithelial cancer would render tumor cells resistant to apoptosis, and in fact, this has been demonstrated in several types of cancers (see below).

ION TRANSPORT AND DRUG RESISTANCE IN CANCER

MULTI DRUG RESISTANCE (MDR)

Chemotherapy resistance—cell-intrinsic or acquired—underlies the failure of most cancer treatments. Many factors are involved in resistance of cancer cells, such as decreased drug uptake, increased drug efflux, detoxification, increased DNA repair, and dys-regulation of apoptotic signaling (Krishna and Mayer, 2000; Stavrovskaya, 2000; Lothstein et al., 2001; Giacomini et al., 2010). One of the most important contributions to drug resistance in solid tumors such as PDAC is a failure to deliver drugs due to poor vascularization of the tumor and impermeability exhibited by dense desmoplasia (see section Chemical/physical properties of the TME for details). The current strategy is to overcome both physical barriers with multi-drug therapy approach (e.g., Provenzano et al., 2012).

ATP-binding cassette (ABC) drug efflux pumps are widely studied in the context of chemotherapy resistance (see e.g., Litman et al., 2001) and will not be discussed here. As described above [sections Loss of epithelial polarity—implications for ion transport and Roles of cell volume regulation in cell proliferation and programmed cell death (PCD)], ion transporters play major roles in shaping the TME, which is, in turn, very important for drug delivery/chemotherapy resistance. The other major contribution of ion transporters in drug resistance in cancer is their role in the resistance to apoptosis, which is one of the major reasons for chemotherapy cross-resistance.

RESISTANCE TO APOPTOSIS

Resistance to apoptosis can develop when the AVD is prevented. This can be mediated by down-regulation of the K^+ and/or Cl^- channels responsible for AVD, as well as of Ca^{2+} channels involved in Ca^{2+} influx and hence modulation of Ca^{2+} sensitive apoptotic steps. Alternatively, resistant cell can develop an enhanced RVI response, which, as described above, counteracts AVD, by up-regulation of NHE1, NKCC1, or hypertonically induced cation channels (HICCS) (**Figure 2**). Accordingly, it was demonstrated that Chinese hamster ovary cells, which do not perform RVI because they lack of NHE1, are more prone to apoptosis compared to cells expressing NHE1 (Rotin and Grinstein, 1989). Moreover, in HeLa cells HICCS rescue cells from staurosporine-elicited apoptosis (Numata et al., 2008). These studies underscore the critical role of volume regulation mechanisms in apoptotic resistance. Finally, although a detailed account of the roles of intracellular channels and transporters in PCD resistance is beyond the scope of this review, it may be noted that the mitochondrial voltage-dependent anion channel, VDAC-1, has been identified as a protein associated with resistance to cisplatin chemotherapy (Tajeddine et al., 2008) and has, although this remains controversial, been suggested to be part of the mitochondrial permeability transition pore, mPTP (see Javadov et al., 2011).

THE ROLE OF ION CHANNELS IN CHEMOTHERAPY RESISTANCE

Ion movements are important in the regulation of apoptosis, but exactly how they are involved in the development of chemotherapy resistance is not always clear; in **Figure 2** and text below we summarize some molecular candidates. *Decreased* K^+

permeability seems to be important cause of cancer cell resistance to apoptosis (Prevarskaya et al., 2010). For example, in PDAC, expression of Kv1.3 is down-regulated, presumably due to aberrant methylation of the Kv1.3 gene promoter, and it is postulated that this may render cells resistant to apoptosis (Brevet et al., 2009). Furthermore, the K^+ ionophore amphotericin B counteracts cisplatin resistance in cancer cell lines (Morikage et al., 1993; Beketic-Oreskovic and Osmak, 1995) by introduction of a high K^+ permeability, and Amphotericin B in conjunction with the NKCC blocker bumetanide was shown to augment cisplatin-induced caspase 3 activation (Marklund et al., 2000, 2001, 2004). The TASK-2 K^+ channel blocker clofilium prevents AVD and abrogates cisplatin-induced caspase 3 activity in a cell line derived from mammary gland adenocarcinomas, Ehrlich ascites tumour cells (EATCs) (Poulsen et al., 2010). Targeting BK (KCa1.1) channels with teraethylammonium or iberiotoxin similarly attenuates cisplatin-induced apoptosis in spiral ligament fibrocytes of the cochlea (Liang et al., 2005). Several human cancers are characterized by a reduced expression of the redox-sensitive K^+ channel Kv1.5 (Bonnet et al., 2007) and down-regulation of Kv1.5 channels in human gastric cancer cells enhances resistance to apoptosis-inducing drugs such as cisplatin (Han et al., 2007). In PDAC, Kv1.3 is down-regulated (Brevet et al., 2009). In addition, TASK-3 (Kcnk9) has been shown to have oncogenic potential in several types of human carcinomas (Pei et al., 2003). Since K^+ channels control cell membrane potential and thus Ca^{2+} influx, the effect of down-regulating K^+ channels on resistance to apoptosis can be also mediated by a decreased Ca^{2+} influx (see also below).

Decreased Cl^- permeability

Induction of apoptosis involves activation of VRAC in several cell types (d'Anglemont de et al., 2004, 2008; Ise et al., 2005; Poulsen et al., 2010). Moreover, some studies have shown a decrease in Cl^- permeability in various MDR cell models (Gollapudi et al., 1992; Lee et al., 2007; Poulsen et al., 2010; Min et al., 2011). The MDR-EATC and the KCP-4 human epidermoid cancer cells, which exhibit acquired resistance to cisplatin, both have strongly decreased VRAC activity (Lee et al., 2007; Poulsen et al., 2010). In KCP-4 cells it was further shown that restoration of the channel's functional expression leads to a decrease in the cisplatin resistance (Lee et al., 2007). Similar results were obtained in human lung adenocarcinoma cells (Min et al., 2011). In wild type EATC, cisplatin treatment induced an AVD response, whereas MDR-EATC showed almost no AVD response when treated with cisplatin (Poulsen et al., 2010). This indicates that impaired activity of VRAC channels contributes to the cisplatin resistance in MDR-EATC by preventing the necessary AVD process.

Ca^{2+} influx

The roles of Ca^{2+} transport in cancer and chemotherapy resistance have been excellently reviewed elsewhere (Prevarskaya et al., 2010, 2013; Dubois et al., 2013) and will only be briefly outlined here. As excessive Ca^{2+}-influx contributes to PCD, conversely, preventing Ca^{2+} influx tends to help the cell to avoid PCD. In agreement with this, apoptosis-resistant prostate cancer cells have strongly reduced levels of store-operated calcium entry (SOCE) (Vanden Abeele et al., 2002; Vanoverberghe et al., 2004; Prevarskaya et al., 2013). The Orai protein is an important component of SOCE, thus down-regulation of Orai will protect the cancer cells from apoptosis. Accordingly, Orai1 was shown to contribute to the establishment of an apoptosis-resistant phenotype in prostate cancer cells (Flourakis et al., 2010).

pH-REGULATORY ION TRANSPORT PROTEINS IN DRUG RESISTANCE IN CANCER CELLS

A growing body of evidence implicates pH-regulatory ion transporters in drug resistance in cancer. The contributions of these transporters to resistance occurs at several levels. Firstly, the acidic extracellular environment in solid tumors, including the creation of a strongly acidic pericellular subdomain due to rapid H^+ efflux (Stock et al., 2007), will, all things equal, decrease the uptake by diffusion across the plasma membrane, of chemotherapeutic drugs which are weak bases, such as doxorubicine and vinblastine, and can alter the carrier-mediated uptake of drugs via pH sensitive uptake carriers (Tredan et al., 2007). Once the drug is inside the cell, the normal-to-alkaline pH_i, created in the tumor cytoplasm through rapid acid extrusion, impacts on the cell death machinery via multiple pathways (Pedersen, 2006). Most work in this context has been done on NHE1, inhibition or knockdown of which has been shown to enhance chemotherapeutically induced cell death in a number of cancer types (Reshkin et al., 2003; Rebillard et al., 2007; Lauritzen et al., 2010; Jin et al., 2011). Also proton pump inhibitors have been effectively used to combat chemotherapy resistance in some cancers (for a review, see De Milito and Fais, 2005), although the mechanisms are less clear, as the H^+ V-ATPases generally predominantly localize to the endosomal/lysosomal compartments, and at least in some cancers appear to contribute little to cytosolic pH regulation (Lauritzen et al., 2010; Hulikova et al., 2013). Finally, inhibition of monocarboxylate carriers (MCTs) in cancer cells that strongly dependent on these transporters should also in principle sensitize cells to chemotherapy, however, little work has so far been done to address this directly (see Halestrap, 2013).

SUMMARY AND PERSPECTIVES

Epithelial cells are endowed with specific sets of ion channels and transporters that are organized in a polarized fashion specific for the function of the given epithelium. The molecular identities, regulation and roles of these channels and transporters in the physiology of epithelial transport and cell volume regulation are relatively well understood. Epithelial cells, no doubt due to their high proliferative rate, but perhaps also due to their continuously challenged cell volume regulation, walk a thin line between physiology and pathophysiology. We suggest, speculatively, that this may endow them with an inherently increased risk of undergoing key events contributing to development of carcinomas. It is interesting to note that in particular epithelia capable of secretion, such as prostate, mammary glands, colorectum, lung/bronchi, pancreas, stomach, and uterus seem to be frequent sites of cancer (Siegel et al., 2013). Does dys-regulation

of existing ion channels/transporters, or changes in the expression of the channels lead to altered cell volume regulation and thus increased proliferation, resistance to apoptosis and chemotherapy? In this review, we have summarized existing evidence for dys-regulation of some of the important ion channels/transporters, generally from cell culture models. However, much more knowledge is needed on genuine epithelial cancer models as well as on epithelial cancers *in vivo*. The complex TME contains a number of local auto- and paracrine agents, and exhibits marked changes in pH, oxygen levels, and probably ion concentrations, compared to the normal epithelial extracellular environment. Moreover, transformed epithelial cells frequently undergo EMT, the basal membrane is degraded, and the epithelial cells come into contact with cell types they would not normally encounter. Future studies should map and functionally characterize the complete ion "transportomes" for the different cell types within the tumor, in order to uncover novel multi-therapeutic approaches to carcinoma chemotherapy.

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Intracellular ion channels and cancer

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Several types of channels play a role in the maintenance of ion homeostasis in subcellular organelles including endoplasmatic reticulum, nucleus, lysosome, endosome, and mitochondria. Here we give a brief overview of the contribution of various mitochondrial and other organellar channels to cancer cell proliferation or death. Much attention is focused on channels involved in intracellular calcium signaling and on ion fluxes in the ATP-producing organelle mitochondria. Mitochondrial K^+ channels (Ca^{2+}-dependent BK_{Ca} and IK_{Ca}, ATP-dependent K_{ATP}, Kv1.3, two-pore TWIK-related Acid-Sensitive K^+ channel-3 (TASK-3)), Ca^{2+} uniporter MCU, Mg^{2+}-permeable Mrs2, anion channels (voltage-dependent chloride channel VDAC, intracellular chloride channel CLIC) and the Permeability Transition Pore (MPTP) contribute importantly to the regulation of function in this organelle. Since mitochondria play a central role in apoptosis, modulation of their ion channels by pharmacological means may lead to death of cancer cells. The nuclear potassium channel Kv10.1 and the nuclear chloride channel CLIC4 as well as the endoplasmatic reticulum (ER)-located inositol 1,4,5-trisphosphate (IP_3) receptor, the ER-located Ca^{2+} depletion sensor STIM1 (stromal interaction molecule 1), a component of the store-operated Ca^{2+} channel and the ER-resident TRPM8 are also mentioned. Furthermore, pharmacological tools affecting organellar channels and modulating cancer cell survival are discussed. The channels described in this review are summarized on **Figure 1**. Overall, the view is emerging that intracellular ion channels may represent a promising target for cancer treatment.

Keywords: cancer, organelles, ion channel, apoptosis, pharmacology

MITOCHONDRIA

The "impermeable" mitochondrial inner membrane (IMM) allows the formation of an electrochemical proton gradient which drives the aerobic synthesis of ATP. The "semipermeable" outer membrane (OMM) encloses a periplasmic space where proteins with fundamental roles in cell death are stored until a sufficiently strong pro-apoptotic signal arrives. Mitochondria have assumed a peculiar role in cancer cell physiology (Ralph and Neuzil, 2009). They are crucial for the control of intracellular Ca^{2+} homeostasis, and produce reactive oxygen species (ROS). ROS are involved in the regulation of physiological processes, but may also be harmful if produced excessively. Mitochondria are the checkpoint of the intrinsic pathway of apoptosis: the release of caspase cofactors, such as cytochrome c (cyt c) and SMAC/Diablo, results in the assembly of the apoptosome and in commitment of the cell to apoptosis. In cancer cells mitochondrial metabolism is deregulated to optimize the production of glycolytic intermediates for anabolic reactions. Much effort has been devoted to discover drugs inducing cancer cell death by targeting tumor-specific alterations of mitochondrial metabolism or by stimulating OMM permeabilization and thus, allowing the release of apoptotic cofactors (Fulda et al., 2010). Mitochondrial ion channels play a role in this process by influencing organellar membrane potential, ROS production, volume, calcium homeostasis, and possibly morphology. The mitochondrial channels characterized over the last two decades include outer membrane-located VDAC and MAC in the IMM, potassium channels mtK_{ATP}, $mtBK_{Ca}$, $mtIK_{Ca}$, mtKv1.3, TASK-3, the non-selective permeability transition pore PTP, chloride channels, and the calcium uniporter (e.g., Zoratti et al., 2009; Shoshan-Barmatz et al., 2010; Rizzuto et al., 2012; Szabò et al., 2012).

CHANNELS OF THE OUTER MITOCHONDRIAL MEMBRANE INVOLVED IN APOPTOSIS/CANCER

Mitochondrial apoptosis-induced channel (MAC)

OMM permeabilization has been proposed to involve oligomers of pro-apoptotic Bax, which display ion channel activity in phospholipid bilayers (e.g., Tait and Green, 2010). However, the hypothesis that Bax alone is sufficient to induce cyt c release has been challenged, given that a single point mutant of Bax did not mediate cell death in Bax/Bak-less mouse embryonic fibroblasts despite forming channels with properties similar to WT Bax (Brustovetsky et al., 2010; Szabò et al., 2011). A pore (mitochondrial apoptosis-induced channel, MAC) with an estimated diameter sufficient to allow the passage of cyt c was detected by patch clamp (Martinez-Caballero et al., 2009). The timing of cyt c release in apoptotic cells correlated with the onset of MAC activity and with the translocation of Bax to mitochondrial membranes. MAC, whose formation requires Bim-induced activation of Bax and a still unidentified protein, is considered as a target for novel

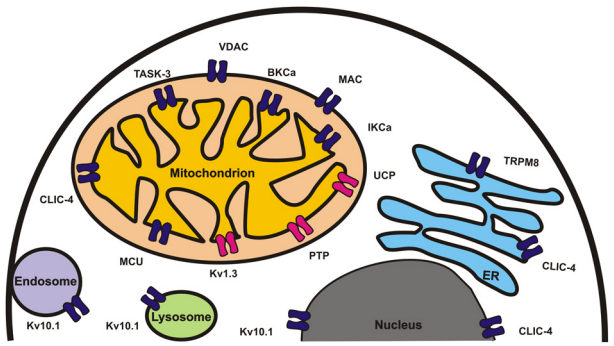

FIGURE 1 | Ion channels involved in regulation of apoptosis and/or tumorigenesis are shown in different organelles. Channels for whose crucial role pharmacological and/or genetic *in vivo* evidence is available are shown in red.

cancer therapies (Peixoto et al., 2012) but specific MAC activators are not available yet. The BH3 mimetic ABT-737, an efficient anticancer agent *in vivo*, activates MAC by disrupting Bcl-2/Bax/Bim complexes (Dejean et al., 2010).

Mitochondrial voltage dependent anion channel (VDAC)

The major protein of the OMM, porin or VDAC is deeply involved in apoptosis. The role of VDAC1 and of the other isoforms VDAC2 and VDAC3 in cell death is multi-faceted and complex (e.g., McCommis and Baines, 2012; Shoshan-Barmatz and Golan, 2012; Shoshan-Barmatz and Mizrachi, 2012). Formation of a large pore comprising VDAC and Bax/Bak was proposed to account for cyt c release (Tsujimoto and Shimizu, 2000; but see Martinez-Caballero et al., 2009). Alternatively, dimers and higher oligomers of VDAC1 might form the conduit for the efflux of cyt c (Shoshan-Barmatz et al., 2010). Binding of anti-apoptotic Bcl-2 and BclxL to VDAC1 (with resulting inhibition of porin) (Shimizu et al., 2000) has an anti-apoptotic action (e.g., Arbel et al., 2012). In contrast, block of VDAC1 by the phosphorothioate oligonucleotide G3139 (Tan, 2012) or by avicins (plant saponins with anticancer activity) is pro-apoptotic, presumably by reducing flux of metabolites across the OMM (Haridas et al., 2007). VDAC2 inhibits Bak activation and apoptosis (Cheng et al., 2003), and Bak reportedly relocates from the OMM to the ER in the absence of VDAC2 (Raghavan et al., 2012). In contrast, Bax-induced cyt c release from mitochondria isolated from WT or VDAC1^{-}, VDAC3^{-} and VDAC1/VDAC3-null cells was reported to be the same (Baines et al., 2007).

VDAC may inhibit apoptosis and promote tumorigenesis through specific interactions with enzymes favoring glycolysis. It is being examined as a cancer-specific target since tumor cells have elevated glycolysis and increased expression of VDACs (Grills et al., 2011). Overexpression of Hexokinase-2 (HK2) and its association with VDAC are key features of glycolytic cancers (e.g., Wolf et al., 2011). HK2 binding to the conduit channeling ATP out of mitochondria provides a metabolic benefit to cancer cells (Warburg effect) and it antagonizes cell death via inhibition of Bax-induced cyt c release (Pastorino et al., 2002; Gall et al., 2011) and/or inhibition of the Mitochondrial Permeability Transition (MPT) (Chiara et al., 2008). HK detachment seems to favor cell death by disruption of aerobic glycolysis and of the energy balance of the cell, regulation of ROS production, altered interaction of Bcl2 family proteins with mitochondria, facilitation of VDAC oligomer formation (e.g., Shoshan-Barmatz et al., 2010; Shoshan-Barmatz and Golan, 2012). Therefore, a major oncological target is the HK-VDAC complex (e.g., Galluzzi et al., 2008; Simamura et al., 2008; Fulda et al., 2010; Mathupala and Pedersen, 2010). HK2 can be dissociated from mitochondria by peptides interfering with HK-VDAC association, by erastin (Yagoda et al., 2007) and by 3-bromopyruvate (e.g., Cardaci et al., 2012; Ko et al., 2012; Pedersen, 2012; Shoshan, 2012). Antifungal drugs clotrimazole and bifonazole and the plant hormone methyl jasmonate (MJ) are also effective. MJ is particularly promising since it has selective anticancer activity in preclinical studies (Fulda et al., 2010). Finally, the anti-cancer agent furanonaphthoquinone (FNQ) induces caspase-dependent apoptosis via the production of ROS, which is enhanced by VDAC1 overexpression (Simamura et al., 2008). A systematic search for compounds acting at the level of VDAC to antagonize cancer remains to be performed.

ION CHANNELS OF THE INNER MITOCHONDRIAL MEMBRANE INVOLVED IN APOPTOSIS/CANCER

Permeability transition pore (MPTP)

When the IMM becomes freely permeable to solutes, the consequences for the cell can be catastrophic. Thus, the selective induction of IMM permeabilization in cancer cells is a strategy worth pursuing in oncotherapy. A number of cellular stresses and cytotoxic agents trigger the prime example of such a catastrophe, i.e., the mitochondrial permeability transition (MPT), considered as a final common pathway of cell death (Brenner and Grimm, 2006; Bernardi, 2013). The MPT is caused by the opening of a large Ca^{2+}- and oxidative stress-activated pore [the mitochondrial megachannel, MMC, with a conductance of up to 1.5 nS (Szabó and Zoratti, 1991)] which makes the IMM permeable to ions and solutes up to about 1500 Da MW, leading to matrix swelling.

MPT is considered to bear substantial responsibilities in the tissue damage caused by, e.g., ischemia/reperfusion and oxidative stress. In cancer cells, instead, signaling pathways are activated which desensitize the mitochondria to MPT induction (Rasola et al., 2010; Matassa et al., 2012; Traba et al., 2012), while chemotherapeutic agents causing oxidative stress may activate signals causing death via the MPT (Chiara et al., 2012). Cyclosporin A (CSA), a cyclic endecapeptide, is a powerful inhibitor of the MPTP (Fournier et al., 1987; Crompton et al., 1988; Broekemeier et al., 1989) (and also of calcineurin and thus is a widely used immunosuppressant). CSA inhibits the MPTP via its binding to matrix cyclophilin (CyP) D, a peptidyl-prolyl cis-trans isomerase (PPIase). Patients treated with CSA to prevent transplant rejection have a high incidence of cancer not only because of the drug's immunosuppressive action, but also because CSA inhibits the MPTP (Norman et al., 2010). The molecular nature of the MPTP is being finally delineated: the dimeric form of ATP synthase and CypD as regulator are currently proposed as components (Baines et al., 2005; Bernardi, 2013; Giorgio et al., 2013).

For oncological applications MPT inducers are relevant, despite the likelihood of noxious side-effects, for example on the nervous system. A large number of compounds, often used at relatively high concentrations, have been shown to induce

the MPT in cultured cells, often as a consequence of oxidative stress and/or disruption of Ca^{2+} homeostasis. Some MPTP-targeting molecules such as 4-(N-(S-glutathionylacetyl) amino) phenylarsenoxide are currently being evaluated in clinical trials for cancer treatment of refractory tumors (Brenner and Moulin, 2012; Elliott et al., 2012).

Signaling pathways which modulate occurrence of the MPT have been elucidated, a key component being GSK3α/β whose activation, e.g., by induction of oxidative stress by gold complex AUL12, favors MPTP opening (Chiara and Rasola, 2013). A large portion of MPTP openers are natural compounds like jasmonates (e.g., Raviv et al., 2013), betulinic acid, the synthetic retinoid CD437 (Lena et al., 2009; Javadov et al., 2011), berberine (Pereira et al., 2007, 2008), honokiol (Li et al., 2007), α-bisabolol (Cavalieri et al., 2009) and shikonin (Han et al., 2007), just to name a few. Data on *in vivo* anti-tumor activities are available for all these compounds (Fulda et al., 2010). Mitochondria-penetrating peptides, such as mastoparan-like sequences, peptides of the innate immunity systems, or the molecules developed by Kelley's group (e.g., Risso et al., 2002; Jones et al., 2008; Horton et al., 2012) also induce MPT. Some MPTP-targeting molecules such as 4-(N-(S-glutathionylacetyl) amino) phenylarsenoxide are currently being evaluated in clinical trials for cancer treatment of refractory tumors (Brenner and Moulin, 2012; Elliott et al., 2012).

IMM potassium channels Kv1.3, BKca, IKca, and TASK-3 in the regulation of apoptosis/cancer

A functional mitochondrial counterpart of the potassium channel Kv1.3 has been identified in the IMM of several cell types (mtKv1.3) (Szabò et al., 2005; Gulbins et al., 2010). It is expected to participate in regulation of mitochondrial membrane potential, volume, and ROS production. A crucial role of mtKv1.3 in apoptosis became evident since expression of a mitochondria-targeted Kv1.3 construct was sufficient to sensitize apoptosis-resistant CTLL-2 T lymphocytes, which lack Kv channels. MtKv1.3 has been identified as a target of Bax and physical interaction between the two proteins in apoptotic cells has been demonstrated (Szabó et al., 2008; Szabò et al., 2011). Incubating Kv1.3-positive isolated mitochondria with Bax triggered apoptotic events including membrane potential changes (hyperpolarization followed by depolarization due to the opening of MPTP), ROS production and cyt c release, whereas Kv1.3-deficient mitochondria were resistant. Highly conserved Bax lysine 128 protrudes into the intermembrane space (Annis et al., 2005) and mimics a crucial lysine in Kv1.3-blocking peptide toxins. Mutation of Bax at K128 (BaxK128E) abrogated its effects on Kv1.3 and mitochondria, as well as in Bax/Bak-less double knockout (DKO) mouse embryonic fibroblasts, indicating a toxin-like action of Bax on Kv1.3 to trigger mitochondrial phenomena.

Psora-4, PAP-1 and clofazimine, three membrane-permeant inhibitors of Kv1.3, can induce death by directly targeting the mitochondrial channel, while membrane-impermeant Kv1.3 inhibitors ShK or Margatoxin did not induce apoptosis (Leanza et al., 2012a,b). Importantly, the membrane-permeant drugs killed cells also in the absence of Bax and Bak, in agreement with the above model. Genetic deficiency or siRNA-mediated downregulation of Kv1.3 abrogated the effects of the drugs. Intraperitoneal injection of clofazimine reduced tumor size by 90% in an orthotopic melanoma B16F10 mouse model *in vivo*, while no adverse effects were observed in several healthy tissues. Similar results were obtained with primary human cancer cells from patients with chronic lymphocytic leukemia (Leanza et al., 2013). The selective action of these drugs on tumor cells is related to a synergistic effect of a higher expression of Kv1.3 and of an altered redox state of cancer cells. The fact that clofazimine is already used in the clinic for the treatment of e.g., leprosis (Ren et al., 2008) and shows an excellent safety profile supports the feasibility of targeting mtKv1.3 for therapy.

The large conductance calcium- and voltage-activated K^+ channel BK_{Ca} (KCa1.1) has been revealed also in intracellular membranes, including nuclear membrane, ER, Golgi and mitochondria (Xu et al., 2002; O'Rourke, 2007; Singh et al., 2012). Patch clamp experiments with recombinant Bax showed an inhibition of BK_{Ca}, which might contribute to opening of the MPTP during cell death (Cheng et al., 2011).

The intermediate conductance potassium channel (IK_{Ca}; KCa3.1), selectively inhibited by clotrimazole and TRAM-34, has been recorded from the inner mitochondrial membranes of human cancer cells (De Marchi et al., 2009; Sassi et al., 2010). TRAM-34 used alone did not induce apoptosis (Sassi et al., 2010; Quast et al., 2012), but it synergistically increased sensitivity to the death receptor ligand TRAIL in melanoma cells (Quast et al., 2012). Given that both TRAM-34 and TRAIL have a relatively good safety profile, co-administration of the two drugs might be exploited for melanoma treatment.

Recently TASK-3 (KCNK9), a two-pore potassium channel, was identified in mitochondria of melanoma and keratinocyte (Rusznák et al., 2008) as well as healthy intestinal epithelial cells (Kovács et al., 2005). Reduced expression of TASK-3 resulted in compromised mitochondrial function and cell survival in WM35 melanoma cells (Kosztka et al., 2011). Whether TASK-3 protein gives rise to a functional channel in the IMM and whether it will become an oncological target remain to be determined.

OTHER IMM CHANNELS LINKED TO TUMORIGENESIS: UNCOUPLING PROTEIN UCP, Mg^{2+} CHANNEL Mrs-2 AND CALCIUM UNIPORTER MCU

Uncoupling protein-2 (UCP-2), which mediates proton leak (Cannon and Nedergaard, 2004; Fedorenko et al., 2012), has been proposed to regulate cell survival by decreasing mitochondrial ROS, since a depolarizing proton leak is expected to diminish superoxide production (Baffy et al., 2011). UCP2 over-expression reportedly prevents oxidative injury, thereby possibly contributing to a higher apoptotic threshold assisting survival of cancer cells. Over-expression of UCP2 was found in numerous types of tumors and has been shown to protect cells from oxidative stress (Arsenijevic et al., 2000; Zhang et al., 2007) and even to abolish chemotherapeutic agent- induced apoptosis (Derdak et al., 2008). Ectopic expression of UCP2 in MCF7 breast cancer cells leads to a decreased mitochondrial membrane potential and increased tumorigenic properties as measured by cell migration, *in vitro* invasion, and anchorage independent growth. Interestingly, UCP2 over-expression has also been proposed to directly contribute to the Warburg phenotype (Samudio et al., 2008) and to development of tumors in an orthotopic model of breast cancer (Ayyasamy et al., 2011). Cisplatin downregulated the expression of UCP2 in colon cancer

cells (Santandreu et al., 2010), suggesting that UCP2 overexpression is involved in the development of a variety of cancers. UCP2 can be considered as a promising oncological target.

Mitochondria accumulate Mg^{2+} via Mrs2, a Mg^{2+}–selective channel of the IMM (Kolisek et al., 2003). An early increase in cytosolic Mg^{2+} occurs during apoptosis (Chien et al., 1999) and this ion seems to be required for cytochrome c release (Kim et al., 2000). Long-lasting knock-down of Mrs2 caused cell death by inducing loss of respiratory complex I and mitochondrial membrane depolarization (Piskacek et al., 2009). A subtractive hybridization method applied on vincristine or adriamycin resistant and parental human gastric adenocarcinoma cell lines highlighted upregulation of Mrs2 (Chen et al., 2009), suggesting that high expression of Mrs2 may protect against death (Wolf and Trapani, 2009).

The molecular identification of the mitochondrial Ca^{2+} "uniporter" (MCU), responsible for the low-affinity uptake of calcium into the mitochondrial matrix (Kirichok et al., 2004), has recently been achieved (Baughman et al., 2011; De Stefani et al., 2011). MCU participates in the control of Ca^{2+} signaling in the whole cell, and may thus be a very useful tool to influence the myriad cellular calcium-dependent processes, including cell death (Rizzuto et al., 2012). Subthreshold apoptotic signals were shown to synergize with cytosolic Ca^{2+} waves (Pinton et al., 2001) resulting in opening of MPTP. Cells overexpressing MCU underwent more pronounced apoptosis upon challenging with H_2O_2 and C2-ceramide (De Stefani et al., 2011). Overexpression of an MCU-targeting microRNA, miR-25, in colon cancer cells resulted in MCU downregulation, impaired calcium uptake and increased resistance to apoptosis (Marchi et al., 2013). Thus, MCU seems to be a crucial protein for tumorigenesis and its specific pharmacological activators, if identified, might become useful tools.

ION CHANNELS IN OTHER ORGANELLES WITH A ROLE IN APOPTOSIS/TUMORIGENESIS

The intracellular chloride channel CLIC4/mtCLIC has both a soluble and a membrane-inserted form and can be localized to the mitochondrial inner membrane (Fernández-Salas et al., 1999), cytoplasm, ER membrane, and the nucleus. CLIC4 overexpression induced apoptosis associated with loss of mitochondrial membrane potential, cytochrome c release, and caspase activation (Fernández-Salas et al., 2002). On the other hand, inhibition of CLIC4 expression triggered mitochondrial apoptosis under starvation and enhanced autophagy in glioma cells (Zhong et al., 2012). Marked changes in expression and subcellular localization of CLIC4 occur early in tumorigenesis. In particular, reduced CLIC4 expression and nuclear localization in cancer cells is associated with the altered redox state and CLIC4 acts as an important suppressor of squamous tumor development and progression (Suh et al., 2012).

A functional "oncogenic" potassium channel, Kv10.1 has been described in the nuclear inner membrane (Chen et al., 2011) where it might participate in setting nuclear $[K^+]$ thereby affecting gene expression. The PM Kv10.1 is also rapidly internalized to lysosomes (Kohl et al., 2011), whose patch clamping has been achieved (Wang et al., 2012). The possible influence of these channels on cancer cell survival remains to be determined.

Finally, we should briefly mention other intracellular channels involved in Ca^{2+} signaling, (Ca^{2+} permeable channels are discussed in detail by other contributions in this special issue). For example the calcium-permeable ion channel TRPM8, overexpressed in several tumors, has been located to the ER (Zhang and Barritt, 2004), resulting in decreased ER $[Ca^{2+}]$ and increased resistance to apoptosis (Bidaux et al., 2007). Patients suffering of breast cancers with high ER-located STIM1 levels have significantly reduced survival (McAndrew et al., 2011). The PM-located other component, ORAi1 contributes to altered calcium homeostasis as well (Monteith et al., 2012). Expression of ER-resident IP_3 receptors acting as Ca^{2+} store release channels is altered in glioblastoma (Kang et al., 2010). Repression of IP_3- mediated Ca^{2+} elevation by Bcl-2 has been proposed to contribute to the pathophysiology of chronic lymphocytic leukemia (Zhong et al., 2011).

In summary, while considerable further work is required to clarify the mechanisms by which intracellular channels contribute to tumorigenesis and tumor progression, or intervene in cell death, a few *in vivo* studies targeting these channels underline the importance of pursuing this line of research.

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Nicotinic acetylcholine receptors mediate lung cancer growth

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Ion channels modulate ion flux across cell membranes, activate signal transduction pathways, and influence cellular transport—vital biological functions that are inexorably linked to cellular processes that go awry during carcinogenesis. Indeed, deregulation of ion channel function has been implicated in cancer-related phenomena such as unrestrained cell proliferation and apoptotic evasion. As the prototype for ligand-gated ion channels, nicotinic acetylcholine receptors (nAChRs) have been extensively studied in the context of neuronal cells but accumulating evidence also indicate a role for nAChRs in carcinogenesis. Recently, variants in the nAChR genes *CHRNA3, CHRNA5,* and *CHRNB4* have been implicated in nicotine dependence and lung cancer susceptibility. Here, we silenced the expression of these three genes to investigate their function in lung cancer. We show that these genes are necessary for the viability of small cell lung carcinomas (SCLC), the most aggressive type of lung cancer. Furthermore, we show that nicotine promotes SCLC cell viability whereas an α3β4-selective antagonist, α-conotoxin AuIB, inhibits it. Our findings posit a mechanism whereby signaling via α3/α5/β4-containing nAChRs promotes lung carcinogenesis.

Keywords: nicotinic acetylcholine receptor, ligand-gated ion channel, lung cancer, small cell lung carcinoma, CHRNA5

INTRODUCTION

Lung cancer remains the leading cause of cancer-related deaths worldwide (WHO, 2011). Despite considerable research efforts to elucidate the molecular underpinnings of the disease, the 5-year survival rate for lung cancer has not changed appreciably over the past three decades and persists at a dismal 15%. The two major types of lung cancer are non-small cell lung carcinoma (NSCLC) and SCLC. The former consists of a heterogeneous group of tumors that account for 80% of lung cancer cases while the latter is less common (15–20% of cases) but is particularly aggressive (Rom et al., 2000; Sandler, 2003). SCLC is characterized by rapid growth and early dissemination resulting in an extremely poor prognosis for which no effective treatments are currently available (Rudin et al., 2008).

Cigarette smoking is the major risk factor associated with lung cancer. This is not surprising given that tobacco contains ∼250 damaging chemicals and ∼50 carcinogens (Hecht, 1999). In the United States alone, over 45 million adults continue to smoke while globally, 10 million cigarettes are sold every minute, making tobacco use the leading cause of preventable deaths (WHO, 2011). Prevention efforts are hampered, however, by the strong reinforcing effects of nicotine, the primary psychoactive component in tobacco.

Nicotine's effects are mediated by nAChRs that are expressed in the reward circuitry and other areas of the brain (Albuquerque et al., 2009). nAChRs are also activated by the endogenous ligand acetylcholine (ACh), hence their name. Additionally, nAChRs are activated by the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the most potent carcinogen in tobacco (Schuller, 2007). Receptor activation allows the flow of sodium, potassium and calcium ions down their electrochemical gradients.

nAChRs are composed of transmembrane subunits that share a common evolutionary origin (Le Novere et al., 2002). In mammalian systems, these subunits are encoded by eleven genes located across different chromosomes (**Table 1**). The genes encoding the human α3, α5, and β4 subunits are found in a gene cluster in chromosome 15q24 and are thought to be both independently and coordinately regulated (Boulter et al., 1990; Scofield et al., 2008). The α3 subunit is usually co-expressed with the β4 subunit while α5 serves as an auxiliary subunit, whose incorporation modifies the calcium permeability of the receptor and its affinity to and desensitization by agonists (Ramirez-Latorre et al., 1996; Yu and Role, 1998). α3β4-containing nAChRs exhibit lower affinity for nicotine and are less desensitized by it, suggesting that this receptor subtype may mediate nicotine's rewarding effects after high affinity nAChR subtypes have

Abbreviations: NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; ACh, acetylcholine; nAChRs, nicotinic acetylcholine receptors; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; GWAS, genome-wide association studies.

been desensitized (Paradiso and Steinbach, 2003; Rose, 2007). Moreover, α3β4α5 nAChRs are thought to play a role in nicotine withdrawal and consistently, are highly expressed in brain regions associated with nicotine withdrawal, such as the medial habenula and the interpeduncular nucleus (Damaj et al., 2003; Salas et al., 2009).

Multiple genome-wide association studies (GWAS) have implicated the *CHRNB4/A3/A5* locus in nicotine dependence and lung cancer (Amos et al., 2008; Hung et al., 2008; Thorgeirsson et al., 2008). A particularly interesting variant in this locus is the non-synonymous single nucleotide polymorphism (SNP) that lies in the fifth exon of *CHRNA5* (rs16969968). This variant encodes a change from an aspartic acid to an asparagine residue at amino acid position 398 (D398N). The asparagine risk allele is associated with decreased maximal response to agonists, indicating altered receptor function (Bierut et al., 2008; George et al., 2012). Additionally, the genotype in this locus appears to correlate with mRNA levels suggesting that rs16969968 may influence *CHRNA5* expression as well (Falvella et al., 2009; Wang et al., 2009). In α3β4α5 nAChRs, the 398 residue also lies close to a β4 residue that is necessary for β4′s ability to increase nicotine-evoked currents, which subsequently leads to nicotine aversion (Frahm et al., 2011). Notably, this increase in current is maximally competed by the D398N variant, resulting in reversal of nicotine aversion. Altogether, these results support the functional relevance of the rs16969968 variant.

The association of nAChR variants with both nicotine dependence and lung cancer susceptibility prompts two hypotheses regarding the role of nAChRs in lung cancer. One hypothesis is that nicotine mainly influences nAChRs in the brain, such that increased levels of nicotine dependence consequently lead to greater exposure to tobacco carcinogens and to lung cancer development (Le Marchand et al., 2008). Consistently, α3, α5, and β4 nAChR subunits are predominantly expressed in select neural circuits that control nicotine intake in rodent nicotine dependence models (Fowler et al., 2011; Frahm et al., 2011). An alternative hypothesis is that the association between nAChR variants and lung cancer is direct, in that altered nAChR function, as encoded by risk alleles, promote carcinogenic processes in the lungs and airway tissues (Schuller, 2009). The following sections discuss evidence in the literature as well as primary data that support a direct role for nAChRs in lung cancer.

Table 1 | Chromosomal locations of genes encoding nAChR subunits.

Subunit*	Gene	Chromosome location**		
		Mouse	Rat	Human
α2	*CHRNA2*	14	15p12	8p21
α3	*CHRNA3*	9	8q24	15q24
α4	*CHRNA4*	2	3q43	20q13.2-q13.3
α5	*CHRNA5*	9	8q24	15q24
α6	*CHRNA6*	8	16q12.3	8p11.21
α7	*CHRNA7*	7	1q22	15q14
α9	*CHRNA9*	5	14p11	4p14
α10	*CHRNA10*	7	1q32	11p15.5
β2	*CHRNB2*	3	2q34	1q21.3
β3	*CHRNB3*	8	16q12.3	8p11.2
β4	*CHRNB4*	9	8q24	15q24

*α8 is expressed only in avian species.

**Summarized from the Entrez Gene Database (Maglott et al., 2011).

EXPRESSION AND FUNCTION OF nAChRs IN LUNG CANCER

The first hint that nAChRs play a direct role in lung cancer comes from several studies demonstrating nAChR expression in several types of cancers (**Table 2**). In lung cancer, we have detected the expression of several nAChR subunit genes, in particular *CHRNA3, CHRNA5,* and *CHRNB4* (Improgo et al., 2010). Differences in nAChR gene expression between smokers and non-smokers have also been reported (Lam et al., 2007).

Another line of evidence stems from studies showing that nAChR ligands promote several hallmarks of cancer (Hanahan and Weinberg, 2000; Schuller, 2009). Nicotine induces cell proliferation in lung cancer cells via protein kinase C (Schuller, 1989; Codignola et al., 1994) and Akt (West et al., 2003; Tsurutani et al., 2005) activation. Nicotine's carcinogenic metabolites have also been shown to promote cell proliferation in lung cancer cells via serotonin-induced stimulation of the Raf-1/MAPK/c-myc pathway (Schuller and Orloff, 1998; Jull et al., 2001) and the Akt pathway (West et al., 2003; Tsurutani et al., 2005).

In addition, nicotine has been shown to inhibit apoptosis by phosphorylation of Bcl-2 family members (Jin et al., 2004a). Apoptotic evasion potentially contributes to nicotine-induced chemoresistance (Maneckjee and Minna, 1990, 1994). Similarly,

Table 2 | Types of cancer cells expressing nAChR subunits.

Cancer type	nAChR subunits	References
Cervical cancer	α5, α7, α9	Calleja-Macias et al., 2009
Colon cancer	α7	Ye et al., 2004
Leukemia	α2, α3, α5, α6, α7, α9, β2, β4	Sato et al., 1999; Chernyavsky et al., 2009
Lung cancer: NSCLC	α3, α4, α5, α6, α7, α9, β2, β4	West et al., 2003; Tsurutani et al., 2005; Lam et al., 2007; Improgo et al., 2010
Lung cancer: SCLC	α3, α5, α7, α9 β2, β4	Codignola et al., 1994; Song et al., 2003; Improgo et al., 2010
Mesothelioma	α7	Trombino et al., 2004
Medulloblastoma	α7	Siegel and Lukas, 1988
Neuroblastoma	α3, β4	Lukas, 1993

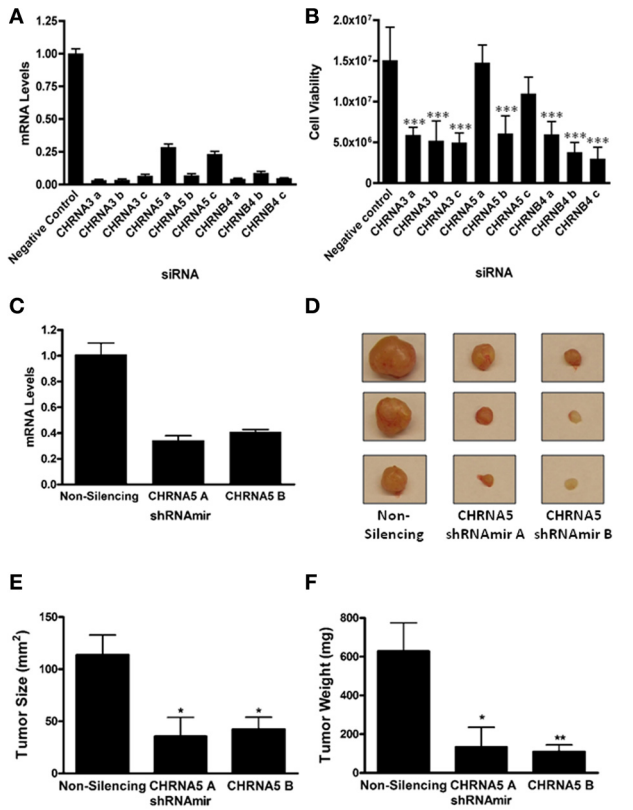

FIGURE 1 | *CHRNA3, CHRNA5,* and *CHRNB4* depletion decreases SCLC cell growth. DMS-53 cells were treated with three distinct *CHRNA3, CHRNA5,* and *CHRNB4* siRNAs or a negative control siRNA (Applied Biosystems). **(A)** Quantitative RT-PCR was performed to determine mRNA levels upon knockdown. **(B)** A bioluminescence-based cell viability assay was performed to determine the effect of siRNA treatment on SCLC cell viability (***$p < 0.001$, ANOVA, Tukey *post-hoc* test). **(C)** *CHRNA5* mRNA levels in DMS-53 cells stably expressing a non-silencing shRNAmir or two *CHRNA5* shRNAmirs (Open Biosystems). shRNAmirs were transduced into DMS-53 cells using a lentiviral delivery system for stable expression (Open Biosystems Trans-Lentiral Packaging System). **(D)** DMS-53 cells stably expressing a non-silencing shRNAmir or two CHRNA5 shRNAmirs were subcutaneously injected into the hind flanks of athymic nude mice. Tumors were harvested after 2 months (representative images shown). Tumor size **(E)** and weight **(F)** were significantly lower in samples treated with CHRNA5 shRNAmirs vs the non-silencing control (*$p < 0.05$; **$p < 0.01$, ANOVA, Tukey *post-hoc* test).

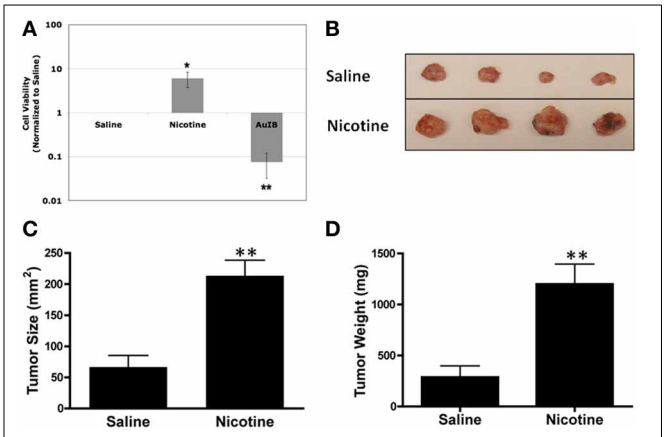

FIGURE 2 | Pharmacological activation or inhibition of nAChRs modulates SCLC growth. (A) DMS-53 cells were treated daily for 1 week with 1 μM nicotine or 2 μM α-conotoxin AuIB. Cell viability assays show that nicotine increases while AuIB decreases SCLC cell viability (values normalized to saline control; *$p < 0.05$, **$p < 0.01$). **(B)** DMS-53 cells were injected subcutaneously into the hind flanks of athymic nude mice. Mice were then implanted with osmotic minipumps that delivered either saline or 24 mg/kg of nicotine daily. Tumors were harvested after 1 month (representative images shown). **(C,D)**. Chronic nicotine exposure increased both tumor size and weight (**$p < 0.01$, Student's *t*-test).

NNK inhibits apoptosis by Bcl-2 phosphorylation (Jin et al., 2004b). Moreover, both nicotine and NNK promote cell survival via the NF-kB pathway (Tsurutani et al., 2005).

Intriguingly, the complete cholinergic system is expressed and functional in lung cells, where ACh acts both as an autocrine and paracrine growth factor (Song et al., 2003; Proskocil et al., 2004). Once activated, this growth loop may provide endogenous mitogenic signaling without further nicotine activation. Such a mechanism may explain residual risk for lung cancer even after smoking cessation.

ACh is also thought to act as a pro-angiogenic signal via autocrine and paracrine signaling in endothelial cells (Heeschen et al., 2002). Nicotine promotes angiogenesis in a PI3-kinase and MAPK-dependent manner (Heeschen et al., 2001). Moreover, nicotine and its metabolite cotinine have been shown to up-regulate the expression of the pro-angiogenic factor VEGF (Conklin et al., 2002).

Many of these cancer-promoting processes are abrogated by pan-nAChR or subtype-selective antagonists. Nicotine-induced cell proliferation via fibronectin up-regulation can be abolished by α-BTx (Zheng et al., 2007). Both α-BTx and the non-selective nAChR inhibitor, mecamylamine, also hinder angiogenic growth *in vitro* (Heeschen et al., 2002). Additionally, nicotine-induced Akt activation and airway cell transformation can be inhibited by the α4β2-selective antagonist, DHβE (West et al., 2003). These observations collectively indicate that many of the above cancer-related processes are mediated by nAChRs.

CHRNA3/A5/B4 IN SCLC

Given the GWAS association between the *CHRNB4/A3/A5* locus and lung cancer risk, we pursued the hypothesis that nAChRs containing the α3, α5, and β4 subunits play a direct role in the development of lung cancer. We focused on SCLC as we had previously observed high expression of *CHRNA5* and upregulation of *CHRNA3* and *CHRNB4* in SCLC (Improgo et al., 2010). We investigated the role of *CHRNA3, CHRNA5,* and *CHRNB4* in SCLC by silencing the expression of these three genes in the SCLC cell line, DMS-53. Three distinct siRNAs against each gene were used (**Figure 1A**). We assessed the effect of knockdown using a bioluminescence-based cell viability assay as previously described (Improgo et al., 2011). All the siRNAs that yielded > 90% knockdown levels produced corresponding decreases in SCLC cell viability ($n = 5$ each, **Figure 1B**), suggesting that expression of *CHRNA3*, *CHRNA5*, and *CHRNB4* is vital for SCLC cell viability, at least in the context of the DMS-53 cell line.

The two siRNAs that yielded the least knockdown (CHRNA5 a and c) did not significantly affect cell viability, suggesting that certain *CHRNA5* depletion thresholds may need to be reached to obtain an observable phenotype. To therefore obtain more robust silencing, we utilized shRNAmirs, hairpins that are designed with flanking miRNA sequences that can harness the cell's endogenous RNAi machinery and promote more efficient knockdowns. In addition, we introduced these shRNAmirs to DMS-53 cells via lentiviral delivery to allow stable expression. For a more physiological approach, we used a tumor xenograft model for *in vivo* propagation of tumors. Cells were implanted into immunocompromised mice and tumor growth was monitored. *CHRNA5* shRNAmir A treatment ($n = 5$) caused a 59% decrease in *CHRNA5* levels while *CHRNA5* shRNAmir B treatment ($n = 9$) caused a 66% decrease in mRNA levels (**Figure 1C**). Quite strikingly, tumor size and tumor weight (**Figures 1D–F**) were significantly lower in cells treated with the *CHRNA5* shRNAmirs vs. the non-silencing shRNAmir control ($n = 9$). These results further support the *in vitro* data described above.

We next tested the effect of nAChR ligands on SCLC cell viability. Using the same bioluminescence assay as above, we observed that nicotine treatment increased SCLC cell viability (**Figure 2A**), consistent with aforementioned reports. To perform the converse experiment, we utilized the α3β4-selective ligand, α-conotoxin AuIB. α-conotoxins are derived from the venom of cone snails, a valuable source for disulfide-bonded peptides that target nAChRs in a highly subtype-selective manner (Azam and McIntosh, 2009). α-conotoxin AuIB, in particular, was isolated from the snail-eating cone *Conus aurilicus* and blocks α3β4 nAChRs with > 100-fold higher potency compared to other nAChR subtypes (Luo et al., 1998). Treatment with α-conotoxin AuIB led to decreased viability of DMS-53 cells (**Figure 2A**), indicating that functional α3β4 nAChRs are present in SCLC cells and are important for the maintenance of SCLC cell viability. In agreement with our genetic approach, this pharmacological approach suggests that activation and blockade of α3α5β4 nAChRs modulates SCLC cell viability.

We then tested the effect of chronic nicotine treatment on tumor growth *in vivo*. We used osmotic minipumps to deliver nicotine as these devices allow continued dosing of drugs while eliminating repeated injections (Salas et al., 2004). Using the same xenograft tumor model as above, we found that chronic nicotine treatment increased tumor size and weight *in vivo* compared to saline controls (**Figures 2B–D**). This is in line with previous findings showing that nicotine promotes tumor growth in various *in vivo* models (Davis et al., 2009; Al-Wadei et al., 2012).

Our results show that *CHRNA3, CHRNA5,* and *CHRNB4* expression is critical for SCLC cell viability. These findings lend mechanistic support to the correlative link between the *CHRNB4/A3/A5* locus and lung cancer susceptibility. That α3β4α5 nAChRs play a direct role in lung cancer, in addition to their role in the brain, points to the pleiotropic function of these genes. Along with published reports, our work suggests a mechanism by which cholinergic signaling via α3β4α5 nAChRs promotes SCLC growth. Though this may raise questions regarding the use of nicotine-based smoking cessation approaches, it also indicates the potential of nAChR antagonists for SCLC therapy.

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The effect of carbonic anhydrase IX on focal contacts during cell spreading and migration

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Carbonic anhydrase IX is a hypoxia-induced transmembrane enzyme linked with solid tumors. It catalyzes the reversible hydration of CO_2 providing bicarbonate ions for intracellular neutralization and protons for extracellular acidosis, thereby supporting tumor cell survival and invasiveness. CA IX is the only human CA isoform containing the proteoglycan (PG) domain in its extracellular part. The PG domain appears to enhance the catalytic activity of CA IX and mediate its binding to the extracellular matrix. Moreover, manipulation of the CA IX level by siRNA or overexpression modulates cell adhesion pathway so that in the presence of CA IX, cells display an increased rate of adhesion and spreading. Here we show that deletion of the PG domain as well as treatment with the PG-binding monoclonal antibody M75 can impair this CA IX effect. Accordingly, CA IX-expressing cells show more prominent and elongated maturing paxillin-stained focal contacts (FC) than CA IX-negative controls, proving the role of CA IX in cell spreading. However, during active cell movement, CA IX is relocalized to lamellipodia and improves migration via its catalytic domain. Thus, we examined the influence of CA IX on FC turnover in these structures. While the lamellipodial regions lacking CA IX display dash-like adhesions, the CA IX-enriched neighboring regions exhibit dynamic dot-like FCs. These results suggest that CA IX can promote initial adhesion through its PG domain, but at the same time it facilitates formation of nascent adhesions at the leading edge of moving cells. Thereby it may allow for transmission of large forces and enhanced migration rate, presumably through catalytic activity and impact of pHe on FC dynamics. Thus, we provide the first evidence that CA IX protein localizes directly in focal adhesion (FA) structures and propose its functional relationship with the proteins involved in the regulation of FC turnover and maturation.

Keywords: carbonic anhydrase IX, focal adhesion, migration, paxillin, cell spreading, ROCK1

INTRODUCTION

The process of focal adhesion (FA) is a necessary step accompanying the migration and invasion of tumor cells during their metastatic dissemination. The adhesome, a network of multiprotein complexes that coordinates FA signaling, includes at least 180 proteins (Zaidel-Bar and Geiger, 2010). Carbonic anhydrase IX is structurally predisposed to participate in this network. It is a cell surface protein consisting of the extracellularly exposed proteoglycan-like region (PG) and catalytic (CA) domain, which is anchored in the plasma membrane by single-pass transmembrane region and short intracytoplasmic tail (IC). Protein kinase A phosphorylates threonine in the IC tail of CA IX and regulates its enzymatic activity (Ditte et al., 2011). The PG-domain is a unique feature of CA IX as it is present only in this isoform of all known carbonic anhydrases (Opavsky et al., 1996). Full-length PG domain exhibits 38% homology with a keratan-sulfate region of aggrecan, a cartilage protein which can interact with ECM components hyaluronan and collagen (Opavsky et al., 1996; Nishimura et al., 1998; Hedlund et al., 1999). This similarity between CA IX and aggrecan indicates possible CA IX involvement in cell-ECM interactions. Moreover, the FA process is also influenced by the extracellular pH (pHe) which is, among other proteins, also modulated by CA IX via its enzymatic activity (Svastova et al., 2004; Stock et al., 2005; Ludwig et al., 2013).

CA IX is expressed in many solid tumors, though lacking in the corresponding normal counterparts (Pastorekova et al., 2006). In different tumor types, CA IX is considered a clinically useful biomarker with prognostic and/or predictive values and as a therapy target. Particularly high and frequent expression of CA IX is present in renal cell carcinomas, where the CA IX-specific monoclonal antibody G250 is evaluated as a promising tool for immunotherapy (Oosterwijk-Wakka et al., 2013). *CA9* gene is strongly regulated by hypoxia as a direct target of the hypoxia-inducible transcription factor (HIF-1) binding to its core promoter (Wykoff et al., 2000). Hypoxic tumors are among the most aggressive ones as hypoxia leads to microenvironmental

changes, such as acidosis and lack of nutrients, which promote the development of promigratory and proinvasive cell phenotype (Chiche et al., 2010). Hypoxia is also functionally linked to altered matrix properties (Erler and Weaver, 2009) through e.g., upregulation of collagen synthesis and remodeling of the ECM by prolyl 4-hydroxylase (P4H) and lysyloxidase (LOX) (Fahling et al., 2004; Postovit et al., 2008). Extracellular acidosis enhances the activity of matrix metalloproteases (MMP) either directly by protonating them or their substrates or indirectly by affecting their exocytosis (Holman et al., 1999; Monaco et al., 2007; Iessi et al., 2008). All these hypoxia-induced changes of the extracellular matrix and pHe facilitate escape of tumor cells from hostile conditions.

CA IX is well-known for its role in pH regulation and acidification of tumor microenvironment, which is based on its ability to catalyze conversion of CO_2 to H^+ and HCO_3^-. The underlying mechanism includes CA IX-generated bicarbonate ions that directly feed bicarbonate transporters for the neutralization of intracellular pH (Swietach et al., 2009; Orlowski et al., 2012). On the other hand, simultaneously produced protons support extracellular acidosis, particularly in hypoxic tumors (Svastova et al., 2004). We recently proved the importance of the catalytic activity of CA IX for the enhancement of cell migration and direct interaction of CA IX with the bicarbonate transporters NBCe1 and AE2 in migratory organelles known as lamellipodia (Svastova et al., 2012). Interestingly, several proteins involved in the adhesome are either pH sensors and/or their activity is influenced by pH (Srivastava et al., 2007; Stock and Schwab, 2009).

The formation and strength of FA are also influenced by the extracellular (pHe) and intracellular pH (pHi) (Lehenkari and Horton, 1999; Stock et al., 2005; Srivastava et al., 2008; Paradise et al., 2011). Assembly of FA sites is a gradual process requiring the step-by-step recruitment of individual proteins that connect integrins and other ECM receptors with actin cytoskeleton. Integrins recruit adaptor and signaling proteins, such as paxillin, vinculin, talin, focal adhesion kinase (FAK), Rho GTPases, etc. (Webb et al., 2002; Parsons, 2003). Focal contacts (FCs) grow and dissolve in close relation to actin polymerization and myosin II-generated tension (Vicente-Manzanares et al., 2009). A central molecule for both assembly and turnover of FCs is the adaptor protein paxillin, which directly binds to integrins (Zaidel-Bar et al., 2007). It can also recruit FAK into an adhesion plaque and trigger its autophosphorylation at Tyr397 which creates a binding site for Src family kinases (Worth and Parsons, 2008). This leads to further FAK phosphorylation at other residues to attain the maximal kinase activity. RhoA-associated protein kinase (ROCK) is essential for myosin II-generated tension and represents a key mechanism of FA maturation. Specific inhibition of ROCK1 or downregulation of the myosin II activity decreases the size of FAs (Rottner et al., 1999; Pasapera et al., 2010). It is therefore interesting that microarray results with HT1080 cells silenced for CA IX showed approximately 50% downregulation of ROCK1 accompanied with the inhibition of FA pathway (Radvak et al., 2013).

FA in migrating cells differs from that in quiescent cells. The migratory cycle consisting of the repetitive adhesion-deadhesion of the front and rear ends of moving cells requires dynamic assembly and disassembly of FCs (Webb et al., 2002). Arising FCs in the leading edge of the lamellipodium undergo the maturation process from new, nascent adhesions to mature FAs. They differ in cellular localization, size, type of actin they are connected to, levels of individual proteins and their phosphorylation (Ridley et al., 2003; Choi et al., 2008). Nascent adhesions formed in the protruding lamellipodia contain, among other proteins, integrin receptors, talin, vinculin, paxillin, α-actinin, FAK, and are enriched in phosphotyrosine. They have a short lifespan (∼60 s) and can either rapidly turnover or grow into focal complexes depending on the cell type (Parsons et al., 2010). Nascent adhesions are connected with dendritic actin and are responsible for the transmission of strong propulsive forces leading to the advancement of lamellipodia (Beningo et al., 2001). Focal complexes developing in the region located right behind the nascent adhesion area are bigger in size and can continue to mature into larger, elongated FAs anchoring thick actin bundles. Actin-crosslinking activity of the myosin II which generates acto-myosin tension, contributes considerably to adhesion maturation. Matured focal adhesions exert weaker forces sufficient for passive anchorage which are important for maintaining a spread cell morphology. Changes, to which FC are subjected during the cell migration cycle, reflect the changes of their function, strictly defined by their localization along the apical-basal axis of a migrating cell.

Here we investigated the contribution of CA IX to cell spreading and adhesion. Firstly, we showed that downregulation of the CA IX level decreases cell attachment and spreading in part by reducing expression of the ROCK1 kinase, the activity of which is critical for the myosin II-driven maturation of FA. Secondly, we provided evidence that the enhancement of CA IX-mediated cell adhesion in quiescent cells depends on the proteoglycan-domain in its extracellular part. Further, we proved that CA IX is directly localized in FC where it colocalizes with the adhesion protein paxillin from the very early stages of the cell attachment to the fully spread state. Moreover, we demonstrated that CA IX colocalizes with paxillin in nascent FAs of lamellipodia in migrating cells where the pH-modulating activity of CA IX can play a role in FC turnover. Finally, through *in silico* analysis of the gene-profiling database of clinical studies we found that different types of tumors with up-regulated *CA9* display activated FA pathway. Altogether, our findings suggest the involvement of CA IX in FA of quiescent as well as migrating cells and thereby disclose a new function for this pH-regulating, hypoxia-induced enzyme.

MATERIALS AND METHODS

CELL CULTURE

HT1080, SiHa, HeLa, MDCK, C-33 A (C33) cells and their transfected derivates were cultured in DMEM with 10% fetal calf serum (Lonza BioWhittaker) at 37°C in humidified air with 5% CO_2. Hypoxic experiments were performed at an anaerobic workstation (Ruskinn Technologies) in 2% O_2, 2% H_2, 5% CO_2, 91% N_2 atmosphere at 37°C.

The transduction of HT1080 cells by lentivirus system has been described elsewhere (Radvak et al., 2013). The conditional shRNA system in HT1080 cells was activated with 0.5 μg/ml doxycycline (Clontech, Mountain View, StateCA, USA) in culture medium.

Preparation of MDCK cells transfected with wt CA IX and with its deletion mutant (ΔPG) has been described previously (Svastova et al., 2004).

TRANSFECTION

For transient silencing of *CA9* gene, HeLa cells were seeded at 1.1×10^6 cells in a 6 cm Petri dish. After 4 h, cells were transfected with pSUPER-sh*CA9* to silence CA IX expression and with pSUPER-shScr as a control (Radvak et al., 2013) using TurboFect Transfection Reagent (Fermentas) according to the manufacturer's recommendations. The next day cells were trypsinized, seeded in a new 6 cm Petri dish at 9×10^5 cells and incubated in hypoxic conditions (2% O_2). Two days later cells were lysed in RIPA buffer containing a cocktail of protease inhibitors (Roche), and analyzed by western blot.

C33 cell line constitutively expressing CA IX protein was prepared by cotransfection of recombinant plasmid pSG5C-CA IX with pSV2neo in 10:1 ratio using TurboFect™ *in vitro* Transfection Reagent (Fermentas). Cells carrying an empty plasmid pSG5C were prepared and used as the negative control in cotransfection with pSV2neo. Transfected cells were subjected to selection in 900 μg/ml G418 for 2 weeks. A mixture of clones was obtained by isolation with magnetic beads (Dynabeads M-450 Tosylactivated, Invitrogen) coupled to M75 antibody specific to CA IX protein. The mixture was expanded and expression of CA IX was analyzed by FACS and immunofluorescence.

ANTIBODIES AND REAGENTS

For immunoblotting, protein concentrations were quantified using BCA kit (Thermo Scientific). Target proteins were detected by the following specific primary antibodies: anti-human CA IX mouse monoclonal antibody M75 in undiluted hybridoma medium (Pastorekova et al., 1992), rabbit anti ROCK1 monoclonal antibody, diluted 1:1000 (Cell Signaling Technology), goat anti β-actin polyclonal antibody, diluted 1:1000 (Santa Cruz Biotechnology). Secondary antibodies were from Sigma: goat anti-mouse IgG peroxidase-conjugated polyclonal antibody, diluted 1:8000, goat anti-rabbit IgG peroxidase-conjugated polyclonal antibody, diluted 1:12000, rabbit anti-goat IgG peroxidase-conjugated polyclonal antibody, diluted 1:5000 (Dako). The following antibodies and reagents were used for immunofluorescence: M75 antibody described above, rabbit anti-paxillin polyclonal antibody, diluted 1:250 (Santa Cruz Biotechnology), secondary antibodies from Invitrogen, diluted 1:1000: Alexa Fluor® 488 donkey anti-rabbit IgG; Alexa Fluor® 488 donkey anti-mouse IgG; Alexa Fluor® 594 goat anti-rabbit IgG, and nuclear stain DAPI (Invitrogen). Collagen was isolated from rat tails per standard procedures.

IMMUNOBLOTTING

HT1080 cells were grown for 3 days in medium containing doxycycline to induce *CA9* silencing. On the fourth day, the cells were trypsinized and seeded in a new 6 cm Petri dish at 9×10^5 cells with or without doxycycline and moved to hypoxia (2% O_2) for 48 h to induce expression of CA IX protein. The cells were then lysed in RIPA buffer containing a cocktail of protease inhibitors (Roche). Samples consisting of 80 μg total proteins were separated in 10% SDS-PAGE and immunoblotting was performed as described elsewhere (Svastova et al., 2003).

IMMUNOFLUORESCENCE

Cells grown on glass coverslips with or without collagen coating were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100. Non-specific binding was blocked with PBS containing 1% BSA for 30 min at 37°C. The cells were sequentially incubated with primary antibodies diluted in PBS with 0.5% BSA (PBS/BSA), each for 1 h at 37°C, washed four times with PBS containing 0.02% Tween 20 for 10 min, incubated with fluorescent secondary antibodies (always added together in one step) diluted in PBS/BSA for 1h at 37°C, washed once with PBS, incubated with DAPI (1:36 000) in PBS to stain nuclei, and then washed three times with PBS for 10 min. Finally, the cells were mounted onto slides in Fluorescent Mounting Medium (Abcam) and analyzed by Zeiss LSM 510 Meta confocal microscope in multitrack scanning mode.

QUANTIFICATION OF FOCAL ADHESIONS IN IMMUNOFLUORESCENCE ASSAYS

C33 CA IX transfected and control neo cells were seeded on collagen coated coverslips at sparse density in serum-free medium, grown for 4 h, fixed and stained as described above. Images of paxillin stained FC were taken using a Zeiss LSM 510 Meta confocal microscope at 400× magnification, zoom 3× at the same microscope settings for all samples, together with accompanying differential contrast images (DIC) to determine cell shape. The length and area of paxillin stained FCs at the cell periphery were measured using intensity thresholding in ImageJ. All measured FCs were within the designated cell area. The spreading area of cells was determined from DIC images in ImageJ software (http://rsb.info.nih.gov/ij/). Significant differences between samples were determined by *t*-test.

To quantify the degree of colocalization between CA IX and paxillin staining in FAs in HT1080 and SiHa cells during the process of initial spreading, the areas of flattened, adhered cells edges were selected using ROI tools in ImageJ software. The values of Pearson's coefficient, which measures the degree of correlative variation of the two channels in a double-stained immunofluorescence image, was calculated for these ROIs by JACoP plugin in ImageJ. Ten cells were analyzed for each phase of two cell lines used.

An analysis of lamellipodial FCs was performed on hypoxic SiHa cells. SiHa cell islands were incubated in hypoxia (2% O_2) in DMEM with 10% FCS for 2 days. For the duration of the last 18 h the cells were starved in 0.5% FCS in DMEM and stimulated into migration by HGF (40 ng/ml) for 3 h, fixed and stained for paxillin and CA IX as detailed above. Cells with developed lamellipodia were identified, lamellipodia were dissected into CA IX—containing areas and adjacent CA IX—free areas according to CA IX fluorescent staining using ROI tools in ImageJ program. Twenty cells were used for the analysis. Analysis of FC area and length based on the intensity thresholding of paxillin staining was performed in relevant ROIs. Samples were statistically compared by *t*-test. All experiments were repeated twice.

CELL SPREADING ASSAY

MDCK and C33 transfectants and their control counterparts, respectively, were seeded on uncoated or collagen-coated coverslips in 12-well plates at the density of 100,000 cells/well and left to attach for 15 min, 30 min, 1 h, and 2 h. At the end of these time intervals, media were removed and attached cells were washed with PBS, fixed in 4% paraformaldehyde, stained with 0.5% Coomassie blue for 5 min at RT and washed 3 times with PBS. Duplicates were used in all experiments which were repeated twice. Images of the fixed cells were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). At least 200 cells were analyzed for each sample, and cell spreading areas of relevant samples were calculated and compared using *t*-test. In all cases, the mean cell diameter of trypsinized cells in suspensions was measured for various transfectants used in the same experiment by the counter (Beckman Coulter) prior to seeding. In case of different cell sizes, cell areas measured at designated times after seeding were normalized by dividing them by corresponding circular cell areas calculated using the mean cell diameter of cells in suspension. When M75 antibody was used to block the PG domain of CA IX, the procedure carried out was as follows: live cells were pre-incubated with M75 antibody (10μg/ml in DMEM with 10% FCS) for 1 h at 37°C at the rotational shaker. Cells were then washed 3 times in serum-free medium. After washing, cells were counted, seeded, fixed at indicated times and stained as described above. Control samples were subjected to the same procedure.

TIME-LAPSE MICROSCOPY

HT1080 cells were grown for 3 days in medium containing doxycycline to induce *CA9* silencing. On the fourth day, the cells were trypsinized, seeded, and exposed to hypoxic conditions (2% O_2) for 48 h to induce expression of CA IX protein. Cells were then counted and seeded at sparse density into 12-well plate, in DMEM with 10% FCS, with or without 0.5 μg/ml doxycycline. Time-lapse acquisition was performed with a Zeiss Cell Observer System (Observer.Z1 microscope with motorized stage), magnification 100×, in the incubation chamber at 37°C in 21% O_2, 5% CO_2 atmosphere. Imaging was managed by Axiovision 4.8 software, using the Multidimensional Acquisition settings. The experiment started approximately 15 min after cell seeding (designated as time 0min), and images were taken in transmitted light every 3 min at 10 different positions for each sample. For single cell tracking analysis only unspread, round cells, very soon after their attachment to the substrate, were selected, tracked manually, and their morphometric features were calculated from time-lapse images in ImageJ program. The overall cell population, control and silenced cells, was characterized at designated times, parameters of all attached cells in the fields of view (more than 200 cells, 10 different positions for each sample) were processed and compared by *t*-test.

META-ANALYSIS OF GENE EXPRESSION PROFILING DATA

Oncomine (https://www.oncomine.org) was examined to identify microarrays datasets with significantly over-expressed CA9 (FC $>$ 2; p-value $<$ 0.0001). The Oncomine is a repository of publicly available gene expression profile data (Rhodes et al., 2007). It can be queried according to various criteria and filters. The raw data from corresponding experiments were retrieved from NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) (Barrett et al., 2013). Statistical analysis of microarrays expression data was performed using R/Bioconductor through user-friendly graphical interface of Chipster software (http://chipster.csc.fi/) (Kallio et al., 2011). Expression data were pre-processed with an RMA procedure and filtered by flags. Differentially expressed genes between two sample groups were identified using Student's t-test ($\alpha = 0.05$) with Benjamini and Hochberg adjustment of p-values. BH adjustment controls the false discovery rate (FDR) by controlling the certainty level. Sets of differentially expressed genes were subjected to Signaling Pathway Impact Analysis (SPIA) (Tarca et al., 2009). SPIA algorithm allows for the identification of KEGG pathways impacted by these differentially expressed genes. A global cut-off p-value was set to 0.1 in order to eliminate false discovery and at the same time preserve sufficient sensitivity of the analysis.

RESULTS

LOSS OF CA IX DECREASES ROCK1 PROTEIN EXPRESSION AND SLOWS DOWN CELL ATTACHMENT AND SPREADING

Our previous microarray analysis revealed the inhibition of the FA pathway in the CA IX-protein-depleted HT1080 cells (Radvak et al., 2013). In order to gain deeper insight into this phenomenon, we decided to explore one branch of this pathway with a direct impact on the cell spreading process. Indeed, the loss of CA IX resulted in a significant transcriptional down-regulation of the ROCK1 kinase, which plays an essential role in the maturation of FC during cell adhesion and spreading on the substrate as well as in the FA turnover during cell migration.

In line with this idea, we first analyzed protein lysates from the *CA9* mRNA-silenced HT1080 cells incubated for 24 h in hypoxia and found that these cells exhibit reduced protein levels of ROCK1 (**Figure 1A**). A similar decrease in ROCK1 protein expression was observed in HeLa cells with a transient *CA9* silencing, whereas no such change occurred in any of the control cells. The observation of the CA IX-related modulation of the ROCK1 protein level in both cell lines supports the functional relationship between these two proteins.

As the main function of the ROCK1-dependent pathway is to activate formation of FC through the myosin II-driven actomyosin tension, we investigated the effect of ROCK1 downregulation, connected with *CA9*-silencing, in HT1080 cells during the process of their adhesion and spreading.

To elucidate the initial spreading process in more detail, we performed a time-lapse experiment using HT1080 cells that had been incubated in hypoxia to induce endogenous expression of CA IX and simultaneously subjected to silencing. The time-lapse experiment itself was run in normoxia for 90 min on a plastic dish, and the images were taken every 3 min, starting at the time point 0 that corresponded to ∼15 min after seeding. We first compared entire populations of the *CA9*-silenced and control cells. At the beginning of the experiment (0 min) the number of control CA IX expressing cells attached to the support was higher than the number of CA IX-depleted cells and they exhibited larger

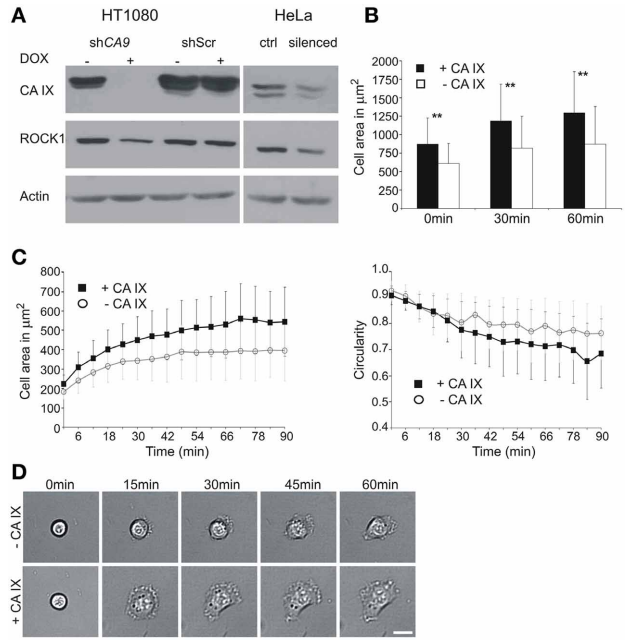

FIGURE 1 | Effect of *CA9* silencing on the expression of ROCK1 protein and initial cell adhesion and spreading. (A) HT1080 cells were pre-incubated with or without doxycycline (±DOX) to induce expression of shRNA. Cells were then trypsinized and placed to hypoxia (2% O_2) for 24 h. The level of ROCK1 protein was reduced in the *CA9*-silenced cells. Control HT1080 Scr shRNA had the same level of ROCK1 as non-silenced *CA9* shRNA (–DOX). The addition of doxycycline to HT1080 Scr shRNA cells did not cause any change in CA IX protein level, nor in ROCK1 expression. HeLa cells transiently silenced for *CA9* (pSUPER-shCA9) also showed a decreased level of ROCK1 protein compared to control cells (ctrl, pSUPER-shScr). **(B,C)** Time-lapse analysis of the entire population **(B)** and individual cells **(C)** of *CA9*-silenced and control HT1080 cells. **(B)** *CA9*-silenced cells (–CA IX) exhibited significantly smaller spreading areas than control non-silenced cells (+CA IX). Graph shows the mean ± standard deviation values for at least 200 cells, significance of differences among samples was tested by *t*-test (** $p < 0.01$). **(C)** In the analysis of individual cells, HT1080 expressing CA IX show faster spreading and achieve larger spreading area when compared with silenced cells (–CA IX). The graph of circularity demonstrates that silenced cells preserve a circular morphology for a longer amount of time, CA IX positive cells, on the opposite, became more polarized. Graphs give mean ± standard deviation values for designated time-points for 29 CA IX expressing cells and 39 silenced cells (–CA IX). **(D)** Representative pictures of characteristic morphology of *CA9*-silenced (–CA IX) and control cells (–CA IX) during the process of initial adhesion and spreading at given time-points. Scale bar 20 μm.

spreading areas. The same tendency was observed in the later time-points (**Figure 1B**). These results were then confirmed by a single-cell tracking for 90 min (**Figures 1C,D**). From the populations we selected only the cells in the very initial stage of the spreading, shortly after their attachment and monitored them for a subsequent 90 min. At the beginning of the experiment (0 min), all selected cells were round, with the spherical shape clearly visible in the transmitted light. Throughout the course of the experiment, CA IX-expressing cells spread faster, reached larger spreading area, often became flattened, formed lamellipodia and exhibited a polarized shape (**Figures 1C,D**). On the other hand, CA IX-silenced cells spread more slowly, rarely formed lamellipodia and their circular morphology with a thicker central area was preserved for a longer period.

It is well-conceivable that the differences in spreading process can be attributed to CA IX-related effect on ROCK1 expression, but due to the complexity of adhesome we can expect that changes of other proteins identified in our microarray data can also contribute to the CA IX impact on FA.

CA IX INCREASES CELL ATTACHMENT AND SPREADING IN THE PG-DOMAIN-DEPENDENT MANNER

To confirm the role of CA IX in FA, we subsequently analyzed transfected MDCK cells constitutively expressing CA IX and compared them with the mock-transfected controls (Svastova et al., 2003). In the cells seeded on collagen-coated coverslips, expression of the full length CA IX protein was associated with an enhanced rate and enlarged area of spreading (**Figure 2A**). Interestingly, the MDCK cells ectopically expressing a deletion variant of the CA IX lacking the N-terminal PG domain spread at a similar rate to the control cells, but their spreading area was in between the areas of the full-length CA IX-expressing cells and mock controls. This observation indicated that PG domain is required for the full effect of CA IX on cell spreading. In accordance with this assumption, we found that the M75 antibody which binds to a repetitive epitope in PG domain (Zavada et al., 2000), was able to reduce both the rate and area of spreading of MDCK CA IX cells on the glass support (**Figures 2B,C**). These results indicate that CA IX protein can mediate the direct interaction of cells with their support.

CA IX COLOCALIZES WITH PAXILLIN IN FOCAL CONTACTS DURING INITIAL ADHESION AND SPREADING

CA IX involvement in the process of cell adhesion and spreading was also confirmed using C33 cells transfected either with cDNA coding for CA IX or with the mock plasmid. The cells were seeded on collagen-coated coverslips and allowed to attach for 4 h. As expected, CA IX-expressing cells displayed a larger spreading area than the control cells (**Figures 3A,B**).

Analysis of the length and number of FAs by immunofluorescence staining of paxillin supported the conclusion of stronger adhesion of CA IX-expressing cells to collagen substrate as they displayed larger, elongated, and dash-like FAs (**Figure 3C**). The overall number of FCs in control neo cells was lower and FCs were smaller and more rounded (FCs per cell: 26 vs. 93 for CA IX-positive cells, $n = 20$ cells). This finding indicates that CA IX contributes to the formation of more mature and stronger contacts capable of connecting with actin stress fibers, which stabilize cells on a substrate.

To confirm the importance of PG domain for the CA IX-increased spreading, we performed the short time adhesion experiment in the presence of M75 antibody. Addition of M75 antibody suppressed the effect of CA IX on the cell spreading, similarly as in MDCK cells (**Figure 3D**).

To characterize the CA IX localization during initial attachment and spreading, we analyzed HT1080 and SiHa cells expressing endogenous CA IX protein in response to hypoxia. The cells were first incubated for 2 days in hypoxia, then re-plated on collagen and fixed at different time points after seeding. Since

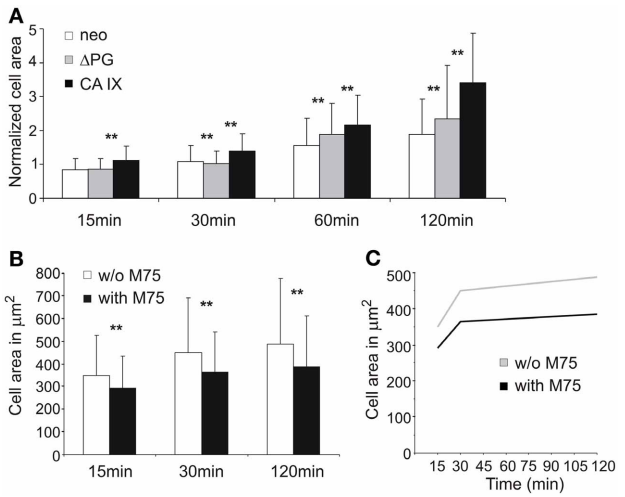

FIGURE 2 | The impact of CA IX expression on adhesion and spreading of stably transfected MDCK cells. (A) MDCK cells were seeded on collagen-coated substrate and fixed at indicated time points. Expression of full length CA IX enhanced spreading area when compared with control neo cells. MDCK cells expressing Δ PG mutant decreased spreading area against cells expressing intact CA IX. Cell area was normalized to compensate for different cell sizes of used transfectant and control cell lines. **(B)** Addition of M75 antibody targeting the PG domain of CA IX led to the reduction of spreading area of adhering MDCK CA IX cells on glass coverslips. Both graphs show the mean ± standard deviation values for at least 200 cells, significance of differences among samples was tested by *t*-test (**$p < 0.01$).**(C)** Presence of M75 antibody inhibits the rate of cell spreading.

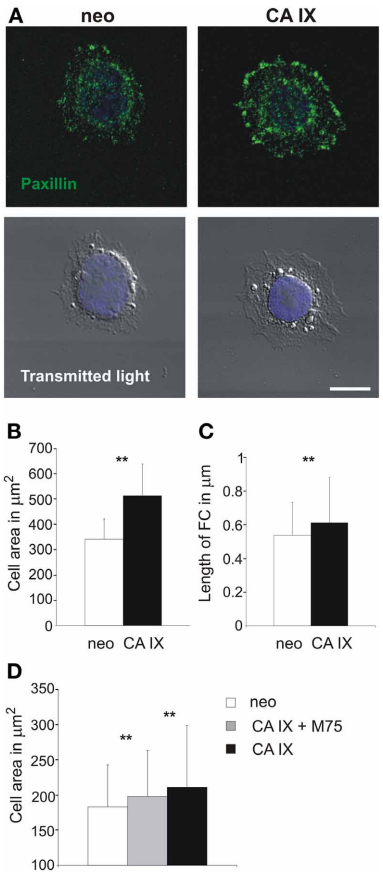

FIGURE 3 | Paxillin-based analysis of focal contacts and spreading of C33 cells with respect to CA IX expression and inhibition of PG domain. (A) Immunofluorescence analysis of paxillin staining of C33 cells stably transfected with CA IX and their neo counterparts 4 h after their seeding on collagen-coated coverslips in serum-free medium. CA IX positive cells displayed larger, dash-like focal adhesions, indicating the existence of more mature focal contacts and stronger adhesion to a substrate. Scale bar 10 μm. **(B,C)** Cells were individually analyzed for spreading area, which was significantly larger for C33-CA IX cells ($n = 20$, *t*-test, **$p < 0.01$), and also for the average length of immunofluorescently stained focal contacts ($n = 20$, *t*-test, **$p < 0.01$). **(D)** Effect of presence of M75 antibody to PG region of CA IX on C33 transfectants. One hour after seeding on a glass coverslip, CAIX expressing C33 cells reached larger spreading areas than control neo cells, and this effect was reduced by the addition of M75 antibody. All graphs show the mean ± standard deviation values for at least 300 cells, significance of differences among samples was tested by *t*-test (**$p < 0.01$).

HT1080 cells attached more quickly than SiHa cells, similar stages of spreading were selected and designated as phase I, II, and III instead of time indications (**Figure 4**). In phase I, shortly after the attachment when the cells still had a round morphology, CA IX was accumulated in the cell periphery where FCs had just begun to form. In phase II, immediately after the cells had begun the process of spreading, the colocalization of CA IX and paxillin in emergent FCs was clearly visible. In phase III, when the cells had just spread and flattened, the number of elongated dash-like FAs increased and colocalization of paxillin with CA IX was preserved. We calculated Pearson's coefficient for regions of cell edges where cells were thinned and where they had started to adhere to the surface. The values of Pearson's coefficient confirmed the high degree of colocalization for HT1080 cells in all observed phases (mean ± standard deviation values: 0.656 ± 0.049, 0.612 ± 0.045, 0.647 ± 0.052 for phases I, II, and III, respectively, $n = 10$ cells) as well as for SiHa in phases I and II (0.772 ± 0.092 and 0.683 ± 0.087, $n = 10$) and partial colocalization for phase III of SiHa cells (0.45 ± 0.16, $n = 10$). Thus, we showed that CA IX localizes in FC during the initial phases of spreading and improves cell adhesion to the substrate. Our findings imply a direct role for CA IX in these processes.

LAMELLIPODIAL CA IX ASSOCIATES WITH SITES OF NASCENT FOCAL CONTACTS DURING CELL MIGRATION

Formation, maturation, and disassembly of FAs represent a highly dynamic process which accompanies cell migration. Indeed, migratory speed is modulated by the rate of adhesion turnover and strength of FCs. As we describe here, CA IX enhances FA but on the other hand, as we described earlier it facilitates cell migration (Svastova et al., 2012). This may appear contradictory because migration is diminished in cells that are strongly attached.

In order to understand this seeming paradox, we followed the CA IX localization during cell migration in relation to paxillin staining. SiHa cells were grown into cell islands in hypoxia and then induced to migration with hepatocyte growth factor (HGF). CA IX displayed extensive redistribution to the leading

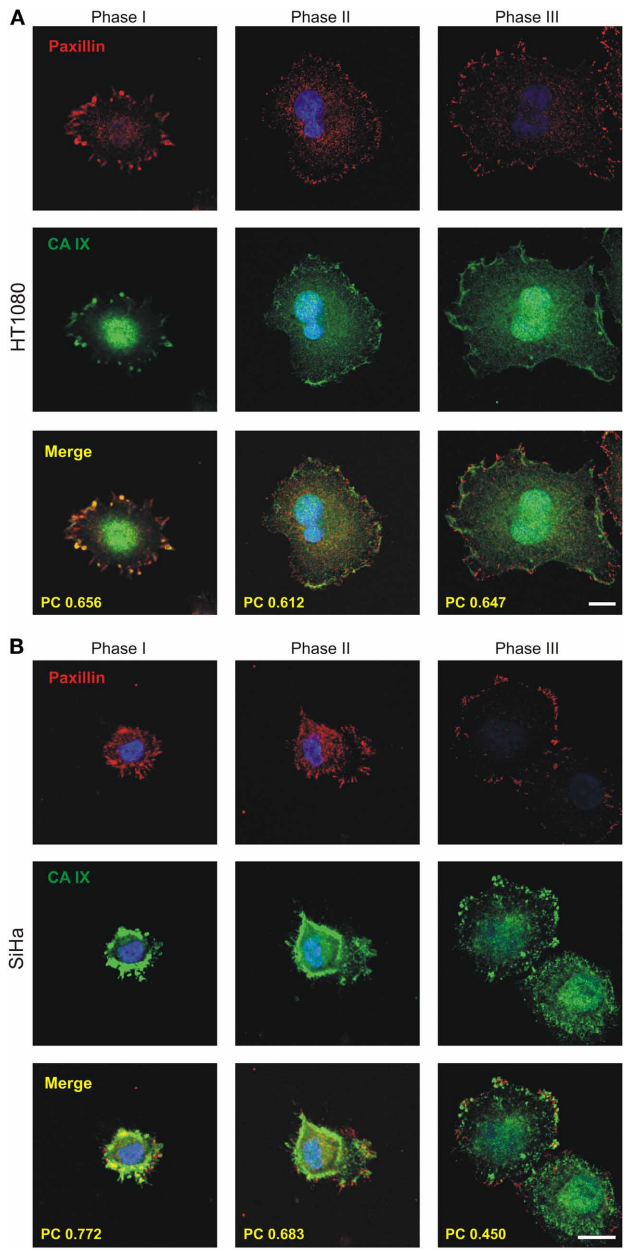

FIGURE 4 | Immunofluorescence analysis of the localization of CA IX during the process of initial spreading of HT1080 and SiHa cells with endogenous expression of CA IX. Cells were cultivated in hypoxia (2% O_2) for 3 days, replated on collagen-coated coverslips and fixed at different times. Samples were double immunostained for CA IX (green) and paxillin (red) and imaged by confocal microscopy. Nuclear DAPI staining is blue. Phases I-III depict cells in different time points from initiation of adhesion. During phase I, shortly after cell attachment, CA IX was accumulated at the cell periphery where focal contacts had just begun to form. In phase II, soon after cells began to spread, the colocalization of CA IX and paxillin (shown in yellow) in forming focal contacts was clearly visible. In phase III (max. 2 h after seeding), characterized by spread and flattened cells, the maturating focal contacts were represented by more elongated paxillin staining with well-preserved colocalization with CA IX. Degree of colocalization between CA IX and paxillin in each phase is expressed by Pearson's coefficient (PC) in the merged images. **(A)** HT1080 cells **(B)** SiHa cells. Scale bars 10 μm.

edges where it was intensively stained together with paxillin (**Figure 5A**). Detailed analysis of lamellipodia (from 20 cells) showed that places where CA IX colocalized with paxillin were characterized by small nascent adhesions but the adjacent CA IX-free areas displayed longer, more mature paxillin contacts (**Figure 5A** inset). Processing of data from confocal microscope images of the lamellipodia dissected into CA IX-positive and CA IX-negative regions of interest (ROI) revealed significant differences in the pattern of paxillin contacts (**Figure 5B**). It is obvious that accumulation of CA IX in advancing lamellipodia occurs at the same place where cells must form transient adhesions to allow for rapid turnover of FC and thereby increase the speed of migration. Thus, the contrasting CA IX roles in FC are clearly dependent on circumstances that require either cell attachment/spreading or cell movement.

DISCUSSION

The maturation of FAs depends on the recruitment of adhesion proteins and their phosphorylation, matrix stiffness as well as on pericellular pH. One of the main regulators of FAs maturation is myosin II-mediated cytoskeletal tension, which regulates FAs growth, composition and phosphorylation of the constituent proteins. In turn, actomyosin-induced tension is regulated by the phosphorylation and activation of the myosin II regulatory light chain by RhoA-dependent ROCK kinases that act through inhibition of the myosin phosphatase (Vicente-Manzanares et al., 2009). Our results show that depletion of CA IX in HT1080 cells significantly decreases the protein level of ROCK1 and attenuates cell adhesiveness and spreading. This also leads to diminished staining of paxillin which is a key component of FC.

Apart from the effect on actomyosin forces, ROCK1 phosphorylates the FAK. In particular, direct FAK phosphorylation by ROCK1 on Ser732 is required for the recruitment of vinculin and subsequent maturation of the focal complex (Lock et al., 2012). Alternatively, ROCK1-induced myosin II activation can promote the FAK-mediated phosphorylation of paxillin, which facilitates vinculin recruitment into FAs to strengthen the connection between actin cytoskeleton and extracellular matrix, and thereby drive the FA maturation (Pasapera et al., 2010). Downregulation of ROCK1 protein level in two different cell lines, HT1080 and HeLa, induced by depletion of CA IX indicates that such ROCK1 mediated modulation of adhesion is a general and functionally important mechanism.

Multiple ROCK1 functions support the assumption that the ROCK1 downregulation caused by CA IX silencing can have a broader impact on adhesion-migration-invasion phenomena. In the previous paper, we documented a decreased expression of MMP9 in *CA9*-silenced cells (Radvak et al., 2013). Notably, reduction of ROCK1 level in these cells could be the mechanism responsible for MMP9 downregulation. The specific inhibitor of ROCK1 (Y27632) as well as RhoA/ROCK pathway inhibitor (simvastatin) can reduce the level of MMP9 mRNA (Turner et al., 2005). Since MMP9 plays an essential role in invadopodia formation and degradation of the basement membrane, its reduction in *CA9*-silenced cells augments the CA IX relevance in the adhesion/invasion process. In addition, CA IX has previously been shown to regulate the activity of MMP9 presumably through

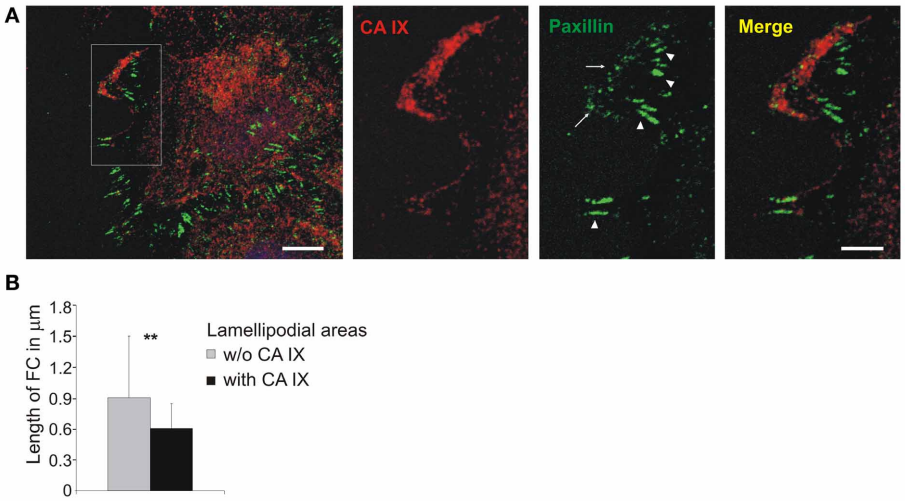

FIGURE 5 | Localization of CA IX in nascent focal adhesions in lamellipodia. (A) Immunofluorescence analysis of SiHa cells grown into cell islands in hypoxia (2% O_2), induced into migration with HGF (40 ng/ml) for 3 h. CA IX (stained red) was massively relocalized to the leading edge where it was co-stained with paxillin, a marker of focal contacts (green). The inset of a magnified lamellipodial area shows that small nascent focal adhesions were formed in the region with accumulated CA IX (arrows), whilst paxillin-stained contacts were more elongated and mature in the neighboring CA IX-free regions (arrowheads). Scale bars: 10 µm (5 µm for the inset). **(B)** Numerical analysis of area and length of focal contacts in lamellipodia from 20 cells dissected into CA IX containing regions and CA IX-free regions. Results confirmed that CA IX containing areas displayed significantly smaller and less elongated focal contacts (compared by *t*-test, $^{**}p < 0.01$).

extracellular production of protons (Sansone et al., 2009). Indeed, transfectants expressing an ion-translocation defective mutant of pH regulator NHE1 (Na^+/H^+ exchanger) also show decreased gene expression of *MMP9* (Putney and Barber, 2004). This indicates that pHe and pHi-related effects of CA IX protein also contribute to changes in the gene expression profile in *CA9*-silenced cells.

Collagen IV is another gene downregulated in response to *CA9* mRNA silencing (Radvak et al., 2013). Both MMP9 and collagen IV are accumulated at the protruding lamellipodia of bronchial epithelial cells (Legrand et al., 1999). Collagen IV promotes migration by providing the substrate for attachment of advancing lamellipodia and MMP9 facilitates turnover and the release of FC by the cleavage of collagen and consequent matrix remodeling. Thus, the influence of CA IX depletion on collagen IV and MMP9 mRNA levels can at least in part explain its effect on FA inhibition.

We previously demonstrated that the catalytic domain is important for the role of CA IX in cell migration/invasion (Svastova et al., 2012). However, our present findings point to the N-terminal PG domain that appears to mediate CA IX effects on cell adhesion to the support especially in relation to the maturation of FC. The evidence is based on our observations of the reduced spreading of cells expressing the truncated form of CA IX that lacks the PG-domain as well as of cells expressing the full length CA IX protein that are treated with the PG domain-binding M75 antibody.

Among all known carbonic anhydrases, only CA IX contains this PG-like N-terminal extension of the extracellular catalytic domain, which displays 38% identity with a keratan sulfate attachment domain of the large aggregating proteoglycan aggrecan (Opavsky et al., 1996). Interestingly, comparative analysis of the core sequence (aa 62–93) of the PG domain containing the pentameric EEDLP repeat revealed even higher, 50% identity to the conserved hexapeptide sequence of the keratan sulfate-enriched region of aggrecan. This part of the aggrecan molecule exhibits a capacity to bind collagen and hyaluronan (Hedlund et al., 1999) and thus it is imaginable that the PG domain of CA IX can behave in a similar manner and bind ECM components.

In the phage display library, Zavada et al. (2000) identified the SASAPVS peptide competing with M75 antibody in binding to PG domain of CA IX. Our alignment analysis performed in BLAST (Basic Local Alignment Search Tool, NIH) showed 100% identity of this peptide with the stromal component mucin (**Table 1**). This is a very interesting finding as mucins are important ECM components contributing to cancer progression. Indeed, aberrant production of mucins is directly connected with the development of pancreatic, breast, colorectal and lung tumors (Hollingsworth and Swanson, 2004; Torres et al., 2012; Valque et al., 2012). Hollingsworth and Swanson (2004) suggest that tumors can use mucins to assemble the local microenvironment during invasion and metastasis in hostile conditions of hypoxia and acidosis.

The idea that CA IX potentially has the ability to bind mucin through its PG domain surely deserves further investigation, especially given the indirect evidence for this relationship provided in several published studies. Saarnio et al. (1998) were the first to notice that CA IX staining in colorectal carcinoma tissues showed the most intense signal in five of six adenocarcinomas with the mucinous component. Similar an observation was later described in ovarian tumors, in which the strongest immunohistochemical

Table 1 | BLAST analysis of peptide SASAPVS competing with M75 binding site in the PG domain of CA IX protein showing 100% identity with gastric mucin.

Alignment statistics for match				
Score	**Expect**	**Identities**	**Positives**	**Gaps**
19.7 bits (39)	304	6/6 (100%)	6/6 (100%)	8
Competing peptide	2	ASAPVS	7	
		ASAPVS		
Mucin	2331	ASAPVS	2336	

reactions detecting CA IX occurred in the borderline mucinous cystadenomas and mucinous cystadenocarcinomas (Hynninen et al., 2006). CA IX is also highly expressed in lung adenocarcinomas with mucinous component (Kon-No et al., 2006). Moreover, a direct association between CA IX and MUC1 protein expression was noted in non-small cell lung cancer (Giatromanolaki et al., 2001). The binding of CA IX to mucins may provide one of possible explanations for these findings.

Returning to the role of CA IX in FA, it is important to be aware that it strongly depends on the situation that leads either to adhesion and spreading or to migration. While the former process involves formation and maturation of FC, the latter requires dynamic remodeling of these contact structures. CA IX participates in FAs during spreading, but there is a role for this protein also in transient FC in migrating cells.

During cell migration, the actin reorganization and attachment of the leading edge to the substrate are the driving forces that regulate movement of the advancing lamellipodia (Pollard and Borisy, 2003; Zaidel-Bar et al., 2003). Nascent adhesions that assemble at the front of the growing lamellipodium are independent of myosin II (the important player in spreading) and are characterized by branched actin arrangement. The rate of assembly and turnover of these adhesions essentially depends on the actin polymerization coinciding with the actin severing activity (Choi et al., 2008). In migrating cells, CA IX colocalizes with paxillin directly in the nascent adhesions. Since these contacts in the leading edge exert transient forces to move the cell forward whilst only the maturation of FAs provides anchors to the substrate, our assumption is that it is the effect of the catalytic activity of CA IX on the acidification of the extracellular space that plays a role in nascent adhesion turnover.

There is increasing evidence that intracellular and extracellular pH gradient influences cell migration in many aspects (Cardone et al., 2005; Stock and Schwab, 2009; Martin et al., 2010). Changes in pHi induced by different cytokines and cellular processes affect protein conformations and macromolecular assemblies (Srivastava et al., 2007). We demonstrated that CA IX interacts with bicarbonate transporters in the lamellipodia of moving cells (Svastova et al., 2012). This interaction seems to be functionally active because coexpression of CA IX with NBC or AE1,2,3 maximizes the rate of bicarbonate transport across the plasma membrane (Morgan et al., 2007; Orlowski et al., 2012). Thus, bicarbonate ions produced by the enzymatic activity of CA IX can contribute to increased pHi in the leading edge in accordance with the established model of pH gradient both inside and around migrating cells.

Cofilin is considered to be one of the intracellular pH sensors which localizes to sites of intensive actin turnover (Srivastava et al., 2007). By severing F-actin it generates actin monomer pool, thus sustaining a high rate of filament elongation and membrane protrusion. The activity of cofilin coincides with pH gradient in migrating cells (Stock and Schwab, 2009). At higher pHi typical for the leading edge, deprotonation of cofilin releases its inhibitory binding to $PI_{(4,5)}P2$ and leads to the acquisition of its active state (Frantz et al., 2008). Released cofilin then generates free-barbed ends of F-actin and promotes advancing lamellipodia. On the other hand, inactivation of cofilin activity stimulates the formation of actin stress fibers and inhibits cell migration (Toshima et al., 2001; Mouneimne et al., 2006).

Proton efflux and consequently increased pHi mediated by the pH regulator of migration NHE1 is necessary for the cofilin-dependent actin filament assembly in response to migratory stimuli (Frantz et al., 2008). CA IX also appears to participate in this process, since lamellipodia form predominantly in the CA IX-positive cells as soon as 15 min after the induction of migration in wounded monolayer (Svastova et al., 2012). Colocalization of CA IX with actin in the places of dynamic actin reorganization and its concurrence with paxillin in nascent adhesions in lamellipodia where CA IX directly interacts with bicarbonate transporters indicate that pH modulation is the method by which CA IX can contribute to actin dynamics and adhesion turnover during migration.

Interestingly, the generation of free barbed ends by cofilin activity defines the location of actin-based protrusions and initiates this process. It is documented that exactly the loss of cofilin binding to $PI_{(4,5)}P_2$, which is also regulated by pHi, is the key mechanism for cofilin activation in the leading edge of mammary carcinoma cells (Van Rheenen et al., 2007). In addition to cofilin, other proteins including talin, vinculin, and gelsolin display pH-dependence of actin binding properties, thus modulating FA remodeling and migration rates (Srivastava et al., 2008).

As we mentioned before, the turnover of FC in the lamellipodia and the rear end is the limiting process regulating migration rate. In the cell rear, a proton-sensitive TRPM7 channel (Ca^{2+} and Mg^{2+} permeant ion channel, transient receptor potential melastatin 7) regulates FA through calcium-dependent protease m-calpain inducing loss of adhesion via cleavage of the adaptor protein talin from integrin-cytoskeleton connection (Su et al., 2006; Stock and Schwab, 2009). In the cell front, i.e., in the lamellipodium, NHE1 as one of the most effective extruder of protons causing extracellular acidification, strongly modulates $\alpha 2\beta 1$ integrin-dependent adhesion and migration of human melanoma cells (Stock et al., 2005). It is important to note that NHE1 and integrin receptors often accumulate at the leading edge of lamellipodia and thus NHE1 could generate acidic microdomains (Grinstein et al., 1993). The local pHe in close proximity to focal contact sites then influences the strength of FA and migration (Stock et al., 2005).

In summary, based on this work we propose that CA IX is a functional component of the FA process contributing to

Table 2 | Cancer gene expression profiling studies with up-regulated CA9 (FC>2) related to significantly activated focal adhesion pathway as identified by SPIA algorithm.

Cancer type	Oncomine dataset	GEO ID	Study type*	SPIA—Focal adhesion (KEGG: 04510)	
				pGFDR**	Status***
Colorectal carcinoma progression	–	GSE1323	T/M	7.19E-02	Activated
Colorectal carcinoma progression	–	GSE2509	T/M	7.24E-02	Activated
Colorectal carcinoma	Skrzypczak Colorectal	GSE20916	N/T	8.11E-03	Activated
Clear cell renal carcinoma	Yusenko Renal	GSE11151	N/T	4.24E-07	Activated
Clear cell renal carcinoma	Lenburg Renal	GSE781	N/T	4.41E-02	Activated
Clear cell renal carcinoma	Gumz Renal	GSE6344	N/T	5.25E-06	Activated
Cervical squamous cell carcinoma	Scotto Cervix 2	GSE9750	N/T	3.48E-03	Activated
Esophageal carcinoma	Hu Esophagus	GSE20347	N/T	1.37E-06	Activated
Pancreatic carcinoma	PlaceStatePei Pancreas	GSE16515	N/T	6.45E-05	Activated

*Study type—T/M, primary tumor/metastasis; N/T, normal tissue/tumor.

**pGFDR—false discovery adjusted global probability testing of the hypothesis that the differentially expressed genes significantly perturb this pathway. The lower the pGFDR value is the lower the probability of observing this or a higher number of differentially expressed genes just by chance.

***Status—indicates whether the pathway is activated or inhibited in the conditions of the study.

both maturation and dynamics of focal complexes depending on the situation that requires either cell spreading or migration. To accomplish this role, CA IX employs its extracellular catalytic domain that appears to be more relevant for the nascent adhesions during migration as well as its N-terminal PG-like domain that is rather required for the adhesion during spreading, although we cannot exclude the crosstalk of these domains in either situation. Indeed, *in vitro* data from the kinetics measurements of the recombinant enzymes suggest that the PG domain considerably increases the catalytic activity of CA IX and makes it the most active CA isoform (Hilvo et al., 2008).

In contrast to other CA isoenzymes that mostly occur in healthy tissues, CA IX is predominantly expressed in tumors in response to hypoxia. Hypoxia also stimulates other constituents of the migration machinery (including ion transporters and pH regulators, such as NHE1) (Cardone et al., 2005; Gatenby et al., 2007) and increases the ability of CA IX to regulate pH through the bicarbonate transport metabolon (Svastova et al., 2004; Morgan et al., 2007). Therefore, it is not surprising that it also participates in cell migration and invasion, thus contributing to the escape of tumor cells from the primary tumor mass in the pH-dependent manner. On the other hand, when tumor cells released to circulation reach the secondary site, they need to adhere and spread to initiate the growth of metastatic lesion, and this could be facilitated by CA IX-improved maturation of the FC.

Although these assumptions are based on experimental results obtained with cultured cells, they clearly have support from *in vivo* studies as found by data mining of the Oncomine and GEO databases (described in M&M). SPIA in nine independent clinical studies of several tumor types with at least 2.0-fold up-regulated transcription of *CA9* revealed the FA pathway as one of the most consistently activated pathways in these tumors (**Table 2**). Conclusions of this study implicate that it could be reasonable to use a combined treatment strategy against CA IX, targeting both catalytic and PG domains, thus maximizing the effect on CA IX action in cell adhesion-migration-invasion and thereby preventing tumor dissemination.

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Calcium-permeable ion channels in control of autophagy and cancer

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Autophagy, or cellular self-eating, is a tightly regulated cellular pathway the main purpose of which is lysosomal degradation and subsequent recycling of cytoplasmic material to maintain normal cellular homeostasis. Defects in autophagy are linked to a variety of pathological states, including cancer. Cancer is the disease associated with abnormal tissue growth following an alteration in such fundamental cellular processes as apoptosis, proliferation, differentiation, migration and autophagy. The role of autophagy in cancer is complex, as it can promote both tumor prevention and survival/treatment resistance. It's now clear that modulation of autophagy has a great potential in cancer diagnosis and treatment. Recent findings identified intracellular calcium as an important regulator of both basal and induced autophagy. Calcium is a ubiquitous secondary messenger which regulates plethora of physiological and pathological processes such as aging, neurodegeneration and cancer. The role of calcium and calcium-permeable channels in cancer is well-established, whereas the information about molecular nature of channels regulating autophagy and the mechanisms of this regulation is still limited. Here we review existing mechanisms of autophagy regulation by calcium and calcium-permeable ion channels. Furthermore, we will also discuss some calcium-permeable channels as the potential new candidates for autophagy regulation. Finally we will propose the possible link between calcium permeable channels, autophagy and cancer progression and therapeutic response.

Keywords: calcium, autophagy, TRP, ion channels, cancer

AUTOPHAGY

Autophagy is a cellular catabolic process for the degradation and recycling of protein aggregates, long-lived proteins and damaged organelles to maintain cellular homeostasis (Ravikumar et al., 2010; Chen and Klionsky, 2011). Normally, autophagy occurs under basal conditions but it can be stimulated in response to different types of cellular stress, such as nutrient starvation, hypoxia, endoplasmic reticulum (ER) stress, oxidative stress, mitochondrial damage as well as treatment with some pharmacological agents (Kroemer et al., 2010). To date, three types of autophagy have been described, including macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Klionsky, 2005). These types differ in their functions and regulatory mechanisms. During macroautophagy, further mentioned as autophagy, cytoplasmic components are engulfed by double membrane structures termed autophagosomes that mature by fusing first with late endosomes or directly with lysosomes to form autolysosomes. Finally, the content of autolysosome together with its inner membrane is degraded by lysosomal hydrolases to be reused in the cell (Ravikumar et al., 2010; Chen and Klionsky, 2011).

The process of autophagosome formation consists of several stages, namely initiation, elongation and maturation and fusion. At present, around 30 autophagy-related genes are identified, and most of them have mammalian orthologs (Ravikumar et al., 2010). These genes are implicated in different stages of autophagy. Thus, the autophagosome formation requires the activity of the class III phosphatidylinositol 3-kinase (PI3K), Vps34. Mammalian Atg18 homolog WIPI-1 binds to PI3P, the product of Vps34 activity, and is recruited to autophagosomal membrane. Vps34 is a part of a protein complex containing Beclin1/Atg6, p150/Vps15 and Atg14/barkor proteins (He and Levine, 2010). Another complex implicated in the initiation stage of autophagosome formation is the focal adhesion kinase family interacting protein FIP200-ULK1/2/Atg1-Atg13 complex, where Atg13, ULK1, and ULK2 proteins have been shown to be direct targets of the mammalian target of rapamycin (mTOR) (Mizushima, 2010). Under nutrient-rich conditions, mTOR is associated with this complex, whereas under starvation conditions inhibition of mTOR causes its dissociation from the complex and leads to activation of ULK1/2, subsequent phosphorylation of FIP200 and autophagy induction (Mizushima, 2010; Kim et al., 2011). The elongation stage requires cleavage of the microtubule-associated protein 1 light chain 3 (Atg8/LC3) by Atg4, resulting in the formation of cytosolic LC3-I protein, which is conjugated to phosphatidylethanolamine (PE) to form membrane bound LC3-II (Tanida et al., 2004). Another stage is the formation of Atg5–Atg12–Atg16L1 protein complex which

facilitates LC3-I conjugation to PE and determines the sites of LC3 lipidation. Thus, LC3-II is specifically targeted to the autophagosome membrane and remains associated with autophagosome even after fusion with the lysosomes (Geng and Klionsky, 2008; Ravikumar et al., 2010). This peculiarity makes it a useful marker for autophagy research (Klionsky et al., 2012).

AUTOPHAGY IN CANCER

Autophagy is thought to be predominantly a cell-survival mechanism. Under basal conditions, autophagy helps to maintain cellular homeostasis through the elimination of damaged organelles and protein aggregates, whereas in stress autophagy provides nutrients from macromolecules, produces energy, removes potentially dangerous elements thus assures cytoprotective response to support cell survival (Mizushima et al., 2008; Ravikumar et al., 2010). Deregulation of autophagy is known to affect many physiological processes and has been implicated in a number of diseases, such as neurodegenerative disorders and cancer (Choi et al., 2013). In cancer, current evidence indicate that autophagy may act as a tumor suppressor, in particular at the early stages of tumor initiation (White, 2012). Indeed, a number of autophagy related proteins, including Beclin1 (Liang et al., 1999; Yue et al., 2003), ATG5 (Yousefi et al., 2006; Takamura et al., 2011), ATG4c (Marino et al., 2007), and ATG7 (Takamura et al., 2011) as well as some accessory proteins (Bif1, UVRAG) (Liang et al., 2007a; Takahashi et al., 2007) have been shown to be tumor suppressors. In addition, several oncosuppressor proteins, such as DAPK (Inbal et al., 2002), PTEN (Arico et al., 2001), TSC1/2, p53 (Feng et al., 2005) and LKB1 (Liang et al., 2007b) have been recently shown to stimulate autophagic process, while a number of oncoproteins, like Bcl-2 (Pattingre et al., 2005), AKT and class I PI3K (Arico et al., 2001) negatively regulate autophagy (Rubinsztein et al., 2012). One of the possible mechanisms for antitumorigenic functions of autophagy is based on its cytoprotective role. More specifically, inhibition of autophagy will favor DNA damage, genomic instability and thus tumorigenesis through the accumulation of protein aggregates, damaged mitochondria and reactive oxygen species (ROS) (White, 2012).

In contrast, in established tumors, autophagy being a primarily survival mechanism can support cancer cell survival in harsh conditions, characterized by nutrient deficiency and hypoxia (Degenhardt et al., 2006; White, 2012). Indeed, accumulated data suggest that autophagy inhibition by genetic or chemical means facilitates apoptotic cell death and inhibits tumor cells growth in several cancers (Amaravadi et al., 2007; Takamura et al., 2011; Yang et al., 2011a,b). In addition, elevated autophagy is often detected in cancer cells in response to radiation and chemotherapy (Ito et al., 2005; Kondo et al., 2005; White, 2012). Furthermore, autophagy seems to contribute to the therapeutic resistance of some cancers, as inhibition of autophagy has been shown to sensitize tumor cells to chemotherapy treatments (Guo et al., 2012; Selvakumaran et al., 2013). Therefore, autophagy inhibition as an adjuvant to chemotherapy represents a promising strategy in the treatment of some cancers (Amaravadi et al., 2011; White, 2012). Indeed, more than 20 clinical trials are currently evaluating the efficacy of chloroquine and hydroxychloroquine (autophagy inhibitors) in treatment of different cancers either in monotherapy or in combination with other anticancer agents. The preliminary results of many of these trials show apparent antitumour activity (Yang et al., 2011b; Kimura et al., 2013). In addition to chloroquine and hydroxychloroquine another autophagy inhibitors, including 3-Methyladenine, bafilomycin A1 and pepstatin A have been shown to enhance the antitumour efficacy of chemotherapeutic drugs both *in vitro* and *in vivo* (Hsu et al., 2009; Li et al., 2010; Cheong et al., 2012; Lamoureux and Zoubeidi, 2013). However, it should be noted that all these autophagy inhibitors are not specific and can modulate other cellular processes, such as endocytosis, lysosomal function etc. Hence unexpected side effects could occur when treating patients with these drugs. Therefore, more specific and potent autophagy inhibitors are clearly needed.

Thus, figuring out whether to stimulate or inhibit autophagy in each particular case will provide a powerful approach to treat cancer.

CALCIUM, Ca^{2+}-PERMEABLE ION CHANNELS AND CANCER

Changes in the cytosolic free Ca^{2+} concentration play a central role in many fundamental cellular processes including muscle contraction, transmitter release, cell proliferation, differentiation, gene transcription and cell death (Berridge et al., 2000). Giving that Ca^{2+} controls so many vital processes, disturbance of the Ca^{2+} homeostasis regulatory mechanisms leads to a vast variety of severe pathologies, including cancer. Indeed, the role of Ca^{2+} is well-established in many cell signaling pathways involved in carcinogenesis (Monteith et al., 2007, 2012; Prevarskaya et al., 2011).

Increase in cytosolic calcium can occur as a result of Ca^{2+} influx from the extracellular space and Ca^{2+} release from intracellular sources. Both Ca^{2+} influx and Ca^{2+} release are tightly controlled by numerous regulatory systems that provide the specific spatial and temporal characteristics of an intracellular calcium signal that are required for sustaining certain cellular functions (Berridge et al., 2000).

Mitochondrial, ER, lysosomal and cytosolic calcium levels are regulated by calcium permeable ion channels localized either on the membranes of the intracellular organelles or on the plasma membrane (Berridge et al., 2003; Rizzuto et al., 2012). The calcium permeable channels, including families of transient receptor potential (TRP) channels, store-operated channels (SOCs), voltage-gated calcium channels, two-pore channels, mitochondrial permeability transition pore (MPTP), mitochondrial calcium uniporter (MCU), IP3 and ryanodine receptors and others contribute to changes in $[Ca^{2+}]_i$ by providing Ca^{2+} entry pathways, by modulating the driving force for the Ca^{2+} entry, and also by providing intracellular pathways for Ca^{2+} uptake/release into/from cellular organelles (Berridge et al., 2003; Pedersen et al., 2005; Bernardi and von Stockum, 2012; Rizzuto et al., 2012).

Thus, modulation of calcium permeable ion channel's expression/function affects intracellular Ca^{2+} concentrations and consequently calcium dependent processes, such as proliferation, apoptosis and autophagy (Flourakis and Prevarskaya, 2009; Decuypere et al., 2011a; Dubois et al., 2013). Indeed, defects in Ca^{2+} channels expression/function are involved in a number of pathologies, including tumorigenesis, since increased expression

of Ca^{2+} channels could lead to elevated cytosolic Ca^{2+} levels and promotion of Ca^{2+}-dependent proliferative pathways (Nilius, 2007; Prevarskaya et al., 2010). As an example, several members of the TRP family of ion channels, namely TRPC1, TRPC3, TRPC6, TRPV1, TRPV6, TRPM1, TRPM4, TRPM5, TRPM7, and TRPM8, show altered expression in cancer cells (Shapovalov et al., 2011). The involvement of SOCs, MPTP, MCU, IP3 receptors and ryanodine receptors in the regulation of cell death has also been described (Hajnoczky et al., 2000; Boehning et al., 2004; Flourakis et al., 2010; Wong et al., 2012b; Bernardi, 2013; Curry et al., 2013; Dubois et al., 2013; Qiu et al., 2013).

ROLE OF Ca^{2+} IN AUTOPHAGY

Recent findings identified intracellular calcium as a key regulator of both basal (Cardenas et al., 2010) and induced (Hoyer-Hansen et al., 2007) autophagy. The complex role for Ca^{2+} in autophagy regulation has become obvious since 1993, when the first report linking autophagy and intracellularly sequestered calcium was published (Gordon et al., 1993). Indeed, Gordon et al. demonstrated that decrease as well as increase in cytosolic Ca^{2+} levels inhibited autophagy in rat hepatocytes (Gordon et al., 1993). And till now, the data on the mechanisms by which calcium controls autophagy remain rather controversial. Several groups reported inhibitory actions of calcium on autophagy, while another proposed mechanisms for calcium to activate autophagy (Decuypere et al., 2011a; Cardenas and Foskett, 2012; Parys et al., 2012). Indeed, Hoyer-Hansen and colleagues provided evidence that a rise in the free cytosolic calcium is a potent inducer of macroautophagy (Hoyer-Hansen et al., 2007). They demonstrated that Ca^{2+} mobilizing agents, namely vitamin D3, thapsigargin, ATP and ionomycin, stimulate autophagy via a signaling pathway involving Ca^{2+} -activated kinase CAMKK-beta, which directly activates AMPK to inhibit mTOR (Hoyer-Hansen et al., 2007). Recently, this pathway was shown to be required for amyloid-beta peptide induced autophagosome formation (Son et al., 2012). Ca^{2+}/CAMKK-beta/AMPK pathway, although mTOR-independent, has been found to be involved in the leucine-rich repeat kinase-2 (LRRK2) induced autophagy (Gomez-Suaga et al., 2012). Authors proposed the mechanism in which LRRK2 activates NAADP receptors, in particular TPC2, leading to Ca^{2+} mobilization from acidic stores that in turn stimulates Ca^{2+}-induced Ca^{2+} release from ER and subsequent CAMKK-beta/AMPK pathway activation (Gomez-Suaga et al., 2012). In all the cases discussed above, buffering of cytosolic Ca^{2+} with BAPTA-AM effectively inhibited autophagosomes accumulation, confirming the role of Ca^{2+}. Another evidence supporting stimulatory effect of Ca^{2+} on autophagy is that exogenously introduced calcium in the form of calcium phosphate precipitates induces macroautophagy, which is Beclin1, Atg5 and PI3K class III dependent (Gao et al., 2008). This effect could be antagonized by extra- or intra-cellular calcium chelation.

In line with activatory role of calcium in autophagy, Sakaki et al. showed that Ca^{2+}-dependent activation of protein kinase Ctheta is required for ER-stress induced autophagy but not for starvation induced autophagy (Sakaki et al., 2008).

In another studies calcium/calmodulin dependent death associated protein kinase (DAPK) was shown to positively regulate autophagy in a Beclin1 dependent manner. Thus, DAPK phosphorylates Beclin1, thereby promoting its dissociation from Bcl-X_L and Bcl-2 inhibitory proteins (Zalckvar et al., 2009a,b).

On the contrary, Khan et al suggested that basal autophagic flux may be negatively regulated by IP3R-dependent Ca^{2+} release from the ER (Khan and Joseph, 2010). The authors proposed a mechanism in which cytosolic Ca^{2+} elevation acts to maintain an elevated mTORC1 activity through AMPK independent pathway (Khan and Joseph, 2010). Furthermore, amino acids were shown to induce an increase in $[Ca^{2+}]_i$, supposedly through the influx of extracellular Ca^{2+}, which acts to enhance the binding of Ca^{2+}/CaM to hVps34, resulting in mTOR activation (Gulati et al., 2008). This pathway could also lead to autophagy inhibition.

Thus, calcium is likely to have different regulatory effects on autophagy, depending on spatial and temporal parameters of Ca^{2+} signaling, nutrient and growth factor availability, as well as pathology (cancer, neurodegenerative disorders, inflammation etc.) (Decuypere et al., 2011a).

CALCIUM PERMEABLE CHANNELS IN THE CONTROL OF AUTOPHAGY

Modulation of calcium permeable channels expression/function affects intracellular Ca^{2+} concentrations and, consequently calcium dependent processes, such as proliferation, apoptosis and autophagy. The role of calcium-permeable channels for proliferation and apoptosis is largely recognized (Monteith et al., 2007; Flourakis and Prevarskaya, 2009; Prevarskaya et al., 2010; Dubois et al., 2013), whereas the information about molecular nature of channels regulating autophagy and the mechanisms of this regulation is still limited.

Hereafter, we will provide an overview of the literature on this subject and discuss the possible involvement of calcium permeable ion channels in the regulation of autophagy.

Most reports considering calcium permeable channels as autophagy regulators focused on the inositol trisphosphate receptor (IP3R), the main intracellular Ca^{2+} release channel (Parys et al., 2012). Together these reports suggested a complex role for IP3R, since both stimulatory as well as inhibitory functions for IP3R toward autophagy have been described.

Thus, in one paper it was suggested that cadmium (Cd^{2+}) induces autophagy through elevation of cytosolic calcium via IP3R and subsequent extracellular signal-regulated kinase (ERK) activation (Wang et al., 2008). As a proof for this, the authors showed that 2-aminoethoxydiphenil borate (2-APB), a blocker of IP3R, suppressed, while knockdown of calcineurin, a putative IP3R inhibitor, increased Cd^{2+}-induced autophagy (Wang et al., 2008). However, 2-APB was shown to modulate a number of TRP channels, SERCA pump and SOCs (Peppiatt et al., 2003; Clapham, 2007) and as to calcineurin, its role in the IP3R regulation is debated at present (Bultynck et al., 2003).

In another study, IP3R was shown to be required for differentiation factor DIF-induced autophagic cell death in *Dictyostelium discoideum* (Lam et al., 2008). Through random insertional mutagenesis, the authors showed that inactivation of the *iplA* gene, the only gene encoding an IP3R in this organism, prevented autophagic cell death (Lam et al., 2008).

The stimulatory role of IP3R on starvation-induced autophagy has been recently shown (Decuypere et al., 2011b). The authors showed that the Ca^{2+} chelator BAPTA-AM as well as the IP3R inhibitor xestospongin B abolished starvation induced increase in LC3 lipidation and GFP-LC3-puncta formation. Moreover, starvation lead to IP3R sensitization through increased Beclin1 binding to the IP3R (Decuypere et al., 2011b).

To date, most reports on IP3R-dependent regulation of autophagy suggest inhibitory role for IP3R toward autophagy (Parys et al., 2012).

Thus, lithium (Li^+) was found to induce mTOR independent autophagy through inhibition of inositol monophosphatase and further decrease in IP3 levels (Sarkar et al., 2005). Consistently, another study demonstrated that IP3R inhibitor xestospongin (XeB) or IP3R knockdown induced autophagy in HeLa cells (Criollo et al., 2007). One of the mechanisms, by which XeB and starvation induce autophagy was proposed by Vicencio et al. (2009). The authors suggested that xestospongin B and nutrient starvation disrupt a molecular complex formed by the IP3R, Beclin 1 and Bcl-2, and presented evidence that the IP3R represses autophagy through Bcl-2-mediated binding of Beclin 1, thus suggesting Ca^{2+}-independent mechanism (Vicencio et al., 2009).

As three IP3R isoforms exist, some groups studied the impact of IP3R on autophagy in the triple IP3R-deficient DT40 cells (Cardenas et al., 2010; Khan and Joseph, 2010). These cells demonstrate higher basal autophagy levels, compared to wild-type. Interestingly, expression of IP3R3, but not of ryanodine receptor type 2, rescued elevated autophagy in these cells (Cardenas et al., 2010). In contrast, expression of Ca^{2+} impermeable mutant D2550A-IP3R3 failed to suppress constitutive autophagy, suggesting a necessity of the Ca^{2+}-release activity for IP3R. The authors proposed the mechanism in which constitutive IP3R mediated Ca^{2+} release and uptake of this Ca^{2+} by mitochondria is fundamentally required to maintain mitochondrial bioenergetics and ATP production in resting cells thereby suppressing autophagy. Absence of this Ca^{2+} transfer results in inhibition of pyruvate dehydrogenase and activation of AMPK, which activates prosurvival macroautophagy in mTOR independent manner (Cardenas et al., 2010).

Along with IP3R some other calcium permeable channels were shown to be involved in autophagy regulation. Among them, TRPML1, also known as mucolipin-1, ubiquitously expressed TRP channel primarily localized to the late endosomal and lysosomal compartments (Zeevi et al., 2009; Cheng et al., 2010). Direct patch-clamp of enlarged lysosomes revealed that TRPML1 is a Ca^{2+} permeable channel (Dong et al., 2008). The main physiological function of TRPML1 channel is considered to serve as a late endosomal/lysosomal Ca^{2+} release channel. Loss-of-function mutations in the human TRPML1 gene result in mucolipidosis type IV, a neurodegenerative lysosomal storage disorder characterized by mental retardation and retinal degeneration (Bach, 2001; Altarescu et al., 2002). Fibroblasts from mucolipidosis type IV patients exhibit enlarged vacuoles with accumulated lipids and acid mucopolysacharides, suggesting the role for TRPML1 in trafficking of proteins and lipids (Riedel et al., 1985; Goldin et al., 1999; Slaugenhaupt et al., 1999). Loss of TRPML1 has been shown to be accompanied by impairment in the lysosomal pH, accumulation of autophagosomes and abnormal mitochondria, accumulation and aggregation of p62 and ubiquitin proteins, all of which suggested a defective autophagy (Jennings et al., 2006; Soyombo et al., 2006; Vergarajauregui et al., 2008; Curcio-Morelli et al., 2010). Indeed, several studies have proposed TRPML1 as autophagy regulator (Vergarajauregui et al., 2008; Venugopal et al., 2009; Curcio-Morelli et al., 2010; Wong et al., 2012a; Venkatachalam et al., 2013). Vergarajauregui et al. showed that accumulation of autophagosomes in TRPML1-deficient fibroblasts obtained from mucolipidosis type IV patients was due to increased Beclin-1 dependent autophagosome formation and delayed fusion of autophagosomes with late endosomes/lysosomes. The authors claimed that TRPML1 is necessary for efficient fusion of both autophagosomes and late endosomes with lysosomes although it is not clear if the Ca^{2+}-channel function of the TRPML1 is essential here (Vergarajauregui et al., 2008). In another study, group of S. Slaugenhaupt showed that CMA is impaired in mucolipidosis type IV fibroblasts (Venugopal et al., 2009). The authors showed that TRPML1 directly interacts with Hsc70 and Hsp40, members of molecular chaperone complex required for CMA, and hypothesized that this interaction may be required for intralysosomal Hsc70 to facilitate the translocation of CMA substrate proteins across the lysosomal membrane. The authors also speculated that TRPML1 channel activity is required for CMA (Venugopal et al., 2009). In 2010 same group investigated macroautophagy in neurons isolated from cerebellum of $TRPML1^{-/-}$ mouse embryos (Curcio-Morelli et al., 2010). These cells displayed higher levels of basal autophagy markers compared to wild-type ones. In addition, LC3-II clearance was affected in these cells, suggesting impairment of lysosomal function. However, the link between observed defects in autophagy and functionality of TRPML1 as a Ca^{2+} permeable channel is missing. Recently, Wong et al showed that Drosophila TRPML is required for TORC1 activation (Wong et al., 2012a). Authors demonstrated defects in amphisomes/lysosomes fusion and elevated late endosomal/lysosomal Ca^{2+} levels in flies lacking TRPML1. Authors also showed decreased TORC1 activity and increased induction of autophagy in $TRPML1^{-}$ mutants. Moreover, authors suggested that TORC1 regulates the subcellular localization of TRPML1. Thus, this study points out to TRPML1 as a Ca^{2+} channel present in amphisomes which releases luminal Ca^{2+} to facilitate Ca^{2+}-dependent fusion of amphisomes with lysosomes (Wong et al., 2012a).

In addition to TRPML1, another member of mucolipin family, TRPML3 has been shown to be involved in autophagy regulation. In contrast to TRPML1, TRPML3 exhibits more restrictive tissue distribution, and is primarily localized to early as well as late endosomes/lysosomes and less to the plasma membrane (Zeevi et al., 2009; Cheng et al., 2010).

It has been shown that overexpression of TRPML3 leads to increased autophagy in HeLa cells (Kim et al., 2009). Moreover, TRPML3 is recruited to autophagosomes upon induction of autophagy. Additionally, expression of dominant negative mutant TRPML3 (D458K) or knockdown of endogenous TRPML3 by siRNA reduces autophagy. Thus, it has been proposed that TRPML3 provides Ca^{2+} that is required for fusion and fission events in autophagy (Kim et al., 2009). Further,

heteromultimerization of TRPML channels was shown to affect autophagy (Zeevi et al., 2010).

Also TRPV1 was proposed to regulate autophagy in thymocytes (Farfariello et al., 2012). The authors showed that capsaicin, an activator of TRPV1, induce Beclin-1 dependent accumulation of LC3-II protein. This effect can be antagonized by capsazepine, a blocker of TRPV1 and compound C, an AMPK inhibitor, suggesting AMPK involvement. The authors proposed that capsaicin induced autophagy is calcium dependent, as cotreatment with EDTA markedly reduced LC3-II accumulation. Moreover, it was shown that capsaicin induces accumulation of ATG4C and triggers its oxidation in a ROS-dependent manner, thus regulating LC3 lipidation levels (Farfariello et al., 2012). However, capsaicin was shown to have TRPV1-independent effects, such as inhibition of voltage-gated calcium channels (Hagenacker et al., 2005), cancer cell growth inhibition and apoptosis induction (Mori et al., 2006; Chow et al., 2007). Additionally, upon prolonged exposure to capsaicin, TRPV1 desensitization occurs and its activity decreases (Caterina et al., 1997). Thus, additional experiments using more specific agonists and antagonists as well as siRNA knockdown are needed to confirm the role of TRPV1 in autophagy regulation. It would be interesting as well to compare the effect of capsaicin on autophagy in TRPV1-expressing and TRPV1-null cells.

Some ion channels, which do not belong to the family of TRP channels, were also proposed to regulate autophagy. Williams et al. found that L-type calcium channels antagonists, namely verapamil, loperamide, nimodipine, nitrendipine and amiodarone induce mTOR-independent autophagy (Williams et al., 2008). Conversely, the L-type Ca^{2+} channel agonist (±)-BAY K 8644 that increases cytosolic Ca^{2+} levels, inhibits autophagy. Authors demonstrated that elevated cytosolic Ca^{2+}, presumably due to activity of L-type calcium channels on the plasma membrane, can activate calpains, a family of Ca^{2+}-dependent cysteine proteases, which cleave and activate the α-subunit of heterotrimeric G proteins $G_s\alpha$. $G_s\alpha$ activation, in turn, increases adenylyl cyclase activity leading to increase in cAMP levels. Next, elevated intracellular cAMP levels negatively regulate autophagy by promoting IP3 production via cAMP-Epac-Rap2B-PLC-ε pathway. Finally, IP3, via IP3R influence cytosolic Ca^{2+} levels, which can again activate calpains, thus creating a potential positive feedback loop for autophagy inhibition (Williams et al., 2008). Again, it is important to mention that although several different L-type calcium channel inhibitors as well as agonist were used in the study, the data showing the effect of siRNA mediated knockdown of L-type calcium channels and/or channel-dead mutants on autophagy are missing. It would be interesting as well to check the effect of these inhibitors on "negative control" cells lacking L-type calcium channels. In addition, verapamil is known to passively diffuse into the lysosome, where it becomes protonated and could cause an increase in lysosomal pH (Lemieux et al., 2004). This could lead to the inhibition of lysosome function and thus block fusion with the autophagosome.

Two-pore channels (TPC) have been also proposed to regulate autophagy (Pereira et al., 2011; Gomez-Suaga et al., 2012). Mammalian TPC family comprise two members TPC1 and TPC2, widely expressed in humans and localized intracellularly on endolysosomes, with TPC2 being specifically targeted to lysosomes. Several groups proposed TPC as a mediator of endolysosomal calcium release in response to the elevation of the second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) (Calcraft et al., 2009; Galione et al., 2009). Recently Pereira et al. demonstrated that NAADP stimulates autophagy via TPCs in rat astrocytes (Pereira et al., 2011). The authors showed that NAADP mediated increase in the number of LC3-GFP puncta was reduced in cells, transfected with dominant negative TPC2 L265P construct, suggesting the importance of TPC2 for autophagy (Pereira et al., 2011). TPC2 channel has been also proposed to be involved in LRRK2 induced autophagy (Gomez-Suaga et al., 2012).

The MCU that was recently identified as a channel responsible for mitochondrial Ca^{2+} uptake (Baughman et al., 2011; De Stefani et al., 2011) has been demonstrated to have the role in autophagy regulation. Indeed, Cardenas et al. showed that the uniporter inhibitor Ru360 inhibited cell O_2 consumption rate, activated AMPK, and induced autophagy (Cardenas et al., 2010). In line with these data, MCUR1(mitochondrial calcium uniporter regulator 1) was shown to regulate autophagy (Mallilankaraman et al., 2012). MCUR1 represents an integral membrane protein that is required for MCU-dependent mitochondrial Ca^{2+} uptake. Knockdown of MCUR1 in HeLa and HEK293T cells reduced cell O_2 consumption rate, activated AMPK, and induced macroautophagy (Mallilankaraman et al., 2012). Importantly, stable knockdown of MCU in HeLa cells elicited essentially the same effects, confirming the regulatory role for MCU toward autophagy (Mallilankaraman et al., 2012).

MPTP has been also suggested to be implicated in autophagy regulation. Elmore et al. proposed that mitochondrial permeability transition (MPT) initiates autophagy in rat hepatocytes. Although the mechanism by which the MPT signals autophagic sequestration was not investigated in this work, the authors hypothesized that factors released from the mitochondrial intermembrane space as a consequence of MPT could stimulate autophagy (Elmore et al., 2001). It is not clear if the Ca^{2+}-release channel function of the MPTP is essential here as well. A functional MPTP was also shown to be required for starvation-induced mitochondrial autophagy (Carreira et al., 2010). The authors demonstrated that starvation induced mitochondrial depolarization in cardiac cells. This depolarization was prevented by cyclosporin A (MPT inhibitor). Further, the authors showed that cyclophilin D a component of the MPTP, is required for mitochondrial removal by starvation-induced autophagy. Interestingly, cardiomyocytes from cyclophilin D deficient mice failed to upregulate autophagy in response to nutrient deprivation, suggesting that MPTP is essential here (Carreira et al., 2010). Again the role of calcium and the importance of calcium permeability for MPTP in the regulation of autophagy were not assessed in this study.

POTENTIAL NEW CANDIDATES FOR AUTOPHAGY REGULATION IN CANCER TREATMENT

Aside from ion channels, described above, all the other calcium permeable channels could potentially be involved in autophagy regulation, as they contribute to the changes in cytosolic calcium

levels. Here, we will provide several hypotheses for autophagy regulation by some of the calcium permeable channels that have not been shown to be directly involved in autophagy regulation. Further, we will propose the possible link between calcium permeable channels, autophagy and cancer progression and therapeutic response. Considering both physiological roles as well as cellular localization we selected several calcium permeable channels, which in our opinion could have an impact on autophagy.

TRPML2 channel, a member of the mucolipin family, has been shown to localize to late and recycling endosomes as well as lysosomes (Zeevi et al., 2009; Cheng et al., 2010). Recent study claimed that TRPML2 does not appear to play a role in starvation-induced autophagy (Zeevi et al., 2010). However, TRPML2 knockdown was demonstrated to induce lysosomal inclusions accumulation in HEK cells (Zeevi et al., 2009). This fact along with the endolysosomal distribution of TRPML2 indicates the potential role of TRPML2 in the regulation of basal as well as other types of autophagy.

TRPM2 is known as a chanzyme, combining two functions: of an ion channel and an enzyme, since the C-terminal of TRPM2 contains enzymatically active adenosine diphosphoribose (ADPR) hydrolase domain (Sumoza-Toledo and Penner, 2011). TRPM2 has been shown to be activated and regulated by variety of stimuli including ADPR, H_2O_2, NAADP, pH, and cytosolic calcium. It is involved in numerous physiological processes, such as production of cytokines, insulin secretion, oxidative stress, apoptosis (Jiang et al., 2010). TRPM2 functions as a Ca^{2+}-permeable channel on the cell surface, but recently TRPM2 has been shown to be also localized intracellularly on the late endosomal and lysosomal membranes where it functions as a lysosomal Ca^{2+} release channel (Lange et al., 2009). Thus, it can possibly affect autophagy in the same manner as TRPML and TPC channels. Interesting that both Ca^{2+}-entry and Ca^{2+}-release channel functions of TRPM2 were shown to be important in H_2O_2-induced beta-cell death (Lange et al., 2009). Further, H_2O_2 is a known activator of autophagy (Chen et al., 2008). Thus, potentially TRPM2 could be involved in H_2O_2-induced autophagy.

Another interesting candidate is the cold receptor TRPM8, which is found in sensory neurons, where it constitutes the principal detector of cold ($<\sim$28°C) (Bautista et al., 2007). In addition to its role as plasmalemmal Ca^{2+} channel, TRPM8 could function as intracellular Ca^{2+}-release channel on the ER membrane (Zhang and Barritt, 2004; Thebault et al., 2005). Initially, TRPM8 was cloned from the human prostate as prostate-specific gene, which is upregulated in malignant tissues (Tsavaler et al., 2001). The role of TRPM8 in cancer was extensively studied in recent years, and published data suggest that TRPM8 could be involved in proliferation, differentiation and apoptosis in cancer cells (Zhang and Barritt, 2004; Thebault et al., 2005). Given the localization of TRPM8 on ER, it would be interesting to study the possible autophagy regulation by TRPM8-mediated Ca^{2+} release from the ER. The potential mechanisms could be the same as for IP3 receptor.

It is worth to note that TRPV1 channel, discussed above, has also been found to be expressed intracellularly at the ER and trans-Golgi network (Turner et al., 2003), so apart from the autophagy modulating mechanism provided above, TRPV1 could also be involved in another autophagy related signaling pathways.

TRPP2, the product of the gene mutated in autosomal dominant polycystic kidney disease (ADPKD), is another possible candidate for autophagy regulation. It is widely expressed, with a highest level in the kidney, and primarily localized in cilia, where it seems to function as a mechanosensor involved in the nodal ciliary movement (Delmas et al., 2004). TRPP2 might function as a plasma membrane calcium-permeable channel (when interacting with TRPP1) or as a calcium release channel located in the ER (Hanaoka et al., 2000; Cahalan, 2002; Koulen et al., 2002). Interesting, TRPP2 was shown to function as a calcium-activated intracellular calcium release channel—property reminiscent of IP3 receptors and ryanodine receptors (Koulen et al., 2002). Thus, potentially TRPP2 could regulate autophagy in a way similar to IP3R.

Interesting, although ryanodine receptor (RyR) constitutes the major cellular mediator of calcium-induced calcium release, the data on its role in autophagy is very scarce. As it was mentioned above, overexpression of ryanodine receptor type 2 in triple IP3R-deficient DT40 cells was without effect on constitutive autophagy (Cardenas et al., 2010). Despite this, we believe that more experiments are required to unravel the role of RyR in autophagy regulation.

The next candidate, TRPV2, is a Ca^{2+} permeable non-selective cationic channel, which has been found to be activated by noxious heat (>50°C), growth factors (i.e., IGF) and stretch (Caterina et al., 1999; Kanzaki et al., 1999; Muraki et al., 2003). It was shown that insulin induced translocation and insertion of TRPV2 into the plasma membrane in a PI3K-dependent manner (Aoyagi et al., 2010). Recent studies revealed the role for TRPV2 in promoting prostate cancer migration and progression to androgen resistance (Monet et al., 2010). Interestingly, Saito et al. demonstrated the function of 2-APB-activated and Rutenium Red-inhibited calcium-permeable ion channel in early endosomes (Saito et al., 2007). The authors reported that this channel has similar pharmacology to that of TRPV2. As early endosomes, and fusion of autophagosomes with functional early endosomes have been shown to be essential for autophagy (Razi et al., 2009), we hypothesize that TRPV2, which apparently forms early endosomal Ca^{2+}-release channel, could be involved in autophagy where it may regulate fusion between autophagosomes and early endosomes. Function on the plasma membrane as well as dependence on PI3K suggest possible complex role in autophagy regulation.

ORAI1 (the calcium release-activated calcium channel protein 1) which constitutes a major molecular component of store-operated calcium (SOC) channels (Hewavitharana et al., 2007) also represents an attractive candidate for autophagy regulation. Recently, Abdelmohsen et al. reported that microRNA miR-519 stimulates autophagy through the downregulation of ORAI1 and ATP2C1 proteins, increase in the cytosolic Ca^{2+} levels, activation of Ca^{2+}-activated calmodulin kinase II (CaMKII) as well as glycogen synthase kinase 3β (GSK3 β) and subsequent p21 upregulation (Abdelmohsen et al., 2012). However, the direct link between autophagy stimulation and functionality of ORAI1 as a Ca^{2+} permeable channel is missing. Thus, additional experiments using specific agonists and antagonists as well as siRNA

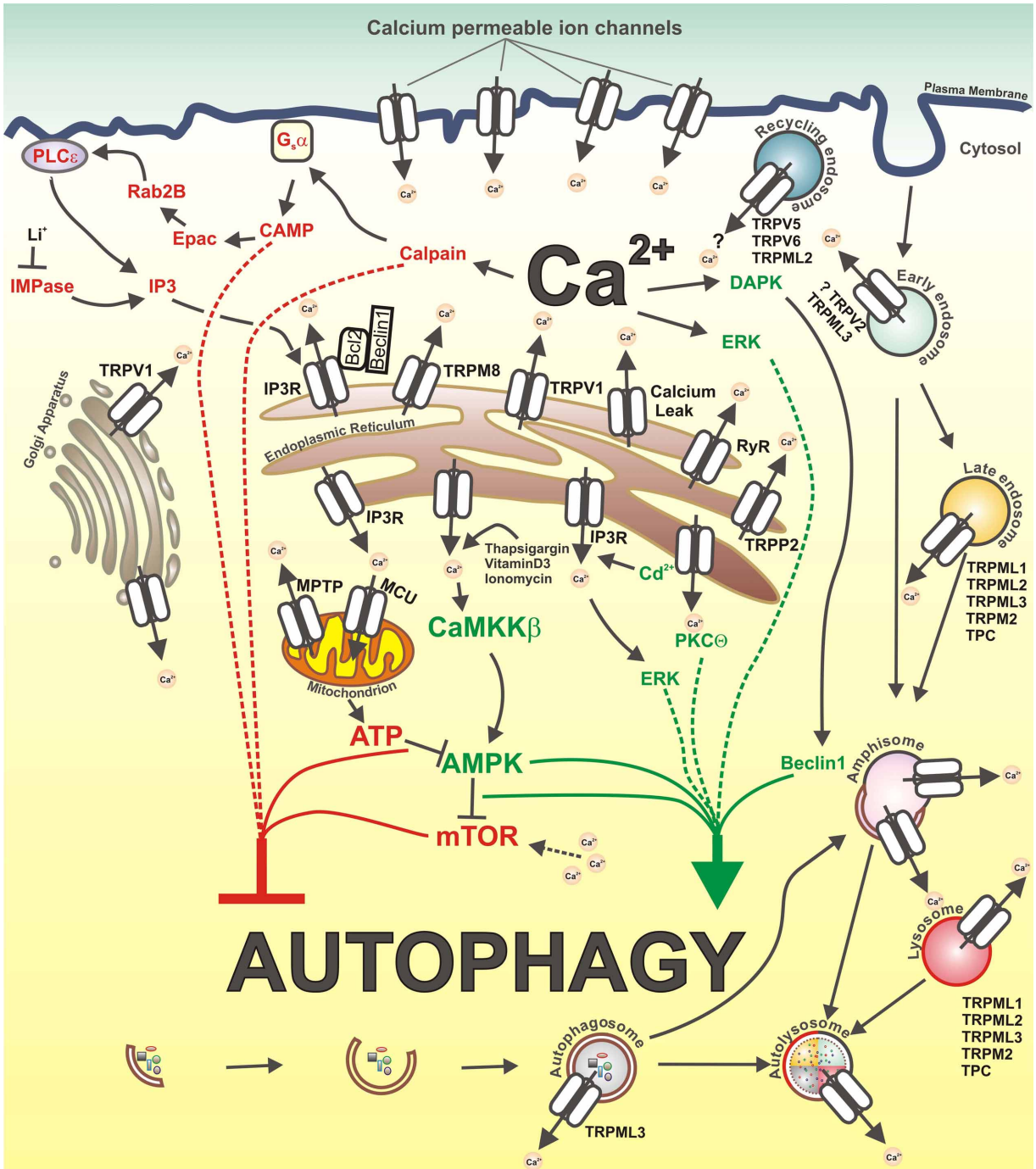

FIGURE 1 | Calcium and calcium-permeable channels in the control of autophagy. Inhibitory and stimulatory actions of Ca^{2+} on autophagy as well as calcium-permeable channels that could be potentially involved in autophagy regulation are depicted. Ca^{2+} mobilizing agents, such as vitamin D3, thapsigargin and ionomycin, lead to increase in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) concentration and subsequent activation of CAMKK-beta, followed by AMPK-dependent mTOR inhibition and autophagy stimulation. ER-stress induced elevation of $[Ca^{2+}]_{cyt}$ levels activate PKCtheta which stimulates autophagy. Additionally, increased $[Ca^{2+}]_{cyt}$ induce activation of DAPK, which phosphorylates Beclin1, thereby promoting its dissociation from Bcl-X_L and Bcl-2 inhibitory proteins, and thus stimulate autophagy. Cadmium induces autophagy through elevation of cytosolic Ca^{2+} via IP3R and subsequent ERK activation. In contrast, constitutive IP3R mediated Ca^{2+} release to mitochondria maintains ATP production and AMPK inhibition, thereby suppressing autophagy. The inhibition of IMPase by Li^+ causes a decrease in IP3 levels and autophagy induction. Further, IP3R-dependent Ca^{2+} release from the ER as well as amino acids-induced increase in $[Ca^{2+}]_{cyt}$ maintain an elevated mTORC1 activity, thus inhibiting autophagy. In addition, IP3R represses autophagy through Bcl-2-mediated binding of Beclin1. Also, increased Ca^{2+} influx through L-type calcium channels on the plasma membrane activates calpains and consequently Gsalpha/Adenylyl cyclase/cAMP/Epac/Rap2B/PLC-ε pathway which negatively regulates autophagy by promoting IP3 production, IP3R activation and Ca^{2+} release.

knockdown of ORAI1 are needed to confirm its role in autophagy regulation.

A subset of other channels, found to be localized both on plasma membrane and intracellular vesicles (i.e., recycling endosomes), including TRPC3, TRPV6, and TRPV5, could also potentially modulate autophagy, although at present it's not clear if these channels are functional intracellularly (Dong et al., 2010; Toro et al., 2011). A graphic overview of the calcium-related mechanisms of autophagy regulation is presented in **Figure 1**.

Thus, available data strongly suggests that calcium permeable channels represent good candidates for autophagy regulation. Given that both autophagy and calcium-permeable ion channels have a role in cancer, this can be highly valuable in order to achieve specific outcomes in anti-cancer therapy. Ion channels could provide some advantages, when targeting autophagy *in vivo* for cancer treatment. Indeed, most ion channels are localized on cell surface, thus they can be subjected to antibody-based targeting that can be particularly useful in the case of channel upregulation in cancer. Moreover, anti-channel antibodies could be used as carriers for radionuclides, toxic molecules or nanoparticles, which can themselves affect autophagy and as such influence cell fate.

The growing number of studies pointing on the fact that inflammation increases the incidence of cancer (Mantovani et al., 2008). Autophagy has been linked to both cancer and inflammation, and is often defective in the inflammatory conditions (White et al., 2010). Among them, Crohn's disease and pancreatitis have been associated with an increased risk of colorectal and pancreatic cancers, respectively (Freeman, 2008; Raimondi et al., 2010). Accordingly, these pathological states are characterized by the accumulation of damaged organelles and polyubiquitinilated protein aggregates, ROS production and DNA damage, the factors that create a cancer-promoting environment (Gukovsky et al., 2012; Nguyen et al., 2013). Hence, this implies that functional autophagy stimulation, to eliminate dangerous garbage, may constitute an effective approach to cancer prevention. Interestingly, a number of calcium-permeable ion channels, including TRPV1, TRPV4, TRPA1, and TRPM8, were shown to be regulated by inflammatory mediators (Nilius et al., 2007; Kochukov et al., 2009; Zhang et al., 2012). Thus, these channels can represent potential targets to stimulate autophagy in inflammatory conditions in order to avert tumorigenesis initiation. Additionally, lysosomal dysfunctions have been reported in pancreatitis (Gukovsky et al., 2012), therefore it could be interesting to consider lysosomal ion channels as well.

On the other hand, in existing tumors autophagy may favor survival and progression. Thus, the possible anticancer therapy should be focused on autophagy inhibition. In this case, considering calcium-permeable channels as a potential tool to target autophagy could also be useful. More specifically, as we discussed above, a number of calcium-permeable ion channels exhibit altered expression in cancer cells. For instance, TRPM8 is upregulated in androgen-dependent prostate cancer cells (Zhang and Barritt, 2004; Thebault et al., 2005). This could possibly influence intracellular calcium levels and consequently autophagy. Hence, targeting TRPM8 as well as another channels overexpressed in cancers could provide an additional control over autophagy, particularly during chemotherapy, and as such contribute to cancer treatment.

CONCLUSIONS

Calcium-permeable ion channels have emerged as important regulators of autophagy and the effect of such regulation most likely depends on Ca^{2+} signals in a spatially restricted subcellular domains. Apparently, such regulation can represent a fundamental mechanism of fine tuning the autophagy. However, the data concerning this subject is very limited, thus further studies are needed to understand the variety of mechanisms, by which calcium channels can influence autophagy.

Accumulated data proves that both calcium-permeable ion channels and autophagy are implicated in cancer initiation and progression as well as chemotherapy resistance. Paradoxically, autophagy has opposite roles in cancer, with both tumorigenesis suppressor action, in particular at the early stages of tumor initiation and cancer promotion effect resulting in tumor cell survival, chemotherapy resistance and cancer progression. Thus, it is important to unravel autophagy regulating pathways to most effectively target autophagy to cure cancer. Identification of the connections between calcium channels and autophagy could define a new strategy in cancer treatment, and identify useful tools and biomarkers for the elaboration of effective anti-cancer therapies. Moreover, as malfunction of autophagy has been linked to a wide range of human pathologies including liver disease, neurodegeneration, Crohn's disease and cancer, uncovering novel mechanisms of autophagy regulation by calcium permeable ion channels could have a broad impact on the "Autophagy" field and contribute to the developing of autophagy as a potential clinical approach to cure diseases.

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The voltage-dependent K^+ channels Kv1.3 and Kv1.5 in human cancer

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Voltage-dependent K^+ channels (Kv) are involved in a number of physiological processes, including immunomodulation, cell volume regulation, apoptosis as well as differentiation. Some Kv channels participate in the proliferation and migration of normal and tumor cells, contributing to metastasis. Altered expression of Kv1.3 and Kv1.5 channels has been found in several types of tumors and cancer cells. In general, while the expression of Kv1.3 apparently exhibits no clear pattern, Kv1.5 is induced in many of the analyzed metastatic tissues. Interestingly, evidence indicates that Kv1.5 channel shows inversed correlation with malignancy in some gliomas and non-Hodgkin's lymphomas. However, Kv1.3 and Kv1.5 are similarly remodeled in some cancers. For instance, expression of Kv1.3 and Kv1.5 correlates with a certain grade of tumorigenicity in muscle sarcomas. Differential remodeling of Kv1.3 and Kv1.5 expression in human cancers may indicate their role in tumor growth and their importance as potential tumor markers. However, despite of this increasing body of information, which considers Kv1.3 and Kv1.5 as emerging tumoral markers, further research must be performed to reach any conclusion. In this review, we summarize what it has been lately documented about Kv1.3 and Kv1.5 channels in human cancer.

Keywords: K^+ channels, cancer, agressiveness, tumor markers, proliferation

VOLTAGE-DEPENDENT K^+ CHANNELS Kv1.3 AND Kv1.5

Potassium channels are one of the most diverse and ubiquitous families of membrane proteins and are encoded by more than 75 different genes (Caterall et al., 2002). Voltage-dependent K^+ channels (Kv), a superfamily comprised of 12 subfamilies (Kv1-Kv12), play a key role in the maintenance of resting membrane potential and the control of action potentials (Hille, 2001). Kv channels also contribute to a wide variety of cellular processes including the maintenance of vascular smooth muscle tone (Yuan et al., 1998), cell growth (DeCoursey et al., 1984), the regulation of cell volume (Deutsch and Lee, 1988), adhesion (Itoh et al., 1995), mobility, epithelial transport (Kupper et al., 1995), homeostasis (Xu et al., 2003), insulin release (Xu et al., 2004), and apoptosis (Storey et al., 2003). Kv channels also control leukocyte membrane potential and play a role in immune system responses (Cahalan and Chandy, 2009). Accordingly, several studies have reported that Kv channels are involved in the activation, proliferation, differentiation, and migration of leukocytes (Cahalan and Chandy, 1997; Wulff et al., 2003; Panyi et al., 2004; Beeton et al., 2005; Felipe et al., 2006). Given their pivotal role in cell physiology, abnormalities in Kv functions can lead to several channelopathies (Ashcroft, 2000).

The voltage dependent K^+ channels Kv1.3 and Kv1.5 are members of the *Shaker* (Kv1) family of K^+ channels and are implicated in tissue differentiation and cell growth (Felipe et al., 2006). Although Kv1.3 was first cloned from brain tissue, its expression is widely distributed throughout the body (Swanson et al., 1990; Bielanska et al., 2009, 2010). This channel is highly expressed in lymphocytes and the olfactory bulb (Stuhmer et al., 1989), and several studies have reported that it is also expressed in the hippocampus (Veh et al., 1995), epithelia (Grunnet et al., 2003), adipose tissue (Xu et al., 2004), and both skeletal, and smooth muscle (Villalonga et al., 2008; Bielanska et al., 2012a,b).

Kv1.3 currents exhibit a characteristic cumulative inactivation and a marked C-type inactivation. The single channel conductance of Kv1.3 is 13 pS, and the voltage required for activation is −35 mV. In contrast, the Kv1.5 channel was first isolated from the human ventricle and is also expressed in the atria (Tamkun et al., 1991). Similar to the Kv1.3 channel, Kv1.5 is also ubiquitously expressed (Swanson et al., 1990; Bielanska et al., 2009, 2010). For example, Kv1.5 is expressed in the immune system, the kidney, skeletal and smooth muscle and, to a lesser extent, the brain (Coma et al., 2003; Vicente et al., 2003, 2006; Villalonga et al., 2008; Bielanska et al., 2012a,b). Kv1.5 currents contribute to the ultra-rapid activating K^+ current in the heart known as I_{kur}, which plays a role in the repolarization of an action potential (Lesage et al., 1992). The conductance of the Kv1.5 channel is 8 pS, and the voltage required for activation is ∼24 mV. Unlike Kv1.3, Kv1.5 inactivation is slow and lacks cumulative inactivation. Such a different biophysical features may explain their distinct regulation in a number of cell types.

Kv1.3 and Kv1.5 are inhibited by 4-aminopyridine (4-AP) and tetraethylammonium (TEA), which are general K^+ channel blockers (Grissmer et al., 1994). Psora-4 is another potent chemical inhibitor of both Kv1.3 and Kv1.5 and has a comparatively lesser effect on the rest of the Kv isoforms (Vennekamp et al., 2004). Highly specific toxins such as charybdotoxin and margatoxin (Leonard et al., 1992; Garcia-Calvo et al., 1993) as well as the anemone peptide ShK and their derivatives (Cahalan and Chandy, 1997) have proven to be highly effective for Kv1.3. On the other hand, Kv1.5 is highly insensitive to Kv1.3 blockers and has no known specific pharmacology. However, new chemicals such as S0100176 (from Sanofi-Aventis) (Decher et al., 2004) or diphenyl phosphine oxide-1 (DPO-1) have been discovered to potently inhibit Kv1.5 (Du et al., 2010).

Leukocytes express a diverse and unique repertoire of Kv proteins, however, Kv1.3 and Kv1.5 are considered the major Kv channels (Cahalan et al., 2001; Vicente et al., 2003, 2006; Wulff et al., 2004; Beeton et al., 2005; Cahalan and Chandy, 2009; Rangaraju et al., 2009; Sole et al., 2009; Felipe et al., 2010). In macrophages, dendritic cells and B lymphocytes, Kv currents are mainly mediated by Kv1.3, however, in contrast to T-lymphocytes, they also express Kv1.5 (Douglass et al., 1990; Vicente et al., 2003, 2006; Wulff et al., 2004; Mullen et al., 2006; Villalonga et al., 2007a,b; Zsiros et al., 2009; Villalonga et al., 2010a,b). We have previously shown that Kv1.5 subunits can coassemble with Kv1.3 subunits to generate functional heterotetrameric channels in macrophages. Interestingly, changes in the stoichiometry of the heterotetramers lead to the formation of new channels, which display different biophysical and pharmacological properties and influence the activation of specific cellular responses (Vicente et al., 2003, 2006, 2008; Villalonga et al., 2007a,b). The voltage for activation of Kv1.3 channel is more hyperpolarized than for Kv1.5. Thus, at physiological membrane potentials of most mammalian cells (from −30 to −60 mV), Kv1.3/Kv1.5 heteromeric channels with a high Kv1.3 ratio would be much more activated than those with low ratios of Kv1.3. The distinct voltage activation threshold of the two channels would explain why different subunit composition in Kv1.3/Kv1.5 complexes can lead to specific alteration of cellular excitability and determine different cell responses. Thus, the expression level of both subunits can influence the degree of cell proliferation, differentiation or activation. In this context, the Kv1.3/Kv1.5 ratio may be an accurate indicator of cell activation. For example, high levels of Kv1.5 would suggest a cell was maintaining an immunosuppressive state, whereas increased ratios of Kv1.3/Kv1.5 might indicate cell activation (Villalonga et al., 2007a,b; Felipe et al., 2010; Villalonga et al., 2010a,b). Leukocytes also express several regulatory subunits (Vicente et al., 2005; Sole et al., 2009) which may associate with Kv1.3/Kv1.5 complexes to enhance diversity and modulate a wide variety of physiological activities (McCormack et al., 1999). In fact, both channels Kv1.3 and Kv1.5, are able to assemble with Kvβ subunits to form functional Kv channels. Kvβ subunits alter current amplitude and gating, confer rapid inactivation, and promote Kv surface expression (Nakahira et al., 1996; Sewing et al., 1996; McCormack et al., 1999). In addition, heterologous expression of Kv1.3 and Kv1.5 with Kvβ subunits in *Xenopus* oocytes and mammalian cells, dramatically modifies the rate of inactivation (Sewing et al., 1996) and the K^+ current density (McCormack et al., 1999), respectively.

Although most studies have been performed in adult tissues, Kv channels are differentially expressed throughout development. To date, several important differences in Kv expression during neonatal development have been reported (Roberds and Tamkun, 1991; Lesage et al., 1992; Felipe et al., 1994; Coma et al., 2002; Grande et al., 2003; Tsevi et al., 2005). We have recently studied the expression pattern of Kv1.3 and Kv1.5 in detail during the early stages of human development, and we have noted the following observations: (1) numerous tissues express Kv1.3 and Kv1.5 channels, (2) both channels are abundantly expressed in fetal liver (Bielanska et al., 2010), which serves as a hematopoietic tissue during early gestation, (3) adult hepatocytes did not express Kv1.3 (Vicente et al., 2003), (4) Kv1.5 is strongly expressed in fetal muscle and heart, whereas Kv1.3 abundance is low, (5) human fetal skeletal muscle expresses slightly more Kv1.3 than adult muscle fibers (Bielanska et al., 2010), and (6) the Kv1.5 channel is predominantly located in adult skeletal muscle and exhibits a cell cycle-dependent regulation pattern (Villalonga et al., 2008). We also examined brain tissue because it undergoes profound changes during the early fetal stages, such as cell proliferation, differentiation and migration. Kv1.3 localizes to the central and peripheral nervous systems, while Kv1.5 overlaps mostly with the central nervous system (Bielanska et al., 2010). In summary, we concluded that Kv1.3 and Kv1.5 channels followed a differential developmental expression profile, which eventually defines an adult phenotype and influences final physiological functions (Roberds and Tamkun, 1991; Lesage et al., 1992).

THE ROLE OF Kv1.3 AND Kv1.5 IN CELL PROLIFERATION

Accumulating evidence suggests that many drugs and toxins that specifically block the activity of Kv channels decrease cell proliferation (Amigorena et al., 1990; Day et al., 1993; Wonderlin and Strobl, 1996; Chittajallu et al., 2002; Conti, 2004; Pardo, 2004; Kunzelmann, 2005; Felipe et al., 2006; Arcangeli et al., 2009; Wulff et al., 2009). For example, non-specific K^+ channel blockers such as 4-AP, TEA and quinidine exert anti-proliferative effects in several different mammalian cell models (Mauro et al., 1997; Hoffman et al., 1998; Liu et al., 1998; Vaur et al., 1998; Wohlrab and Markwardt, 1999; Faehling et al., 2001; Wohlrab et al., 2002; Roderick et al., 2003).

Although the underlying mechanisms regarding how these channels promote proliferation is still a subject of debate (Roura-Ferrer et al., 2008; Villalonga et al., 2008), there are several events that may be controlled by Kv during cell growth, including membrane potential, Ca^{2+} signaling and cell volume (Wonderlin and Strobl, 1996; Conti, 2004; Pardo, 2004; Felipe et al., 2006). For example, during the early phases of cell cycle progression (G1/S), cells undergo a transient hyperpolarization which involves Kv channel activity (Wonderlin and Strobl, 1996). Interestingly, cancer cells are typically more depolarized in comparison with terminally differentiated cells (Pardo, 2004; O'Grady and Lee, 2005), although a transient hyperpolarization is required for the progression of the early G1 phase of the cell cycle (Wonderlin and Strobl, 1996). Thus, one would hypothesize that a blockage of K^+ flux, which would lead to depolarization,

should interfere with cell proliferation by inhibiting transient hyperpolarization.

Conversely, during lymphocyte proliferation, The combined action of Kv1.3 and KCa1 provides enough hyperpolarization to allow the Ca^{2+} influx required for proliferation. The resultant negative shift in membrane potential generates the required driving force for Ca^{2+} entry through Ca^{2+} channels (CRAC) from the extracellular space and its release from the inner stores (Arcangeli et al., 2009). Furthermore, cell growth is associated with cell volume increases throughout the G1 phase of the cell cycle (Lang et al., 2000). In fact, glioma cells exhibit their highest proliferation rates within a relatively narrow range of cell volumes, with decreased proliferation both over and under this optimal range. So, the rate of cell proliferation it is optimal within a cell volume window and appears to be controlled by low and high cell size checkpoints (Rouzaire-Dubois et al., 2004). Changes in membrane potential and cell volume are necessary for cell cycle progression, both of which require the action of K^+ channels (Pardo, 2004).

The role of Kv channels in cell growth has been extensively studied in cells of the immune system. For instance, Kv1.3 is known to play an essential function in the activation of T lymphocytes (Panyi, 2005), which is dependent upon an increase in voltage-gated K^+ conductance (McCormack et al., 1999). Moreover, a selective inhibition of Kv1.3 channels prevents cell activation and has been shown to exhibit immunosuppressive effects (Shah et al., 2003). Although previous studies have argued against a role of Kv1.3 in proliferation and activation of B-lymphocytes (Amigorena et al., 1990; Partiseti et al., 1993), it has been published that Kv1.3 protein levels increase in proliferating hippocampal microglia and control macrophage proliferation (Kotecha and Schlichter, 1999; Vicente et al., 2003; Villalonga et al., 2007a,b). Kv1.5 channels also play a crucial role in the activation and proliferation of oligodendrocytes, hippocampal microglia, macrophages and myoblasts (Attali et al., 1997; Kotecha and Schlichter, 1999; Villalonga et al., 2007a,b, 2008, 2010a,b). In macrophages, Kv1.3 depletion impairs cell growth and migration, both of which are characteristic features of cancer development (Villalonga et al., 2010a,b). Recently, we have determined that Kv1.5 is involved in the proliferation and migration of human B-cells (Vallejo-Gracia et al., 2013).

Several studies have demonstrated that Kv1.5 channels play a definitive role in muscle cell signaling. In this context, we have reported that regulation of Kv1.3 and Kv1.5 expression is cell cycle-dependent in L6E9 myoblasts. In fact, Kv1.5 expression changes throughout cell cycle progression with maximum expression occurring during the G1/S phase (Villalonga et al., 2008) and increased expression has also been noted during myogenesis (Vigdor-Alboim et al., 1999). Furthermore, our pharmacological evidence implies a role for Kv1.5 in the cell proliferation process (Villalonga et al., 2008). An alternative theory suggests that the role of the Kv1.3 channel in skeletal muscle could be connected to insulin sensitivity (Xu et al., 2004). However, Kv1.5 is thought to inhibit skeletal muscle cell proliferation through a mechanism involving the accumulation of cyclin-dependent kinase inhibitors (such as $p21^{cip-1}$ and $p27^{kip1}$) and a marked decrease in the expression of cyclins A and D1 (Villalonga et al., 2008).

It is well established that glial cells abundantly express Kv channels, including those that are part of the *Shaker* (Kv1) subfamily (Verkhratsky and Steinhauser, 2000), and different Kv channels are closely related to the cell cycle progression of human glia (Sontheimer, 1994). For instance, in rat oligodendrocyte precursor cells, the transition of quiescent cells into the G1 phase of the cell cycle is accompanied by increased levels of Kv1.3 and Kv1.5 proteins (Chittajallu et al., 2002). Moreover, the specific inhibition of Kv1.3 elicited a G1 arrest, while a reduction in Kv1.5 protein mediated by antisense oligonucleotide transfection had no effect on cell growth (Attali et al., 1997; Chittajallu et al., 2002). In contrast, Kv1.5 antisense treatment inhibited cell growth in astrocytes (MacFarlane and Sontheimer, 2000). Because blockage of Kv1.5 sufficiently decreased the proliferation of astrocytes but not oligodendrocytes, this channel may play different functional roles in different types of cells. In fact, these differential results, together with the involvement of Kv1.5 in cell growth (Attali et al., 1997; MacFarlane and Sontheimer, 2000; Soliven et al., 2003; Wang, 2004; Lan et al., 2005; Villalonga et al., 2008), argue against a singular role for these channels in cell proliferation. In addition, both Kv1.3 and Kv1.5 have been shown to be involved in promoting apoptosis. Psora-4, PAP-1 and clofazimine, three distinct membrane-permeable inhibitors of Kv1.3, induce cell death by directly targeting the mitochondrial channel in multiple human and mouse cancer cell lines (Leanza et al., 2012) and efficiently induce apoptosis of chronic lymphocytic leukemia cells (Leanza et al., 2013).

There is no clear understanding how K^+ channels actually promote cell proliferation but possible mechanisms such as membrane voltage changes, cell volume regulation and the effect of mitogenic signals have been proposed (Wonderlin and Strobl, 1996). Exists a correlation between membrane potential and mitotic activity. Thus, terminally differentiated cells in G0 phase are very hyperpolarized whereas rapidly cycling tumor cells never entering G0 and are very depolarized (Binggeli and Weinstein, 1986). Mitogenic stimulation induces a short hyperpolarization at early G1, followed by depolarization. Although subsequent hyperpolarization during G1 has not been reported for all cell types, it is frequently observed and is believed to be essential for proliferation. Changes in membrane potential also alters the $[Ca^{2+}]_i$ concentration and promotes nutrient transport. Cell growth (increase in cell size) and proliferation (increase in number) are also closely related processes (Rouzaire-Dubois and Dubois, 1998). Thus, during progression through the cell cycle, the cell volume continuously changes. Particularly during G1/S transition and around the M phase large volume changes occur. It affects the concentration of enzymes that controls cell growth. Alteration in cell volume also regulate concentration of nutrients as well as cell-cycle effectors. Finally, cell cycle is controlled by distinct effectors such as oscillating cyclins and cyclin-dependent kinases. Inhibition of K^+ currents causes membrane depolarization and accumulation of the cyclin-dependent kinase inhibitors p27 and p21 (Ghiani et al., 1999). Thus, cell cycle-relevant proteins may be directly regulated by membrane voltage. Current evidence points that voltage-sensitive K^+ channels control cancer cell proliferation but the pathways involved are still unclear.

Same channels can participate in the stimulation of both cell proliferation and apoptosis. This paradox may depend on the temporal pattern of K^+ channel activation. Thus, oscillating K^+ channel activity typical of proliferating cells has completely different effects as sustained K^+ channel activation typical of apoptotic cells. Activation of K^+ channels during apoptosis is much more pronounced than during proliferation causing a drastic fall in the $[K^+]_i$ compared to during cell cycling (Cain et al., 2001; Bock et al., 2002). Since many of the growth-and mitosis-related enzymes require a minimal $[K^+]_i$, a loss of K^+ reduce the proliferative activity (Hughes et al., 1997; Bortner and Cidlowski, 1999; Cain et al., 2001; Bock et al., 2002). Therefore, activation of both Cl^- and K^+ channels must stay within a certain conductance to support proliferation, otherwise programmed cell death is triggered (Bock et al., 2002). Another important factor could be in Ca^{2+} signaling. Oscillatory Ca^{2+} rises were associated with proliferation and have not been observed during apoptosis. In contrast, a steady Ca^{2+} increase appears to be needed for apoptotic enzymes activity (Kunzelmann, 2005).

Kv1.3 AND Kv1.5 IN SOLID CANCERS

In addition to their role in proliferation, migration and invasion (Conti, 2004; Pardo, 2004; Felipe et al., 2006; Villalonga et al., 2010a,b), potassium channels appear to contribute to the development of cancer (Kunzelmann, 2005). Kv channels are expressed in a number of tumors and cancer cell lines (Nilius and Wohlrab, 1992; Chin et al., 1997; Skryma et al., 1997; Laniado et al., 2001). Moreover, induced tumors in experimental models also exhibit high levels of several voltage-gated K^+ channels, including Kv1.3 and Kv1.5 (Villalonga et al., 2007a,b).

Over the past decade, many studies have found that these channels are aberrantly expressed in different human tumor cells (**Table 1**), and the expression of both Kv1.3 and Kv1.5 channels is altered in a variety of human cancers including prostate cancer (Abdul and Hoosein, 2002a,b, 2006), colon cancer (Abdul and Hoosein, 2002a,b), breast cancer (Abdul et al., 2003; Brevet et al., 2009; Liu et al., 2010), lung cancer (Pancrazio et al., 1993; Wang et al., 2002), liver cancer (Zhou et al., 2003), smooth muscle cancers (Bielanska et al., 2012a), skeletal muscle cancers (Bielanska et al., 2012b), kidney cancer, bladder cancer, skin cancers (Bielanska et al., 2009) and gliomas (Preußat et al., 2003).

The number of tumor cells diminishes when K^+ channels are blocked with toxins or drugs. For example, K^+ channel blockers exhibit anti-proliferative effects in several human cancers such as prostate tumors (Rybalchenko et al., 2001; Abdul and Hoosein, 2002a; Fraser et al., 2003), hepatocarcinoma (Zhou et al., 2003), mesothelioma (Utermark et al., 2003), colon cancer (Abdul and Hoosein, 2002b), breast carcinoma (Strobl et al., 1995; Abdul et al., 2003), glioma (Preußat et al., 2003), and melanoma (Nilius and Wohlrab, 1992; Allen et al., 1997; Artym and Petty, 2002).

GASTROINTESTINAL CANCERS

Many Kv channels, including Kv1.3, Kv1.5, Kv1.6, Kv2.1, and Kv2.2, are present in immortalized gastric epithelial cells and several gastric cancer cells (AGS, KATOIII, MKN28, MKN45, MGC803, SGC7901, SGC7901/ADR, and SGC7901/VCR). Interestingly, downregulation of Kv1.5 significantly inhibits cell proliferation and the tumorigenicity of SGC7901 cells. However, the authors conclude that Kv1.5 is necessary, but not sufficient, for gastric cancer cell proliferation (Lan et al., 2005). In addition, siRNA-mediated depletion of Kv1.5 abolished the depolarization-induced influx of Ca^{2+}. Thus, Kv1.5 channels may be involved in tumor cell proliferation by controlling Ca^{2+} entry. In addition, I_{Ks} currents are related to the development of multi-drug resistance in gastric cancer cells (Wu et al., 2002). Therefore, these studies could provide a novel strategy to reverse the malignant phenotype of gastric cancer cells.

Proliferation of several human colon cancer cell lines (SW1116, LoVo, Colo320DM, and LS174t) was increased by two K^+ channel activators, minoxidil and diazoxide. In contrast, several Kv blockers, including dequalinium and amiodarone, caused a marked growth-inhibition of human colon cancer cell lines. Glibenclamide is another Kv channel blocker that inhibits cellular proliferation (Abdul and Hoosein, 2002a,b). Proliferation of the colorectal carcinoma cell line DLD-1 is drastically reduced in the presence of 4-AP. However, inhibition of Ca^{2+}-sensitive K^+ channels and ATP-sensitive K^+ channels did not have an effect on cell proliferation. Interestingly, K^+ channel inhibitors blocked $[Ca^{2+}]_i$ influx, suggesting that K^+ channel activity may control the proliferation of colon cancer cells by modulating Ca^{2+} entry (Yao and Kwan, 1999). Although colon biopsies exhibited an increase in Kv1.3 and Kv1.5 expression, this phenomenon may be an artifact of the massive presence of inflammatory cells, which express high levels of both channels (Bielanska et al., 2009).

BREAST CANCER

Kv1.3 expression has been examined by immunohistochemistry in healthy human breast samples and their matched cancer tissue counterparts. While Kv immunostaining is not observed in normal human breast tissues, most cancer specimens show moderate staining in the epithelial compartment. In addition, the K^+ channel activator minoxidil stimulates the growth of MCF-7 human breast cancer cells. On the contrary, K^+ channel blockers such as dequalinium and amiodarone have marked inhibitory effects on MCF-7 cell proliferation (Abdul et al., 2003). Other K^+ channel-blockers also inhibit breast cancer growth (Strobl et al., 1995) and potentiate the growth-inhibitory effects of tamoxifen on human breast (MCF-7, MDA-MB-231), prostate (PC3, MDA-PCA-2B), and colon (Colo320DM, SW1116) cancer cell lines (Abdul et al., 2003). However, the expression of Kv1.3 in breast cancer is not well defined. Kv1.3 expression is increased in breast cancer biopsies in comparison with healthy breast tissues (Abdul et al., 2003). However, Brevet et al. argues that less Kv1.3 expression is found in cancerous samples, and claims that Kv1.3 gene promoter methylation is increased. Because Kv1.3 expression correlates with both poorly differentiated tumors and a younger age of patients with tumors, the authors suggest that a loss of Kv1.3 may be a marker for poor prognosis of breast tumors (Brevet et al., 2009). Immortalized human mammary epithelial cells with different tumorigenic properties demonstrated that the expression of Kv1.3 varies depending on the tumorigenicity and stage of the breast cancer (Jang et al., 2009). In addition, we have recently found that Kv1.3 and Kv1.5 expression increases concomitantly with an elevation of infiltrating inflammatory cells surrounding

Table 1 | Expression of Kv1.3 and Kv1.5 in solid cancers and tumoral cells.

Tissues	Tumors and cell lines	Features	Kv1.3	Kv1.5	References
Stomach	Stomach cancer epithelium cells	Positive in infiltrating inflammatory cells	Absent	Low	Bielanska et al., 2009
Colon	Colon adenocarcinoma	Positive in infiltrating inflammatory cells	Moderate (75%)	Moderate (80%)	Bielanska et al., 2009
Breast	Brast cancer	N.A.	High (30%) Moderate (58%) Low (12%)	N.D.	Abdul et al., 2003
	Breast adenocarcinoma	Grade I tumor Grade II tumor Grade III tumor	High* High* Low*	N.D.	Brevet et al., 2009
	Mammary epithelial M13SV1 cells mammary epithelial m13sv1r2 cells Mammary epithelial M13SV1R2-N1cells	Inmortalized Weakly tumorigenic Highly tumorigenic	Low * High * High *	N.D.	Jang et al., 2009
	Mammary duct carcinoma	Positive in infiltrating inflammatory cells	Absent	Absent/ Low (30%)	Bielanska et al., 2009
Prostate	Prostate cancer PC3, DU145, LNCaP, MDA-PCA-2B cell lines	N.A.	High (47%) Moderate (29%) Low (24%)	N.D.	Abdul and Hoosein, 2002a
	LNCaP cell lines	High K^+ currents Weakly metastatic	High*	N.D.	Laniado et al., 2001
	PC3 cell lines	Low K^+ currents strongly metastatic	Low*		
	AT-2 cell lines	High K^+ currents weakly metastatic	High*	N.D.	Fraser et al., 2000
	Mat-LyLu cell lines	Low K^+ currents strongly metastatic	Low*		
	Prostatic hyperplasia Human prostate cancer	Benign Primary	High (89%) High (52%)	N.D.	Abdul and Hoosein, 2006
Smooth muscle	Leiomyoma Leiomyosarcoma	Benign Aggressive	Low High	Low Low	Bielanska et al., 2012a
Skeletal muscle	Embryonal rabdomyosarcoma Alveolar rabdomyosarcoma	Low aggressiveness High aggressiveness	Low High	Low High	Bielanska et al., 2012a
	Rabdomyosarcoma	N.A.	Absent	Low (30%)	Bielanska et al., 2009
Brain	Astrocytoma Oligodendroglioma Glioblastoma	Low malignancy High malignancy High malignancy	Low* Low* Low*	High* Moderate* Low*	Preußat et al., 2003
	Astrocytoma Glioblastoma	Low malignancy High malignancy	Absent/Low Absent/Low	Low (70%) Low (40%)	Bielanska et al., 2009
Kidney	Kidney carcinoma	N.A.	Absent	Moderate (60%)	Bielanska et al., 2009
Bladder	Bladder carcinoma	N.A.	Absent/ Low	Low (60%)	Bielanska et al., 2009
Lung	Lung adenocarcinoma	Positive in infiltrating inflammatory cells	Absent	Low (60%)	Bielanska et al., 2009

(Continued)

Table 1 | Continued

Tissues	Tumors and cell lines	Features	Kv1.3	Kv1.5	References
Pancreas	Pancreas adenocarcinoma	Positive in infiltrating inflammatory cells	Absent	Moderate (90%)	Bielanska et al., 2009
Ovary		Positive in infiltrating inflammatory cells	Absent	Absent	Bielanska et al., 2009
Skin	Squamous skin cell carcinoma	N.A.	Absent	High (100%)	Bielanska et al., 2009

*Parenthesis indicates the percentage of expressing cells. *Expression compared to healthy and control samples. N.D, not determined; N.A, not available.*

the tumor nodule in breast carcinoma samples (Bielanska et al., 2009). This finding could shed light on the debate, however, more studies must be undertaken to elucidate these mechanisms.

PROSTATE CANCER

Expression of Kv1.3 and Kv1.5 channels has also been extensively studied in prostate cancer cells. Four different human prostate cancer (Pca) cell lines, two of which were androgen-unresponsive (PC3, DU145) and two of which were androgen-responsive (MDA-PCA-2B, LNCaP), were examined by immunohistochemistry to determine expression levels of Kv1.3. Strong immunostaining for Kv1.3 was detected in normal prostate samples, whereas most of the Pca specimens showed strong and moderate Kv1.3 staining (Abdul and Hoosein, 2002a). In addition, different K^+ channel activators, such asminoxidil, 1-ethyl-2-benzimidazolinone, and diazoxide, had significant growth-stimulatory effects on PC3 cells. In contrast, K^+ channel-blockers such as dequalinium, amiodarone, and glibenclamide, caused a dose-dependent growth inhibition of both androgen-unresponsive and androgen-responsive Pca cell lines. Furthermore, channel blockers triggered morphological feature changes such as nuclear shrinkage and fragmentation, suggesting an activation of apoptotic signaling mechanisms (Abdul and Hoosein, 2002a). Although the highly metastatic PC3 cell line expressed Kv1.3 (Laniado et al., 2001), its expression was inversely related to metastasis in prostate cancer (Abdul and Hoosein, 2002a). In another report, Kv density inversely correlated with the metastasis of human prostate cancer cell lines (Laniado et al., 2001). Thus, lower Kv-staining in clinical Pca specimens compared to Kv-staining levels in normal prostate cells may correlate with an increased probability of metastatic disease (Abdul and Hoosein, 2002a). Voltage-gated K^+ currents have been characterized by electrophysiology in rat (Mat-LyLu and AT-2) and human (PC3 and LNCaP) PCa cell lines (Skryma et al., 1997; Rane, 2000; Laniado et al., 2001; Rybalchenko et al., 2001). Both the strongly metastatic MAT-LyLu and the weakly metastatic AT-2 cell lines expressed Kv1.3 currents. Interestingly, Kv1.3 currents had different biophysical properties in the two rat prostate cancer cell lines, which displayed markedly different metastatic abilities. Thus, MAT-LyLu cells displayed significantly smaller maximal K^+ current densities and an increased negative resting potential when compared to AT-2 cells. Taken together, these data suggest that K^+ currents in the MAT-LyLu cells may be less active than those in the AT-2 cells (Fraser et al., 2000). Therefore, human prostate cancer cells with different metastatic ability displayed a differential modulatory action of K^+ channels. This finding, together with the exclusive expression of voltage-gated Na^+ channels in MAT-LyLu cells (Grimes et al., 1995), suggests a role for voltage-dependent ion channels in metastatic cell behavior (Laniado et al., 1997; Fraser et al., 2003). High epithelial Kv1.3 expression has also been observed in all normal prostate and benign prostatic hyperplasias (BPH), whereas only half of primary human prostate cancer (Pca) samples express Kv1.3. Furthermore, reduced Kv1.3 protein levels in Pca correlated with high tumor grade and a poor prognosis. Because there was a significant inverse correlation between Kv1.3 levels and prostate tumor stage, Kv1.3 expression may be a useful diagnostic or prognostic marker for prostate cancer (Abdul and Hoosein, 2006).

MUSCLE SARCOMAS

Kv channels are crucial for the modulation of arterial tone and the control of vascular smooth muscle cell proliferation (Michelakis et al., 1997) and migration (Cidad et al., 2010; Cheong et al., 2011). Although myofibers are terminally differentiated, some myoblasts may proliferate by re-entering the cell cycle. Margatoxin, a specific blocker of Kv1.3, reduces proliferation and migration of mouse and human vascular smooth muscle cells. However, margatoxin does not fully abrogate migration, suggesting that a Kv1.3-independent component is involved in this process (Cheong et al., 2011). During vascular smooth muscle proliferation, Kv1.3 expression increases while Kv1.5 expression decreases (Cidad et al., 2010). Thus, Kv1.3 expression is altered during myoblast proliferation and differentiation, although it does not play a substantial role in either process (Villalonga et al., 2008). Conversely, Kv1.5 channel expression seems to contribute to vascular smooth muscle tone (Yuan et al., 1998).

In a recent study, we analyzed Kv1.3 and Kv1.5 expression in human samples of smooth muscle tumors [such as leiomyoma (LM) and leiomyosarcoma (LMS)] and compared the tumor samples to their healthy specimen counterparts. LM and LMS are a benign uterus tumor and an aggressive retroperitoneal neoplasm, respectively. Kv1.3 is poorly expressed in healthy muscle and in indolent LM samples but was significantly induced in malignant LMS. Similar to Kv1.3, Kv1.5 is almost absent in healthy biopsies, but Kv1.5 staining was heterogeneous and faint in LM samples. In contrast, Kv1.5 displayed a poor and homogeneous expression in aggressive LMS samples. Interestingly, a clear positive correlation between the expression of Kv1.3 and Kv1.5 and the aggressiveness

of the smooth muscle neoplasm was noted. These results suggested that Kv1.3 and Kv1.5 could be used as potential molecular targets for the treatment of aggressive smooth muscle sarcomas (Bielanska et al., 2012a,b).

Kv1.3 and Kv1.5 undergo alterations in different types of human skeletal muscle sarcomas (Bielanska et al., 2009, 2012b). Kv1.3 is absent in the less aggressive embryonal rhabdomyosarcoma (ERMS), whereas its expression in the aggressive alveolar rhabdomyosarcoma (ARMS) is notable and equivalent to that found in fetal muscle. Kv1.5, which weakly stains healthy adult skeletal muscle, strongly stains fetal tissues in a manner similar to that of Kv1.3. In addition, ERMS specimens show heterogeneous expression of Kv1.5, which could indicate different stages of proliferation and/or differentiation of individual cells. In contrast, ARMS samples express homogeneous and highly expressed levels of Kv1.5 (Bielanska et al., 2012b). Therefore, the expression of both Kv1.3 and Kv1.5 channels increased significantly with respect to the tumor aggressiveness grade in ERMS and ARMS.

GLIOMAS

In addition to contributing to the proliferation of normal glia, Kv1.3 is detected in human gliomas, which are brain tumors arising from glial cells. Gliomas emerge from both astrocytic and oligodendrocytic lineages and consist of low and high malignancy grades, respectively. Preußat and coworkers have reported that some Kv1.3 and Kv1.5 differential expression occurs with respect to the malignancy grade of the tumor. For example, Kv1.5 expression is elevated in astrocytomas, moderate in oligodendrogliomas, and low in glioblastomas. Although the expression of Kv1.5 inversely correlates with glioma malignancy, no such correlation is evident for Kv1.3. These data suggest that reduced levels of Kv1.5 protein in biopsies when compared to the levels found in adjacent healthy tissues may be a good candidate biomarker for both glioma detection and outcome prediction (Preußat et al., 2003). Other studies have revealed abundant Kv1.3 and Kv1.5 expression in brain tumors and suggested that while Kv1.3 expression is notable in astrocytomas, Kv1.5 expression is elevated in glioblastomas (Bielanska et al., 2009). Although it is not known whether Kv1.3 and Kv1.5 expression is increased in gliomas vs. healthy cells, Kv1.5 expression also occurred more in diffuse astrocytoma than in high grade ones. Moreover, glioblastoma patients with higher Kv1.5 expression had slightly better survival (Arvind et al., 2012).

OTHER SOLID CANCERS

Recently, we have performed an extensive analysis of Kv1.3 and Kv1.5 protein expression in a wide variety of human tumors. Our results indicated that most cancers experienced an alteration of Kv gene expression. We found that Kv1.3 is present in healthy stomach, kidney, skeletal muscle, and lymph node, whereas expression of Kv1.3 was low in the breast, ovary, pancreas, bladder, lung, colon, and brain. Taken together, these data demonstrated that Kv1.3 is more ubiquitously expressed than was suggested by previous studies. In addition, most tumors showed no major differences in Kv1.3 expression when compared to healthy tissues. However, Kv1.3 expression was downregulated in kidney, bladder, and lung carcinomas (Bielanska et al., 2009). On the other hand, Kv1.5 expression was evident in most of the analyzed tissues but not in the breast. The abundance of Kv1.5 was low in the ovary, urinary bladder, and lung. Interestingly, unlike Kv1.3, Kv1.5 expression was increased in most human tumors. For example, stomach, pancreatic, and bladder tumors expressed more Kv1.5 than healthy specimens, however, Kv1.5 expression was decreased in renal adenocarcinoma when compared to healthy tissues. Because this channel is involved in K^+ transport and cell volume regulation (Felipe et al., 1993), a decrease in Kv1.5 expression would likely be accompanied by a loss of renal function. Finally, Kv1.5 expression was unaffected in ovary and lung tumors (Bielanska et al., 2009).

The immunohistochemical analysis of Kv1.3 and Kv1.5 channels in all of these cancers demonstrated that in most cases, stronger Kv1.3 and Kv1.5 expression is mainly confined to the inflammatory cells surrounding the tumors (Bielanska et al., 2009).

It is tempting to speculate that because these human specimens are usually from patients who have already been diagnosed with some type of cancer, most of them show histological signs of reactivity and should be interpreted with caution. This was the case for stomach, pancreatic, and breast cancers (Bielanska et al., 2009) and it may explain differences between our studies and those performed by Hoosein and coworkers in breast cancer (Abdul et al., 2003). Contrary to this hypothesis, other cancers such as those of the bladder, skin, ovary, and lymph node, exhibited Kv1.5 induction in the tumorigenic cells (Bielanska et al., 2009).

Kv1.3 AND Kv1.5 IN BLOOD CANCERS

LYMPHOMAS

Because Kv channels control neoplastic processes in leukocytes such as cell activation, proliferation, migration and apoptosis (DeCoursey et al., 1984; Gollapudi et al., 1988; Khanna et al., 1999; Wickenden, 2002; Vicente et al., 2003, 2005; Cahalan and Chandy, 2009; Wulff et al., 2009), these proteins are thought to be involved in the mechanisms underlying lymphoma invasiveness (Cruse et al., 2006; Bielanska et al., 2009; Felipe et al., 2012) and malignancy.

In a preliminary study, we have shown that Kv1.3 and Kv1.5 are differentially altered in human non-Hodgkin's lymphomas (Bielanska et al., 2009), however, a more complete study was required to confirm these initial findings. Recently, we have examined the expression of Kv1.3 and Kv1.5 in a panel of human non-Hodgkin lymphomas. To our knowledge this was the first study to examine Kv1.3 and Kv 1.5 expression in diffuse large B-cell, follicular B-cell, mantle, anaplastic and T-cell lymphomas in comparison with control lymph nodes. Furthermore, because these human cancers exhibited different grades of malignancy, we determined whether there was a correlative relationship between Kv1.3 and Kv1.5 expression and the clinical aggressiveness of these human lymphomas (**Figure 1**). Kv channels have previously been proposed as tumorigenic markers and therapeutic targets (Conti, 2004; Kunzelmann, 2005; Pardo et al., 2005; Felipe et al., 2006; Stuhmer et al., 2006), although in most cases there was no clear correlation between channel expression and tumorigenicity (Preußat et al., 2003). In these studies, we found that control

lymph nodes expressed high levels of heterogeneous Kv1.3, which could indicate a certain mechanism of action, while Kv1.5 abundance was low and homogeneous. Interestingly, Kv1.3 and Kv1.5 were differentially altered in non-Hodgkin's human lymphomas. For example, indolent follicular lymphomas expressed noticeable levels of Kv1.5, while aggressive diffuse large B cell lymphomas showed low Kv1.5 expression. Thus, Kv1.5 expression is inversely correlated with clinical aggressiveness in non-Hodgkin's lymphomas. Preuβat and coworkers found a similar inverse correlation between the level of Kv1.5 immunostaining and tumor grade in gliomas (Preußat et al., 2003). Although further studies with a larger number of subjects for each tumor type must be performed, the level of Kv1.5 protein may be useful in the diagnosis or prognosis of some lymphomas (**Table 2**).

In contrast, the expression of Kv1.3, did not correlate with either the state of de-differentiation or the nature of the lymphomas, although its expression was decreased in most cancers (Bielanska et al., 2009) (**Figure 1**). Previous studies have also demonstrated that Kv1.3 expression showed no apparent connection with the tumorigenic state when considering the prognosis of the tumor (Arcangeli et al., 2009; Bielanska et al., 2009). Kv1.3 expression showed no apparent correlation with malignancy or clinical aggressiveness, similar to the findings in gliomas (Preußat et al., 2003). Taken together, these data suggest that Kv1.3 may act as a tumor suppressor. Hypoxia, which occurs commonly in solid tumors and is associated with malignant progression (Vaupel et al., 2004), decreased Kv1.3 protein levels and activity in human T lymphocytes (Conforti et al., 2003). Moreover, suppression of Kv1.3 prevents apoptosis, which would favor tumor development (Bonnet et al., 2007).

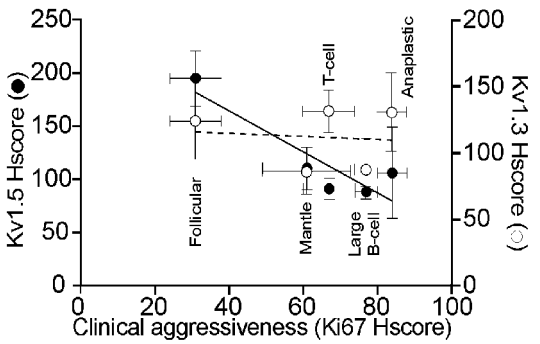

FIGURE 1 | Expression of Kv1.3 and Kv1.5 in human non-Hodgkin lymphomas. A Histoscore (Hscore) was calculated to establish a phenotypical correlation of clinical agressiveness based in the expression of Ki67 (Castellvi et al., 2009). (○) Kv1.3 Hscore in non-Hodgkin's lymphomas. (●) Kv1.5 Hscore. Results are mean ± SEM of Kv1.3 and Kv1.5 Hscore plotted against Ki67 Hscore as a marker of the clinical aggressiveness of the lymphoma. While a Pearson's correlation coefficient of $r = 0.106$ with a $P < 0.866$ indicated a complete absence of correlation of Kv1.3, a Pearson's correlation coefficient of $r = 0.895$ with a $P < 0.040$ indicated an inverse correlation between Kv1.5 abundance and the aggressiveness of tumor.

LEUKEMIAS

Distinct K^+ channel blockers have anti-proliferative effects on human myeloblastic leukemia cells (Wang et al., 1997; Xu et al., 1999). Moreover, membrane-permeable K^+ channel inhibitors, such as Psora-4, PAP-1 and clofazimine, induce apoptosis of chronic lymphocytic leukemia cells. In contrast, these cells are resistant to the membrane-impermeable inhibitor ShK, which clearly suggests that the plasma membrane-located Kv1.3 is not responsible for the observed apoptotic response. In fact, pathologic B cells showed higher Kv1.3 protein expression and were sensitive to treatment, whereas healthy cells express less Kv1.3 and were resistant to the drugs (Leanza et al., 2013). Clofazimine treatment also significantly reduced tumor size in an orthotopic melanoma mouse model (Leanza et al., 2012). Therefore, clofazimine might be a promising new therapeutic tool to treat chronic lymphocytic leukemia patients (Leanza et al., 2013).

Because Kv1.3 expression was decreased in most cancers, some authors have suggested that this channel may act as a tumor suppressor. In this context, hypoxia decreased Kv1.3 protein levels and inhibited proliferation of T-lymphocytes (Conforti et al., 2003; Chandy et al., 2004; Vaupel et al., 2004). Surprisingly, the Kv1.3 channel is also thought to play an important role in apoptosis in T-cells (Arcangeli et al., 2009) because elevated Kv1.3 facilitates an apoptotic response (Bock et al., 2002; Szabo et al., 2008). Thus, it is thought that Kv1.3 promotes proliferation in oligodendrocytes (Vautier et al., 2004) but also controls leukocyte activation and is crucial for the induction of apoptosis in lymphocytes (Storey et al., 2003; Szabo et al., 2004, 2008, 2010; Gulbins et al., 2010). These interesting findings seem rather contradictory with respect to the cellular function of Kv1.3. However, it has been suggested that the environmental conditions in which channel activation takes place and the magnitude of the activated conductance could determine whether the channel supports

Table 2 | Expression of Kv1.3 and Kv1.5 in blood cancers.

Tissue	Kv1.3	Kv1.5	Tumors and Cell lines	Features	References
Lymph node	Low	High	Follicular B-cell lymphoma	Low aggressiveness non-Hodgkin's lymphoma	Vallejo-Gracia et al., 2013
Lymph node	Low	Low	Mantle lymphoma	High aggressiveness non-Hodgkin's lymphoma	Vallejo-Gracia et al., 2013
Lymph node	Low	Moderate	T-cell lymphoma	High aggressiveness non-Hodgkin's lymphoma	Vallejo-Gracia et al., 2013
Lymph node	Moderate	Low	Diffuse large B-cell lymphoma	High aggressiveness non-Hodgkin's lymphoma	Vallejo-Gracia et al., 2013
Lymph node	Low	Moderate	Anaplastic lymphoma	High aggressiveness non-Hodgkin's lymphoma	Vallejo-Gracia et al., 2013
Lymph node	High	N.D.	Chronic lymphocytic leukenia		Leanza et al., 2013

N.D, not determined.

proliferation or apoptosis (Kunzelmann, 2005). In this context, it is tempting to speculate that suppression of Kv1.3 activity would slow apoptosis and favor tumor development. Moreover, increasing the expression of Kv1.5 with dichloroacetate triggers apoptosis in lung, breast, glioblastoma and endometrial cancer cell lines (Bonnet et al., 2007; Wong et al., 2008). Furthermore, the K^+ channel blocker clofilium induces apoptosis in human promyelocytic leukemia (HL-60) cells (Choi et al., 1999). However, these hypotheses are supported by little evidence, and further research is required to confirm these conclusions.

CONCLUSION

Several K^+ channels are essential for cell proliferation and appear to play a role in the development of cancer. In this context, further investigation is needed to fully understand the role of membrane ion channels in normal and neoplastic cell proliferation. A large body of data indicates that tumor cells differentially altered the expression of voltage dependent K^+ channels. Furthermore, Kv1.5 and to some extent Kv1.3, are aberrantly expressed in many human cancers. We can conclude that the abundance of Kv1.5 expression mostly increases in tumor cells, whereas Kv1.3 expression is generally downregulated. Interestingly, both Kv1.3 and Kv1.5 have displayed an apparent connection between their expression and the tumorigenic state of different cancer cells that may be attributable to remodeling mechanisms. To date we know that there is a clear positive correlation between the expression of both Kv1.3 and Kv1.5 channels and the clinical aggressiveness of smooth muscle neoplasms. In contrast, an inverse correlation between the levels of Kv1.3 and prostate cancer tumor stage/metastatic capacity and an inverse correlation between Kv1.5 expression and glioma tumor grade have been described. In addition, Kv1.5 expression exhibits a significant correlation with the degree of malignancy of rhabdomyosarcomas, renal tumors and lymphomas. These findings suggest that Kv1.3 and Kv1.5 channels could be used not only as tumor biomarkers but also as prognostic and diagnostic indicators.

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Emerging role of TRP channels in cell migration: from tumor vascularization to metastasis

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Transient Receptor Potential (TRP) channels modulate intracellular Ca^{2+} concentrations, controlling critical cytosolic and nuclear events that are involved in the initiation and progression of cancer. It is not, therefore, surprising that the expression of some TRP channels is altered during tumor growth and metastasis. Cell migration of both epithelial and endothelial cells is an essential step of the so-called metastatic cascade that leads to the spread of the disease within the body. It is in fact required for both tumor vascularization as well as for tumor cell invasion into adjacent tissues and intravasation into blood/lymphatic vessels. Studies from the last 15 years have unequivocally shown that the ion channles and the transport proteins also play important roles in cell migration. On the other hand, recent literature underlies a critical role for TRP channels in the migration process both in cancer cells as well as in tumor vascularization. This will be the main focus of our review. We will provide an overview of recent advances in this field describing TRP channels contribution to the vascular and cancer cell migration process, and we will systematically discuss relevant molecular mechanism involved.

Keywords: cancer cells, tumor angiogenesis, cell migration, TRP channels, Orai/Stim1

INTRODUCTION

Metastasis is the main cause of mortality in cancer and depends on two key processes: cell migration of cancer cell to invade adjacent tissues followed by intravasation into blood/lymphatic vessels and tumor vascularization, which give access to bloodstream. During the metastatic process cell migration of both epithelial and endothelial cells (EC) is an essential step leading to the spread of the primary tumor and to the invasion of neighboring connective tissue, lymphatic system and blood vessels. Cell migration and tumor vascularization are often accompanied by changes in ion channel expression and/or activity and, consequently, by abnormal progression of the cellular responses with which they are involved. In particular Ca^{2+} channels are of utmost importance since Ca^{2+} is the key messenger regulating signaling pathways important in cellular processes as proliferation, apoptosis, gene transcription, migration and angiogenesis (Roderick and Cook, 2008; Monteith et al., 2012).

In this context the relatively recent Ca^{2+} channel family of Transient Receptor Potential (TRP) have been associated with several cancers and their role has been increasingly clarified the two last decades (Bödding, 2007; Nilius et al., 2007; Gkika and Prevarskaya, 2009; Prevarskaya et al., 2011). TRP proteins display an extraordinary diversity of functional properties and have profound effects on a variety of physiological and pathological conditions (Ramsey et al., 2006; Nilius et al., 2007; Montell, 2011). Indeed TRP channels modulate intracellular Ca^{2+} concentrations, controlling critical cytosolic and nuclear events that are involved in the initiation and progression of cancer. It is not therefore surprising that the expression and function of some TRP channels is altered during tumor growth and metastasis. In particular, a typical feature of TRP channels is their ability to be activated by a wide range of external stimuli (including light, sound, chemicals, temperature, and touch) as well as of changes in the local environment (Venkatachalam and Montell, 2007; Nilius and Owsianik, 2011). As such, they can be envisioned as polymodal molecular sensors suggesting that the physiologically relevant stimulus for any given TRP will be governed by the specific cellular context (i.e., phosphorylation status, lipid environment, interacting proteins and concentration of relevant ligands), which dramatically changes during carcinogenesis. Indeed, recent evidences increasingly clarify the role for different TRP channels making them very promising players since their expression and/or activity mark and regulate specific stages of cancer progression (Nilius et al., 2007; Gkika and Prevarskaya, 2011; Prevarskaya et al., 2011; Ouadid-Ahidouch et al., 2013).

On the other hand, TRP channels are widely expressed in ECs and their functions have been related to critical steps of

tumor vascularization as well as *in vivo* angiogenesis (Fiorio Pla et al., 2012a; Munaron et al., 2013). TRP channels-mediated Ca^{2+} influx can be triggered by the release from intracellular Ca^{2+} stores giving rise to store-operated Ca^{2+} entry (SOCE). An alternative route is second messenger, store-independent Ca^{2+} entry (NSOCE) (Ambudkar and Ong, 2007).

Due to the essential role of cell migration of both epithelial and EC in the so-called metastatic cascade that leads to the spread of the disease within the body, we provide here an overview of recent advances in this field describing TRP channels contribution to migration process systematically discussing relevant molecular mechanism involved.

TRPC CHANNELS

TRPC channels are tetrameric, non-selective cation channels, which are central constituent of both store-operated Ca^{2+} entry (SOCE) as well as receptor-activated Ca^{2+} entry (ROCE). TRPC channels have been described to be functionally coupled to different tyrosine kinase receptor (i.e., VEGF, bFGF) and G protein-coupled receptors (Ambudkar and Ong, 2007).

Increasing evidences show the involvement of these channels in chemotaxis and directional migration processes (Schwab et al., 2012).

TRPC1

The role of TRPC1 in cell migration has been shown by several groups. In particular TRPC1 channels determine polarity and persistence of different cell types and are involved in stimuli-mediated directional cues in both *in vivo* and *in vitro* (Wang and Poo, 2005; Fabian et al., 2008; Schwab et al., 2012).

As concerning cancer cell migration, TRPC1 is expressed in several glioma cell lines, including D54, D65, GBM62, STTG1, U87, and U251 and in Grade IV malignant glioma patient tissue (Bomben and Sontheimer, 2008). In glioma cells TRPC1 has been correlated with EGF-mediated directional migration. In particular EGF-mediated chemotactic migration is lost when TRPC channels are inhibited pharmacologically and reduced when the expression of TRPC1 is compromised through shRNA knockdown. Interestingly, TRPC1 channels localize to the leading edge of migrating glioma cells where they co-localize with markers of caveolar lipid rafts. This raft association appears important since disruption of lipid rafts by depletion of cholesterol impaired TRPC1channel-mediated Ca^{2+} entry and EGF mediated chemotaxis (Bomben et al., 2011) (**Table 1**). Interestingly TRPC1-mediated Ca^{2+} entry seems to colocalize with Chloride Channel ClC-3 in caveolar lipid rafts of glioma cells. This interaction is functionally relevant during EGF-induced chemotaxis. Therefore the authors propose that Cl^- channels (most likely ClC-3) are important downstream target of TRPC1 in glioma cells, coupling elevations in $[Ca^{2+}]i$ to the shape and volume changes associated with migrating cells (Cuddapah et al., 2013) (**Table 1**; **Figure 1**).

Beside the described role on cancer cell migration, a proangiogenic role for TRPC1 has been described *in vivo*. Knockdown of zebrafish TRPC1 by morpholinos causes severe angiogenic defects in intersegmental vessel sprouting, presumably due to impaired filopodia extension and EC migration. Furthermore, *in vivo* time-lapse imaging of cellular behaviors showed that the angiogenic defect caused by TRPC1 deficiency is associated with markedly impaired filopodia extension, migration, and proliferation of intersegmental vessels (ISV) tip cells (Yu et al., 2010) (**Table 1**). On the other hand TRPC1 is expressed in different endothelial cell types, and promotes capillary-like tube formation in primary human umbilical vein EC (HUVEC) cells but not on EA.hy926 cells, an endothelial cell line derived from HUVECs fused with human lung adenocarcinoma cell line A549 (Antigny et al., 2012) (**Table 1**).

Beside the role of resident (EC), great interest has been recently focused on circulating endothelial progenitor cells (EPCs), a subpopulation of bone marrow-derived mononuclear cells also found in peripheral blood as important players in tumor vascularization. They promote vessel formation in adult, and it was recently reported that they have the ability to incorporate into tumor tissues (Carmeliet, 2005; Bussolati et al., 2011). In this regard, TRPC1 has been suggested to contribute to regulation of cell migration of EPCs isolated from rats bone marrow. Interestingly TRPC1 downregulation dramatically reduces SOCE in EPC (Kuang et al., 2012) (**Table 1**). These data are in accordance with data obtained from EPC isolated from peripheral blood of patients affected by renal cellular carcinoma (RCC; RCC-EPCs) and control EPCs (N-EPCs). In this study in fact Lodola and co workers report that TRPC1 is upregulated in RCC-EPCs where it's involved in SOCE (Lodola et al., 2012). On the other hand no data are at the moment available for a direct role for TRPC1 in human EPC.

In conclusion, form the data available, TRPC1 seems to be a general player involved in both cancer as well as endothelial cell migration although the molecular mechanism is still elusive.

TRPC3

Not many data are available about the role of TRPC3 in cancer cell or EC migration. However recently a functional expression of TRPC3 has been described in MCF-7 breast cancer cell line. TRPC3 mediates store-operated Ca^{2+} entry (SOCE) as shown by TRPC3 knock down. Moreover PUFA (both arachidonic acid (AA) and Lysophosphatidic Acid) inhibits TRPC3-mediated Ca^{2+} entry, which correlates with inhibition of MCF-7 migration. The data suggest a possible role for TRPC3-mediated Ca^{2+} entry in breast cancer cell migration although a direct link is still missing (Zhang et al., 2012) (**Table 1**).

TRPC5—TRPC6

TRPC6 was known till recently to increase proliferation of epithelial cells in prostate (Thebault et al., 2006; Yue et al., 2009), breast (Guilbert et al., 2008; Aydar et al., 2009), liver (El Boustany et al., 2008) and renal cancer (Song et al., 2013). Since TRPC6 protein over expression in breast cancer is not correlated with tumor grade, estrogen receptor expression or lymph node positive tumors (Guilbert et al., 2008), one could think that TRPC6 plays a role primarily in proliferation and not in metastasis. However, two recent studies show that TRPC6 promotes cancer cell migration in head and neck squamous cell carcinomas (Bernaldo de Quirós et al., 2013) and glioblastoma (Chigurupati et al., 2010). In fact TRPC6 expression is increased in head and neck squamous

Table 1 | TRP/Orai1 functions in cancer and endothelial cell migration.

Channel	Cell type	Cell migration		Techniques used	Proposed mechanism	References
		Epithelial	Endothelial			
TRPC1	Glioma cell lines; zebrafish; HUVEC	+	+	Boyden chamber; morfolinos on zebrafish; tubulogenesis *in vitro*	EGF-mediated migration, involvement of lipid raft, ClC-3 interaction/ filopodia extention	Yu et al., 2010; Bomben et al., 2011; Antigny et al., 2012; Cuddapah et al., 2013
TRPC3	MCF-7 (breast cancer)	+	ND	Boyden chamber	Indirect link	Zhang et al., 2012
TRPC6	Head and neck carcinoma cell line; glioblastoma multiple; HMEC; HPAEC; HUVEC;	+	+	Wound healing; matrigel invasion assays on transwell; soft agar colonogenic assay; tubulogenesis in vitro	Notch activation under hypoxia in turn promote TRPC6 expression; in EC PTEN regulates TRPC6 expression	Hamdollah Zadeh et al., 2008; Ge et al., 2009; Chigurupati et al., 2010; Kini et al., 2010; Bernaldo de Quirós et al., 2013
TRPC5/ TRPC6	BAEC, MAEC	ND	–	Wound healing	Lysophosphatidylcholine activate TRPC6 which in turn promote TRPC5 membrane expression	Chaudhuri et al., 2008
TRPV1	Human hepatoblastoma cells (HepG2); cervical and bladder cancer cell	±	–	Random cell migration, boyden chamber, matrigel invasion assays, in vivo xenografts on nude mice	TRPV1 antagonist capsazepine inhibits both cannabidiol-induced tissue inhibitors of the matrix metalloproteinase 1 (TIMP-1) expression and activation of the MAPKs p38 and p42/44; capsaicin promotes IGF (insulin-like growth factor)-1 release, GZMA and MMP9 activation, a-tubulin disassembly and cytoskeleton degradation. The effect is reverted by TRPV1 overexpression	Waning et al., 2007; Ramer and Hinz, 2008; Ramer et al., 2010; Caprodossi et al., 2011
TRPV2	Prostate cancer cells (PC3), urothelial cancer vells (T24/83)	+	ND	Migration assays on transwell, matrigel invasion assays on transwell, in vivo xenografts on nude mice	Lysophospholipids and adrenomedullin activate TRPV2 via PI3K pathway. TRPV2 activation induce MMP2, MMP9 and cathepsin B52 expression	Monet et al., 2010; Oulidi et al., 2013
TRPV4	Hepatoblastoma cell line (HepG2); bovine capillary endothelial (BCE) cells and human dermal microvascular endothelial (HMVE); BHMEC; TEC	+	+	Random motility; wound healing; live cell microscopy after mechanical shear stress application	Ultrarapid activation by b1integrin; activation by AA via actin remodeling	Thodeti et al., 2009; Matthews et al., 2010; Fiorio Pla et al., 2012b
TRPM7	Breast cancer, lung cancer, nasopharingeal cancer and pancreatic ductal adreno-	+	ND	Matrigel invasion assays on transwell	TRPM7 activation of Src-MAPK signaling pathway, focal adhesion number; EGF-mediated TRPM7 membrane expression	Gao et al., 2011; Middelbeek et al., 2012; Meng et al., 2013

(Continued)

Table 1 | Continued

Channel	Cell type	Cell migration		Techniques used	Proposed mechanism	References
		Epithelial	Endothelial			
TRPM8	Carcinoma prostate metastatic cancer; glioblastoma; squamous carcinoma cell lines	–	–	Wound healing; transwell; random motility; *in vivo* xenografts on nude mice	Activation by icilin and PSA; TRPM8 diminish PFAK levels	Wondergem et al., 2008; Yang et al., 2009b; Gkika et al., 2010; Zhu et al., 2011; Okamoto et al., 2012; Valero et al., 2012
ORAI1/ STIM1	Breast cancer; cervical cancer; HUVEC; EA.hy926 cells; EPC	+	+	Transwell; matrigel invasion assays on transwell random migration; *in vivo* xenografts on nude mice; *in vitro* tubulogenesis; wound healing	Stimulation of focal adhesion turnover via ras and rac GTPases; downstream to VEGF.	Abdullaev et al., 2008; Yang et al., 2009a; Chen et al., 2011; Dragoni et al., 2011; Li et al., 2011; Beech, 2012

HMEC, human microvascular EC; HPAEC, human pulmonary artery EC; HUVEC, human umbilical vein EC; EA.hy926, EC line derived from HUVECs fused with human lung adenocarcinoma cell line A549; BTEC, tumor derived EC from breast carcinoma; MAEC, Mause Aortic EC; BHMEC, brain microvascular EC; EPC, endothelial precursors cells; RCC-EPC, EPC isolated from renal carcinoma patients; EGF, epithelial Growth Factor; ClC-3, chloride channel; PTEN, phosphatase and tensin homolog protein; TIMP1, metallopeptidase inhibitor 1; MAPK, mitogen activated protein kinase; IGF, insulin-like growth factor; GZMA, Granzyme A; MMP9, Matrix metalloproteinase 9; PI3K, Phosphatidylinositol 3-kinase; MMP2, Matrix metalloproteinase 2; AA, arachidonic acid.

cell carcinomas tumor samples and cancer cell lines. In this type of carcinomas knockdown of TRPC6 expression does not significantly alter cell proliferation but dramatically inhibits invasion (Bernaldo de Quirós et al., 2013). The authors showed by the means of wound-healing assays and matrigel invasion assays that the effect on invasion is much more pronounced than in migration: knock down of TRPC6 expression by siRNA resulted in a 36% decrease in cell migration and in a ∼90% decrease in invasiveness. These data suggest an essential role of TRPC6 in the 3D motility of cancer cells (Bernaldo de Quirós et al., 2013). On the other hand, in glioblastoma multiforme, the most common primary brain tumor in humans and one of the most angiogenic tumors, TRPC6 expression is markedly upregulated compared to normal brain tissue. This increase in the channel expression is dependent on Notch activation under hypoxia conditions. Both pharmacological inhibition of Notch and knockdown of TRPC6 expression reduce in a similar way glioma migration and invasion *in vitro* (Chigurupati et al., 2010) (**Table 1**).

Beside its role on cancer cell migration, inhibition of the hypoxia-induced TRPC6 expression has an effect in endothelial cell tube formation *in vitro* as it reduced the number of branch points (Chigurupati et al., 2010), indicating that TRPC6 is essential for the angiogenic potential of glioma cells. In this regard, TRPC6 has been now largely accepted as a key player in cell migration in both macrovascular as well as microvascular EC. In particular it has been related to VEGF signaling pathway as the responsible for Ca^{2+} influx and consequent downstream effects (Cheng et al., 2006; Ge et al., 2009). Dominant negative TRPC6 significantly reduces EC number, migration and sprouting (Hamdollah Zadeh et al., 2008). Moreover, TRPC6 promotes both proliferation and tubulogenesis induced by VEGF, but not bFGF, in HUVECs (Ge et al., 2009). Phosphatase and tensin homolog (PTEN) interacts with TRPC6 and regulates cell surface expression of TRPC6 and consequently Ca^{2+} entry, endothelial permeability, and *in vitro* angiogenesis in human pulmonary arterial ECs (HPAECs) (Kini et al., 2010) (**Table 1**; **Figure 1**). Interestingly PTEN-TRPC6-mediated migration and tubule formation *in vitro* does not require the PTEN phosphatase domain, pointing out an interesting new role for PTEN as scaffolding protein (Kini et al., 2010).

Interestingly, TRPC6 can also inhibit EC migration acting in concert with TRPC5: when ECs are incubated in lysophosphatidylcholine (LPC), rapid translocation of TRPC6 initiates Ca^{2+} influx that results in externalization of TRPC5. Activation of this TRPC6–5 cascades cause a prolonged increase in intracellular Ca^{2+} concentration ($Ca^{2+}{}_i$) that inhibits EC movement. This effect is largely attenuated in EC harvested from aortas of TRPC6-/- mice: although LPC caused a prolonged rise in $Ca^{2+}{}_i$ in wild-type EC, it had no effect on $Ca^{2+}{}_i$ in TRPC6-deficient ECs (Chaudhuri et al., 2008) (**Table 1**; **Figure 1**). Moreover LPC-mediated TRPC6 and TRPC5 activation is mediated by tyrosine phosphorylation. This is an interesting finding since fyn and src tyrosine kinases have been described to regulates TRPC6 activity in COS-7 cells (Hisatsune et al., 2004).

Thus the final role of TRPC6 channels on EC migration is closely associated with cellular microdomains composition: when associated with VEGF receptor TRPC6 may function as downstream player and activate EC migration; on the other hand when localized in proximity with TRPC5, TRPC6-TRPC5 cascade results in attenuation of EC migration (**Figure 1**).

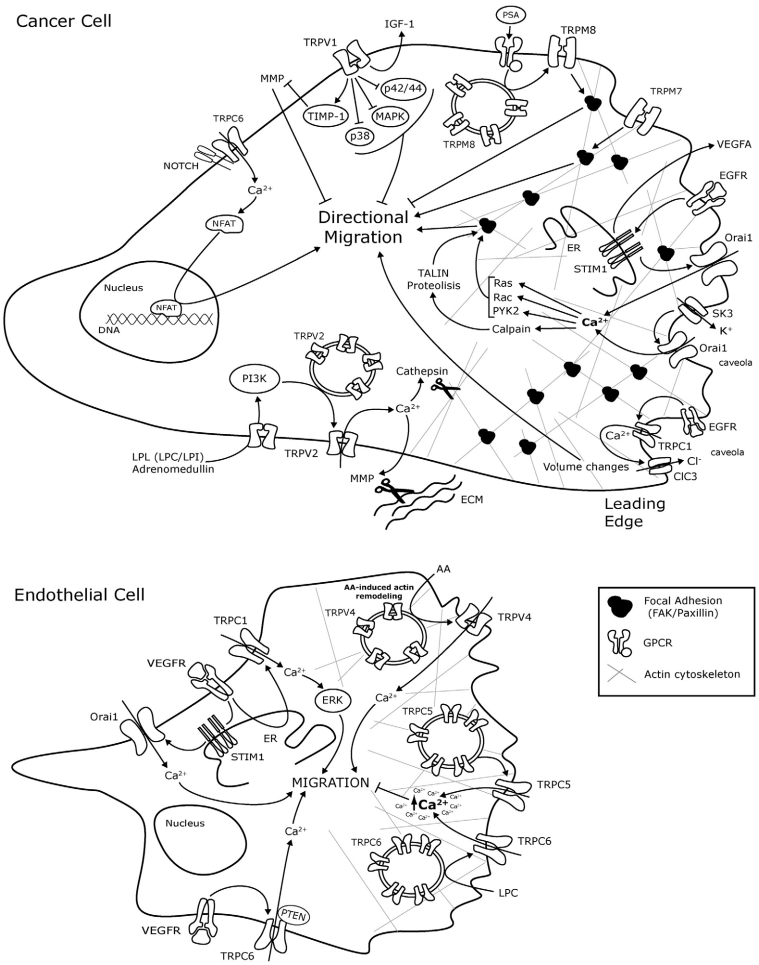

FIGURE 1 | Schematic representation of TRP and ORAI1 channels molecular mechanisms involved in cancer cell and endothelial cell migration. The mechanisms are presented in representative Cancer cells and endothelial cells without any tissue specification. AA, arachidonic acid; ClC-3, Chloride channel-3; EC, endothelial cells; ER, endoplasmic reticulum; bFGF, basic Fibriblast Growth Factor; GZMA, Granzyme A; GPCR, G-protein coupled receptor; IGF, insulin-like growth factor; LPL, lysophospholipids; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; MAPK, mitogen activated protein kinase; MMP, Matrix metalloproteinase; MMP2, Matrix metalloproteinase 2; NFAT, Nuclear factor of activated T-cells; PI3K, Phosphatidylinositol 3-kinase; PTEN, Phosphatase and tensin homolog; Pyk2, Protein tyrosine kinase Pyk2; SK3, K + channel; TIMP1, metallopeptidase inhibitor 1; VEGF, Vascular Endothelium Growth Factor; VEGFR, VEGF Receptor.

TRPV CHANNELS

TRPV1

A growing set of recent evidence using the agonists and antagonists of TRPV1 channel suggest that this channel could be implicated in the modulation of migration and invasion of several types of cancer cells (Waning et al., 2007; Ramer and Hinz, 2008; Ramer et al., 2010; Caprodossi et al., 2011). The most known agonist of TRPV1 capsaicin (the main component in chili pepper) has been shown to lead to an acceleration of human hepatoblastoma cells (HepG2) pretreated with hepatocyte growth factor (HGF). In contrast, HepG2 cells kept in the absence of HGF are not accelerated by capsaicin while the TRPV1 antagonist capsazepine prevents the stimulation of migration. Patch-clamp experiments of the treated cells suggest that the sustained stimulation of migration by capsaicin is probably due to a sustained elevation of TRPV1 channel activity (Waning et al., 2007) (**Table 1**). However, three other studies propose an anti-migratory and anti-invasive role for TRPV1 in lung, cervical and bladder cancer cell (Ramer and Hinz, 2008; Ramer et al., 2010; Caprodossi et al., 2011). In particular, in the two studies by Ramer et al., it is shown that the TRPV1 antagonist capsazepine restores invasiveness of lung and cervical cancel cell, the inhibition of which is due to cannabinoid treatment. It has to be noted that cannabinoids were shown to have an antitumorigenic role inhibiting cell metastasis and angiogenesis (Portella et al., 2003; Blázquez et al., 2004) most likely due to stimulation and consequent desensitization of TRPV1 channel (De Petrocellis et al., 2011). The molecular mechanism of this anti-invasive effect was further studied and it was shown that the TRPV1 antagonist capsazepine causes a significant suppression of both cannabidiol-induced tissue inhibitors of the matrix metalloproteinase 1 (TIMP-1) expression and activation of the MAPKs p38 and p42/44 (Ramer et al., 2010) (**Figure 1**).

TRPV1-mediated inhibition of migration has been analyzed more in detail in a recent study in bladder cancer (Caprodossi et al., 2011) (**Table 1**; **Figure 1**). In this study two urothelial cell lines were used, the low grade RT4 cells and the moderately differentiated 5637 cells of a higher grade in which TRPV1 mRNA and protein levels are dramatically reduced as compared with RT4 cells. Capsaicin promotes the invasiveness of 5637 cells by triggering IGF (insulin-like growth factor)-1 release, GZMA and MMP9 activation, a-tubulin disassembly and cytoskeleton degradation. Interestingly, TRPV1 transfection of these cells reverts the capsaicin-induced migration and MMP9 activation, suggesting an inhibitory role played by TRPV1 in urothelial cancer cell invasion and metastasis.

TRPV2

TRPV2 is expressed in aggressive prostate and bladder cancer cells and tissue samples in which its activation stimulates the migration and invasive phenotype of these cells (Caprodossi et al., 2008; Monet et al., 2009, 2010). In particular two factors, lysophospholipids and adrenomedullin, were shown to increase cell motility by activating the channel. Lysophospholipids are significant actors in tumor development, since they stimulate angiogenesis, growth, survival and migration of malignant cells from various origins (Raj et al., 2004; Hao et al., 2007). Likewise lysophospholipids (LPC and lysophosphatidylinositol, LPI) were shown to act as new physiological stimuli for TRPV2 channel inducing channel translocation to the plasma membrane through activation of Gi or Go proteins and phosphatidylinositol 3,4-kinase (PI3,4K signaling). This accumulation of the TRPV2 channel in the plasma membrane results into higher Ca^{2+} entry which in turn promoted prostate cancer cell migration by induction of key invasion markers, such as the matrix metalloproteinases MMP2, MMP9 and cathepsin B52 (Monet et al., 2009). Further it was shown that siRNA- mediated TRPV2 silencing reduces the size and invasive properties of xenografted prostate tumors in nude mice and downregulates the expression of MMP2, MMP9 and cathepsin B52, suggesting that TRPV2 is a viable pro-metastatic target *in vivo* (Monet et al., 2010) (**Table 1**; **Figure 1**).

The second factor characterized so far acting on cell migration through TRPV2 is adrenomedullin, a peptide originally isolated from a human pheochromocytoma (Kitamura et al., 1993). A number of studies have implicated adrenomedullin in tumor growth, progression and metastasis by affecting angiogenesis, cell proliferation, apoptosis and migration (Zudaire et al., 2003; Nakamura et al., 2006; Nikitenko et al., 2006). In this regard, it was recently shown that adrenomedullin, induces prostate and urothelial cancer cell migration and invasion through TRPV2 translocation to plasma membrane and the subsequent increase in resting Ca^{2+} level (Oulidi et al., 2013) (**Table 1**; **Figure 1**).

TRPV4

TRPV4 is an interesting emerging player in cell migration. In particular, an increasing amount of literature is accumulating on vascular EC: the high selectivity of the pharmacological compounds acting as antagonists for this channel makes it a promising molecular target for antiangiogenic treatments (Everaerts et al., 2010). TRPV4 is widely expressed in the vascular endothelium where it is proposed to act as a mechanosensor. The channel is indeed activated by changes in cell morphology, during cell swelling and shear stress (Vriens et al., 2004; Everaerts et al., 2010). In particular fluid shear stress regulates cell re-orientation in a TRPV4 dependent manner, while in larger arteries the channel is a key player in shear stress-induced vasodilation (Hartmannsgruber et al., 2007; Thodeti et al., 2009). More recently the molecular mechanism underlie ultrafast TRPV4 by shear stress has been investigated: mechanical force applied through b1 integrin induces a near instantaneous and localized transient TRPV4 mediated Ca^{2+} influx in intact capillary EC expressing both native and genetically engineered integrin receptors. The ultra rapid response of the Ca^{2+} signal (within 4 msec) observed using whole cell Ca^{2+} imaging strongly suggests that TRPV4 channels are activated in the absence of second messengers, and are directly mechanosensitive (Matthews et al., 2010). Both shear stress and agonist-activation of TRPV4 enhance EC proliferation and trigger collateral growth after arterial occlusion (Troidl et al., 2009). Fiorio Pla et al. recently demonstrated that AA-activated TRPV4 is essential for breast tumor-derived EC (BTEC) migration: the expression of endogenous TRPV4 was significantly higher in BTEC compared to "normal" EC (HMEC) (Fiorio Pla et al., 2012b). TRPV4 plays a key role in mediating Ca^{2+} entry in BTEC as loss of TRPV4 expression resulted in complete inhibitions of $Ca^{2+}{}_i$ responses and migration induced by the specific agonist phorbol ester 4α-phorbol 12,13-didecanoate (4αPDD) and AA. Finally, AA induces actin remodeling and increases surface expression of TRPV4 in BTEC (Fiorio Pla et al., 2012b) (**Table 1**; **Figure 1**). It is important to stress the use of BTECs in this study as suitable model to investigate the role of proangiogenic factors and their related cell signaling triggered in ECs, compared to the widely used ECs obtained from normal tissues. In fact the great amount of detailed studies on proangiogenic endothelial signaling have been performed *in vitro* on different types of primary or immortalized macrovascular and microvascular EC lines from human or animal normal tissues; on the other hand much less information is available so far about tumor-derived ECs (Fiorio Pla et al., 2012a). As previously reported for TRPC6, the dynamics of a single TRP should be considered in a more integrated framework: in this regard recent data reported that the trafficking to the plasma membrane of TRPV4-TRPC1 heteromeric complex is enhanced by Ca^{2+} store depletion in HUVEC, resulting in an enhanced Ca^{2+} influx upon exposure to shear flow (Ma et al., 2010). Moreover, enhanced TRPV4-C1 trafficking to the plasma membrane contribute to SOCE and I_{SOC} in the EC (Ma et al., 2011). On the other hand, after a heated debate, it is now largely accepted thet TRPC1 channels contribute to SOCE in different cells type including HUVECs (Cheng et al., 2013).

As regarding cancer cells, not much is known about its role in migration. However a study from Schwab and co-workers has described a role for TRPV4 in hepatoblastoma cell line (HepG2): 4αPDD led predominantly to increased lamellipodial dynamics and velocity in HGF treated HepG2 cells, although the displacement, a measure of the cell persistence, was not statistically different from control conditions (Waning et al., 2007).

TRPM CHANNELS

TRPM1

TRPM1 has been suggested to be a tumor suppressor and a decrease in TRPM1 expression appears to be a prognostic marker for metastasis in patients with localized malignant melanoma (Duncan et al., 1998; Fang and Setaluri, 2000). Taking in consideration the loss of expression of TRPM1 channel during the progression of melanomas toward more invasive forms it could be hypothesized that TRPM1 presence and/or activation inhibits migration. A very interesting recent study shed light to that issue and the authors report that tumor suppressive activity is not mediated by TRPM1 directly but by a microRNA (miR-211) hosted within an intron of TRPM1. Increasing expression of miR-211 but not TRPM1 reduces migration and invasion of malignant and highly invasive human melanomas characterized by low levels of melastatin and miR-211. Thus this intronic miRNA assumes a tumor suppressive function previously ascribed to *trpm1*, its host gene (Levy et al., 2010). However, miRNA genes are frequently hosted by protein coding genes, phenotypes attributed to genetic deletion of protein-coding genes may actually be attributable to abrogated expression of the hosted miRNAs (Moffett and Novina, 2007). It has to be noted that a single miRNA may target thousands of genes: it is therefore possible that altered expression of a single miRNA can regulate complex phenotypes. Indeed, a network analysis of melanoma-expressed genes filtered for their roles in metastasis identified three central node genes: IGF2R, TGFBR2, and NFAT5 (Levy et al., 2010). Expression of these genes is reduced by miR-211 and knockdown of each gene phenocopied the effects of increased miR-211 on melanoma invasiveness. In conclusion, this study suggest miR-211 as a suppressor of melanoma invasion whose expression is silenced (or selected against) via suppression of the entire melastatin locus during human melanoma progression (Levy et al., 2010).

TRPM7

TRPM7, as TRPV4, is another stretch-activated Ca^{2+} and Mg^{2+} permeable channel, which present a kinase domain at its C-terminus and allowing its involvement both in cellular Mg^{2+} status and in intracellular signaling (Bates-Withers et al., 2011; Paravicini et al., 2012). TRPM7 has been described as a regulator of actomyosin contractility, cell adhesion, and directed cell migration (Clark et al., 2006, 2008; Su et al., 2006; Prevarskaya et al., 2011; Schwab et al., 2012). In particular TRPM7 is responsible for the Ca^{2+} flickers at the front lamellipodia of migrating fibroblast mediating the polarized migration toward a chemo attractant (Wei et al., 2009). However, a role for this bifunctional channel in cancer progression has been proposed only recently: TRPM7 is critical for cell migration of different cell cancer models such as breast cancer, lung cancer, nasopharingeal cancer and pancreatic ductal adrenocarcinoma (Chen et al., 2010; Gao et al., 2011; Middelbeek et al., 2012; Rybarczyk et al., 2012; Meng et al., 2013). Silencing TRPM7 with specific small interfering RNA lead to a significant reduction in migration and invasion capability of MDA-MB-435 via Src and p38, JNK and ERK1/2 signaling pathway (Meng et al., 2013) as well as by regulating myosin II–based cellular tension, thereby modifying focal adhesion number, cell–cell adhesion and polarized cell movement (Middelbeek et al., 2012) (**Table 1**; **Figure 1**). Interestingly in lung cancer cells A549, EGF functionally regulates TRPM7 expression at the plasma membrane thus increasing cell migration rate (Gao et al., 2011) while TRPM7 protein downregulation significantly interferes with the metastatic potential of human breast cancer *in vivo* (Middelbeek et al., 2012).

On the other hand TRPM7 is involved in a number of vascular disorders such as hypertension and dysfunction of endothelial and smooth muscle cells (Yogi et al., 2011).

Opposite data have been reported for different ECs: while TRPM7 silencing significantly impairs HMEC motility (Baldoli and Maier, 2012), TRPM7 inhibition stimulates HUVEC to migrate (Baldoli et al., 2013). These results further underscores that TRPM7 serves different functions in EC of different origins. On possible explanation for this descrepancy is that HUVEC and microvascular EC might express different Mg transporters. In particular, Baldoli and coworkers discuss the possibility that other Mg channels vicariate TRPM7 functions in HUVEC.

TRPM8

TRPM8 expression is strongly up-regulated in numerous cancers such as prostate, breast, colon, lung, pancreas and skin while it is dramatically reduced during metastasis in the androgen-independent prostate cancer (Tsavaler et al., 2001; Henshall et al., 2003; Yee et al., 2010). This pattern of variation in TRPM8 channel expression makes it an interesting candidate as a diagnostic marker for detection of cancer and as prognosis marker for evaluating the outcome of epithelial cancers (Zhang and Barritt, 2006). Concerning the role of this channel in migration Gkika et al have recently propose that it could have a protective role in prostate metastatic cancer (Gkika and Prevarskaya, 2011), since recent data show that it blocks cancer cell migration in prostate cells (Yang et al., 2009b; Gkika et al., 2010; Zhu et al., 2011; Wang et al., 2012). In particular overexpression of TRPM8 in prostate cancer cells reduces the cell motility through the inactivation of FAK (Yang et al., 2009b) (**Figure 1**). Moreover, it seems that only the presence of TRPM8 on the plasma membrane is sufficient to reduce migration, suggesting a basal activity of the channel possibly affecting FAK phosphorylation while TRPM8 activation by icilin, one of its agonists, further reduced cell motility (Gkika et al., 2010). Interestingly, the well-known prostate cancer marker, Prostate Specific Antigen (PSA) that is secreted in the prostatic acini and is therefore in contact with the extracellular part of TRPM8, activates the channel and decelerates cell migration by inducing its plasma membrane accumulation (Gkika et al., 2010) (**Table 1**; **Figure 1**). Furthermore, TRPM8 expression has a negative effect in angiogenesis as it was recently shown in nude mice. Mice transplanted with prostate cancer cells over-expressing TRPM8 develop tumours that are less vascularized than control. The lower microvascular density of the TRPM8 xenografts can be explained by their lower expression of FAK and VEGF, which is one of the most potent angiogenic factor (Zhu et al., 2011). Taken together these three studies suggest that TRPM8 could play a protective role in prostate cancer progression by reducing both cell migration and angiogenesis.

It has to be noted that contrasting results on the role of TRPM8 in cell migration have been shown in glioblastoma, prostate cancer and squamous carcinoma cell lines (Wondergem et al., 2008; Okamoto et al., 2012; Valero et al., 2012;). In particular, Nilius and co-workers suggest that menthol (TRPM8 agonist) accelerates cell migration of glioblastoma cells (Wondergem et al., 2008); on the other hand, pharmacological agents inhibiting TRPM8 reduce cell speed of prostate cancer cells (Valero et al., 2012). However, the authors used a pharmacological approach and no direct data using siRNA or overexpression that would reinforce the involvement of the channel in cell migration was provided. The discrepancy in the results in between these different studies can be due to several reasons: (i) the pharmacological agents used are not the same, (ii) agonists/antagonists can involve the activation of other channels (or proteins), (iii) the cell systems were not always the same.

It would be interesting to investigate the *in vivo* effects of the activators and inhibitors of TRPM8, as well as the use of pore mutants in addition to the wild-type channel overexpression used in mice xenografts (Zhu et al., 2011). These experiments together with *in vitro* studies could give some insight on which is the critical factor in migration during carcinogenesis: the activity of the TRPM8 channel or the changes in conformation of the TRPM8 protein during its interaction with pharmacological agents and its subsequent changes in protein-protein complexes? However, the present divergence in the results is as puzzling as in the case of TRPV1 and makes it difficult to draw conclusions concerning the use of the channel agonists or antagonists as pharmacological candidates in clinics.

ORAI1/STIM1 COMPLEX

Orai1 and STIM1 are components of the so-called Ca^{2+} release activated currents (CRAC) channels (Yeromin et al., 2006; Cahalan et al., 2007; Hewavitharana et al., 2007). Since they are closely linked to TRP channel function, we will include the discussion for these proteins here. In this regard Recently, TRPC proteins have been shown to associate with Orai1 and STIM1 in a dynamic ternary complex regulated by the occupation of membrane receptors in several cell models, which might play an important role in the function of TRPC proteins (Salido et al., 2011; Cheng et al., 2013).

Reflecting the eminent importance of CRAC current following receptor stimulation, several recent studies addressed the role of Orai1/STIM1 in chemotactically or chemokinetically stimulated migration with a particular focus on cell adhesion both in cancer cells as well as in vascular endothelium. Orai1/STIM1 complex is implicated in breast, nasopharyngeal carcinoma, cervical and glioblastoma multiforme tumor cell migration *in vitro* and in a mouse model of metastases generated by tumor xenografts (Yang et al., 2009a; Chen et al., 2011; Motiani et al., 2013; Zhang et al., 2013). The inhibition of store-operated Ca^{2+} entry (SOCE) by a pharmacological agent, SKF96365, or by siRNA-mediated STIM1 or Orai1 silencing is able to inhibit MDA-MB- 231 cell migration and matrigel invasion, as well as reduce the spread of xenografted tumor cells in mice; on the other hand, reexpression of siRNA-resistant STIM1 or Orai1 constructs rescued the invasion of the STIM1 or Orai1 siRNA-treated cells. STIM1- and Orai1-mediated SOCE regulates cell migration at least partly through modulating focal adhesion turnover, which in turn facilitates cell migration of metastatic cancer cells; on the other hand blocking Ca^{2+} influx affects both the assembly and disassembly of focal adhesions, which may impair traction force generation in migrating cells. The defects of focal adhesion turnover and cell migration induced by SOCE impairment can be rescued by constitutively active forms of the small GTPases RAS and RAC, suggesting the involvement of these regulators of focal adhesions in the modulation of cell migration by SOCE (Yang et al., 2009a) (**Table 1**; **Figure 1**). Similar results were obtained on hepatoblastoma cell line where STIM1 play a key role in focal adhesion turnover (Yang et al., 2013). STIM1 is also a key player in EGF-mediated cervical cancer and nasopharyngeal carcinoma cell migration by inhibiting calpain activity and focal adhesion turnover (Chen et al., 2011; Zhang et al., 2013); STIM1 play also a role in stimulating angiogenesis by regulating VEGF-A release from cancer cells thus proposing a multiple function for STIM1 in tumor biology (Chen et al., 2011). On the other hand recently the association of Orai1 and K+ channels have been involved in cancer cell migration: SK3 channels functionally associate with the Orai1 channel in a breast cancer MDA-MB-435s within lipid rafts. This localization of an SK3–Orai1 complex seemed essential to control cancer cell migration and bone methastases. Interestingly, STIM1 seems not to be involved in this effect (Chantôme et al., 2013) (**Figure 1**).

The role of ORAI1/STIM1 complex has been studied recently also in EC migration and tumor vascularization. In particular it concurs to the VEGF-mediated SOCE in HUVECs (Abdullaev et al., 2008; Li et al., 2011). VEGF stimulation promotes STIM1 clustering which in turn activates Orai1 (Li et al., 2011). Moreover, knock-down of Orai1 inhibits HUVEC migration, proliferation and *in vitro* tubulogenesis subsequent to the sustained intracellular Ca^{2+} elevation triggered by VEGF (Abdullaev et al., 2008; Li et al., 2011; Beech, 2012) (**Figure 1**). Interestingly, it has been recently reported that STIM1, as well as TRPC1 and TRPC4 knockdown, reduces tube formation in both HUVECs and EA.hy926 cells (Antigny et al., 2012). These data are of particular interest since functional interaction between Orai1 and TRPC1 has been described as previously stated (Ong et al., 2007; Salido et al., 2011; Cheng et al., 2013).

Notably, suppression of Orai1 expression or expression of dominant negative mutants of Orai1 abolish SOCE in EPCs as well as *in vitro* tubule formation (Li et al., 2011). Blockade of SOCE affects EPC proliferation, migration and *in vitro* tubulogenesis induced by VEGF (Dragoni et al., 2011). Moreover, EPCs isolated from RCC patients (RCC-EPCs) display a greater SOCE, which correlates with the over-expression of Orai1, Stim1, and TRPC1 as compared to control cells (EPCs from healthy patient) (Lodola et al., 2012).

CONCLUSIONS

In the two last decades the progressive understanding of the molecular mechanisms that regulate the establishment and progression of different tumors is leading to even more specific and efficacious pharmacological approaches. In this picture, TRP channels represent a very promising player since their expression and activity mark and regulate specific stages of cancer

progression (Gkika and Prevarskaya, 2009; Prevarskaya et al., 2011; Schwab et al., 2012; Ouadid-Ahidouch et al., 2013). On the other hand, the downside of migration is represented by disease conditions in which cell migration constitutes a major pathophysiological element. The most prominent example is the formation of tumor metastases. Tumor cell migration is an essential step of the so-called metastatic cascade that leads to the spread of the disease within the body (Yamaguchi et al., 2005; Lohela and Werb, 2010).

The data presented in this review firmly proof the concept that different members of the TRP family of channels constitute integral components of the cellular migration machinery linking in many cases the plasma membrane (or intracellular membranes) signals to cytoskeletal migration motor or cell adhesion proteins functions in cancer cells. Indeed, locomotion involves lamellipodial extensions at the leading edge, new attachments to stabilize the extension, transcellular contractility, and detachment at the rear. These processes involve a multitude of molecules such as growth factors and their receptors, matrix metalloproteinases, integrin, small GTPases, FAK and SRC kinases, elements of the cytoskeleton, all of which underlie biochemical events of the moving cells. In particular, data on TRPM7 and Orai1/STIM1 complex clearly link these channels to this dynamic process leading to directional migration (Yang et al., 2009a; Middelbeek et al., 2012). It is also clear that the study of TRP channels in cellular migration is a young field (as denoted by the fact that the majority of data are from the last 10 years) so that studies mechanistically linking the channel role to the migration machinery are still limited and more research is needed. On the other hand, the scientific interest on this topic is largely increasing as pointed out by PubMed search and we aspect the knowledge of the molecular mechanisms responsible for TRP channels role in migration will be largely unveiled in the next ten years.

Another issue to be considered is the wide distribution as well as the multiplicity of cancer hallmarks controlled by a given TRP or Orai1 protein which requires careful consideration of its therapeutic potential. This problem could be overcome by directed targeted therapies taking advantage from nanobiomedicine: for example, nanoparticle functionalization with peptide cyclic RGD for angiogenesis-specific targeting (Cheng et al., 2011) together with a specific channel modulator could be successfully employed. Another good example of such a strategy is the 'smart bomb' for prostate cancer, which combines a sarcolemmal and endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (which induces apoptosis through the activation of ER stress and Ca^{2+} entry pathways) with a targeting peptide that is a substrate of the serine/protease prostate-specific antigen (PSA) (Denmeade and Isaacs, 2005).

The wide expression of TRP channels has also to be taken in account regarding vascular endothelium. In this regard when considering TRPV4 potential role in tumor vascularization (Fiorio Pla et al., 2012b) it's important to notice that TRPV4 is ubiquitary in healthy vascular endothelium and plays a physiological role both in large arteries and microvessels: these relevant activities require careful consideration of its therapeutic potential. On the other hand, an overexpression on TEC could be exploited for a tumor targeted therapy based on lower inhibiting doses of TRPV4 antagonists which could selectively affect TEC and not normal EC.

Moreover ubiquitous expression of the channels could be used as a positive feature, due to the redundancy of the signaling pathways which regulates the different hallmarks of cancer: in other words, the use of specific channels to selective co-target different key steps of carcinogenesis beside tumor vascularization, could result in more effective and long lasting therapies. For example, TRPC6 channels targeting could affect VEGF release from tumor cells as well as EC migration and tumor vascularization (Hamdollah Zadeh et al., 2008; Ge et al., 2009; Chigurupati et al., 2010).

In addition as it was described above for some channels (TRPV1 and TRPM8) pharmacological action has not always the same result with siRNA and/or overexpression approaches due to broad action on several cellular components. There is therefore a real need for detailed studies *in vivo* in order to test which are the most effective and suitable molecules to target in therapeutics. Another reason for the possible differences in the cellular responses observed in different tumors could be due to some peculiar features of these channels in certain cellular environments. It is now well described that TRP channel can interact in specific microdomains giving rise to different signal transduction pathways and cellular signals. In this regard an example is given by TRPC6: when associated with VEGF receptor TRPC6 may function as downstream player and activate EC migration (Chigurupati et al., 2010); on the other hand when localized in proximity with TRPC5, TRPC6-TRPC5 cascade results in attenuation of EC migration (Chaudhuri et al., 2008). On the other hand TRP and ORAI1 channels functionally interact with other family of channels: a nice example is TRPC1 which is functionally related to ClC-3 in caveolar lipid rafts of glioma cells to promote EGF-induced chemotaxis (Cuddapah et al., 2013). Similarly ORAI1 interacts with K+ channels in breast cancer (Chantôme et al., 2013). Differences in channels complexes and/or microdomain localization could therefore account for different cellular effect in tumors.

In conclusion we expect that the more detailed understanding together with a more integrative view on TRP channels in cell migration, as well as careful studies using appropriate *in vivo* and *in vitro* models, will facilitate the advances in this exiting field of cellular physiopathology

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