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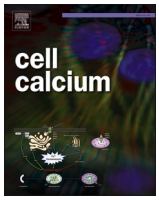

Relevance of lysosomal Ca^{2+} signalling machinery in cancer

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A B S T R A C T

In comparison with normal cells, cancer cells are equipped with a higher number of lysosomes, involved in degradative and non-degradative roles. In particular, the lysosome is a Ca^{2+}signalling hub, and the enhancement of this interconnected machinery in cancer cells has recently prompted investigations into the role that lysosomal ion channels play in oncology. The present review reports findings about the emerging role of lysosomal Ca^{2+}channels: Two-Pore Channels (TPCs), Transient Receptor Potential Cation Channels (TRPMLs; mucolipins), and Purinergic X Receptor 4 (P2×4R), in a variety of cancer models, highlighting their impact on crucial functions such as the regulation of autophagy and the composition of the tumour microenvironment, including the secretion-mediated interplay with immune and endothelial cells. Notably, recent evidence indicates that, by regulating tumour secretome, lysosomal Ca^{2+} signalling can affect the composition of the tumour-infiltrating immune cell repertoire. Intriguingly, the data so far available show that the protumoral/antitumoral role of lysosomal Ca^{2+} channels can differ according to the specific genetic context, types of cancer and the malignancy stage, and signals from the microenvironment.

Introduction

Pathophysiological processes in the human body are regulated by a diversity of extracellular and intracellular signalling pathways. Changes in intracellular calcium (Ca^{2+}) constitute one of the key signalling mechanisms in the body, mediating such a diversity of processes that it has been stated that '$Ca^{2+}$is everything' [1]. Changes in intracellular Ca^{2+}can be induced by influx of extracellular Ca^{2+}, this influx being regulated by voltage-operated calcium channels (VOCCs), receptor-operated calcium channels (ROCCs), and storage-operated calcium entry (SOCE) [2]. The other main source of intracellular Ca^{2+}signals is internal stores, which release $Ca^{2+}$when their receptors are activated by Ca^{2+}-mobilizing messengers, the primary ones being inositol 1,4,5-trisphosphate (IP_3), cyclic adenosine diphosphate ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP) [3]. While IP_3 and cADPR activate IP_3 receptors (IP_3Rs) and ryanodine receptors (RyRs), respectively, both of which regulate Ca^{2+}release from the endo/sarcoplasmic reticulum (ER/SR), NAADP targets two-pore channels (TPCs) to mediate Ca^{2+}release from acidic endolysosomal organelles [4]. NAADP is such a potent Ca^{2+}-mobilizing messenger that it can exert its action even at low nanomolar concentrations. NAADP-induced Ca^{2+}signalling also triggers Ca^{2+}release from the ER/SR, via Ca^{2+}-induced Ca^{2+}release (CICR) [5–7]. Although TPCs have been shown to be integral components of the NAADP-regulated Ca^{2+}channel system, there is also evidence that they can act as sodium (Na^+) channels regulated by phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) [8]. There are three subtypes of TPCs, TPC1, TPC2 and TPC3; however, only TPC1 and TPC2 are present in human and mouse cells [9]. TPC1 and TPC2 differ in their pattern of distribution; thus TPC1 is found in a range of endolysosomal organelles, while TPC2 is distributed more predominantly in late endosomes and lysosomes [10]. In 2016, the 3D structure of TPC1 from Arabidopsis thaliana (At-TPC1) was determined using X-ray crystallography [9], and more recently, the 3D structures of mouse TPC1 (MmTPC1) and human TPC2 (HsTPC2) were determined with single-particle electron cryo-microscopy (cryo-EM) [11, 12]. Such studies have revealed differences and similarities between TPC1 and TPC2 in terms of both the structure of the 'pore' region and interactions with the intracellular regulator $PI(3,5)P_2$.

A key unsolved issue until recently was the mechanism of interaction of TPCs with another key regulator, NAADP. As already mentioned,

; VOCCs, voltage-operated calcium channels; TPCs, Two-Pore Channels; TRPMLs, Transient Receptor Potential Cation Channels; P2X4R, Purinergic X Receptor 4; NAADP, nicotinic acid adenine dinucleotide phosphate.

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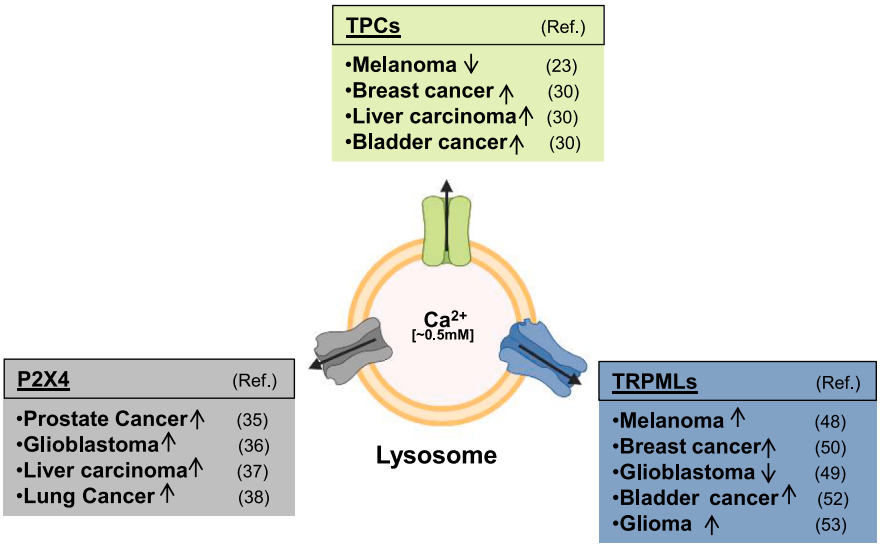

Fig. 1. Overview of pro- or anti-tumoral role of lysosomal Ca^{2+}channels in tumour cells. The panel reports references on data from experimental inhibition or stimulation of the different channels in different tumour cell types. Arrows synthetically indicate the pro-tumoral (↑) or anti-tumoral (↓) role of each channel, showing it can be opposite in different tumours.

NAADP can endogenously regulate Ca^{2+}release via TPCs. Previous findings showing that NAADP mediates endolysosomal Ca^{2+}release via TPCs were contradictory, and it was suggested that a separate unidentified NAADP-binding protein exists to accessorize TPC activation [13].

Recently, two such NAADP-binding proteins have been identified and shown to physically interact with TPCs: Jupiter microtubule-associated homolog2 (JPT2) and Like-Sm protein 12 (LSM12) [14]. Exactly how JPT2 and LSM12 interact with TPCs at the molecular level remain to be characterized, as does the relationship between these two proteins, but study of these issues should help to further understanding of the mechanisms of action of TPCs, their modes of regulation, and precise pathophysiological roles.

In terms of such pathophysiological roles, TPCs have been shown to be involved in a diversity of processes involved in health and disease, including stem cell differentiation, smooth muscle and cardiac contraction, glucose homoeostasis and diabetes, intracellular trafficking and autophagy, immunity, infection and inflammation, lipid metabolism, neurodegenerative disorders, pulmonary hypertension, anaphylaxis, neuroplasticity, neo-angiogenesis, tumorigenesis, and metastasis [4].

In addition to TPCs, another important family of endolysosomal ion channels is the TRPMLs [15]. TRPML1 is a ubiquitously expressed cation channel located in lysosomes. The two other family members, TRPML2 and TRPML3 are less ubiquitously expressed and in contrast to TRPML1, are also located in early and recycling endosomes. P2X4R is an ATP-gated cation channel widely expressed in a variety of tissues, which plays a role in the secretion of inflammatory mediators, and as a regulator of cardiac contractility and vascular remodelling [16]. Unlike other members of the P2X family, which are expressed on the cell surface, P2X4 is unique in being predominantly intracellular, within endolysosomal compartments. This unusual distribution has led to the suggestion that P2X4R might function at endolysosomal membranes in addition to its role at the plasma membrane. Patch-clamp recordings of ATP-evoked currents from enlarged vacuolar lysosomes have supported this view and revealed that lysosomal P2X4 receptors are under the dual regulation of intraluminal ATP and pH. In addition, lysosomal P2X4 channels have been shown to play an important physiological role during the secretion of surfactant from alveolar type II (ATII) epithelial cells [17].

In the rest of this review, we will discuss the mechanisms of action and roles of endolysosomal ion channels such as TPCs, TRPMLs, P2X4R and the Ca^{2+}signals that they regulate, in cancer.

Lysosomal Ca^{2+} channels in cancer cells

Normal cells maintain a very low cytosolic Ca^{2+}concentration at about 100 nM with a 10–15,000 fold gradient with the extracellular environment of 1–1.5 mM; this particular need to keep Ca^{2+} at low levels to avoid precipitates has allowed for the evolutionary development of Ca^{2+}intracellular signalling functions. The regulation of intracellular Ca^{2+}concentration occurs through a complex and interconnected machinery between different organelles thus allowing $Ca^{2+}$to function as an intracellular messenger. Dysfunctional Ca^{2+}signalling in cancer is currently well established from preclinical and clinical evidence [18]. Ca^{2+}levels in endolysosomal compartments (about 0.5 mM) are comparable to those in the endoplasmic reticulum and lysosomes have emerged as fundamental intracellular Ca^{2+}stores [19]. Lysosomal Ca^{2+}homeostasis is regulated through calcium channels and the most studied in cancer are i) the mucolipin subgroup of the TRP ion channel family: TRPML1, 2 and 3; ii) the ATP-gated cation channel P2 × 4; and iii) the two-pore channels: TPC1 and 2 (Fig. 1).

Involvement of TPCs channels in tumour progression

It has been demonstrated that some polymorphisms of TPC2 are associated with hair pigmentation [20]. Melanoma is a malignant tumour originating from transformed versions of melanocytes, cells that normally produce melanin. Different types of melanoma can be classified by their different levels of melanin pigmentation, which is highly deregulated in this pathology [21]. We identified the importance of VEGFR2/NAADP signalling in melanoma progression. Thus, we demonstrated that melanoma tumour volume and lung metastases are reduced in vivo by treatment with Ned-19, the NAADP orthostatic antagonist [22]. In that study, we did not investigate the direct involvement of TPC2 that was previously identified to have a fundamental role in this signalling regulating angiogenesis in vitro and in vivo [23]. In particular, Ambrosio et al. [24] identified TPC2 on melanosomes, which can be identified as modified lysosomes and represent the site of melanin synthesis. The absence of TPC2 can influence melanosome pH and size. Considering this background evidence, we further investigated the role of TPC2 in melanoma in particular using human metastatic cell lines (CHL1 and MeWo, both BRAF wild-type). We found that TPC2 KO affects the metastatic traits of the cells. Performing an adhesion assay that used cell plates coated with collagen type I matrix,

we found that CHL1 TPC2 KO cells showed a drastically reduced ability to bind this matrix. We also compared CHL1 WT and TPC2 KO cells' ability to invade a matrigel substrate, and observed that CHL1 TPC2 KO cells were more invasive than WT controls. In line with this, the TPC2 KO cells showed an increased secretion of matrix metalloproteinase 9 (MMP9), and mesenchymal characteristics such as increased expression of N-cadherin, the transcription factor zinc-finger E-box-binding homeobox1 (ZEB-1), a known inducer of Epithelial Mesenchymal Transition, and vimentin, which is considered a mesenchymal marker [25].

To investigate the underlying molecular basis for this increase in metastatic potential after TPC2 KO in CHL1 cells, we chose to study whether there was any effect of such TPC2 KO on the HIPPO signalling pathway. In recent years, this pathway has been demonstrated to modulate cell proliferation and differentiation and to contribute to the progression of a number of diseases, including cancer [26]. In particular, the HIPPO effectors YAP and TAZ are correlated with cancer progression. High YAP levels correlate with decreased survival in melanoma patients [27] and TAZ activation has been linked to lung cancer brain metastasis [28].

The activation status of YAP/TAZ was analysed as the expression of ankyrin repeat domain-containing protein (ANKRD1), cysteine-rich 61 (CYR61), and connective tissue growth factor (CTGF), which are considered to be main *bona fide* YAP/TAZ target genes. Notably, it was found that all these target genes were strongly up-regulated in CHL1 TPC2 KO cells indicating YAP/TAZ activation [25]. It has been demonstrated that TAZ can regulate the transcription of PD-L1 [29], one of the ligands of PD-1, which represents a target for advanced melanoma immunotherapy [30]. Moreover, we found that CHL1 TPC2 KO cells increased the level of surface expression of PD-L1 compared to WT [25]. Conversely, no activation of YAP/TAZ target genes was detected in TPC2 KO B16-F0 primary melanoma mouse cells (BRAF wild type), supporting previous data [22]. Further investigation will be necessary to understand whether this different behaviour is only related to BRAF mutations or else to broader species-specific differences in YAP/TAZ transcriptional activation.

Moreover, it has been demonstrated that TPC2 is necessary for melanin production and can influence melanoma cell proliferation, migration and invasion: when TPC2 is knocked out or inhibited with some flavonoid compounds (e.g. naringenin) these features are reduced [31]. The model used in this study was the human highly pigmented metastatic melanoma cell line MNT1 (BRAF mutated).

Further studies will be useful to understand the possible different roles of TPC2 related to different genomic backgrounds, since melanoma is a high mutational burden cancer; in this way the presence or the absence of this channel could be beneficial or catastrophic in association with other common mutated genes (BRAF, K-RAS, p53) in melanoma.

Interestingly, Nguyen et al. [32] linked TPC1 and TPC2 to cancer cell malignant behaviour by using as a model a cell line derived from human urinary bladder carcinoma and a well-differentiated hepatocyte-derived carcinoma cell line, Huh7. These authors demonstrated that TPC1 and TPC2 silencing, as well as pharmacological inhibition, reduced the adhesion and migration of invasive tumour cells *in vitro*. In addition, silencing of TPC2 with siRNA or by means of pharmacological inhibitors in an *in vivo* metastatic mouse mammary cancer model, have been shown to significantly decrease the formation of lung metastasis. The results obtained in these cancer models differ from those reported in melanoma, described above. This apparent experimental divergence about the role of TPCs/Ca^{2+}signalling in cancer and whether inhibition of these channels increases or hampers metastatic traits in melanoma cells, might reflect TPCs having distinct roles and functions in different stages of aggressiveness and specific types of cancer. Recently, Muller et al. highlighted the key role of TPC2/Ca^{2+}signalling in cancer cell proliferation and tumour growth by using the RIL-175 mouse hepatocellular carcinoma cell line. These authors showed that TPC2 KO reduces proliferation of cancer cells and developed novel tetrandrine-derived small molecule TPC2 inhibitors [135] . In addition, we have demonstrated that the natural flavonoid naringenin acts as an inhibitor of TPC2, as shown by electrophysiological evidence in a heterologous system, i.e. Arabidopsis vacuoles lacking endogenous TPCs [34, 35]. Notably, in a model of human melanoma cells, Netcharoensirisuk et al. [31] confirmed that naringenin, as well as additional flavonoids, are specific inhibitors of TPC2, since, conversely to TPC2, the endolysosomal cation channel TRPML1 was not blocked by naringenin. Recent work reported the role of endo-lysosomal Ca^{2+}signalling in primary cultures of human metastatic colorectal carcinoma cells in which TPC1 is the most expressed TPC isoform. Using both pharmacological and genetic approaches, these studies revealed that the Ca^{2+} response to NAADP was triggered by TPC1 [36]. Moreover, pharmacological and genetic blockade of TPC1 reduced ERK and Akt phosphorylation, which have long been known to support Ca^{2+}-dependant proliferation in both normal and neoplastic cells. These studies suggest that TPC1 is a druggable target in colorectal cancer cells.

P2X4R in cancer

In studies of the biological significance of endolysosomal ion channels in cancer, P2X4R has been positively associated with tumourigenesis in some cancer types. He et al. [37] have demonstrated that inhibition of P2X4R impaired the growth and mobility of prostate cancer (PCa) cells. In BALB/c immunocompromised nude mice inoculated subcutaneously with the human prostate cancer cell line PC3, the selective P2X4R antagonist 5-BDBD showed anti-tumourigenic effects. In addition, it has been reported that various human glioblastoma multiforme cells expressed high levels of P2X4R when compared with normal human astrocytes and down-regulation of these receptors by siRNA suppressed cell growth and viability through inhibition of the BDNF/TrkB/ATF4 signalling pathway [38]. Similarly, samples of liver cancers such as hepatocellular carcinoma, adenocarcinoma and ampullary carcinoma, exhibited significantly increased P2X4 receptor expression as compared with the control tissue samples [39]. Furthermore P2Y1, P2X4 and P2X7 receptors are upregulated in bronchoalveolar cells in metastatic lung cancer [40]. Altogether, these findings suggest a role for P2X4R in cell proliferation of several carcinomas. Notably, a novel association between polymorphisms in TPCN2 and P2X4R in subtypes of cancer in terms of cancer risk, disease recurrence, malignancy and metastasis, has been reported by us in a recent study that identified novel cancer biomarkers for the development of diagnostic and therapeutic strategies [41]. The involvement of P2X4R in cancer is further demonstrated by data showing that this channel can play a pro-angiogenic role, as shown in a very recent study [42], which will be more extensively reported in a following section. In addition, P2X4R may also play an important role in inflammasome activation in several organs and, given the clinical relevance of inflammasomes in multiple forms of cancer, this additionally points to P2X4R as a therapeutic target for several clinical conditions involving inflammasome activation, including cancer [43].

In oncology, further interest in P2X4R's expression, activation and downstream effectors stems from the key role this receptor plays in the pathogenesis of chronic pain,which makes it interesting as a potential target for cancer patient treatment. At the peripheral level, damaged tissues are known to be particularly rich in extracellular ATP, which results in activation of its receptor P2X4R in nearby cells, particularly macrophages, followed by the release of factors sensitising local sensory nerve endings [43]. The transmission of peripheral nociceptive signals activates microglial cells in the central nervous system [44], triggering a complex process characterised by important changes in gene expression, including a remarkable increase in the expression of P2X4 receptors. Similarly to what is observed in macrophages, in activated microglia Ca^{2+} influx consequent to binding of ATP to P2X4R triggers a series of intracellular signalling cascades resulting in the release not only of proinflammatory cytokines but, interestingly, also of BDNF [45]. Collectively, these factors, acting through both direct and indirect

mechanisms, are capable of enhancing sensory nerve transmission and inducing or exacerbating pain. Highly selective drugs capable of targeting this network of signals are at present the subject of active research [44].

Role of TRPMLs in cancer

TRPML1, TRPML2, and TRPML3 are non-selective cation channels belonging to the TRP channel family. These channels are encoded by *MCOLN* genes (*MCOLN1, MCOLN2*, and *MCOLN3*) that are on human chromosome 1 and chromosome 19 [46]. TRPML1 is expressed in all tissues and it is localized on the lysosome and late endosome (LE) [47]; by releasing lysosomal Ca^{2+}it can regulate several processes, such as autophagosome-lysosome fusion, lysosome exocytosis, vesicle trafficking and, in case of mutation, it can cause a human autosomal recessive disease termed mucolipidosis type IV (MLIV), a lysosomal storage disease [48]. TRPML2 and TRPML3 are present in specific cell types: there are high levels of TRPML2 in thymus, spleen, kidney and high levels of TRPML3 in melanocytes, hair cells of the inner ear, neonatal enterocytes, and bladder epithelial cells (BECs). Intracellularly, TRPML1 is primarily distributed in the later compartments of the endocytic pathway, while TRPML2 is localized on the recycling endosomal membrane and TRPML3 is present in the earlier compartments of the endocytic pathway (the early endosome) [49]. Compared to TRPML1, the other two channels (TRPML2-TRPML3) are less well studied. Emerging evidence links TRPML1 with melanoma, breast cancer, bladder cancer, head-and-neck cancer and glioblastoma [50–53]. Kasitinon et al. [50] have observed that, relative to normal melanocytes, melanoma cells show a higher TRPML1 expression and in patient-derived melanomas loss of this channel results in the arrest of melanoma growth both in culture and in xenografts. Using human glioblastoma primary cancer cells and glioblastoma cell lines Santoni et al. [51] have shown that the elimination of TRPML1 from cancer cells leads to greater ability to proliferate, increased survival and ability to invade the host. On the other hand, an interesting correlation between mutated HRAS and increased TRPML1 expression emerged in some tumours, for instance bladder cancer or head-and-neck cancer [54]. Compared to tumours presenting wildtype HRAS, the mutation in this oncogene appears to result in increased expression of TRPML1 and consequently increased cell vitality and proliferation, determined by the fact that TRPML1 maintains oncogenic HRAS in nanoclusters responsible for signalling on the plasma membrane. Accordingly, in these cells TRPML1 silencing resulted in decreased cell proliferation and viability. Like TPC2, TRPML1 can also therefore either favour malignancy or act against it, depending on a variety of conditions, the specificity of which represents an important challenge.

As for the paralogues of TRPML1, namely TRPML2 and TRPML3, limited information is available. We know that TRPML2 is involved in glioma proliferation and survival. As shown in [55], this channel is more expressed in the most severe forms of glioma and its elimination by siRNA compromises the growth and vitality of these cancer cells.

As for TRPML3, its knock-down has been shown to affect autophagy and membrane trafficking [56], but the exact role that this channel may play in cancer is still to be investigated.

Endolysosomal Ca^{2+} signalling affects the secretome of cancer cells

The cancer secretome includes an assortment of proteins secreted by cancer cells through different secretory pathways and represents a powerful instrument for elucidating cancer biology. For instance, secreted proteins might represent putative tumour biomarkers or therapeutic targets for various types of cancer [57], so understanding how their secretion is regulated is of fundamental importance in this field of research.

Lysosomal exocytosis is a finely regulated process which controls different cellular functions: plasma membrane repair, secretion and transmitter release, neurite outgrowth and particle uptake in macrophages [58]. It has been demonstrated that Ca^{2+}-dependant lysosomal exocytosis has a crucial role in tumour progression and chemoresistance [59]. The inhibition of this process suppresses the invasiveness and chemoresistance of sarcoma cells, while increased lysosomal exocytosis promotes invasiveness and drug-resistance [60]. TRPML1 is a key regulator of lysosomal exocytosis. Indeed, cholesterol recycling by TRPML1-mediated lysosomal exocytosis contributes to the proliferation of oncogenic HRAS-driven cancer cells. TRPML1 knockout cancer cells showed suppressed movement of cholesterol to the plasma membrane, which consequently reduced the proliferation rate of such cells [54].

A very recent article explored the involvement of TPCs in EGF receptor (EGFR) trafficking, understanding of which is fundamental for establishing novel anti-tumour therapies. In particular, TPC deletion causes a delay of endolysosomal EGFR trafficking and a prolonged activation of associated ERK and JNK signalling cascades [33]. Lin-Moshier et al. [61] have shown an interaction between TPC2 and some Rab proteins, such as Rab5, Rab7 and Rab11. Rab-GTPase proteins are crucial for the formation, transport, tethering, and fusion of transport vesicles as a general mechanism for regulating traffic between organelles [62]. In particular, both Rab7 and Rab11 are associated with melanosomes. TPC2 is localized on the melanosome membrane and acts to control melanosome pH and size [24]. Furthermore, Rab11a and Rab11b knockout cells showed accumulation of melanosomes at the cell periphery and had increased melanin content [63]. Thus, studying the involvement of TPC2 in melanosome release and trafficking represents a key strategy for the development of new cancer therapies, since Dror et al. demonstrated that melanosome content is fundamental for the creation of a tumour niche and for reprogramming distant fibroblasts to help metastasis formation [64].

Moreover, it has been demonstrated that TPC2 overexpression or knockdown (KD) affects extracellular vesicles (EVs) secretion in mouse breast cancer 4T1 and HeLa human cervical cancer cell lines [65]. In particular, these authors studying the role of TPC2 in autophagy progression, demonstrated that EV secretion was abated in TPC2 overexpressing cells and, conversely, was increased in TPC2 KD cells, indicating that TPC2 plays a key role in trafficking and exocytosis of EVs.

TPC2 has also been shown to be involved in integrin-β1 recycling in cancer cells [32], affecting cell adhesion ability. Thus, understanding TPC2 involvement in vesicular trafficking represents a further important challenge. As for P2X4R, Palinski et al. [42] showed that EVs from sarcoma patients induced tumour angiogenesis and, interestingly, this effect was due to the increasing traffic of lysosomal P2X4R to the cell membrane.

Since it is now well established that lysosomes represent, besides their primary role in the catabolism of macromolecules, an intracellular signalling and intercellular communication platform, the relevance of lysosomal Ca^{2+}signalling is becoming an intriguing field of research. Movement and spatial distribution of lysosomes towards the periphery of cells is essential for cancer progression, metastasis and activation of stromal cells in the tumour microenvironment, by increasing lysosomal exocytosis and release of exosomes [66]. Given that Ca^{2+} intracellular spikes regulate lysosomal positioning and exocytosis, Ca^{2+} channels expressed in lysosomes may represent novel druggable targets in cancer therapy.

Lysosomal Ca^{2+} channels as players in tumour neo-angiogenesis and vascular mimicry

Angiogenesis is a normal and complex process necessary for the formation of new blood and lymphatic vessels from a pre-existing vasculature. This process is fundamental during embryogenesis but

occurs minimally in healthy adults [67]. A critical role for active angiogenesis has been demonstrated in cancer progression where it is considered a cancer hallmark. This phenomenon (neoangiogenesis) is typically initiated by branching of nearby capillaries and plays an important part in tumour growth, maintenance, and metastasis [68].

Ca^{2+} signalling plays a crucial role in angiogenesis and angiogenic factors generate Ca^{2+} rises via two mechanisms: entry from extracellular milieu, through the opening of Ca^{2+}-permeable channels in the plasma membrane, and release from intracellular Ca^{2+} stores [69].

The presence of TPC2 has been shown to be essential for neoangiogenesis. In particular, the signalling pathway VEGFR2/NAADP/TPC2/Ca^{2+} has been identified as controlling the angiogenic response of endothelial cells to VEGF, which is the major player in controlling angiogenesis [23]. This result was confirmed *in vivo*: the matrigel plug assay revealed that VEGF-induced angiogenesis was blocked using Ned-19, an NAADP antagonist, in wild-type mice and in TPC2 KO mice but not in TPC1 KO mice, indicating the specific role of TPC2 in the regulation of this process. Moreover, naringenin, a direct inhibitor of TPC2, was able to inhibit the formation of the vessel-like structures in vitro and the vascularization of the matrigel plugs in vivo [35]. Of note, in recent years it has been observed that cancer cells can mimic endothelial cells in the formation of vessel-like structures, a phenomenon known as vascular mimicry (VM) [70]. Given the importance of TPC2 in neoangiogenesis and its role in autophagy, which is considered a participating process in VM, it would be interesting to investigate the role that endolysosomal channels might play in the formation of these cancer cell-lined pseudo capillaries.

A very recent study has investigated the role of P2X4R in neoangiogenesis, and demonstrated that tumour-derived micro vesicles (T-MVs) from sarcoma patients can induce neoangiogenesis and this phenomenon is reduced following the pharmacological or genetic inhibition of P2X4R. In particular, the authors have proposed a mechanism related to an increase of cytosolic Ca^{2+} and mitochondrial activity, suggesting that T-MVs induced P2X4R translocation from the lysosome to the plasma membrane where it regulated an increased Ca^{2+} influx, mitochondrial activity and ATP production . Interestingly, they observed a reduction of tumour volume and neoangiogenesis in mice treated with a P2X4R pharmacological inhibitor [42].

Lysosomal Ca^{2+} signalling, autophagy and cancer

This part of our review analyses the interplay between lysosomal Ca^{2+} signalling and autophagy in cancer. Autophagy is an evolutionary lysosome-dependant highly conserved mechanism for the recycling and the degradation of cytoplasmic constituents, such as lipids, protein aggregates and damaged organelles, in response to stressful conditions, nutritional deprivation, and oxidative stress, in order to maintain cellular homeostasis and cell survival. Three different types of cell autophagy have been described [71, 72]: macroautophagy (MA), commonly referred to as autophagy, which involves the formation of a double-membrane structure containing the cytoplasmic material; in this compartment, termed the autophagosome, proteins and organelles are first engulfed, then digested by lysosomal enzymes, and finally recycled to be utilized again for cellular metabolism, development, and homeostasis [73, 74]. In microautophagy (MI) lytic organelles take up cytoplasm directly [75], while in chaperone mediated autophagy (CMA) substrates are selectively recognized by a cytosolic chaperone and then dispatched for degradation in lysosomes [76]. Given its essential role in cellular homeostasis, autophagy is involved not only in different physiological processes but also in several human diseases, including obesity, diabetes, cardiovascular diseases, neurodegenerative disorders, and cancer [77–79]. The role of autophagy in cancer has been considerably studied and found to have dual roles, acting as both a tumour suppressor and as a mechanism of tumour survival. In early tumourigenesis, acting as a quality-control mechanism, autophagy prevents tumour initiation and suppresses cancer progression; in contrast, once the tumour progresses to a later stage, when it is established and subjected to environmental stresses, autophagy, as a dynamic degradation and recycling system, contributes to the survival and growth of the tumour and promote its aggressiveness [80]. In this context, lysosomal Ca^{2+} signalling can play a key role in the autophagic process in cancer.

TRPMLs in autophagy in cancer

Cancer cells are subjected to stress and a lack of nutrients due to the high degree of growth they undergo. Ion channels, like TRPML1, acting on the autophagic process, can therefore determine the survival of cancer cells. Kasitinon et al. [50] have demonstrated that TRPML1 has the function of inhibiting the MAPK pathway and mTORC1 signalling in melanoma cells, increasing tumour survival. Lack of TRPML1 impairs the survival of melanoma cells, but tumour growth and development are restored when these kinases are inhibited . Yang et al. [53] have demonstrated that mTORC1 functions downstream of TRPML1 and CaM (calmodulin) controlling lysosome size. In particular, TRPML1, CaM and mTORC1 form a macromolecular complex necessary to control mTORC1 activity, demonstrating an mTORC1-dependant molecular mechanism for lysosomal membrane fission. Xu et al. have determined the role of TRPML1 in autophagy in triple-negative breast cancer (TNBC) [52]. Their study demonstrated that TRPML1 is specifically increased in TNBC cell lines under stressful conditions, and is associated with increased mTORC1 activity and lysosomal ATP release that promotes TNBC growth and invasion. In an independent study, using glioblastoma primary cancer cells and glioblastoma cell lines, Santoni et al. [51] have demonstrated that TRPML1 silencing induces autophagy, nitric oxide (NO) production and cathepsin B-dependant apoptosis, and that apoptotic-resistant cells proliferate at a higher rate than control cells. In a more long-term fashion, in glioblastoma cell lines, TRPML1 silencing enhanced survival and invasion ability. Rühl et al. [81] have identified the first highly potent and isoform-selective TRPML1 antagonist, the steroid 17β-oestradiol methyl ether (EDME), and reported that in the MDA-MB-231 human breast cancer cell line this compound abated autophagy as well as migration and invasion. TRPML1 can affect the balance between inactive (phosphorylated and cytoplasmic) and active (dephosphorylated) TFEB, the latter being capable of translocating to the nucleus and regulating lysosomal and autophagic genes. The balance between these two TFEB states can affect the regulation of Ca^{2+}-sensitive proteins such as calmodulin and calcineurin [82]. In particular, according to a recent study [83], TRPML1 stimulation triggers the sequential activation of Ca^{2+}-dependant kinases, resulting in acute autophagic response, while TFEB nuclear translocation is necessary for sustained autophagy and is mediated by calcineurin. In addition, it has been shown that the modulation of autophagic flux is a multistep process that could be also independent from TFEB translocation. In addition, a negative feedback regulation has been described between mTORC1 and TRPML1, mediated by calmodulin, which is necessary to prevent uncontrolled loss of mTORC1 and to maintain cellular homeostasis during starvation or disease conditions [84, 85].

The evidence so far available show that TRPML1 can play different roles depending on the tumour type and on the tumour microenvironment. Analogously to its impact on autophagy, loss of this channel can either increase or inhibit tumour growth, depending on the context and stage of the disease, as well as on the genetic background. As for TRPLM2 and TRPML3, to our knowledge there is no evidence about the role of these channels in tumourigenesis, and their functions in this context await further investigation.

TPCs in autophagy in cancer

Two-pore channels (TPCs), which are cation-permeable channels on the membranes of the endolysosomal compartment, are important mediators of intracellular Ca^{2+} signalling, involved in a great number of pathophysiological processes that include cell growth, development,

metabolism, cancer progression, as well as endolysosomal trafficking, lysosomal exocytosis, and autophagy [4, 86]. In particular, the autophagy-lysosomal pathway is associated with hallmarks of cancer, such as escape from cell death pathways, evasion of immune surveillance, and deregulation of metabolism. Recent evidence has contributed to an interesting debate about the role of TPCs in autophagy and in cancer. Thus, on the one hand, TPC2 has been reported to promote or block the autophagic process at different stages in such different cell lines as astrocytes, cardiomyocytes, and skeletal muscle [87–89]. A recent study investigating the role of TPC2 in autophagy in cancer cells reported that overexpression of TPC2 in the 4T1 mouse breast cancer cell line and in the HeLa human cervical cancer cell line reduced autophagosomal–lysosomal fusion, resulting in the accumulation of microtubule-associated protein light chain 3 (LC3)-II and syntaxin17 (STX17)-positive autophagosomes [65]. In addition Lin et al. [88] have observed that in the skeletal muscle of TPC2 KO mice there is an increase of autophagy , that is exacerbated in response to colchicine. On the other hand it has been reported that activation of the lysosomal Ca^{2+}channels TPC1 and TPC2 by NAADP and glutamate induced autophagic flux in immortalized rat astrocytes and SHSY5Y neuroblastoma cells [90]. An explanation for this dualistic role of TPCs could likely result from the analysis of the tumour genetic background, as the deregulation of TPC2 by pharmacological inhibition [31, 86, 91, 92], or by CRISPR/Cas9 gene editing [25], could have a different effect on cell lines displaying different mutations; how tumour genetic background impact on TPCs functions is an issue that deserves further attention [93].

P2X4R in autophagy in cancer

Purinergic signalling receptors for extracellular nucleotides (P1 and P2 receptors) are widely expressed by mammalian cells. The P2 receptors are divided into P2X and P2Y groups and each group contains several members with distinct ion selectivity and regulatory properties. In contrast to cell surface P2 receptors, the P2X4 receptor (P2X4R) is predominantly localized on lysosomal and/or lysosome-related organelles, and controls Ca^{2+} released from lysosomes, which in turn drives lysosomal membrane fusion with late endosomes, autophagosomes, phagosomes, and the plasma membrane, resulting in exocytosis [16]. This channel, the most ubiquitously expressed P2X receptor in mammals, is an ATP-gated receptor, as demonstrated in whole-lysosome patch clamp experiments [94], in which this receptor was activated by luminal ATP provided that the pH was raised to 7.4. P2X4R is required for endolysosomal membrane fusion with intracellular organelles; in fact it has been demonstrated [95] that overexpression of P2X4R, as well as increasing endolysosomal activity by alkalinization of the endolysosome lumen, promoted vacuole enlargement in cells and endolysosome fusion in a cell-free assay. P2X4R and calmodulin (CaM) form a complex at the endolysosomal membrane where P2X4R activation recruits CaM to promote fusion and vacuolation in a Ca^{2+}-dependant fashion. Moreover, P2X4R activation-triggered fusion and vacuolation were suppressed by inhibiting CaM. In addition, the silencing of P2X4R led to a decrease in viability and proliferation in different glioblastoma multiforme (GBM) cell lines, through the inhibition of BDNF/TrkB signalling in U87 and T98 cells, thereby suppressing the expression of the activating transcription factor 4 (ATF4) [38]. These two molecules, CaM and ATF4, are directly correlated to autophagic flux [40, 96], suggesting a possible role of P2X4R also in autophagy; data linking this channel to the autophagic process can be found in an article [97] in which, through thermal proteome profiling, the membrane-bound P2X4R was identified as a target of the autophagy inhibitor indophagolin. Further studies are still needed to define the role of P2X4R in the autophagic process, both in healthy cells and in cancer.

Endolysosomal Ca^{2+} signalling regulates immune cell repertoire in the tumour microenvironment

As shown in a number of reviews summarizing the most recent findings regarding the endolysosomal Ca^{2+} channels expressed in cancer cells involved in "autocrine" pro- or anti-tumour biological processes in various types of tumours [19, 41, 46, 47, 98, 99], the role of these channels in immune cells in the tumour microenvironment (TME) has been poorly investigated, although accumulating evidence about the importance of endolysosomal channels in immunity and inflammation have recently been emerging [100, 101]. In this context, the crosstalk between innate and adaptive responses is important in maintaining a functional immune system in order to protect the individual against foreign substances such as allergens, toxins, viruses, bacteria, and tumour cells. The innate immune system involves monocytes, macrophages, dendritic cells, mast cells, basophils, neutrophils, eosinophils, and natural killer cells (NK), while the adaptive immune system is composed of B cells and T lymphocytes. On this basis, endolysosomal Ca^{2+}signalling could be an important factor in the regulation of TME immune cells [102–104]. For instance, in the previous section, we described the involvement of lysosomal Ca^{2+}signalling in autophagy and, although detailed mechanistic steps remain to be elucidated, increasing evidence implicates autophagy in the secretory pathway, showing that it plays an active role in modifying the molecular composition of the tumour secretome; this, in turn, can heavily impact on functions of cells in the TME, in particular tumour-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs), resulting in local immunosuppression and defective tumour surveillance [105, 106]. Cancer cells can influence TAMs polarization by releasing cytokines, glucocorticoids, extracellular vesicles, and extracellular matrix components, giving rise to a large spectrum of pro-tumoral macrophages [107]; pro-tumoral macrophages influence cancer progression by controlling adaptive immunity, angiogenesis, tumour cell proliferation and metastasis, playing a crucial role also in cancer resistance to treatment [108]. Based on these data and on the well-known TAM plasticity, TAM re-education toward a pro-inflammatory, anti-tumorigenic functional status has been proposed as a strategy to promote tumour inhibition in prostate cancer [109]. Besides the involvement of endolysosomal Ca^{2+} channel-mediated pathways in autophagic processes, another cellular mechanism hijacked by tumour cells to evade chemotherapeutics, independently of the expression of multidrug resistance (MDR) transporters, is based on their ability to efflux lysosomotropic drugs via upregulation of lysosomal exocytosis. By promoting lysosomal exocytosis, TRPML1 may also participate in drug resistance by releasing sequestrated anticancer drugs. In accordance with this, a study highlighted that inhibiting lysosomal exocytosis by verapamil, an FDA-approved Ca^{2+}channel blocker, rendered rhabdomyosarcoma cells sensitive to doxorubicin [60]. Notably, autophagy induced during cancer therapy coupled to lysosomal degradation has been recognized as a key mechanism of immunosurveillance and resistance to immunotherapy [66]. In two recent reports, activation of autophagy has been linked to selective lysosomal degradation of MHC-I and immune evasion of pancreatic cancer cells. Reduced expression of MHC-I at the cell surface of cancer cells results in failed recognition of these cells by CD8+ T cells, hampering the efficacy of immunotherapy. In contrast, inhibition of autophagy and lysosomal degradation restores surface levels of MHC-I, leading to improved antigen presentation and an enhanced anti-tumour T cell response. In this model, inhibitors of autophagy sensitize tumours to immune checkpoint blockade therapy [110, 111]. The engagement of the host immune system by an evolving malignancy is complex, involving a myriad of cell types and cell signalling phenomena, as a result of dynamic intercellular molecular dialogue between the tumour and stroma; non-neoplastic cells gain specific phenotypes and functions that are pro-tumorigenic. Besides the endolysosomal Ca^{2+} signalling involved in the tumour secretome that influences the immune cell microenvironment, a role has also recently

been shown for these channels, mainly TRPMLs and TPCs, in the immune cells themselves. In fact, a recent study stated that lysosomal Ca^{2+}channels expressed in NK cells regulate effector function; thus activation of the lysosomal Ca^{2+}channel TRPML1 reduces granzyme B production [112, 113]. The same TRPML1 ER-derived Ca^{2+} stores are involved in the classic killing mechanisms of T cells [114]. The expression of TRPMLs in cancer and TRPML-regulated anti-tumour immunity are discussed in a recent review, highlighting the impact of TRPMLs on immune function through regulation of lysosomal exocytosis, endocytosis, and phagocytosis [47]. The founding member TRPML1 is expressed in a number of tissues including adrenal gland, lung, bladder and placenta as well as in thymus, spleen and immune cells [101]. Although TRPML2 is found in most organs, it is abundant in immune cells and tissues, its mRNA being mainly detected in lymphocytes and other cells of the immune system [115]. TRPML2 is also involved in antitumour immunity mediated by dendritic cells (DC). In fact, DCs efficiently present tumour antigen from tumour cell-derived microparticles to CD8+ T cells, through TRPML2 activity [116]. Moreover, the levels of TRPML2 are dramatically upregulated in macrophages upon TLR activation. TRPML2 KO mice displayed impaired recruitment of peripheral macrophages in response to i.e. injections of LPS or live bacteria with a severely reduced production of several chemokines, in particular CCL2, suggesting a novel role for TRPML2 in the innate immune response [115, 117]. M1 and M2 macrophage subsets are different in terms of phenotype and functions, with the classically activated macrophages (M1) having an antitumour activity, whereas M2 macrophages display immunosuppressive and pro-tumour functions. A recent study reported that the TRPML1 pathway regulates an anti-tumour immune response through resetting TAMs towards a tumour-killing M1 phenotype [118]. Regarding TPCs, the other endolysosomalCa^{2+}channels considered to play a role in the TME, recent studies demonstrated that novel TPC agonists appear to regulate the relative Na^{+}/Ca^{2+}permeability and voltage-dependence of TPCs, suggesting that different agonists binding at different sites on TPCs differentially affect endolysosomal functions [119]. Pethő et al. underline the role of pH in cellular physiology and the role of ion channels, such as TPCs, that can determine alterations in intracellular and extracellular pH. These alterations of pH affect cell fate, cell metabolism and growth of the tumour cells and, in the tumour microenvironment, are pivotal for the functions of immune cells both in innate and adaptative immunity [120]. In particular, the upregulation or the downregulation of these channels can affect phagocytosis and micropinocytosis and regulate endocytic traffic in macrophages [121]. Furthermore, Chen et al. have demonstrated that TPCs and TRPMLs are involved in the osmotically-driven changes in the surface-to-volume ratio of endolysosomes in order to promote endocytic and recycling traffic in innate immune cells, also in this case in macrophages [102]. Moreover, Davis et al. have shown that NAADP and Ca^{2+} signalling are needed for the scission of the phagosomes during the last phase of phagocytosis and that this process is correlated with the activation of calcineurin and dynamine [122]. Arlt et al. have analysed the role of TPC1 and endolysosomal Ca^{2+} signalling in the development of the immune response with the release of inflammatory mediators, in the interplay between the endoplasmic reticulum and lysosomes [123]. Pharmacological inhibition or genetic deletion of TPC1 enhanced mastocyte degranulation and release of histamine, while the number of mastocytes was reduced in TPC1 KO mice compared to WT controls [122]. Concerning adaptative immunity, a cytotoxic T lymphocyte (CTL) kills a tumorigenic cell by Ca^{2+}-dependant exocytosis of cytolytic granules at the immunological synapse formed between the two cells. Davis et al. have demonstrated that although IP_3-mediated Ca^{2+} release from the endoplasmic reticulum activates the store-operated Ca^{2+}-influx pathway required for exocytosis, this is not sufficient in itself for this process to occur. These studies have identified a new role for the Ca^{2+}-mobilizing messenger NAADP and for TPCs present on the granules, in T cell receptor signalling in CTLs [100, 124].

The field of immunology related to Ca^{2+} signalling is still largely unexplored, and many aspects of the cellular crosstalk between the tumour and the immune system remain to be understood, therefore further studies are needed in this direction to try to better understand the tumour microenvironment and the role of lysosomal Ca^{2+}channels.

Concluding remarks and perspectives

In recent years the number of cellular functions in which the involvement of lysosomes has been demonstrated has tremendously increased. Interestingly, most novel findings have contributed to the discovery of interactions not only between intracellular compartments or metabolic pathways, but also between the endolysosomal system and the environment surrounding the cell or even distant sites. A few examples are worth mentioning, though these are far from exhaustive:

Nutrient sensing: The mTOR signalling network is hyperactivated in many tumors and its lysosomal component mTORC1 controls autophagy through TFEB (together with Ca^{2+}-binding calcineurin); *Calcium-driven lysosomal exocytosis*: Lysosomal exocytosis is a process leading to the secretion of lysosomal content upon lysosome fusion with plasma membrane causing the release of lytic enzymes and acidification of the microenvironment as well as the release of ATP, which can favour metastasisThe lysosomal TRPML1 channel regulates triple negative breast cancer development by promoting mTORC1 and purinergic signalling pathways [52]; in addition, lysosomal exocytosis modifies the composition of the cell membrane, thereby affecting signalling events and immune cell recognition while contributing to multidrug resistance and cancer evasion, (reviewed in [66]); endolysosomal exocytosis entails also the release of extracellular vesicles and exosomes [125] carrying signals from cancer cells to near and distal sites] [42,126] and from CAFs, fuelling cancer progression through immunomodulation of the TME and the release of growth and invasion promoting factors [127]; recently, involvement of endolysosomal trafficking in viral control [91,128–130] and even in stem cell identity transition, have been reported [131], both of potential oncological interest. In particular, given that TPCs play a role in lysosomal trafficking and exocytosis and that β-coronavirus, like many other viruses, traffic to the lysosome and egress by lysosomal exocytosis [132], these data open the way to novel anti-viral therapeutic approaches by targeting endolysosomal calcium channels. Will the emerging central role which the endolysosomal system and its ion channels are being shown to play, increasingly impact on cancer research and disclose whether cancer cells with altered expression of lysosomal Ca^{2+} channels are more sensitive to pharmacological modulation? No doubt it will, also given the prevalence of lysosomal activity in various types of cancer. The big challenge in anticancer strategies is to be able to target both efficiently and selectively the factors hierarchically responsible for the cascade of processes distorting the physiology of the cell. While convincing evidence demonstrates that the endolysosomal system is a crucial signalling hub in cancer, controlling the autophagic and extracellular release pathways, before this can be translated into therapeutic approaches a great deal of information is needed; for instance, for each cancer type and stage what are the specific upstream activators of each channel or combination of them, and how are fundamental processes like survival, invasion, and exchange of signals, individually controlled by the activation of these triggers? From the variety of functions involving Ca^{2+} release from endolysosomal channels one can expect this machinery to be more finely tuned than presently known, in its individual components as well as in the interactions amongst them and with the master regulators of cancer phenotype.

Increasing evidence is showing that the fate of a cancer cell is strictly linked to the composition and function of its microenvironment: vascularization, regulating nutrient and oxygen supply, cells of the immune system playing against or in favour of the malignant state, cancer associated fibroblasts, even the details of collagen organization [133], all contribute to the onset and progression of the disease. It is in this

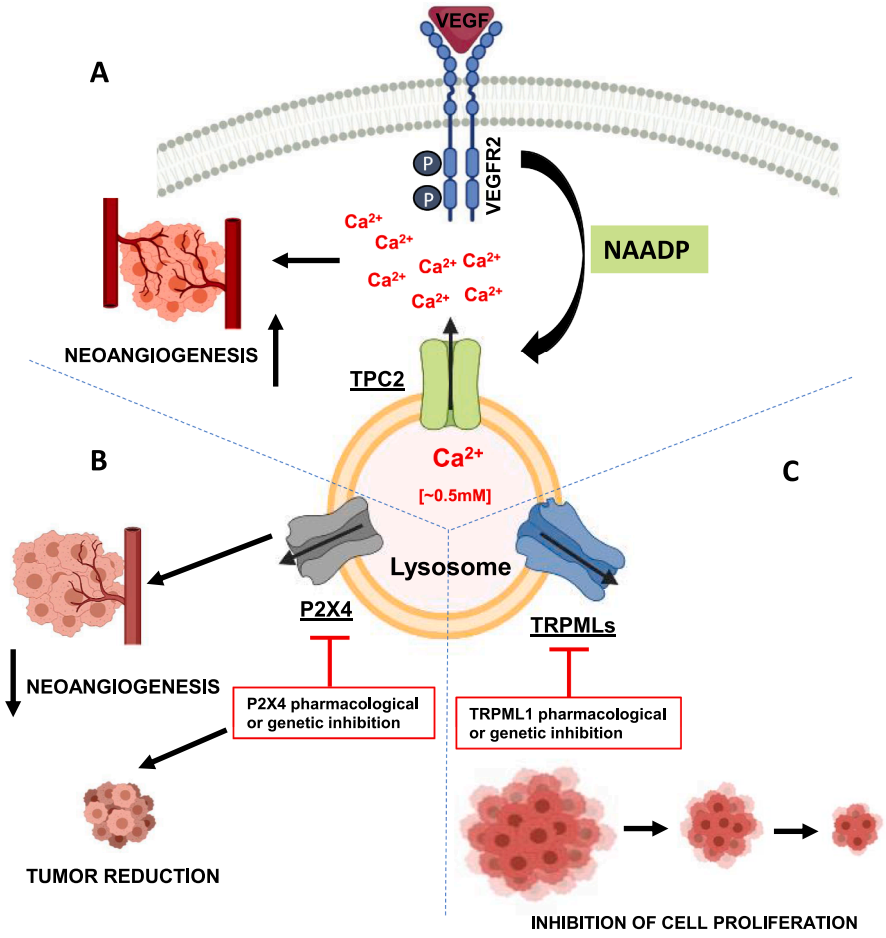

Fig. 2. Role of lysosomal Ca^{2+}channels in neoangiogenesis and tumor progression. (A) Two–pore channel 2 (TPC2) controls neoangiogenesis mediated by the activation of Vascular Endothelial Growth Factor Receptor 2 (VEGFR2), through NAADP-mediated release of lysosomal Ca2+. (B-C). Genetic or pharmacological inhibition of P2X4R and TRPMLs can block neoangiogenesis and reduce cell proliferation and tumour growth.

broad framework that the involvement of lysosomes in cancer is to be investigated, further disclosing the role of this versatile system in the whole array of cell types present in the tumour microenvironment (from endothelial cells to macrophages, through to lymphocytes, fibroblasts etc.) and, importantly, in the exchange of short- and long-range signals. Certainly, methodological restrictions need to be overcome. This richly interwoven network is in fact poorly reproduced in most in vitro models, that are often limited to cell lines and hardly ever utilize human primary tumours. Animal experimental models and genetic manipulation do offer the possibility of studies in a more instructive context, but species differences are a great hurdle, and most animal models used so far to examine human cancer cells have a defective immune system. On the other hand, the increasing wealth of molecular data on such somewhat reductive experimental models can represent an invaluable tool when used in the cross-analysis of clinical data combined with genetic profiling. Molecular analysis of the heterogeneity of cancer cell phenotypes down to the single cell level and statistical screening of clinical datasets, represent powerful allies for the challenge of bridging cell biology and clinical findings. In this respect, screening human cancer data for well identified molecular components of the endolysosomal system, in particular its set of ion channels and their downstream far-reaching effectors, has huge potentialities to shed light on specific cancer pathways. Towards this direction, our very recent study of clinical data from the UK Biobank has analysed TPC2 and P2RX4 polymorphisms, shedding light on their complex role in the onset and progression of different cancers [41]. The use and perspective advancement of technology, e.g. proteomics, nanotechnologies, and particularly bioinformatics could improve our understanding of the hierarchy of linear signalling pathways showing how signalling networks are organized and interact with one another, comparing a huge amount of data coming from the interplay of metabolic, genetic and pharmacological studies. This will certainly contribute to enrich not only our knowledge of specific cancer pathways but also our operational anti-cancer armoury towards a common therapy-orientated goal.

Fig. 2

CRediT authorship contribution statement

Samantha Barbonari: Writing – original draft, Formal analysis, Conceptualization. **Antonella D'Amore:** Writing – original draft, Formal analysis, Conceptualization. **Fioretta Palombi:** Conceptualization, Writing – review & editing. **Paola De Cesaris:** Data curation, Conceptualization. **John Parrington:** Conceptualization, Writing – review & editing. **Anna Riccioli:** Supervision, Conceptualization, Writing – review & editing. **Antonio Filippini:** Supervision, Conceptualization, Writing – review & editing.

Conflicts of interest statement

No potential conflicts of interest are disclosed.

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