Chapter 12
Role of Mechanosensitive TRP Channels in Abnormal Vasculature of Tumors

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Abstract Solid tumors necessitate vascularization for metabolic support and metastasis, relying on the process of angiogenesis to form new blood vessels. However, the constant stimulation of endothelial cells from pro-angiogenic soluble factors and mechanical forces creates a tumor vasculature that is structurally and functionally abnormal. Most anti-angiogenic therapies have focused on targeting VEGF signaling to pursue the tumor vasculature. However, these anti-VEGF therapies have been met with limited success in clinical trials. Hence, recent studies have started to investigate the role of mechanical signaling in tumor angiogenesis as it occurs in a mechanically dynamic environment. This chapter focuses on mechanosensitive ion channels that belong to the transient receptor potential (TRP) superfamily, with special emphasis on the role of TRPV4 in the endothelium, as well as deregulation of TRPV4 signaling within the tumor endothelium, and its potential as a target for normalization of tumor vasculature to improve cancer therapy.

Keywords Angiogenesis • Calcium • Endothelial cells • Extracellular matrix • Mechanotransduction • TRPV4 • Tumor

Introduction

The oxygen and nutrient demands of solid tumors make them dependent on angiogenesis for new blood vessels to grow and metastasize. However, the continuous needs of the tumor create vessels that are structurally and functionally abnormal

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[1, 2] and result in a hypoxic environment due to insufficient delivery of nutrients and reduced waste clearance [3]. Tumor angiogenesis is driven by numerous factors in attempts of sprouting new vessels [4] which include acidosis, inflammatory cytokines, as well as the activation of oncogenes that occur at the cellular level, upstream of VEGF and other tumor promoting hormones [5, 6]. Importantly, the tumor extracellular matrix (ECM) becomes stiffer due to the release of plasma components through the leaky tumor vessels as well as dynamic remodeling of these components by tumor and stromal cells. Further, an increase in interstitial fluid pressure in tumors creates turbid blood flow [7] impeding the delivery of cancer drugs. Together, these mechanical forces create an abnormal micromechanical environment to which the tumor vasculature does not respond properly. Therefore, recent studies have started to target the mechanical aspects of the tumor microenvironment for cancer therapies, with specific interest on ion channels. The transient receptor potential (TRP) family of ion channels has already gained much attention in cancer, especially those that exhibit mechanosensitive properties. This chapter will cover the abnormal tumor vasculature, and aims to examine the role of mechanosensitive ion channels involved in the aberrant environment, with a special focus on TRPV4.

Tumor Vasculature

As with any organ, the endothelium can be subjected to dysfunction and failure. Such is the case of the tumor vasculature, the structure of which is controlled by irregular intrinsic and extrinsic factors. These abnormalities generate microvasculature that contain regions of heterogeneity, with areas within the tumor being "hyper" or "hypo" vascular in both function and density [8, 9]. At the cellular level, pro-angiogenic factors generate the weakening of VE-cadherin-mediated endothelial cell (EC) junctions, which in turn distort the vessel wall structure and promote EC migration [10]. Pericytes that cover the endothelium, generally create a stable and mature vascular network, but in tumors have been found reduced in number, and loosely attached to the EC, causing vessels to become immature and leaky. This effect is the result of increased growth factor signaling, such as vascular endothelial growth factor (VEGF), which has been found to impede the adherence of pericytes to the surrounding endothelium [11–13]. Overall, these new vessels are not only tortuous and dilated, but lack structural support [8], endorsing dysfunctional blood vessels that promote erratic blood flow [8, 14] and hyper-permeability that allow protein and fluid extravasation into the extracellular space, all of which increase matrix deposition [8]. Additionally, the growing tumor mass often times leads to the compression and eventual collapsing of the existing vessels, further diminishing blood supply and increasing hypoxic and acidic conditions within the tumor [8, 15]. Altogether, the structural and functional aberrations of the tumor vasculature are prompted by the persistent needs of the tumor cells to vascularize and the constant stimulation of angiogenesis.

Tumor Angiogenesis

The progression of solid tumors relies heavily on angiogenesis and requires the endothelium to switch from a quiescent, or resting, phenotype to one that is more invasive, termed the "angiogenic switch"[16]. To begin this process, the basement membrane must be injured or wounded, triggering destruction and hypoxia of the surrounding tissue. Pro-angiogenic factors are released to stimulate EC to migrate, proliferate, and stabilize. However, in tumor angiogenesis, not only are pro-angiogenic factors up-regulated, but angiogenic inhibitors must be down-regulated to maintain adequate stimulation of angiogenesis [17]. Overall, the local equilibrium of the tumor microenvironment is unbalanced, and considering the abnormalities of the tumor vasculature, makes it pertinent to address the complexities associated with tumor EC (TEC) themselves.

Tumor Endothelial Cells (TEC)

Altogether, the structural and functional aberrations of the tumor vasculature promote drastic genetic and morphological changes within the cells [18]. These factors can then generate tumor cells that demonstrate a more hostile phenotype with potential for metastasis [19]. It is now known to a large extent that the tumor endothelium is defective, with TEC showing distinct irregularities with respect to shape and size when compared to normal endothelial cells (NEC). These cells tend to have long cytoplasmic projections that can extend across the lumen, with the tips of some TEC protruding into the lumen, creating intercellular gaps within the vessel wall that most often result in the leakage or pooling of blood [20].

At the molecular level, the tumor endothelium, unlike the normal endothelium, express a host of genes, recently identified as transmembrane proteins called tumor endothelial markers (TEM) [21, 22]. In-depth studies on TEC isolated from different carcinomas, show that these cells do not undergo senescence, are resistant to serum starvation and apoptosis, and are structurally abnormal compared to NEC. TEC isolated from mouse xenograft tumors were found to have variable DNA content, not only between its normal counterpart cell types, but between individual cells as well, indicating the existence of heterogeneity within TEC. These cells have larger nuclei and exhibit characteristic cytogenic and structural abnormalities, such as aneuploidy and chromosomal aberrations including deletions, non-reciprocal translocations, and abnormal centrosomes [23–26]. Furthermore, TEC isolated from mouse prostate tumors were found to express both hematopoietic and mesenchymal stem cell markers, verifying the heterogeneity of these cells. Additionally, it was found that these cells were also able to undergo unusual mesenchymal differentiation into cartilage and bone-like tissues in conditioned medium, confirming the ability of TEC to adapt to their surrounding environment [27]. Based on these studies, it can be interpreted that the cellular and molecular aberrations of TEC can invariably contribute to the abnormal angiogenic process and ultimately tumor growth and metastasis.

However, when grown in defined endothelial medium, TEC (derived from mouse prostate tumors) express endothelial markers and show morphological features similar to NEC [27]. Interestingly, we have demonstrated that TEC show aberrant mechanosensing and abnormal angiogenesis *in vitro*, suggesting deregulation of mechanosensing mechanisms in these cells [28]. A fascinating question, that has yet to be considerably studied, pertains to the origin of these TEC. A variety of studies [29, 30] have highlighted that the source is from stem-cell tumor cells, to "circulating $CD34^{+}/VEGFR\text{-}2^{+}$ endothelial progenitor cells"; however, there has been limited progress in conclusively identifying the source of these cells, making it difficult to develop any specific targets as part of an anti-angiogenic strategy.

Current Approaches to Target Tumor Angiogenesis

Since first described by Dr. Folkman [3], the concept of angiogenesis and anti-angiogenic therapies has revolutionized the way cancer has been studied and clinically treated. Inhibition of angiogenic activators such as VEGF, placental growth factor (PIGF), fibroblast growth factor (FGF) [31] and associated growth factor signaling mechanisms, have provided new avenues in successfully treating as well as studying tumor angiogenesis. Anti-VEGF therapies, including anti-VEGF neutralizing monoclonal antibodies and receptor tyrosine kinase inhibitors (RTKIs), dominate current approaches in treating malignant tumors [32, 33], initially achieving great success. One such study found that treating patients of metastatic colorectal cancer with anti-VEGF antibody, Bevacizumab, in combination with systemic chemotherapy, increased patient survival [34], which was later attributed to tumor 'vascular normalization.' The principle of vascular normalization seeks to restore the balance of angiogenic factors within the tumor microenvironment to regulate vessel growth and maturity [8, 35]. This allows for improved delivery of chemotherapeutic agents by reestablishing a more normal vascular network. While anti-VEGF-mediated approaches hold promise, it has a narrow window in terms of the transient nature of the resulting 'normal' vessel. Furthermore, these growth factor mediated therapies have become redundant, ineffective, and in some instances, detrimental to the treatment of cancer, owing to inherent or acquired drug resistance, the potential for metastatic capability, and the general absence of predictive markers to monitor tumor responses in select patient populations [36]. These findings were further substantiated in a recent finding that demonstrated a rapid decrease in delivery of chemotherapy to the tumor after anti-VEGF therapy in non-small cell lung cancer (NSCLC) patients using PET imaging [37].

Limitations in targeting soluble factors have led to the idea that other factors, such as mechanical forces, may be contributing to the abnormal tumor vasculature. In fact, defects in mechanotransduction, the conversion of mechanical forces into biochemical signals, have been reported as the basis of diverse pathological conditions [38, 39]. A number of studies have also described a balance of underlying mechanical forces as driving factors in sensitizing capillary EC to angiogenic

growth factors, to form functional networks of blood vessels. Furthermore, EC are exposed and respond to mechanical forces such as shear stress and cyclic strain imposed by blood flow. In fact, it was postulated that local micromechanical forces modulate the endothelial response to growth factors [40]. One of the fastest responses of EC to mechanical forces is Ca^{2+} influx through mechanosensitive ion channels. Ion channels have already been the subject of various reviews, as they have recently been found up-regulated/down-regulated or simply dysfunctional in different pathological diseases. However, the role of mechanosensitive ion channels in physiological or pathological angiogenesis is largely unknown. Thus, targeting ion channels in the mechanically dynamic tumor microenvironment may lead to novel therapies among the abnormal tumor vasculature.

TRP Channels

The onset of neovascularization and subsequent tumor progression is associated with the generation of cell populations that differ phenotypically, which often arise from a deregulation of key signaling pathways and mutations or deletion of several proteins. Transient receptor potential (TRP) channels represent a superfamily of proteins that have, over the years, been understood to affect or be affected by a variety of pathological conditions, including cancer. In addition to the transcriptional regulation of TRPs by hormones and growth factors produced by the tumor microenvironment, alternative splicing of genes leads to the generation of protein isoforms with altered functions and variations in subcellular localization. Such effects have been documented in diverse tumor types where a decrease in Ca^{2+} influx may be attributed to decreased expression of TRP channels [41].

A growing body of evidence has identified members of the TRP family, mainly members of TRPC (canonical), TRPM (melastatin) and TRPV (vanilloid), as key regulators of mechanotransduction [42–45]. TRP channels have a profound effect on EC function, and any dysregulation of these channels can result in EC dysfunction [46]. Because TRP channels are found in the plasma membrane, they are easily and directly accessible to the blood stream, which make these channels potential molecular targets when vascular diseases arise. Below, we specifically describe mechanosensitive TRP channels expressed in the endothelium, most of which are involved in a variety of cancers. Although we have tried to incorporate all relevant studies of these channels in tumor angiogenesis, much of this field remains vastly unexplored, with potential to investigate new targets.

TRPC

TRP canonical channels (TRPC), the founding member of the TRPs, are made up of seven family members (1–7) that are expressed in the endothelium [47], as well as the surrounding smooth muscle cells [48]. TRPC channels support endothelial

function such as vascular regulation, permeability, and the endothelial-derived nitric oxide (NO) mediated vasorelaxation of smooth muscle cells. While all of these essential functions may be necessary for the angiogenic process, TRPC1 and TRPC6 have been implicated in mediating mechanotransduction. Studies propose TRPC1 is a stretch-activated channel while others have confirmed TRPC6 is activated by pressure as well as osmotically or mechanically induced plasma membrane stretch [47]. While the role of TRPC1 in angiogenesis has been confirmed, most of the evidence suggests that the mechanosensitive properties may not be obligatory, but that TRPC1 may act alongside other TRPC channels to carry out mechanotransduction. Additionally, TRPC1 and TRPC6 have been found to respond to inflammatory agonists resulting in EC cell shape changes [49, 50]. TRPC6 has also been found in human pulmonary arterial endothelial cells (HPAEC) to control endothelial contraction, cell shape, and permeability [49, 51]. In regards to the cancer environment, many TRPC channels have been found to exist among several different types of cancer tissue, including breast cancer [52], ovarian cancer [53, 54], hepatoma [55], prostate cancer [56], basal cell carcinoma [57], renal cell carcinoma [58], malignant gliomas [59], glioblastoma [60], gastric tumors [61], and lung cancer [62]. However, there have not been any studies to date that suggest TRPC channels are directly involved in the tumor vasculature.

TRPM

TRP melastatin channels (TRPM) are the largest and most diverse among the TRP superfamily. Made up of eight family members (1–8), these channels are widely expressed in endothelial cells and vascular smooth muscle [47], making them important for normal vascular function. When it comes to vascular mechanosensing, both TRPM4 and TRPM7 have been found to contribute to mechanotransduction. In cerebral artery myocytes, TRPM4 is activated by membrane stretch [63] and in cerebral artery smooth muscle cells, take part in mediating membrane depolarization and myogenic vascular tone [64]. Furthermore, some studies have suggested that TRPM4 activation by stretch may be secondary to Ca^{2+} responses [63–65]. Additionally, TRPM7, which acts as an ion channel and a functional kinase, can be activated by cell stretch and swelling to carry out mechanotransduction [66]. TRPM7 is highly permeable to Mg^{2+}, which contributes to the role TRPM7 plays in angiogenesis and vascular remodeling due to the diverse effect of Mg^{2+} on EC function [67]. Pathologically, some studies suggest that TRPM4 may be involved in decreased NO production [68], while TRPM7 plays a role in angiogenesis and oxidative stress induced cell death [47, 69]. Recent TRPM7 studies in ovarian cancer have found that TRPM7 is needed for cancer cell growth, migration, and invasion [70], but TRPM4 nor TRPM7 channels have been found to affect the tumor endothelium.

TRPP and TRPA

The TRP polycystin (TRPP) ion channel family is well known in polycystic kidney disease (PKD) [71]. Expressed ubiquitously among vertebrates, these channels are involved in mechanotransduction due to TRPP1 and TRPP2 channel activation by shear stress [47]. These channels are important for flow-induced vascular response and NO production [47, 72].

TRPA1 is the only mammalian member to belong to the TRP ankyrin (TRPA) family. Although predominately expressed in nociceptive neurons of the peripheral ganglia and the mechanosensory epithelium of the inner ear [73], this mechanosensitive ion channel has recently been found expressed in the endothelium as well as the surrounding perivascular cells in cerebral circulation [74]. Overall, TRPA1 mainly plays a role in nociception [75], mechanotransduction [76], and thermal [77], and oxygen sensing [78]. The only studies of TRPA1 in the endothelium were performed by Earley and colleagues [79], which found activated TRPA1 stimulated endothelium-dependent smooth muscle cell hyperpolarization and vasodilation. Pathologically, TRPA1 is involved in acute and chronic pain and possibly chronic inflammatory diseases, and only few studies have been performed in regards to TRPA1 in cancer [80, 81].

TRPV1

The TRP vanilloid (TRPV) family of channels has received the most attention in regards to mechanotransduction in the vasculature, specifically TRPV1 and TRPV4, both of which are expressed in the endothelium [47]. The first family member, TRPV1, is activated by capsaicin [82], anandamide, arachidonic acid (AA) [83], PIP2 hydrolysis [84], acidity, and noxious heat ($T > 43$ °C) [82], and is important for NO production and endothelium-dependent vascular relaxation [85, 86]. TRPV1 has been implicated to mediate the later stages of angiogenesis from various studies. In human umbilical vascular EC (HUVEC), TRPV1 activation inhibits VEGF-induced proliferation, DNA synthesis, and capillary-like tube formation. Additionally, activation of TRPV1 via capsaicin also inhibited VEGF-induced vessel formation and vessel sprouting in mouse Matrigel plug and rat aortic ring assays, respectively [87]. When it comes to mechanosensation, TRPV1 was originally discovered to take part in inflammatory thermal hyperalgesia, nociception, and pain sensation [88, 89]. TRPV1 expression has been found altered in cancers of the prostate [90, 91], colon, pancreas [92, 93], and bladder. Expression is increased in all of these except bladder cancer, in which the cancerous urothelium causes a decrease in TRPV1 due to cells becoming more de-differentiated as the carcinoma cells progress to a more aggressive state [92]. Moreover, TRPV1 in cancer has been often associated with pain, especially due to TRPV1 expression in neurons [94]. These findings make TRPV1 a good target for pharmacological inhibition of cancer pain in several types of cancers.

TRPV4: A Novel Target for Cancer Therapy

In recent years, TRPV4 has emerged as a widely accepted mechanosensor, contributing significantly to the process of mechanotransduction. TRPV4 is a non-specific Ca^{2+} permeable channel activated by a variety of physical and chemical stimuli such as temperature, hypotonicity, phorbol esters, endocannabinoids, arachidonic acid (AA), and epoxy eicosatrienoic acids (EETs) [95–97]. The TRPV4 protein is composed of a cytosolic N-terminal region, six transmembrane domains including the pore region, and an intracellular C-terminal tail (Fig. 12.1). The N-terminal region contains the ankyrin repeat domains (ARD), which consists of six ankyrin repeats (ANK) (ANK1–6) [98, 99]. A proline-rich domain (PRD) has been implicated in the mechanosensitivity of the TRPV4 channel, preceding the first ankyrin repeat. Within this PRD, proline residues at positions 142, 143, and 144, interact with pacsin 3, a protein implicated in vesicular membrane transport, endocytosis, and cytoskeleton reorganization [100, 101]. The TRPV4 C-terminal tail contains additional functional domains such as a TRP box, a calmodulin-binding site, and a binding site for cytoskeletal proteins such as MAP7, actin, and tubulin [102, 103].

TRPV4 is ubiquitously expressed among various tissues including lung, liver, heart, trachea, and the vascular endothelium [104–107]. Structurally similar to the *osm-9* gene found in *Caenorhabditis elegans*, mammalian TRPV4 is thought to

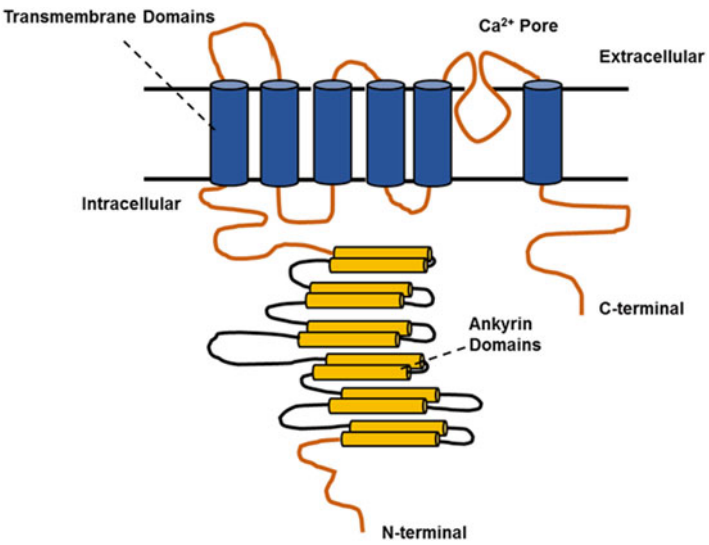

Fig. 12.1 Schematic of the structure of the mechanosensitive ion channel TRPV4. The TRPV4 channel is a tetramer and each subunit is composed of a cytosolic N-terminal region and six transmembrane domains, including the pore region, and an intracellular C-terminal tail. The N-terminal region contains the ankyrin repeat domain (ARD), which consists of six ankyrin repeats

share functional properties in mechanosensation. Previous studies found that when human embryonic kidney (HEK) 293 cells transfected with TRPV4 were exposed to shear stress, the increase in intracellular Ca^{2+} was due to TRPV4 activation, which was inhibited by TRPV antagonist, ruthenium red [108]. Liedtke et al. [109] found that sensory neurons in *C. elegans* responded to osmotic and mechanical stimuli via TRPV4 channels, providing *in vivo* support of the mechanosensitive properties of TRPV4. The first evidence indicating TRPV4 as a mechanosensor in the endothelium was reported by Kohler and coworkers [110], where they showed that shear stress-induced Ca^{2+} influx and vasodilation were mediated by TRPV4, which was confirmed by their later study using TRPV4-null mice [111]. A separate study has also revealed that TRPV4-mediated Ca^{2+} influx is critical for flow-induced release of mitochondrial reactive oxygen species (ROS) and vasodilation in human coronary arteries [112]. We and others have reported that TRPV4-dependent Ca^{2+} influx plays critical role in agonist (acetylcholine)-induced vasodilation [74, 113, 114]. Together, these studies demonstrate that TRPV4 plays an important role in the mechanical force and agonist-induced regulation of vascular tone. TRPV4 also plays a role in lung microvascular EC in which elevated lung hydrostatic pressures caused EC Ca^{2+} influx, an increase in vascular permeability and lung edema; these effects were abolished upon treatment with TRPV4 inhibitors and in TRPV4KO mice [106, 115].

Although the role of TRPV4 as a mechanosensor has been confirmed in the endothelium and other tissue and organ systems, the molecular mechanisms by which TRPV4 transduces mechanical signals is not well known. Both cell and ECM-generated mechanical forces have been critical signals that dictate normal vessel growth and patterning [40, 116, 117]. A critical event that takes place during neovascularization involves the directional migration of EC towards angiogenic stimuli, characterized by the realignment of the actin cytoskeleton and EC reorientation. Endothelial cells have been shown to reorient perpendicular to the direction of cyclic strain; but align parallel to flow in response to shear stress [118]. This reorientation response of EC to cyclic strain was found to be regulated by stretch-activated (SA) channels [119], however the specific identity of the SA channel or molecular mechanism(s) underlying this reorientation response is not known.

We set out to investigate the precise stretch-activated Ca^{2+} channel involved in regulating EC reorientation and our studies revealed that mechanical strain applied to integrins resulted in a rapid influx of Ca^{2+} via TRPV4 in EC. We further identified TRPV4 channels as the specific SA channel that mediates integrin-to-integrin signaling required for the cyclic strain-induced reorientation of EC [120] (Fig. 12.2). Specifically, we demonstrated that cyclic strain-induced TRPV4-mediated Ca^{2+} influx activates PI3K which in turn stimulates the activation of additional β1 integrins that may regulate Rho/Rac signaling required for the reorganization of the actin cytoskeleton and EC reorientation (Fig. 12.3). We further showed that mechanical strain application on β1 integrins activates TRPV4 through its interaction with a transmembrane CD98 protein, located in focal adhesions [121]. Based on these findings, we postulated that the transfer of mechanical force between cell surface molecules (β1 integrins, CD98 and TRPV4) localizes mechanotransduction almost

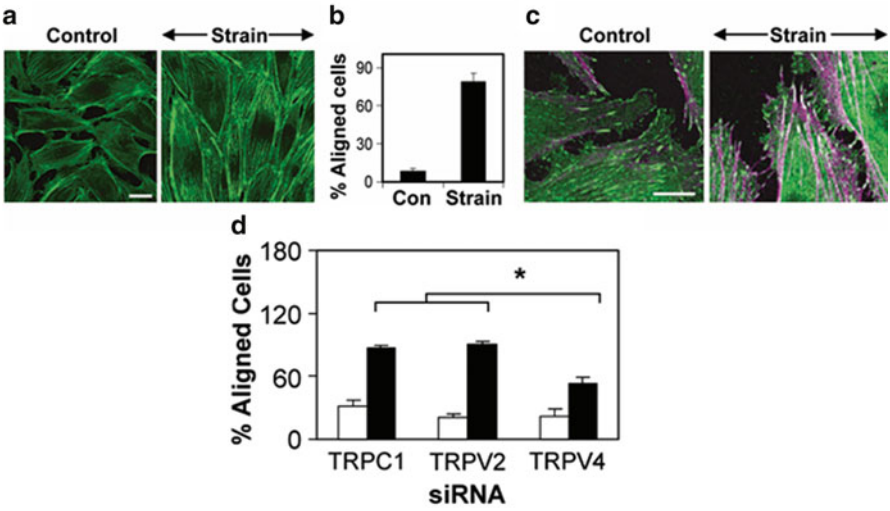

Fig. 12.2 TRPV4 channels mediate cyclic strain-induced endothelial cell reorientation. (**a**) Fluorescence images of endothelial cells (EC) subjected to 0 or 10 % uniaxial cyclic strain (2 h, 1 Hz). Cells were cultured on fibronectin-coated flexible silicone membranes, fixed, and stained with Alexa488-phalloidin to visualize actin stress fibers. *Arrow* indicates the direction of applied strain. Scale bar: 25 μm. (**b**) Quantification of cell alignment in control and strain exposed cells as the percentage of cells oriented 90 ± 30° (aligned) relative to the direction of applied strain ($p < 0.0006$); error bars indicated S.E.M. (**c**) Immunofluorescence images of EC subjected to 0 or 10 % uniaxial cyclic strain. Cells were stained for vinculin (*green*) and actin stress fibers (*magenta*) to show that application of strain causes enhanced recruitment of vinculin to large focal adhesions, that co-localize with the ends of reinforced stress fibers (shown in *white*). Scale bar: 25 μm. (**d**) siRNA knockdown of TRPV4 significantly inhibited cyclic strain-induced EC reorientation, compared to siRNA knockdown of TRPV2 or TRPC1. The quantification of cell alignment was measured as the percentage of cells oriented 90 ± 30° (aligned) relative to the direction of applied strain in control (*white bars*) and strain exposed (*black bars*) in human EC. (*$p < 0.0025$) (reprinted from Thodeti et al., *Circ. Res.* 104:1123–1130, 2009; Fig. 1 and Fig. 6D [120])

instantaneously within focal adhesions [121] and may regulate many complex cell and tissue behaviors. The importance of TRPV4 as a mechanosensor in EC was further validated when we demonstrated that a siRNA-mediated knockdown of TRPV4 in EC resulted in the failure of these cells to reorient perpendicular to the direction of applied cyclic strain [120] (Fig. 12.2d). To our surprise, when tumor-derived endothelial cells (TEC) were exposed to cyclic strain, these cells failed to reorient [28]. This aberrant mechanosensitive response exhibited by TEC was also found manifested in cell-spreading experiments. These cells exhibited uncontrolled spreading on ECM substrates of increasing rigidity (such as within a tumor), as opposed to NEC that stopped spreading at the highest stiffness. Further, *in vitro* angiogenesis assays revealed that TEC exhibited abnormal angiogenic behavior which was demonstrated to be mediated by high basal active-Rho and Rho-kinase (ROCK) [28].

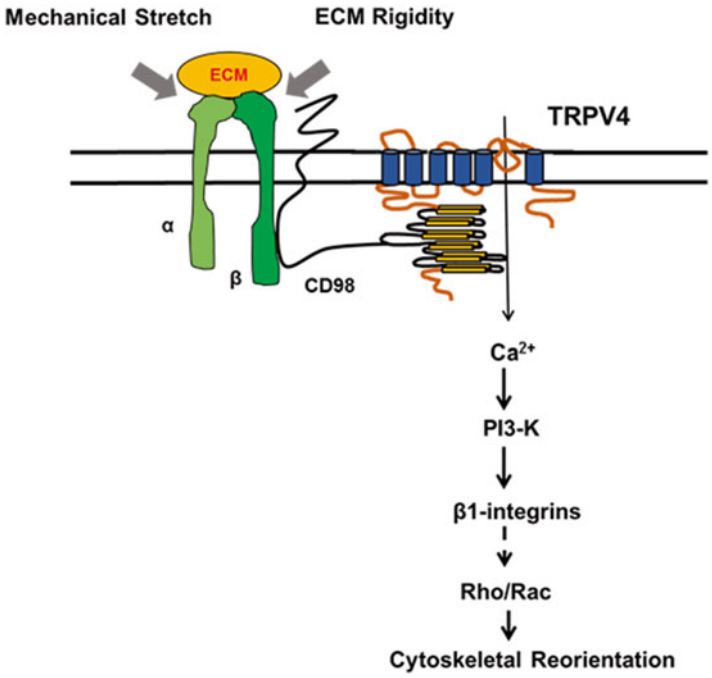

Fig. 12.3 TRPV4 mediated mechanotransduction in endothelial cells. A schematic model showing TRPV4-dependent mechanical signaling in normal endothelial cells. Application of mechanical force (cyclic stretch or ECM stiffness) to integrins activates ultra-rapid calcium influx through TRPV4 via interaction with a transmembrane protein CD98. The released Ca^{2+} activates additional integrins via PI3K. This integrin-to-integrin signaling may further regulate downstream Rho/Rac pathways necessary for reorganization of the actin cytoskeleton and reorientation of EC [120]

Since TEC failed to reorient to applied cyclic strain, a phenomenon reminiscent to NEC subjected to siRNA-mediated TRPV4 silencing, we postulated that aberrant mechanosensitivity in TEC may be caused by altered TRPV4-dependent signaling. Indeed, our recent work has demonstrated that TRPV4 expression and function are impaired in TEC [122]. Specifically, we found a significant reduction in TRPV4 expression (~40 %), while functional assays revealed a 40–50 % decrease in Ca^{2+} influx in TEC when stimulated with specific TRPV4 agonists, GSK1016790A (GSK) and 4α-PDD (Fig. 12.4). An important downstream consequence of tumor growth is an increase in matrix rigidity, owing to the leakage of plasma components from highly permeable blood vessels, and increased synthesis/degradation of matrix components [123]. This increase in ECM stiffness has been shown to influence TEC spreading, migration, and tube formation. Because we have previously shown that TEC display abnormal mechanosensitivity to substrate stiffness [28], and TRPV4 was found functionally impaired, we overexpressed TRPV4 to determine whether we could restore TEC mechanosensitivity towards varying ECM rigidity. We found that overexpression of TRPV4 reduced the abnormal spreading exhibited by TEC

on the highest stiffness gelatin gels (2 kPa; comparable to the stiffness of tumors). The ability of cells to migrate is reliant on the mechanosensing efficiency of the cell in response to ECM rigidity, which is an important step in the angiogenic process [124]. We therefore investigated the migratory ability of TEC and found that these cells exhibit abnormal migration (40 μm/h); and restoring TRPV4-dependent mechanosensitivity normalized TEC migration consistent with the migration of NEC (10 μm/h). Additionally, overexpression of TRPV4 in TEC was able to decrease the high basal Rho activity previously observed [28], as well as normalize the abnormal angiogenesis exhibited in both 2D and 3D *in vitro* angiogenesis assays. We further demonstrated that these effects were similarly achieved by modulating TRPV4 activity pharmacologically using a specific TRPV4 activator, GSK1016790A. These findings suggest that TRPV4 signaling regulates tumor angiogenesis via Rho-dependent mechanosensing mechanisms. Finally, to determine the functional role of TRPV4 in tumor angiogenesis *in vivo*, we induced syngeneic tumors in TRPV4KO mice by subcutaneously injecting LLC (mouse Lewis Lung Carcinoma) cells. Tumor growth and angiogenesis was significantly enhanced in TRPV4KO mice compared to WT mice. Immunohistochemical analysis of tumor sections obtained from TRPV4KO tumors revealed the vessels were immature, i.e. large vessels with decreased pericyte coverage. Further, TRITC-dextran perfusion experiments confirmed that these vessels were leaky, as the dextran fluorescence was enhanced in the tumor tissue in TRPV4KO mice compared to WT mice. Notably, these findings suggest that TRPV4 is critical for maintaining vessel structure and integrity and that absence of TRPV4 not only increases tumor angiogenesis but also inhibits vessel maturity.

Thus, our study has not only provided a unique role for the mechanosensitive TRPV4 channel in the tumor vasculature, but opens up an uncharted therapeutic target in vascular normalization strategies. Specifically we have demonstrated TRPV4 activation with GSK1016790A normalized tumor vasculature (increased pericyte coverage) and reduced tumor growth when given in combination with the anti-cancer drug Cisplatin, but not alone. These findings suggest that normalization of tumor vasculature by TRPV4 activation may have improved the delivery of Cisplatin and reduced tumor growth (Fig. 12.5). Thus, our work is the first to study the functional significance of TRPV4 in tumor angiogenesis *in vitro* and *in vivo* and identify a novel role for TRPV4 in the regulation of tumor vessel growth and maturity.

Conclusion and Perspectives

The neovascularization of solid tumors create dysfunctional endothelium, generating a microenvironment in which the vasculature becomes heterogeneous and erratic blood flow ensues. This ultimately affects the delivery and efficacy of systemically administered agents. Although endothelial cells are generally heterogeneous, they are commonly regulated by many soluble, membrane-bound, and mechanical factors, including ion channels. Because most ion channels are found in

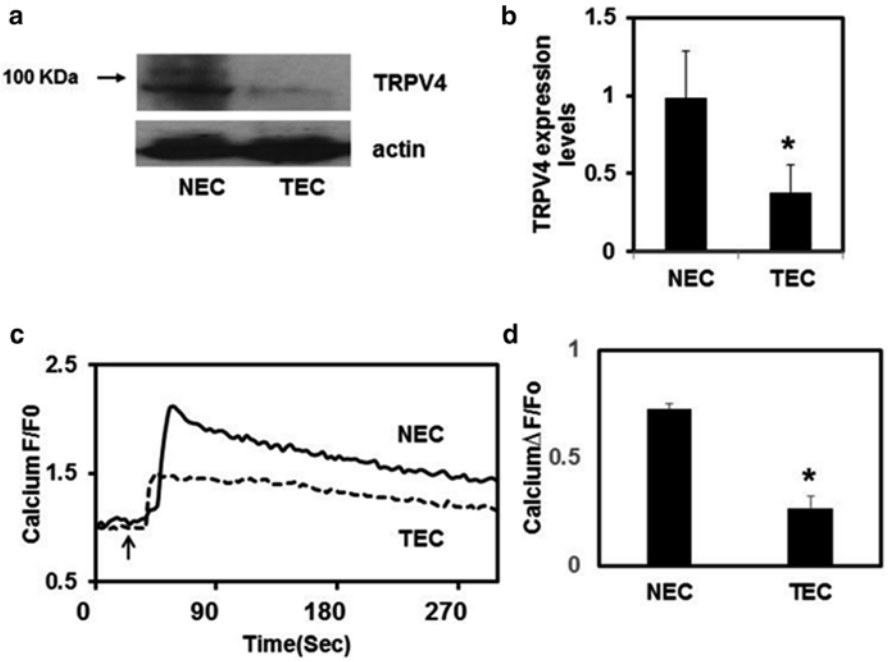

Fig. 12.4 Expression and function of TRPV4 channels in normal and tumor endothelial cells. (**a**) Western blot showing TRPV4 protein expression in normal (NEC) and tumor-derived endothelial cells (TEC). (**b**) Densitometric analysis of the Western blots showing significant ($p \leq 0.05$) reduction in TRPV4 expression in TEC compared to NEC. (**c**) Relative changes in cytosolic calcium in response to a selective TRPV4 agonist, GSK1016790A (100 nM) in Fluo-4 loaded NEC and TEC. *Arrow* denotes the time when the cells were stimulated with the TRPV4 agonist. (**d**) Quantitative analysis of cytosolic calcium influx induced by GSK1016790A in NEC and TEC. (F/F0 = ratio of normalized Fluo-4 fluorescence intensity relative to time 0). The results shown are mean ± SEM from three independent experiments. The significance was set at $p \leq 0.05$ (reprinted from Adapala et al., *Oncogene* 2016; 35:314–22 [122])

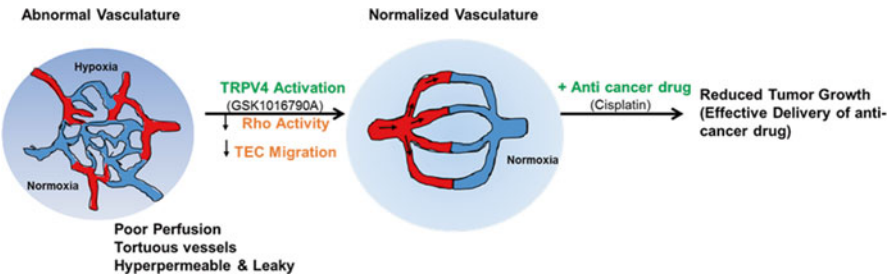

Fig. 12.5 TRPV4 activation normalizes tumor vasculature enhancing chemotherapeutic drug delivery. The tumor vasculature is characterized by tortuous, hyper-permeable, and poorly perfused vessels surrounded by varying regions of hypoxia and normoxia. The treatment with a specific small molecular activator of TRPV4, GSK1016790A, normalizes the tumor vasculature and improves the efficient delivery of chemotherapeutic drug (Cisplatin) leading to reduced tumor growth in WT mice injected subcutaneously with LLC (Lewis Lung Carcinoma) cells (Adapala et al., *Oncogene* 2016; 35:314–22 [122])

the plasma membrane, they are accessible to the blood stream, making them favorable targets when vascular diseases arise. Most current therapies concentrate on targeting the cytokines involved in tumor angiogenesis. Recent pre-clinical and clinical evidence has demonstrated that anti-VEGF drugs, as well as many other direct or indirect angiogenesis inhibitors, can transiently promote the normalization of the tumor vasculature. However, growth factor mediated normalization therapies have largely been unsuccessful due to several challenges, such as drug resistance and redundancy among others. Considering these findings, targeting mechanotransduction may offer a more effective means to treating or reducing solid tumor growth. We have shown mechanosensitive ion channel TRPV4 modulates tumor endothelial function and angiogenesis, and have unraveled a TRPV4-dependent mechanotransduction mechanism in angiogenesis. These findings could lead to the development of novel growth factor-independent therapeutic targets, not only to induce vascular normalization and improve cancer therapy, but also for other angiogenic disorders such as diabetic retinopathy and age-related macular degeneration.

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