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Activation of mechanosensitive ion channel TRPV4 normalizes tumor vasculature and improves cancer therapy

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Abstract

Tumor vessels are characterized by abnormal morphology and hyper-permeability that together cause inefficient delivery of chemotherapeutic agents. Although VEGF has been established as a critical regulator of tumor angiogenesis, the role of mechanical signaling in the regulation of tumor vasculature or tumor endothelial cell (TEC) function is not known. Here, we show that the mechanosensitive ion channel TRPV4 regulates tumor angiogenesis and tumor vessel maturation via modulation of TEC mechanosensitivity. We found that TEC exhibit reduced TRPV4 expression and function, which is correlated with aberrant mechanosensitivity towards ECM stiffness, increased migration and abnormal angiogenesis by TEC. Further, syngeneic tumor experiments revealed that the absence of TRPV4 induced increased vascular density, vessel diameter and reduced pericyte coverage resulting in enhanced tumor growth in TRPV4 KO mice. Importantly, overexpression or pharmacological activation of TRPV4 restored aberrant TEC mechanosensitivity, migration and normalized abnormal angiogenesis *in vitro* by modulating Rho

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activity. Finally, a small molecule activator of TRPV4, GSK1016790A, in combination with anti-cancer drug Cisplatin, significantly reduced tumor growth in WT mice by inducing vessel maturation. Our findings demonstrate TRPV4 channels to be critical regulators of tumor angiogenesis and represent a novel target for anti-angiogenic and vascular normalization therapies.

Keywords

Angiogenesis; Calcium; Endothelial; Extracellular Matrix; Mechanotransduction; Tumor; TRPV4; Vascular normalization

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing capillaries, is critical for solid tumor maintenance, growth and progression as it ensures proper oxygen and nutrient delivery to the tumor. However, the tumor vasculature is structurally and functionally abnormal, as characterized by its high tortuosity, non-uniform pericyte coverage and hyper-permeability[1, 2]. These abnormal vessels cause irregular blood flow and distribution, impaired oxygen delivery and impede immune cell function, which together lead to inefficient delivery of anti-cancer agents causing tumor cell resistance to radiation and chemotherapies[3-5]. Conventional anti-angiogenic therapies focus on either neutralizing the effect of soluble angiogenic factors such as vascular endothelial growth factor (VEGF) using specific antibodies or inhibiting VEGFR kinase activity[6-8]. These conventional anti-angiogenic strategies showed only modest success in clinical trials due to the development of resistance (evasive or intrinsic) as tumor endothelial cells (TEC) became refractory to anti-VEGF therapy over time[3-5]. These findings led to the emergence of a new concept called “vascular normalization”, i.e. transient inhibition of tumor angiogenesis leading to normalization of tumor vasculature which improves the efficacy of chemo- and radio therapies [1, 2]. Although vascular normalization combined with chemotherapy have shown transient benefits, clinically they failed to exhibit long term beneficial effects owing to these strategies still being focused onVEGF-targeted therapies, despite their shortcomings [2, 9, 10]. Infact, recent positron emission tomograpy (PET) imaging results have demonstrated a rapid decrease in the delivery of chemotherapeutic drugs to tumors post anti-VEGF therapy, in non-small cell lung cancer (NSCLC) patients [11].Therefore, an urgent need arises for the development of novel vascular normalization strategies.

In addition to soluble stimuli, such as VEGF and PDGF (platelet derived growth factor) local mechanical cues conveyed by the extracellular matrix (ECM), due to cyclic deformation of blood vessels and hemodynamic forces, are also potent inducers of directional capillary growth and vascular remodeling *in vitro* and *in vivo* [12-16]. Endothelial cells (ECs) sense mechanical forces associated with tissue distortion through integrin receptors that mediate their adhesion to the surrounding ECM[12, 14, 15, 17]. Unlike normal ECM, the tumor ECM becomes stiffer as a result of continuous remodeling of matrix components by tumor cells and stromal fibroblasts. Further, the tumor vasculature is hyper-permeable due to irregular basement membrane and poor pericyte coverage and therefore releases plasma components into the surrounding extracellular space, eventually leading to

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increased ECM stiffness. Cells sense changes in ECM stiffness through integrin receptors which transduce these mechanical signals into the cell through the actin cytoskeleton. As shown previously in tumor epithelial cells[18, 19] and TEC[20], this increase in ECM stiffness also feeds back to enhance integrin-mediated Rho/ROCK (Rho-associated kinase) activity and contraction that can lead to aberrant mechanosensitivity of TEC.

One of the earliest responses of cells to mechanical force is the influx of calcium through the activation of mechanosensitive ion channels[21-23]. Although activation of these channels is thought to be independent of integrin mechanosensing, several lines of evidence indicate that both integrins and mechanosensitive ion channels are well connected[22, 24-28]. We have previously shown that the application of cyclic strain to normal endothelial cells (NEC) induces cell reorientation through activation of mechanosensitive Transient Receptor Potential Vanilloid 4 (TRPV4) ion channel-dependent calcium influx, which in turn activates additional integrins and causes down-stream cytoskeletal reorganization[16]. Importantly, siRNA knockdown of TRPV4 channels inhibited mechanical strain-induced reorientation of NEC, thus confirming their key role in endothelial cell mechanosensitivity. Notably, endothelial cell mechanosensitivity strongly influences vascular formation and patterning[20], and thus, tumor vessel malformations could arise from deregulation of TEC mechanosensing. Indeed, we have previously shown that TEC fail to reorient in response to cyclic strain, exhibit aberrant mechanosensitivity to ECM stiffness and undergo abnormal angiogenesis *in vitro*. Importantly, this abnormal TEC behavior resulted, at least in part, from abnormally high basal Rho activity[20]. However, the upstream signaling molecule or the molecular mechanism(s) governing high Rho-mediated aberrant mechanosensitivity and angiogenesis by TEC remain unknown. In the present study, we explored whether the mechanosensitive TRPV4 ion channel is a key determinant of TEC dysfunction leading to tumor angiogenesis and abnormal vasculature.

Results

Tumor endothelial cells express lower levels of TRPV4 and TRPV4-dependent calcium influx

We and others have previously shown that TRPV4 channels are functionally expressed in endothelial cells and act as mechanosensor of cyclic stretch and flow[16, 29]. We also demonstrated that TRPV4 channels induce mechanical force-dependent calcium influx in integrin specific manner in endothelial cells and this TRPV4-dependent mechanotransduction regulates cyclic strain–induced endothelial cell reorientation[16]. Interestingly, we found that tumor derived endothelial cells (TEC) failed to reorient in response to cyclic strain similar to that of TRPV4 knockdown endothelial cells suggesting TEC may mimic TRPV4 knockdown phenotype [20]. Because mechanical force (cyclic strain)-induced reorientation in endothelial cells is dependent on TRPV4 channels[16], we investigated whether these channels contribute to the abnormal mechanosensitivity of TEC[20]. TEC and normal EC (NEC) used in the present study have been isolated and characterized for EC markers, EC function and mechanosensing at different passages[20, 30]. We first compared TRPV4 expression levels between NEC and TEC using Western blot analysis. While NEC exhibited strong expression of TRPV4,[16, 31] it was significantly lower

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in TEC (Fig.1A, B). To determine the functional implications of lower TRPV4 expression in TEC, we measured calcium influx in Fluo-4/AM loaded cells, in response to the addition of specific TRPV4 activators, GSK1016790A or 4-α-PDD. By using calcium imaging, patch-clamp, siRNA knockdown and TRPV4 null cells, we have previously demonstrated that these compounds specifically activates TRPV4-mediated calcium influx in endothelial cells[16, 31]. GSK1016790A (100 nM) induced calcium influx in both NEC and TEC but the influx in TEC was decreased by almost 40-50% compared to NEC ($p \leq 0.001$) (Fig.1C, D). Another TRPV4 agonist, 4-α-PDD (10 μM) also induced a rapid calcium influx in NEC, which was again significantly reduced by ∼40% ($p \leq 0.01$) in TEC (Supplementary Fig.S1 A, B). These results demonstrate that TRPV4 expression and function (calcium influx) are impaired in TEC.

Tumor Angiogenesis and tumor growth are enhanced in TRPV4 null mice

Next, to confirm if TRPV4 expression level contributes to tumor angiogenesis *in vivo*, we induced tumors in TRPV4 KO and WT mice (C57BL/6) by subcutaneously injecting mouse Lewis lung carcinoma cells (LLC). We found that tumor growth was 2-3 times greater in TRPV4 KO mice compared to WT mice at day 21 (Fig.2A). Importantly, immunohistochemical analysis revealed that tumors in TRPV4 KO mice exhibited a greater fraction of hyper-dilated (malformed) vessels with significantly larger vessel diameters ($p \leq 0.05$) compared to WT mice (Fig. 2, B, C). In contrast to tumor vessels in WT mice, those in TRPV4 KO mice exhibited poor pericyte coverage, as determined by very weak α-SMA staining, indicating the immature nature of these vessels. (Fig. 2D, E). Further, the tumors from TRPV4 KO mice exhibited increased microvessel density compared to tumors from WT mice (Supplementary Fig.S2 A, B). Next, we assessed the leakiness of TRPV4 KO vessels by performing permeability assays using TRITC-dextran (3000 MW) perfusion, via tail vein injections. Immunohistochemical analysis revealed increased TRITC staining within the tumor tissue, surrounding the vessels, in TRPV4 KO mice compared to WT tumors suggesting that TRPV4KO vessels are indeed hyperpermeable (Suppl Fig.S3). Taken together, these results clearly suggest that TRPV4 plays a critical role in modulating tumor angiogenesis and the absence of TRPV4 can lead to abnormal tumor angiogenesis (immature (leaky) vessels), possibly through altered mechanotransduction exhibited by endothelial cells.

TRPV4 overexpression restores mechanosensitivity and reduces abnormal cell migration in tumor endothelial cells

The above findings suggest that lower levels of TRPV4, a known mechanosensor in endothelial cells [16, 22, 32, 33] may contribute to the previously reported aberrant mechanosensitivity of TEC in response to ECM stiffness and cyclic strain [20]. Since ECM stiffness is known to increase in tumors [19], which can also influence TEC spreading, migration and tube formation, we investigated if overexpression of TRPV4 might rescue the abnormal mechanosensitivity exhibited by TEC. To achieve this, we expressed a human TRPV4-EGFP construct in TEC; EGFP fluorescence revealed that more than 80% cells were transfected with TRPV4-EGFP (Supplementary Fig. S4A). We found that overexpression of TRPV4 increased GSK1016790A-induced calcium influx in TEC by almost 4-fold ($p \leq 0.01$) compared to EGFP-alone expressing cells (Supplementary Fig.S4 B, C). The other

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TRPV4 specific activator, 4-α-PDD, also increased calcium influx in these TRPV4-transfected TEC cells compared to EGFP-expressing controls (not shown).

To explore if TRPV4 overexpression influences TEC mechanosensitivity towards ECM stiffness, we next cultured TRPV4-overexpressing TEC on transglutaminase linked gelatin gels of varying stiffness (370 and 2280 Pa; representing intermediate and high stiffness, respectively) which mimic the stiffness of tumor ECM [19] for 6 h and compared their degree of spreading[20] with that of control EGFP-expressing cells (Fig. 3A, B). In past studies, we found that both NEC and TEC spread similarly on low stiffness (98 Pa) gels[20]. However, in that study, NEC exhibited increased spreading on intermediate stiffness (370 Pa) which reached plateau at high stiffness (2280 Pa) while TEC continue to increase their spreading with increased stiffness. Therefore, in the present study, we focused on intermediate (370 Pa) and high (2280 Pa) stiffness gels. We found that NEC (expressing only EGFP) spread to similar extent on both intermediate and high stiffness gels (370 and 2280 Pa) further confirming that the spreading of NEC reached plateau at 370 Pa (Fig.3 A, B), similar to our previous study[20]. On the other hand, spreading of TEC (expressing only EGFP) was significantly more on high stiffness gels compared to intermediate stiffness gels (Fig.3 A,B). In contrast, TEC-TRPV4-EGFP exhibited reduced spreading on high stiffness gels compared to TEC-EGFP alone, with no significant difference between intermediate and high stiffness substrates (Fig. 3A, B) suggesting that TRPV4 overexpression restored substrate mechanosensitivity in these cells. We did not find any change in NEC spreading when TRPV4-EGFP was overexpressed (data not shown). To further confirm that TRPV4 overexpression restores mechanosensitivity, we exposed TEC-EGFP and TEC-TRPV4-EGFP cells to cyclic strain, which is widely used to test endothelial cell mechanosensitivity, as previously described[16]. As shown earlier[20], we found that TEC-EGFP failed to reorient while TEC overexpressing TRPV4-EGFP significantly oriented in response to cyclic strain (data not shown).

Since cell migration is dependent on mechanosensing of matrix stiffness and is a critical component of angiogenesis, we next explored if TRPV4 overexpression which normalized mechanosensitivity also influence TEC migration. To demonstrate this, we chose substrates of intermediary stiffness, as they supported optimal TEC cell spreading with or without TRPV4 overexpression. Consistent with their abnormal mechanosensitivity, TEC exhibited high cell migration speed (40 μm/h; (Fig.3 C, D), which was significantly suppressed (10 μm/h) when cells were transfected with TRPV4 (Fig.3 C, D). Further, we found that NEC migrated at a speed of 10 μm/h (Supplementary Fig.S5) suggesting that overexpression of TRPV4 normalized TEC cell migration to that of NEC. Taken together, all the above findings clearly demonstrate that TRPV4 expression restored mechanosensitivity in TEC and normalized migration.

TRPV4 overexpression or pharmacological activation normalizes abnormal tube formation in vitro via modulation of Rho activity

Since TRPV4 overexpression restored TEC mechanosensitivity to ECM stiffness which reduced abnormal cell migration, we explored whether TRPV4 expression normalizes vessel

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formation and if so, further determine the molecular mechanisms that underlie this process. First, TEC expressing TRPV4-EGFP or EGFP alone were tested for their ability to form capillary networks using a Matrigel® based *in vitro* 2D angiogenesis assays. We have previously shown that TEC form robust tubes when plated at low density (2×10^4 cells/ well) on Matrigel (2D) but at high density (8×10^4 cells/ well), these cells form tubes and then undergo multicellular retraction with disruption of tubular networks[20]. Importantly, TEC cultured within (rather than on top of) Matrigel formed tubular structures that were abnormally dilated and non-uniform, an abnormal morphology [20]; (Fig. 4A) reminiscent of that observed within the cancer microvasculature *in vivo*, whereas NEC reorganized into tubular structures of relatively uniform size under similar culture conditions. These findings suggest that TEC are capable of tube formation in both 2D and 3D Matrigels. However, they collapse on 2D Matrigel due to high contraction mediated by cumulative high Rho activity [34], but form abnormal tubes in 3D. Therefore, we used both 2D and 3D Matrigel assays to determine if TRPV4 overexpression could normalize abnormal tube formation by TEC. To achieve this, first, we plated TEC expressing EGFP alone or overexpressing TRPV4 (expressing TRPV4-EGFP) at high density (8×10^4 cells/ well) on Matrigel for 2D tube formation. As expected, TEC expressing EGFP alone, formed tubes at high density (8×10^4 cells/ well) but these formed tubes underwent multicellular retraction and collapsed (Fig. 4A). In contrast, TEC overexpressing TRPV4 formed a robust tubular network, with quantitative analysis revealing a significant increase (almost 10 fold) in tube length in TRPV4-expressing TEC compared to EGFP-expressing counterparts (Supplementary Fig.S6). Interestingly, we did not find any change in tube formation by NEC either expressing EGFP alone (Fig.4A) or overexpressing TRPV4-EGFP (not shown). We also found that TRPV4 overexpression normalized tube formation in 3D Matrigels (Fig4A).

Next, we examined if pharmacological activation of TRPV4 normalizes abnormal tube formation *in vitro*. For this, we activated TRPV4 in TEC using the small molecule activator of TRPV4, GSK1016790A (GSK; 100 nM). We found that, similar to TRPV4 overexpression, TEC, but not NEC, treated with GSK formed robust tubes at high plating density (8×10^4 cells/ well) (Fig.4B). Since activity-dependent regulation of ion channels was shown in various cancers[35, 36], we then asked if activation of TRPV4 with GSK modulates TRPV4 expression in TEC. We found that treatment of TEC with GSK for 24 h significantly increased TRPV4 protein expression (Fig. 4C). These results demonstrate that TRPV4 overexpression or pharmacological activation by GSK normalizes abnormal angiogenesis exhibited by TEC through the restoration of mechanosensitivity towards ECM stiffness and thus identifies TRPV4 as a critical mediator of angiogenesis.

To understand the molecular mechanism downstream of TRPV4 that mediates normalization of angiogenesis, we focused on Rho which regulates endothelial contraction that is required for partial rounding of endothelial cells during tube formation (34). We have previously demonstrated that TEC (expressing only EGFP) showed high basal Rho activity [20] which is the reason for abnormal collapse of TEC on 2D Matrigel, when plated at high densities. However, we found that overexpression of TRPV4-EGFP significantly reduced this high basal Rho activity exhibited by TEC (approximately ½-fold lower; $p \leq 0.001$) (Fig.4 D). We found that TRPV4 activator GSK1016790A (100 nM) also inhibited high basal Rho activity

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in TEC (Supplementary Fig.S7) suggesting that TRPV4 is a critical modulator of Rho activity in TEC.

TRPV4 specific small molecule activator GSK1016790A normalizes tumor vasculature in vivo and reduces tumor growth in combination with Cisplatin

Finally, we explored if pharmacological activation of TRPV4 induces tumor vascular normalization/maturation *in vivo* and improves efficacy of chemotherapeutic drug (Cisplatin). To achieve this, we injected TRPV4 activator GSK1016790A intraperitonially every day for 14 days in WT mice that had developed palpable tumors (around 100 mm^3, after 7 days). Cisplatin was given once per week starting 2-4 days after GSK treatment and tumor growth was monitored every week until day 21. First, we examined vessel maturity in tumors by staining for pericyte coverage. We observed that the vessels in GSK and GSK-Cisiplatin-treated tumors, but not in control or Cisplatin treated ones, showed increased pericyte coverage (Fig.5A, B) suggesting that TRPV4 activation normalized tumor angiogenesis and induced vessel maturation which may help efficient delivery of Cisplatin. Consistent with this observation, we found that tumor growth was markedly reduced in GSK-Cisplatin treated animals (Fig.5C), but not in control or Cisplatin treated mice, suggesting the improved delivery of Cisplatin due to the normalization of vessels by TRPV4 activation.

Discussion

Tumor angiogenesis has been widely shown to be regulated by soluble growth factors such as VEGF and fibroblast growth factor (FGF). However, regulation of tumor angiogenesis by mechanical forces is not well known. In the present study, we found that tumor endothelial cells express lower levels of TRPV4, a mechanosensitive ion channel, which is a key upstream signaling molecule that regulates tumor endothelial cell mechanosensitivity, tumor angiogenesis and tumor vessel maturation. Importantly, we demonstrate that overexpression of TRPV4 normalizes aberrant TEC mechanosensitivity, migration and angiogenesis through modulation of Rho activity. We further confirmed these findings *in vivo* by demonstrating that TRPV4 KO mice lacking this mechanosensing molecule exhibit severe tumor vessel malformations, characterized by increased vessel diameter, length and density, and enhanced tumor growth. Finally, we demonstrated that administration of the specific small molecule TRPV4 activator, GSK1016790A induced vessel maturation and, in combination with a chemotherapeutic drug, Cisplatin, reduced tumor growth in WT mice. To the best of our knowledge, this is the first report to demonstrate a role for TRPV4 in angiogenesis *in vitro* or *in vivo*.

TRPV4 channels are non-selective calcium ion channels ubiquitously expressed in endothelial cells that act as a mechanosensor of cyclic strain and shear stress in endothelial cells [16, 22, 29, 32, 33, 37]. We have previously shown that endothelial cells express functional TRPV4 channels (Fluo-4 imaging, patch-clamp) and that these channels mediate mechanical signals from integrin to integrin that are critical for reorientation of NEC in response to cyclic strain [16]. The fact that EC isolated from tumors fail to reorient in response to cyclic strain and exhibit aberrant mechanosensitivity towards substrate stiffness[20], suggested that

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TRPV4 channel signaling might be altered in these cells. Indeed, we found that TEC express low levels of functionally active TRPV4 channels compared to NEC. TRPV4 protein usually exhibits two bands that are below and above of 100 kDa in Western blots[16,29,53-56]. Although the upper band has been demonstrated to be the glycosylated form of TRPV4[53] which influences its membrane translocation and activity, we did not find any significant difference in terms of the relative amount of each band in NEC or TEC, suggesting that TRPV4 expression is down-regulated. Consistently, TRPV4 expression has been shown to be modulated by microRNA -203[57]. However, it is not clear how tumor cells or tumor stroma may influence TRPV4 expression in TEC. Importantly, we demonstrate that TRPV4 overexpression or pharmacological activation by a small molecular activator, restored mechanosensitivity to matrix stiffness and cyclic strain, reduced migration and normalized the abnormal angiogenesis exhibited by TEC *in vitro*.

The aberrant mechanosensitivity of TEC is known to be mediated by high basal Rho activity[20]; however, the molecular mechanism up-stream of Rho responsible for this abnormal behavior is not yet known. Here, we show that overexpression of TRPV4 significantly inhibited high basal Rho activity in TEC. This reduction in basal Rho activity could possibly inhibit the high basal contraction of TEC [34] which then may be responsible for the restored mechanosensitivity of these cells towards substrate stiffness and normalization of angiogenesis. In fact, transient inhibition of Rho kinase with Y-27632 can normalize TEC responses to cyclic strain[20]. Although the exact molecular mechanism through which TRPV4 regulates Rho activity is not known, it is plausible that TRPV4 exerts its effects by modulating integrin activation, which has been shown to be sensitive to mechanical force-induced TRPV4-dependent calcium influx[16]. Importantly, binding of integrins to ECM transiently inhibit Rho activity[38, 39] and facilitate cell shape changes, leading to cell spreading and reorientation. Interference with binding of additional integrins using function blocking antibodies also significantly inhibits TRPV4-dependent cyclic strain-induced CE reorientation[16].

Although TRPV4 role in endothelial migration was shown [40], the role of TRPV4 in physiological or pathological angiogenesis is not demonstrated. Our results demonstrating TRPV4 overexpression normalized tube/vessel formation in 2D Matrigels, coupled with increased vessel malformations and enhanced tumor growth in TRPV4 KO mice, confirms that TRPV4 plays a critical role in tumor angiogenesis. Further, combination of GSK and Cisplatin, but not each them alone, inhibited tumor growth suggesting that TRPV4 activation-induced vascular maturation improved the efficacy of Cisplatin. These findings have significant value in vascular normalization therapies directed towards the treatment of cancer. Since the initial proposal that tumors rely on neovascularization for their survival and growth[41, 42], angiogenesis has become a potential target for cancer therapy. In fact, multiple angiogenesis inhibitors have now been identified, and some clinically approved (e.g., Avastin), that inhibit the growth of a wide variety of experimental tumors in many animal models albeit with limited success in patients. Although VEGF is required for normal proliferation and migration of EC, it is important to emphasize that the tumor vasculature in certain cancers, such as in renal cell carcinoma, is largely refractory to therapies designed to alter VEGF signaling, as growth factors are redundant for angiogenesis [3-6, 43]. Nevertheless, current vascular normalization therapies still focus on targeting soluble growth factors such

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as VEGF and PDGF [2, 10, 44-51]. Notably, recently it was shown NSCLC patients treated with humanized VEGF antibody, bevacizumab reduced both perfusion and net influx rate of [(11)C] docetaxel within 5 h as measured by PET. Further, it was demonstrated that these effects persisted even after 4 days[11]. Importantly, these findings show no evidence for a substantial improvement in drug delivery to tumors by anti-VEGF treatment and highlight the importance of drug scheduling and supports further studies to optimize scheduling or the use of anti-angiogenic drugs.

Thus, our results showing that a mechanosensitive ion channel, TRPV4 regulates tumor angiogenesis through the modulation of Rho-dependent mechanosensitivity of TEC and, a small molecule activator of TRPV4, GSK1016790A together with Cisplatin, inhibits tumor growth, suggests TRPV4 as a target for developing a novel mechanotransduction-based therapy for vascular normalization. To our knowledge, TRPV4 channels have not previously been studied as potential therapeutic targets in angiogenesis, therefore we believe that elucidation of TRPV4-dependent mechanotransduction mechanisms and their role in angiogenesis may open entirely new avenues for developmental therapeutics for cancer as well as other angiogenic disorders, such as age-related macular degeneration and diabetic retinopathy.

Materials and Methods

Cell culture

Normal (NEC) and Tumor (TEC) endothelial cells were obtained from a transgenic adenocarcinoma mouse prostate (TRAMP) model, as previously described[20, 30]. Cells were plated on fibronectin (FN) or gelatin-coated tissue culture dishes and grown in a defined medium composed of low glucose DMEM, 10% fetal bovine serum, 10% Nu Serum IV, VEGF(1ng/ml) basic fibroblast growth factor (3 ng/ml), heparin salt (0.1 mg/ml), 1% insulin-transferrin-selenium and antibiotic/mycotic mix. Cells were cultured in a 37°C, 5%CO_2 incubator, split at ∼90-95% confluence, and used between passages 11-22. These cells (NEC and TEC) were characterized for the presence of endothelial markers and function. We found that both of these cells expressed endothelial markers including CD31(PECAM-1), VE-cadherin, Von Wildebrand Factor (vWF), endothelial nitric oxide synthase (eNOS) and bind to isolectin-IB4 as measured by FACS, Western blotting, RT-PCR and immunocytochemistry. In contrast, these cells do not express mesenchymal markers α-SMA and PDGFR-β receptor[20, 30]. NEC and TEC formed robust tubular structures/sprouts in 2D and 3D Matrigel/Fibrin gel angiogenesis assays further confirming that these are functional EC [20, 30]. We have shown these EC retained their endothelial phenotype and TRPV4 expression (not shown) even up to 22 passages[20].

Transfection

Cells were transfected with TRPV4-EGFP (kind gift of Dr. Jendrach, Germany) or EGFP constructs using targefect (targetingsystems)[31]. The transfection efficiency was found to be 80-90%.

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Cell spreading on flexible substrates

Transglutaminase-crosslinked gelatin hydrogels of increasing stiffness were prepared using 3, 5 and 10% (w/v) final gelatin concentration and incubated at 4°C overnight to stabilize crosslinking (19). Cells in regular culture medium were plated at low density (to minimize cell-cell interactions) and allowed to spread for 6 h.

Rho activation assay

Rho activity was determined using the Rhotekin-RBD affinity precipitation assay as described previously[20, 52]. Briefly, TEC overexpressing TRPV4-EGFP or EGFP alone or TEC treated with GSK1016709A (Sigma-Aldrich; 100 nM for 15 min) were lysed in 1% Triton X-100 buffer and centrifuged at 12000× g for 15 min. Equal volumes (compensated for equal protein concentration) of clarified lysate were incubated with GST-Rhotekin-RBD beads (Cytoskeleton Inc.), for 1 h at 4°C. The beads were collected by centrifugation and washed 3 times with wash buffer. The bound GTP-Rho was extracted with SDS-sample buffer and was detected using Rho mAb (SantaCruz) on a Western blot. GTP-Rho levels were calculated from the densitometric analyses of Western blot and normalized to the levels of total-Rho and presented as relative Rho activity with that of TEC transfected with EGFP alone or TRPV4-EGFP.

Calcium Imaging

TEC overexpressing TRPV4-EGFP or EGFP alone or NEC were cultured on MatTek glass bottom dishes and loaded with Fluo-4/AM (1-4 μM) for 30 min, washed 3 times in calcium medium (136 mM NaCl, 4.7 mM KCl,1.2 mM MgSO4, 1.1 mM CaCl2, 1.2 mM KH2PO4, 5 mM NaHCO3, 5.5mM glucose, and 20 mM Hepes. pH 7.4). Cells were stimulated with TRPV4 activators 4-α-PDD (Sigma-Aldrich; 10 μM) or GSK1016790A (100 nM) in calcium medium[16, 22, 31]. Calcium imaging was performed on Leica SP2 Confocal Microscope or Olympus FluoView 300 confocal microscope and analyzed using Leica/Olympus software and Microsoft Excel. We have previously confirmed that TRPV4 channels are expressed in WT and NEC and that TRPV4 activators 4-α-PDD (10 μM) or GSK1016790A (100 nM) specifically induce calcium influx in TRPV4 expressing but not TRPV4 null endothelial cells in the calcium containing media[31].

SDS-PAGE and Western blot analysis

Cells were lysed in TritonX-100 with protease and phosphatase inhibitor cocktail (Boston Bioproducts). Cell lysates were separated by electrophoresis on 8% SDS- polyacrylamide gels and transferred to Immobilon ® polyvinylidene difluoride membrane. The membrane was blocked in 5% milk in TBS with 0.1% Tween-20 (TBS-Tw) for 1 h. The blot was then incubated with the following primary antibodies anti-TRPV4 (1:300) (Alomone), anti-Actin (1:1000). The ECL (Pierce West Pico) method was used with anti-rabbit (Jackson Laboratories) at a dilution of 1:10,000 and developed using Kodak X-ray film or Protein Simple. Results were quantified using Image J software.

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In vitro angiogenesis assays

Growth factor-reduced Matrigel® (BD Biosciences) was plated on 48 well plates and kept at 37°C in an incubator for 30 min. Cells overexpressing TRPV4-EGFP or EGFP alone (2-8 × 10^4 cells/well) were plated on Matrigel and incubated at 37°C for 16-18 h[20]. For 2D assays performed in the presence or absence of TRPV4-specific agonist, cells were treated with GSK1016790A (100nM) prior to plating. Tube formation was quantified by obtaining images as described below.

Microscopy, image analysis and statistics

The expression of EGFP-TRPV4 in EC was visualized using a Nikon Eclipse TE 2000-E microscope (Nikon, Japan) fitted with a CoolSnap HQ digital camera (Photometrics) or Olympus IX72 fluorescence microscope (Olympus, Japan). The cells spread on flexible gelatin hydrogels were fixed with 4% paraformaldehyde while cells forming tubular structures were left untreated, and samples from both studies were imaged using a Nikon Diaphot 300 phase contrast microscope (Nikon, Japan) fitted with a Hamamatsu digital camera (Hamamatsu Photonics, Japan) or Olympus IX72 fluorescence microscope (Olympus, Japan). Image analyses were performed using ImageJ software (NIH). For cell spreading studies, TEC cell areas were measured by tracing cell perimeter and normalized as described previously[20]. At least 30 cells were evaluated. For cell migration experiments, live cell images were recorded with a CCD camera (Hamamatsu, Tokyo, Japan) on a Nikon Eclipse TE 2000-E microscope (Nikon, Japan) or Olympus IX81 fluorescence microscope (Olympus, Japan) equipped with phase contrast optics and processed using the Image J. The microscope was also equipped with an on-stage heater that maintained the temperature at 37°C at all times, and the culture medium was covered with a thin layer of mineral oil to prevent evaporation. Cell migration was measured by marking the centroids of the migrating cell recorded at 20 min intervals over 2 h and then used to calculate the speed of cell migration. All data are expressed as mean ± SEM and evaluated for differences using student's t-test and/or one-way ANOVA.

Syngeneic tumor model in mice and analysis of tumor growth, vascular malformation and angiogenesis

All the experiments were performed according to approved protocol by Northeast Ohio Medical University, IACUC. Mouse Lewis lung carcinoma (LLC) cells (2 × 10^6) were subcutaneously injected in the flank region of wild type C57BL/6 mice (WT) or TRPV4 knockout mice in C57BL/6 background (KO). Tumor size was measured using calipers at 7, 14 and 21 days and tumor volume was calculated according to the formula $V= 4/3*Pi*Length/2*(width/2)^2$. At day 21, mice were euthanized and tumor tissues were collected and fixed for immunohistochemistry or stored at -80°C. To measure tumor angiogenesis, tumor tissue sections of 10 μm thicknesses were stained with anti-CD31 (PECAM-1) to visualize the microvessels, α-SMA to stain pericytes and DAPI to label the nuclei. Images were acquired using Olympus IX72 microscope and the microvessels density, diameter (Feret) and length were calculated using Image J software. For the *in vivo* drug experiments, six to eight mice/group were used and the animals were divided in to four groups: 1) WT (control) 2) WT + TRPV4 activator 3) WT + Cisplatin and 4) WT + TRPV4

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activator + Cisplatin. Once the tumors were palpable (after 7 days), the mice were daily given an intraperitonial (i.p) injection of TRPV4 agonist GSK1016790A (10 μg/kg) to groups 2 and 4 until day 21. The anti-cancer drug Cisplatin (3 mg/kg/week) was administered i.p. once/week to groups 3, and 4, 3 days post treatment with TRPV4 activator, until day 21. The WT control received saline as a vehicle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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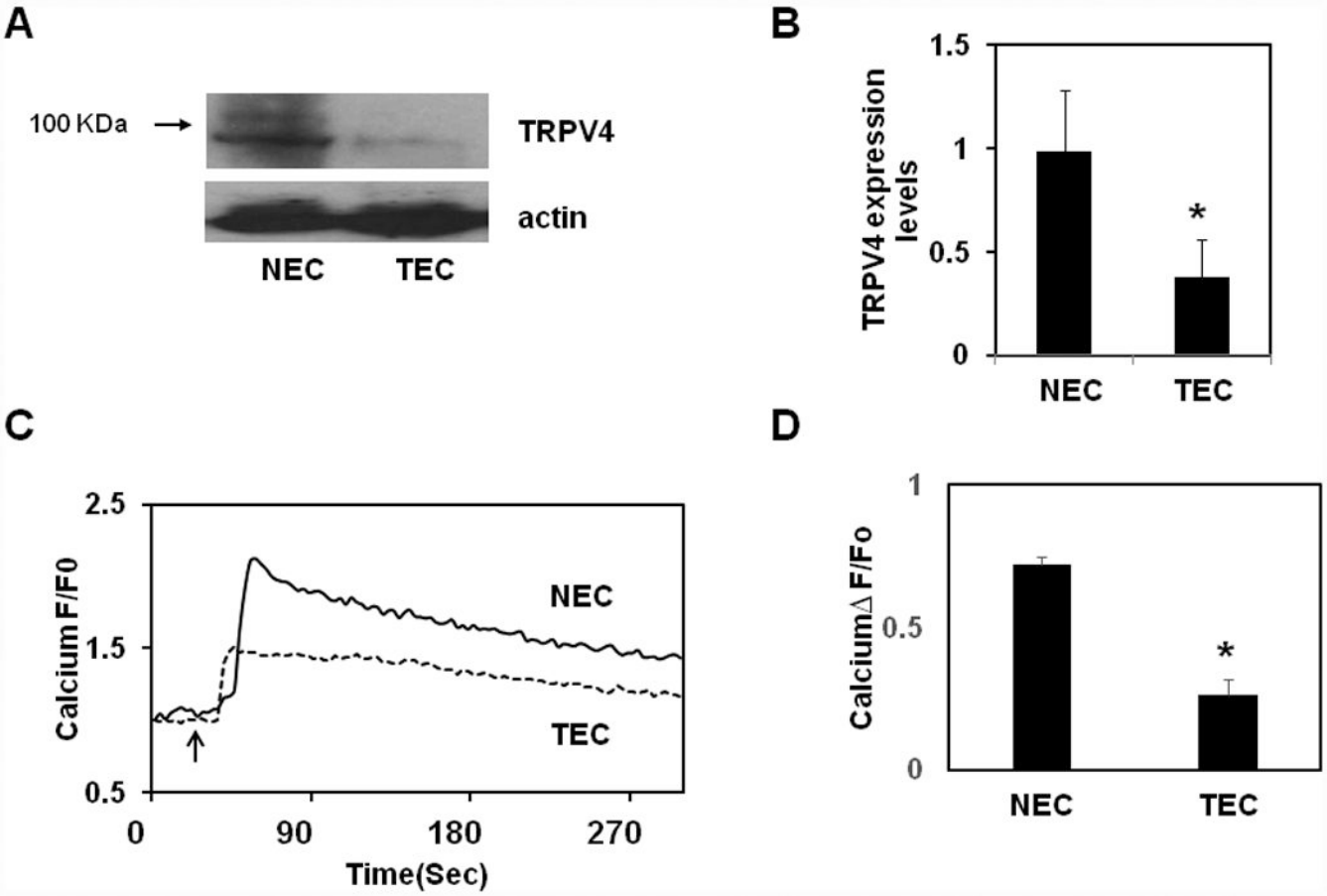

Fig.1. TRPV4 channel expression and function in normal and tumor endothelial cells

A) Western blot analysis of TRPV4 expression in normal (NEC) and tumor-derived endothelial cells (TEC). **B)** Quantitative analysis of the Western blots showing significant ($p \leqq 0.05$) reduction in TRPV4 expression in TEC. **C)** Representative traces showing relative changes in cytosolic calcium in response to a selective TRPV4 agonist, GSK1016790A (100 nM) in Fluo-4 loaded normal and tumor endothelial cells (n=300). 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 3 independent experiments. The significance was set at $p \leqq 0.05$.

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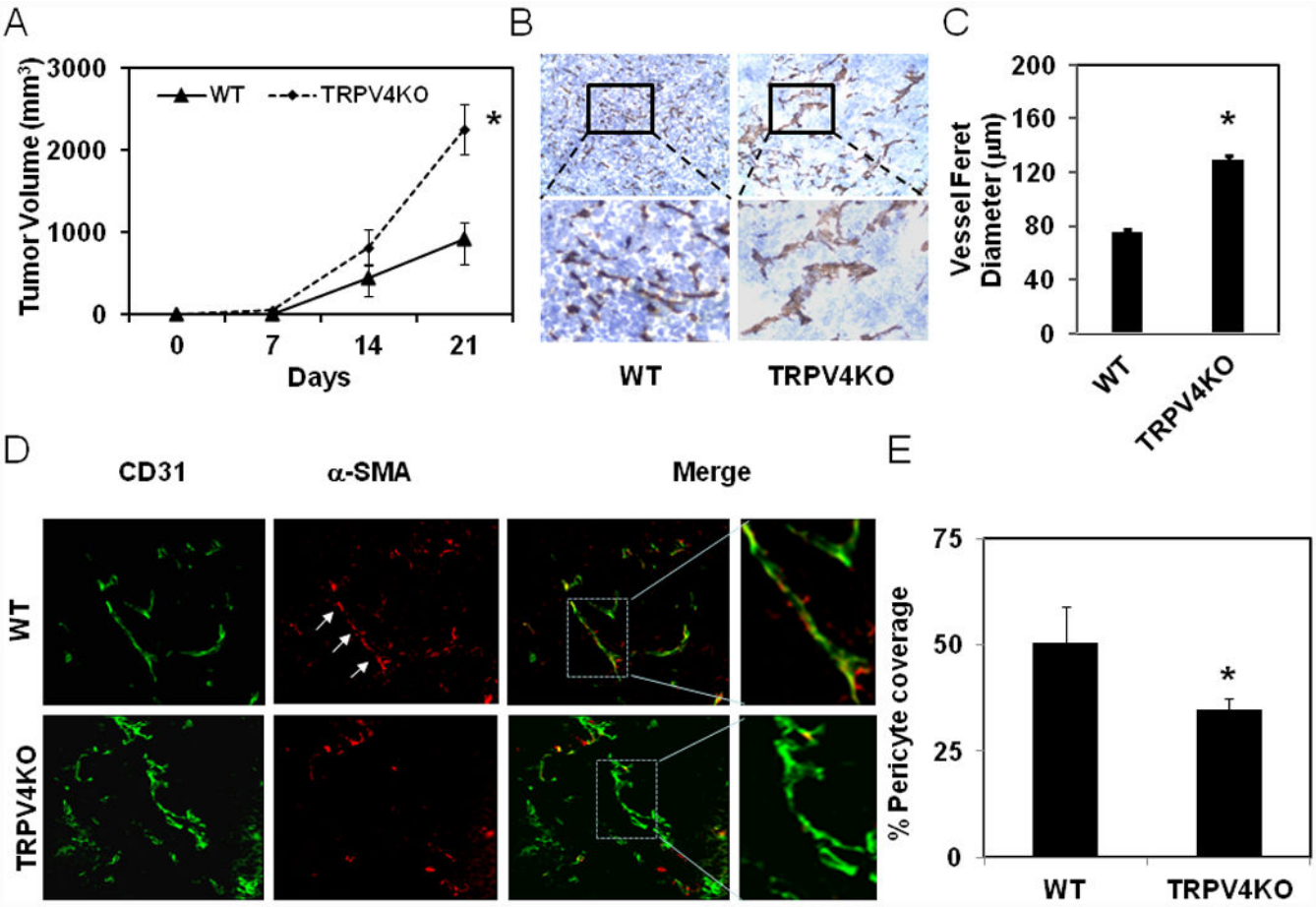

Fig.2. Vessel malformations and tumor growth are enhanced in TRPV4 knockout mice

A) Time-dependent growth of the tumors in WT and TRPV4KO mice. Mouse Lewis lung carcinoma (LLC) cells (2×10^6) were subcutaneously injected in to wild type C57BL/6 mice (WT) or TRPV4 knockout mice in C57BL/6 background (TRPV4KO) and tumor growth was measured using calipers at indicated days. The data shown are ± SEM of three independent experiments (n=8-10 mice for each group). **B)** Immunohistochemical analysis showing increased vessel diameter (feret) in tumors (21 days) from TRPV4 knockout mice (TRPV4KO) compared to wild type mice (WT). **C)** Quantitative analysis of microvessel diameter in tumors from WT and TRPV4KO mice. **D)** Frozen sections of tumors (10 µm thickness) were stained with CD31 (green) and α-SMA (red) to measure pericyte coverage (matured vessels). **E)** Quantitative analysis of pericyte covered microvessels in tumors from WT and TRPV4KO mice. The results shown are mean ± SEM from 3 independent experiments. The significance was set at $p \leq 0.05$.

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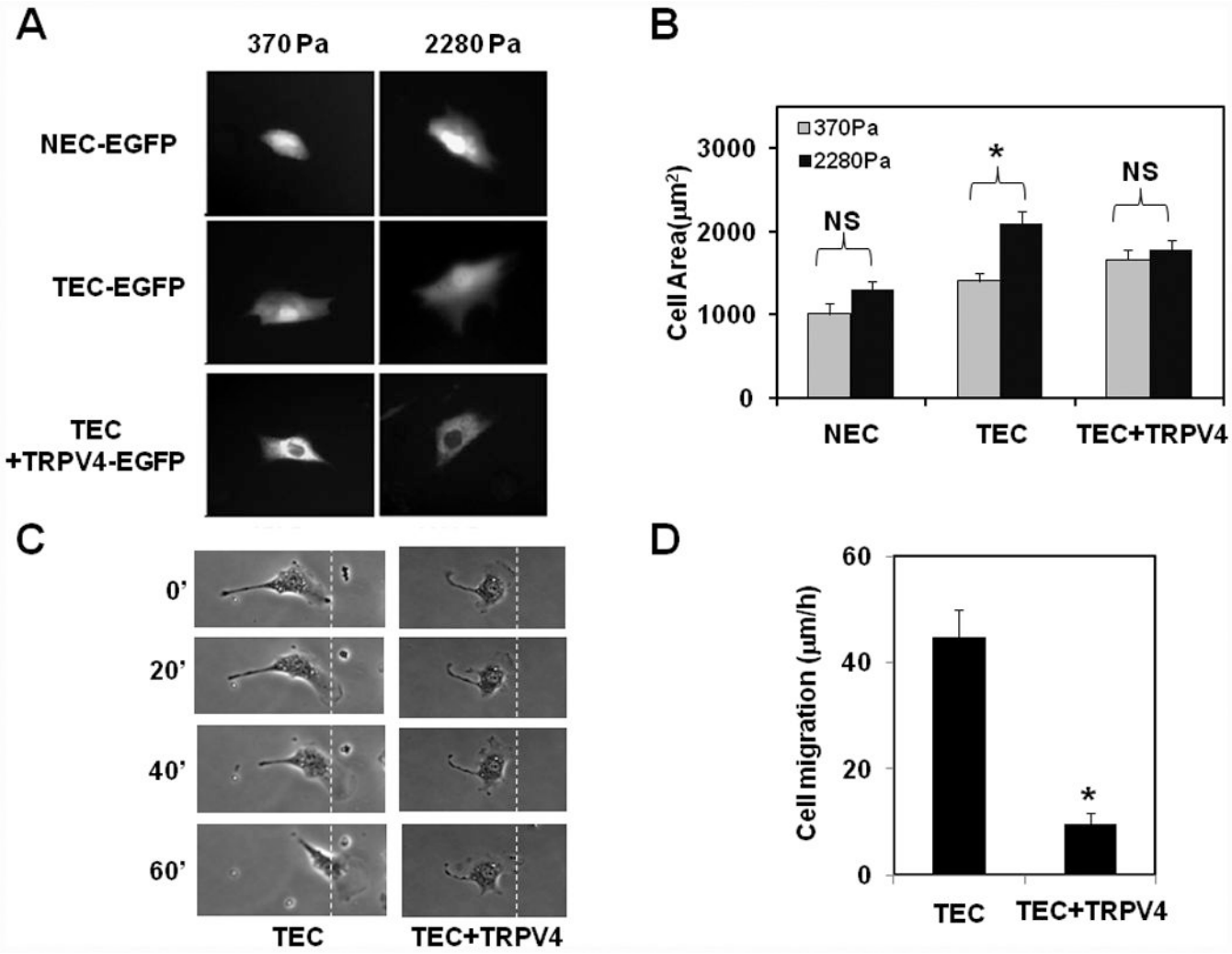

Fig.3. TRPV4 overexpression restores mechanosensitivity towards ECM stiffness, and reduces migration in TEC

A) Representative images showing normal (NEC) and tumor (TEC) endothelial cell spreading (indicative of mechanosensitivity) on intermediate (370 Pa) and high (2280 Pa) stiffness ECM gels. Cells were transfected with either EGFP alone or TRPV4-EGFP **B)** Quantitative analysis of projected cell areas of NEC and TEC (expressing EGFP alone) and TEC+TRPV4 (expressing TRPV4-EGFP) on ECM gels of intermediate and high stiffness (370 and 2280 Pa). The results shown are mean ± SEM from 3 independent experiments. The significance was set at $p \leqq 0.05$. NS= non-significant. **C)** Time lapse phase contrast micrographs showing migration of TEC-EGFP (TEC) and TEC+TRPV4-EGFP (TEC +TRPV4) cells plated on the surface of ECM gels of 370 Pa stiffness. Cells were allowed to spread for 4 h at 37°C and later shifted on to a microscope stage and random cell migration was recorded every 10 min using time lapse microscopy. Dashed line denotes the border of the leading edge at time 0. **D)** Quantification of cell migration as measured by marking the centroid of migrating cells overtime. Note: NEC migrated with a speed of 10 μm/h (Supplementary Fig.S5) suggesting that overexpression of TRPV4 normalized TEC

migration. The results shown are mean ± SEM from 3 independent experiments. The significance was set at $p \leqq 0.05$.

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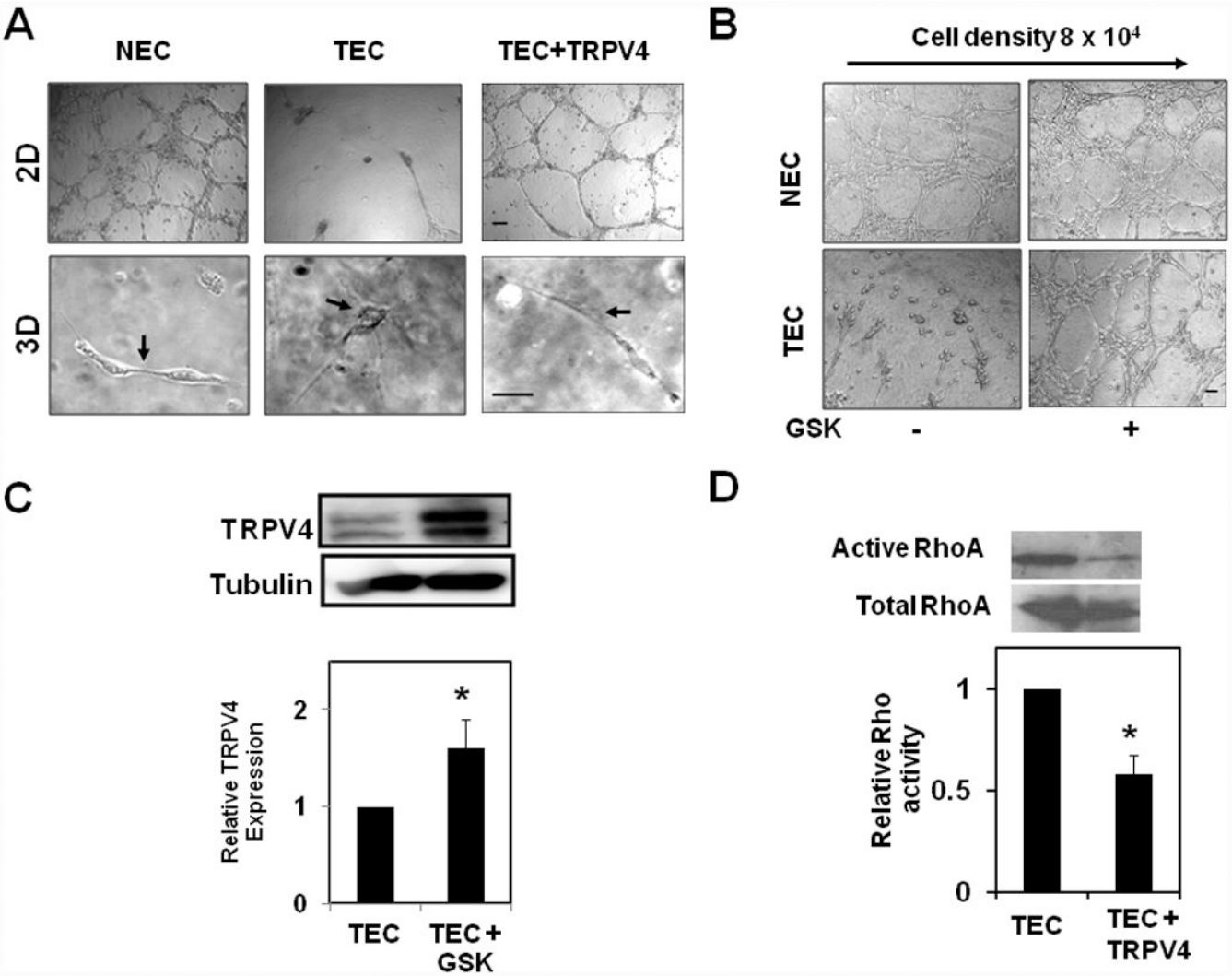

Fig.4. TRPV4 overexpression normalizes abnormal angiogenesis by tumor EC through the inhibition of abnormal Rho activity

A) Phase contrast micrographs showing the normalizing effects of TRPV4 overexpression on TEC angiogenic behavior when plated on 2D Matrigels (at high densities; 8×10^4 well that cause collapse of tubular networks) and 3D Matrigels. NEC-EGFP (NEC), TEC-EGFP (TEC) and TEC-TRPV4-EGFP (TEC+TRPV4) cells were plated and cultured on the surface of Matrigel for 18 h (2D) or mixed in Matrigel and cultured for 14 days (3D). Note that the overexpression of TRPV4 in TEC cells restored tube formation on 2D Matrigel and normalized abnormal tubes in 3D Matrigel. Scale bar= 10 μm. **B)** Phase contrast micrographs showing the normalizing effects of pharmacological activation of TRPV4 with GSK1016790A (100 nM) on TEC angiogenic behavior when plated on 2D (at high densities; 8×10^4/ well that cause collapse of tubular networks). Scale bar= 10 μm. **C)** Representative Western blot showing TRPV4 expression in TEC untreated or treated with GSK1016790A (100 nM) for 24 h. Densitometry analysis of relative changes in TRPV4 expression measured by normalizing the levels of TRPV4 with that of tubulin. **D)** Representative Western blot showing the levels of active-Rho and total Rho for TEC and

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TEC+TRPV4 cells. Rho activity was analyzed in TEC (EGFP) and TEC+TRPV4 cells cultured under regular growth conditions using the Rhotekin-RBD binding assay. Densitometry analysis of relative changes in Rho activity. Rho activity levels were measured by normalizing the levels of active Rho with that of total Rho.

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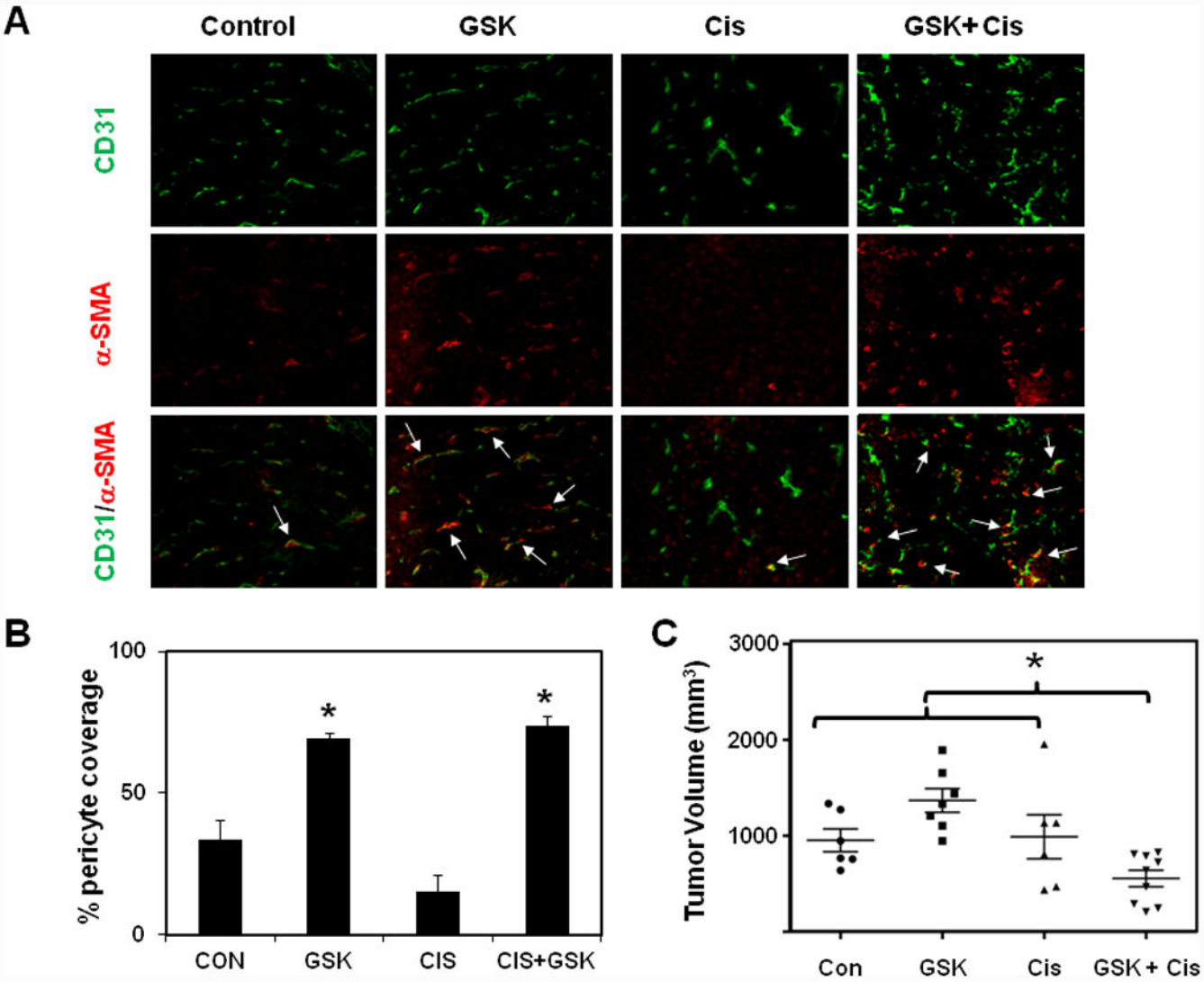

Fig.5. TRPV4 activation with a small molecule activator together with Cisplatin reduces tumor growth in WT mice

A) Syngeneic tumors (LLC) were injected in the back of WT (C57BL/6) mice and tumor growth was monitored for 21 days. TRPV4 activator, GSK1016790A (GSK) was injected i.p. everyday starting from day 7 (after palpable tumors were observed) until 21 days. Cisplatin was injected i.p. (once/week) 3 days after the injection of GSK1016790A. Frozen sections of tumors (10 μm thickness; from 21 day) were stained with CD31 (green) and α-SMA (red) to measure pericyte coverage (matured vessels). **B)** Quantitative analysis of pericyte covered microvessels in tumors from control, GSK, Cisplatin, and GSK + Cisplatin treated mice. The results shown are mean ± SEM from 3 independent experiments. The significance was set at $p \leqq 0.05$. **C)** Tumor volumes among mice groups. Note that tumor growth was reduced in GSK + Cisplatin (*) treated mice. However, treatment with either of the drug alone did not inhibit tumor growth, indicating that GSK treatment improved Cisplatin delivery through the normalization of the abnormal tumor vasculature.