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**ORIGINAL ARTICLE**

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

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Tumor vessels are characterized by abnormal morphology and hyperpermeability that together cause inefficient delivery of chemotherapeutic agents. Although vascular endothelial growth factor 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 transient receptor potential vanilloid 4 (TRPV4) regulates tumor angiogenesis and tumor vessel maturation via modulation of TEC mechanosensitivity. We found that TECs exhibit reduced TRPV4 expression and function, which is correlated with aberrant mechanosensitivity towards extracellular matrix 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 knockout mice. Importantly, overexpression or pharmacological activation of TRPV4 restored aberrant TEC mechanosensitivity, migration and normalized abnormal angiogenesis *in vitro* by modulating Rho activity. Finally, a small molecule activator of TRPV4, GSK1016790A, in combination with anticancer drug cisplatin, significantly reduced tumor growth in wild-type 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.

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## 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 hyperpermeability.[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 anticancer 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 (TECs) became refractory to anti-VEGF therapy overtime.[3–5] These findings led to the emergence of a new concept called 'vascular normalization', that is, transient inhibition of tumor angiogenesis leading to normalization of tumor vasculature that 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 on VEGF-targeted therapies, despite their shortcomings.[2,9,10] In fact, recent positron emission tomograpy 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 patients.[11] Therefore, an urgent need arises for the development of novel vascular normalization strategies.

In addition to soluble stimuli, such as VEGF and platelet-derived growth factor, local mechanical cues conveyed by the extracellular matrix (ECM), owing 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 hyperpermeable owing to irregular basement membrane and poor pericyte coverage and therefore releases plasma components into the surrounding extracellular space, eventually leading to 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 TECs,[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.

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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 (NECs) induces cell reorientation through the activation of mechanosensitive transient receptor potential vanilloid 4 (TRPV4) ion channel-dependent calcium influx, which in turn activates additional integrins and causes downstream cytoskeletal reorganization.[16] Importantly, small-interfering RNA knockdown of TRPV4 channels inhibited mechanical strain-induced reorientation of NECs, thus confirming their key role in EC mechanosensitivity. Notably, EC mechanosensitivity strongly influences vascular formation and patterning,[20] and thus tumor vessel malformations could arise from deregulation of TECs mechanosensing. Indeed, we have previously shown that TECs 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 TECs 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

### TECs express lower levels of TRPV4 and TRPV4-dependent calcium influx

We and others have previously shown that TRPV4 channels are functionally expressed in ECs 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 ECs and this TRPV4-dependent mechanotransduction regulates cyclic strain-induced EC reorientation.[16] Interestingly, we found that tumor-derived ECs (TECs) failed to reorient in response to cyclic strain similar to that of TRPV4 knockdown ECs, suggesting that TECs may mimic TRPV4 knockdown phenotype.[16,20] Because mechanical force (cyclic strain)-induced reorientation in ECs is dependent on TRPV4 channels,[16] we investigated whether these channels contribute to the abnormal mechanosensitivity of TECs.[20] TECs and NECs 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 NECs and TECs using western blot analysis. Although NECs exhibited strong expression of TRPV4,[16,31] it was significantly lower in TECs (Figures 1a and b). To determine the functional implications of lower TRPV4 expression in TECs, we measured calcium influx in Fluo-4/AM loaded cells, in response to the addition of specific TRPV4 activators, GSK1016790A or 4-α-PDD. Using calcium imaging, patch-clamp, small-interfering RNA knockdown and TRPV4 null cells, we have previously demonstrated that these compounds specifically activates TRPV4-mediated calcium influx in ECs.[16,31] GSK1016790A (100 nM) induced calcium influx in both NECs and TECs but the influx in TECs was decreased by almost 40–50% compared with NECs ($P \leqslant 0.001$; Figures 1c and d). Another TRPV4 agonist, 4-α-PDD (10 μM) also induced a rapid calcium influx in NECs, which was again significantly reduced by ~40% ($P \leqslant 0.01$) in TECs (Supplementary Figures S1A and B). These results demonstrate that TRPV4 expression and function (calcium influx) are impaired in TECs.

### Tumor angiogenesis and tumor growth are enhanced in TRPV4 null mice

Next, to confirm whether the TRPV4 expression level contributes to tumor angiogenesis *in vivo*, we induced tumors in TRPV4

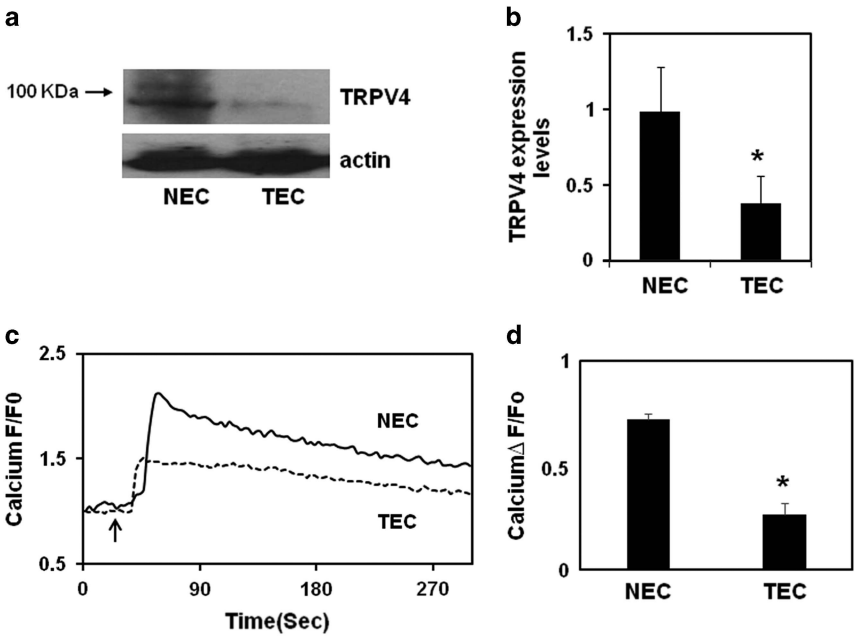


**Figure 1.** TRPV4 channel expression and function in normal and tumor endothelial cells. (**a**) Western blot analysis of TRPV4 expression in normal (NECs) and tumor-derived endothelial cells (TECs). (**b**) Quantitative analysis of the western blots showing significant ($P \leqslant 0.05$) reduction in TRPV4 expression in TECs. (**c**) Representative traces showing relative changes in cytosolic calcium in response to a selective TRPV4 agonist, GSK1016790A (100 nM) in Fluo-4 loaded NECs and TECs ($n = 300$). Arrow denotes the time when the cells were stimulated with the TRPV4 agonist. (**d**) Quantitative analysis of cytosolic calcium influx induced by GSK1016790A in NECs and TECs. (F/F0 = ratio of normalized Fluo-4 fluorescence intensity relative to time 0). The results shown are mean ± s.e.m. from three independent experiments. The significance was set at $P \leqslant 0.05$.

knockout (KO) and wild-type (WT) mice (C57BL/6) by subcutaneously injecting mouse Lewis lung carcinoma cells. We found that tumor growth was 2–3 times greater in TRPV4 KO mice compared with WT mice at day 21 (Figure 2a). Importantly, immunohistochemical analysis revealed that tumors in TRPV4 KO mice exhibited a greater fraction of hyperdilated (malformed) vessels with significantly larger vessel diameters ($P \leqslant 0.05$) compared with WT mice (Figures 2b and 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. (Figures 2d and e). Further, the tumors from TRPV4 KO mice exhibited increased microvessel density compared with tumors from WT mice (Supplementary Figures S2A and B). Next, we assessed the leakiness of TRPV4 KO vessels by performing permeability assays using tetramethylrhodamine isothyocyante-dextran (TRITC) (3000 MW) perfusion, via tail vein injections. Immunohistochemical analysis revealed increased tetramethylrhodamine isothyocyante staining within the tumor tissue, surrounding the vessels, in TRPV4 KO mice compared with WT tumors, suggesting that TRPV4 KO vessels are indeed hyperpermeable (Supplementary Figure S3). Taken together, these results clearly suggest that TRPV4 has 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 ECs.

### TRPV4 overexpression restores mechanosensitivity and reduces abnormal cell migration in TECs

The above findings suggest that the lower levels of TRPV4, a known mechanosensor in ECs[16,22,32,33] may contribute to the previously reported aberrant mechanosensitivity of TECs in response to ECM stiffness and cyclic strain.[20] As ECM stiffness is known to increase in tumors,[19] which can also influence TECs spreading, migration and tube formation, we investigated whether the overexpression of TRPV4 might rescue the abnormal mechanosensitivity exhibited by TECs. To achieve this, we expressed a human TRPV4-enhanced green fluorescent protein (EGFP) construct in TECs; EGFP fluorescence revealed that >80% cells were transfected with TRPV4-EGFP (Supplementary Figure S4A). We found that overexpression of TRPV4 increased GSK1016790A-induced calcium influx in TECs by almost fourfold ($P \leqslant 0.01$) compared with EGFP alone expressing cells (Supplementary Figures S4B and C). The other TRPV4-specific activator, 4-α-PDD, also increased calcium influx in these TRPV4-transfected TECs compared with EGFP-expressing controls (not shown).

To explore whether TRPV4 overexpression influences TEC mechanosensitivity towards ECM stiffness, we next cultured TRPV4-overexpressing TECs 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 (Figures 3a and b). In past studies, we found that both NECs and TECs spread similarly

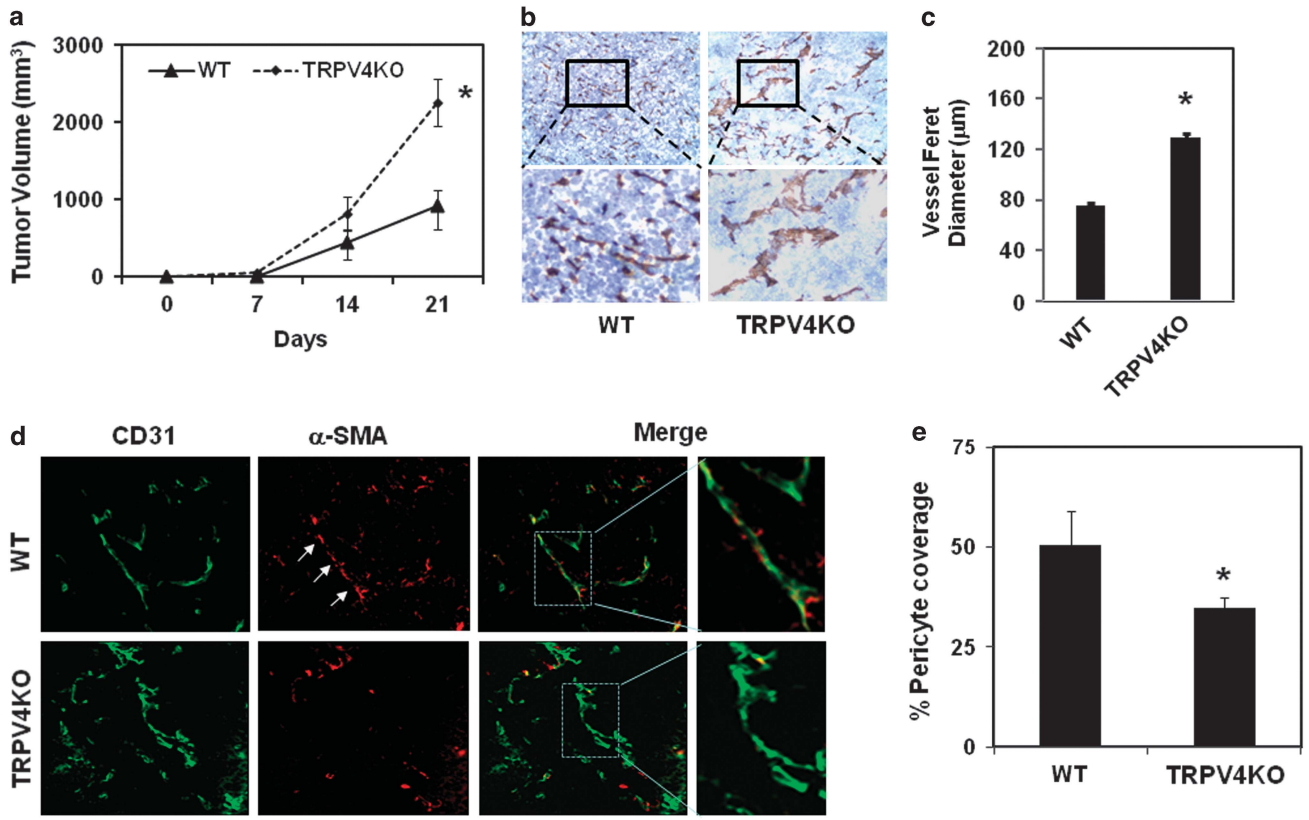


**Figure 2.** Vessel malformations and tumor growth are enhanced in TRPV4 knockout mice. (**a**) Time-dependent growth of tumors in wild-type (WT) and TRPV4 KO mice. Mouse Lewis lung carcinoma (LLC) cells ($2 \times 10^6$) were subcutaneously injected in to WT C57BL/6 mice or TRPV4 knockout (KO) mice in C57BL/6 background and tumor growth was measured using calipers at indicated days. The data shown are ± s.e.m. of three independent experiments ($n = 8$–10 mice for each group). (**b**) Immunohistochemical analysis showing increased vessel diameter in tumors (21 days) from TRPV4 KO mice compared with WT mice. (**c**) Quantitative analysis of microvessel diameter (feret) in tumors from WT and TRPV4 KO 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 TRPV4 KO mice. The results shown are mean ± s.e.m. from 3 independent experiments. The significance was set at $P \leqslant 0.05$.

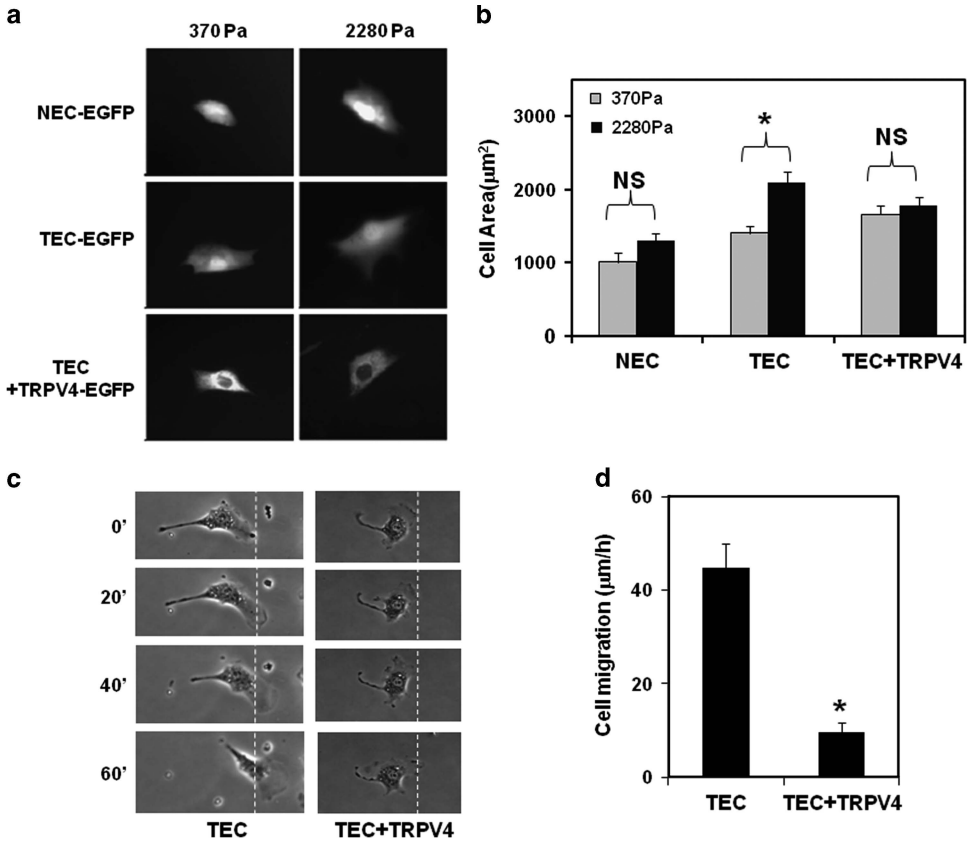


**Figure 3.** TRPV4 overexpression restores mechanosensitivity towards ECM stiffness, and reduces migration in TECs. (**a**) Representative images showing normal (NEC) and tumor endothelial cell (TEC) spreading (indicative of mechanosensitivity) on intermediate (370 Pa) and high (2280 Pa) stiffness ECM gels. Cells were transfected with either EGFP alone or TRPV4-EGFP. (**b**) Quantitative analysis of projected cell areas of NECs and TECs (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 ± s.e.m. from three independent experiments. The significance was set at $P \leqslant 0.05$. 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 Figure S5), suggesting that overexpression of TRPV4 normalized TEC migration. The results shown are mean ± s.e.m. from three independent experiments. The significance was set at $P \leqslant 0.05$.

on low stiffness (98 Pa) gels.[20] However, in that study, NECs exhibited increased spreading on intermediate stiffness (370 Pa), which reached plateau at high stiffness (2280 Pa), whereas TECs 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 NECs (expressing only EGFP) spread to similar extent on both intermediate and high stiffness gels (370 and 2280 Pa), further confirming that the spreading of NECs reached plateau at 370 Pa (Figures 3a and b), similar to our previous study.[20] On the other hand, spreading of TECs (expressing only EGFP) was significantly more on high stiffness gels compared with intermediate stiffness gels (Figures 3a and b). In contrast, TEC-TRPV4-EGFP exhibited reduced spreading on high stiffness gels compared with TEC-EGFP alone, with no significant difference between intermediate and high stiffness substrates (Figures 3a and 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 ECs mechanosensitivity, as previously described.[16] As shown earlier,[20] we found that TEC-EGFP failed to reorient, whereas TECs overexpressing TRPV4-EGFP significantly oriented in response to cyclic strain (data not shown).

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

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

As TRPV4 overexpression restored TEC mechanosensitivity to ECM stiffness, which reduced abnormal cell migration, we explored whether TRPV4 expression normalizes vessel formation and if so,

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

Next, we examined whether pharmacological activation of TRPV4 normalizes abnormal tube formation *in vitro*. For this, we activated TRPV4 in TECs using the small molecule activator of TRPV4, GSK1016790A (GSK; 100 nM). We found that, similar to TRPV4 overexpression, TECs, but not NECs, treated with GSK formed robust tubes at high plating density ($8 \times 10^4$ cells per well; Figure 4b). As activity-dependent regulation of ion channels was shown in various cancers,[35,36] we then asked whether activation of TRPV4 with GSK modulates TRPV4 expression in TECs. We found that treatment of TECs with GSK for 24 h significantly increased TRPV4 protein expression (Figure 4c). These results demonstrate that TRPV4 overexpression or pharmacological activation by GSK normalizes abnormal angiogenesis exhibited by TECs 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 that regulates endothelial contraction, which is required for partial

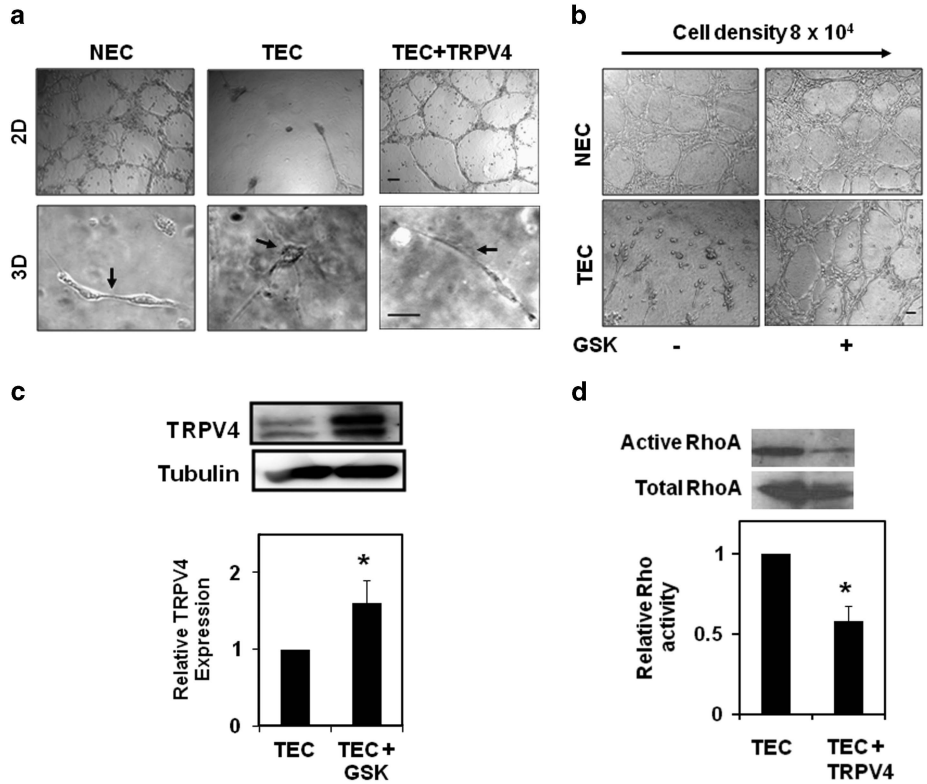


**Figure 4.** TRPV4 overexpression normalizes abnormal angiogenesis by TECs through the inhibition of abnormal Rho activity. (**a**) Phase-contrast micrographs showing the normalizing effects of TRPV4 overexpression on TEC angiogenic behavior when plated on 2D Matrigels (at high densities; $8 \times 10^4$ 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; $8 \times 10^4$ cells per well that cause collapse of tubular networks). Scale bar = 10 μm. (**c**) Representative western blot showing TRPV4 expression in TECs 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 TEC+TRPV4 cells. Rho activity was analyzed in TECs (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.

rounding of ECs during tube formation.[34] We have previously demonstrated that TECs (expressing only EGFP) showed high basal Rho activity,[20] which is the reason for abnormal collapse of TECs 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 TECs (~½-fold lower; $P \leqslant 0.001$; Figure 4d). We found that TRPV4 activator GSK1016790A (100 nM) also inhibited high basal Rho activity in TECs (Supplementary Figure S7), suggesting that TRPV4 is a critical modulator of Rho activity in TECs.

### TRPV4-specific small molecule activator GSK1016790A normalizes tumor vasculature *in vivo* and reduces tumor growth in combination with cisplatin

Finally, we explored whether 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 everyday 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-cisplatin-treated tumors, but not in control or cisplatin-treated ones, showed increased pericyte coverage (Figures 5a and 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 (Figure 5c), but not in control or cisplatin-treated mice, suggesting the improved delivery of cisplatin owing 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. However, regulation of tumor angiogenesis by mechanical forces is not well known. In the present study, we found that TECs

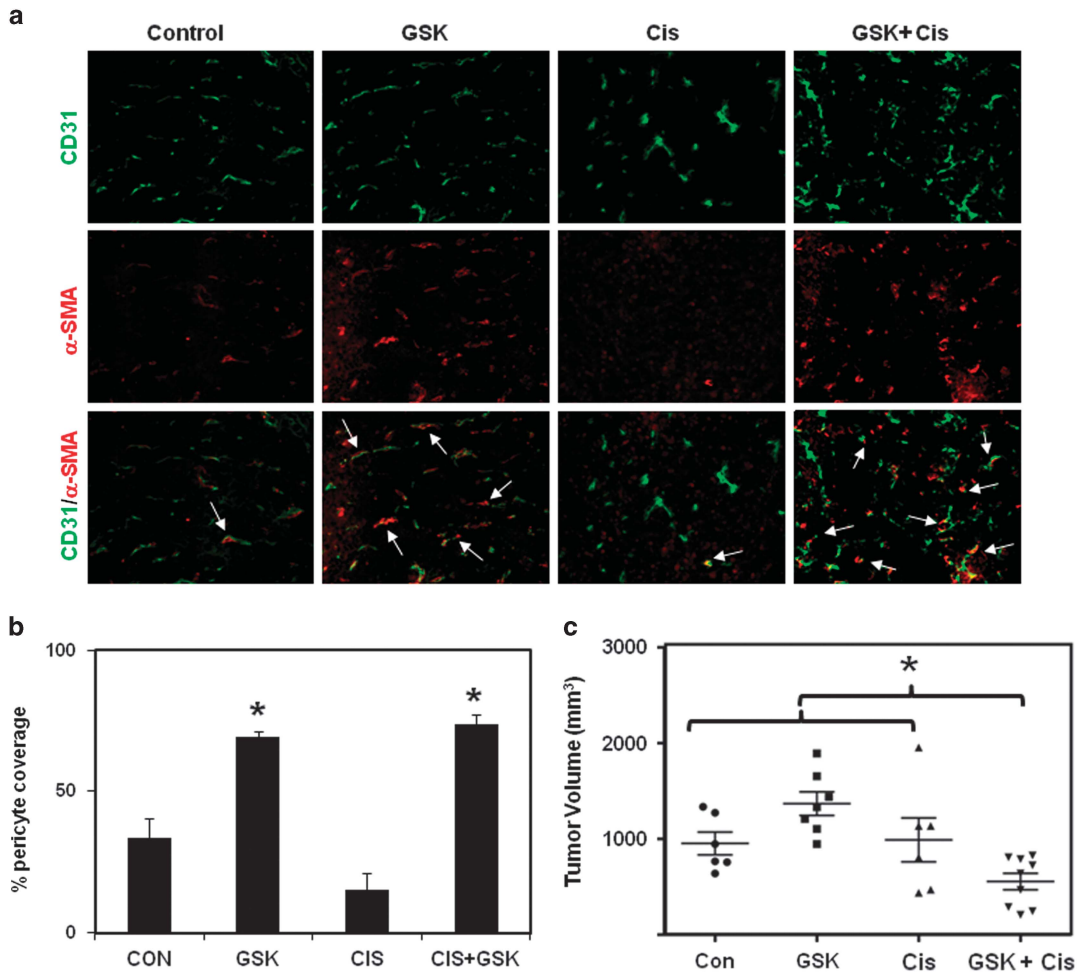


**Figure 5.** TRPV4 activation with a small molecule activator together with cisplatin reduces tumor growth in WT mice. (**a**) Syngeneic tumors (LLC) were injected in the back of WT (C57BL/6) mice and tumor growth was monitored for 21 days. TRPV4 activator, GSK1016790A (GSK) was injected i.p. everyday starting from day 7 (after palpable tumors were observed) until 21 days. Cisplatin was injected i.p. (once per 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 ± s.e.m. from three independent experiments. The significance was set at $P \leqslant 0.05$. (**c**) Tumor volumes among the 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. i.p., intraperitonial; LLC, Lewis lung carcinoma.

express lower levels of TRPV4, a mechanosensitive ion channel, which is a key upstream signaling molecule that regulates TEC mechanosensitivity, tumor angiogenesis and tumor vessel maturation. Importantly, we demonstrate that overexpression of TRPV4 normalizes aberrant TEC mechanosensitivity, migration and angiogenesis through the 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 the 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 ECs that act as a mechanosensor of cyclic strain and shear stress in ECs.[16,22,29,32,33,37] We have previously shown that ECs 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 NECs 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 TRPV4 channel signaling might be altered in these cells. Indeed, we found that TECs express low levels of functionally active TRPV4 channels compared with NECs. TRPV4 protein usually exhibits two bands that are below and above of 100 kDa in western blots.[16,29,38–41] Although the upper band has been demonstrated to be the glycosylated form of TRPV4,[38] which influences its membrane translocation and activity, we did not find any significant difference in terms of the relative amount of each band in NECs or TECs, suggesting that TRPV4 expression is downregulated. Consistently, TRPV4 expression has been shown to be modulated by miR-203.[42] However, it is not clear how tumor cells or tumor stroma may influence TRPV4 expression in TECs. 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 TECs *in vitro*.

The aberrant mechanosensitivity of TECs is known to be mediated by high basal Rho activity;[20] however, the molecular mechanism upstream of Rho responsible for this abnormal behavior is not yet known. Here we show that the overexpression of TRPV4 significantly inhibited high basal Rho activity in TECs. This reduction in basal Rho activity could possibly inhibit the high basal contraction of TECs,[20] 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 TECs 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[43,44] 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 EC reorientation.[16]

Although TRPV4 role in endothelial migration was shown,[45] 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 has 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. As the initial proposal that tumors rely on neovascularization for their survival and growth,[46,47] angiogenesis has become a potential target for cancer therapy. In fact, multiple angiogenesis inhibitors have now been identified, and some clinically approved (for example, Avastin, Roche, Basel, Switzerland), which inhibit the growth of a wide variety of experimental tumors in many animal models, although with limited success in patients. Although VEGF is required for normal proliferation and migration of ECs, 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.[3–6,48] Nevertheless, current vascular normalization therapies still focus on targeting soluble growth factors such as VEGF and platelet-derived growth factor.[2,10,49–56] Notably, recently, it was shown that non-small cell lung cancer patients treated with humanized VEGF antibody, bevacizumab, reduced both perfusion and net influx rate of [(11)C] docetaxel within 5 h as measured by positron emission tomograpy. 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 TECs 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

NECs and TECs were obtained from a transgenic adenocarcinoma mouse prostate model, as previously described.[20,30] Cells were plated on fibronectin or gelatin-coated tissue culture dishes and grown in a defined medium composed of low glucose Dulbecco's-modified Eagle's medium, 10% fetal bovine serum, 10% Nu Serum IV, VEGF (1 ng/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 3 °C, 5% $CO_2$ incubator, split at ~90–95% confluence, and used between passages 11 and 22. These cells (NECs and TECs) 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, endothelial nitric oxide synthase and bind to isolectin-IB4 as measured by fluorescence-activated cell sorting, western blotting, RT–PCR and immunocytochemistry. In contrast, these cells do not express mesenchymal markers α-SMA and platelet-derived growth factor receptor-β.[20,30] NECs and TECs formed robust tubular structures/sprouts in 2D and 3D Matrigel/Fibrin gel angiogenesis assays, further confirming that these are functional ECs.[20,30] We have shown that these ECs 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 (Targeting Systems, El Cajon, CA, USA).[31] The transfection efficiency was found to be 80–90%.

### 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.[20] 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.[20,57] In brief, TECs overexpressing TRPV4-EGFP or EGFP alone or TECs treated with GSK1016709A (Sigma-Aldrich, St Louis, MO, USA; 100 nM for 15 min) were lysed in 1% Triton X-100 buffer and centrifuged at × 12 000 *g* for 15 min. Equal volumes (compensated for equal protein concentration) of clarified lysate were incubated with GST-Rhotekin-RBD beads (Cytoskeleton Inc., Denver, CO, USA), for 1 h at 4 °C. The beads were collected by centrifugation and washed three times with wash buffer. The bound GTP-Rho was extracted with SDS sample buffer and was detected using Rho mAb (Santa Cruz, Dallas, TX, USA) 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 TECs transfected with EGFP alone or TRPV4-EGFP.

### Calcium Imaging

TECs overexpressing TRPV4-EGFP or EGFP alone or NECs were cultured on MatTek glass bottom dishes and loaded with Fluo-4/AM (1–4 μM) for 30 min, washed three 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, 5.5 mM 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 NECs, and that TRPV4 activators 4-α-PDD (10 μM) or GSK1016790A (100 nM) specifically induce calcium influx in TRPV4 expressing but not TRPV4 null ECs in the calcium-containing media.[31]

### SDS–PAGE and western blot analysis

Cells were lysed in Triton X-100 with protease and phosphatase inhibitor cocktail (Boston Bioproducts, Boston, MA, USA). 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 Tris-buffered saline with 0.1% Tween-20 (Tris-buffered saline-Tw) for 1 h. The blot was then incubated with the following primary antibodies anti-TRPV4 (1:300; Alomone, Jerusalem, Israel), anti-actin (1:1000). The enhanced chemiluminescence (Pierce West Pico, Carlsbad, CA, USA) method was used with anti-rabbit (Jackson Laboratories, Bar Harbor, ME, USA) at a dilution of 1:10 000 and developed using Kodak (Rochester, NY, USA) X-ray film or Protein Simple. Results were quantified using Image J software.

### *In vitro* angiogenesis assays

Growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) was plated on 48-well plates and kept at 3 °C in an incubator for 30 min. Cells overexpressing TRPV4-EGFP or EGFP alone ($2–8 \times 10^4$ cells per well) were plated on Matrigel and incubated at 3 °C for 16–18 h.[20] For 2D assays performed in the presence or absence of TRPV4-specific agonist, cells were treated with GSK1016790A (100 nM) before plating. Tube formation was quantified by obtaining images as described below.

### Microscopy, image analysis and statistics

The expression of EGFP-TRPV4 in ECs was visualized using a Nikon Eclipse TE 2000-E microscope (Nikon, Konan, Japan) fitted with a CoolSnap HQ digital camera (Photometrics) or Olympus IX72 fluorescence microscope (Olympus, Tokyo, Japan). The cells spread on flexible gelatin hydrogels were fixed with 4% paraformaldehyde, whereas cells forming tubular structures were left untreated, and samples from both studies were imaged using a Nikon Diaphot 300 phase-contrast microscope (Nikon) fitted with a Hamamatsu digital camera (Hamamatsu Photonics, Tokyo, Japan) or Olympus IX72 fluorescence microscope (Olympus). Image analyses were performed using Image J software (NIH). For cell spreading studies, TEC 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 Photonics) on a Nikon Eclipse TE 2000-E microscope (Nikon) or Olympus IX81 fluorescence microscope (Olympus) 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 ± s.e.m. and evaluated for differences using student's *t*-test and/or one-way analysis of variance.

### 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 cells ($2 \times 10^6$) were subcutaneously injected in the flank region of WT C57BL/6 mice or TRPV4 KO mice in C57BL/6 background. 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 4',6-diamidino-2-phenylindole (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 per group were used and the animals were divided in to four groups as following: (1) WT (control); (2) WT+TRPV4 activator; (3) WT+cisplatin; and (4) WT+TRPV4 activator+cisplatin. Once the tumors were palpable (after 7 days), the mice were daily given an intraperitonial injection of TRPV4 agonist GSK1016790A (10 μg/kg) to the groups 2 and 4 until day 21. The anticancer drug cisplatin (3 mg/kg per week) was administered intraperitonially once per week to the groups 3, and 4, 3 days post treatment with TRPV4 activator, until day 21. The WT control received saline as a vehicle.

## CONFLICT OF INTEREST

CKT and DI have rights in a patent based on some of the results presented in this manuscript. The remaining authors have no conflict of interest.

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