Non-Small Cell Lung Cancer A549 cells induces HUVECs proliferation and migration through TRPV3 promoting the secretion of VEGF

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Abstract

Background Angiogenesis is vital in the process of primary tumor growth and metastasis. Ca2+ signaling is crucial for tumor angiogenesis. This study aimed to detect the potential role of Ca2+ permeable transient receptor potential vanilloid-3 (TRPV3) in the angiogenesis of non-small cell lung cancer (NSCLC). Methods Small interfering RNA was used to down-regulate TRPV3 expression in A549 cells. A laser scanning confocal microscope was used to examine intracellular calcium concentration ([Ca2+]i). HUVECs tube formation and migration assay, Western blot, MTT and ELISA were performed to detect the potential mechanisms of TRPV3 in tumor angiogenesis. A mouse tumor xenograft model was performed to expound the effects of TRPV3 on tumor cell growth. Results Inhibition of TRPV3 reduced [Ca2+]i and protein expression of VEGF and HIF-1α in A549 cells. Moreover, HIF-1α depletion decreased the VEGF secretion level and expression. Depletion of TRPV3 inhibits HUVECs proliferation, tube formation and migration induced by conditioned medium. And TRPV3 inhibition could decrease the volume of xenograft tumors, MVD of CD34+ cells. HIF-1α, VEGF and p-CaMKП expression levels in the xenograft tumors of RuR and siTRPV3 groups was reduced. Conclusions TRPV3 calcium channel protein may play a key role in NSCLC angiogenesis. TRPV3 could promote the angiogenesis through HIF-1α-VEGF signaling pathway. Targeting TRPV3 channel protein by novel approaches would be useful for reversing NSCLC angiogenesis.

Background

Lung cancer is the leading cause of death in malignant tumors in globally [1]. Non-small cell lung cancer (NSCLC) accounts for almost 85% of all lung cancer cases [2].Furthermore, for patients with advanced NSCLC, the 5-year survival rate is no more than 5% [3]. Angiogenesis is vital in the process of primary tumor growth and metastasis [4].Angiogenesis pathways have been identified as important therapeutic targets in many malignant tumors, including lung cancer. However, the angiogenesis molecular mechanisms underlying the development of NSCLC are still not completely understood.

Ca^{2+}signaling is crucial for tumor angiogenesis [5]. Ca^{2+} influx directly modulates VEGF signaling, endothelial proliferation and angiogenesis [6].The expression of amount of Ca^{2+} permeable transient receptor potential (TRP) channels is altered in many malignant tumors [7], such as TRPC1, TRPC3, TRPC6, TRPV1 and TRPV4 [8-11].Transient receptor potential vanilloid-3 (TRPV3), as one of the members of the TRP channels, we have demonstrated that TRPV3 was overexpressed in NSCLC and promoted proliferation of A549 and H1299 lung cancer cells. TRPV3 inhibition decreased intracellular calcium concentration ($[Ca^{2+}]_i$) of lung cancer cells and arrested cell cycle progression in G1/S boundary [12]. TRPV3 belongs to the temperature-sensitive TRP channels, is activated by innocuous warm temperatures in the range of 33–39 °C. Liu et al. revealed that TRPV3 activation plays a central role in cardiac fibrosis induced by pressure overload in rats [13]. Aijima et al. argued that knockdown of TRPV3 suppressed proliferation of oral epithelia in mice [14]. Zhang et al. revealed that TRPV3 promotes hypoxia-mediated

pulmonary artery smooth muscle cells proliferation via increased [Ca^{2+}]i [15].Here, we investigated if TRPV3 plays a role in endothelial cell (EC) proliferation and tumor angiogenesis.

Hypoxia-inducible factors-1α (HIF-1α) is important in the process of angiogenesis, promoting the expression of pro-angiogenic factors such as VEGF and their receptors [16]. High HIF-1α expression is found in hemangioblastoma, glioblastoma multiforme, colonic adenocarcinoma and subtypes of breast, prostate and lung cancer [17]. Calcium signal are demonstrated regulators of HIF-1α at different stages of the HIF-1 pathway, including transcription, translation, stabilisation or nuclear translocation in many cancer types [18]. Hui et al. revealed that calcium antagonist inhibited the expression of HIF-1α under anoxia, and calcium influx was required for HIF-1α mRNA translation [19]. TRPC1 calcium channel was also shown to regulate the constitutive translation of HIF-1α via an Akt dependent pathway in basal breast cancer cells [8]. Zhu et al. showed that TRPC5 calcium channel induced nuclear translocation of HIF-1α from the cytosol to the nucleus by siRNA-mediated silencing of TRPC5 in MCF7 breast cancer cells and xenografts [20]. Furthermore, Lu et al. indicated indirect regulation of Ca^{2+} through HIF-1 has been confirmed to mediate the chemotherapy-stimulated breast cancer stem cell enrichment [21].

In this study, we aimed to determine activation of TRPV3 might induce the expression of HIF-1α and VEGF, promote EC proliferation and NSCLC angiogenesis both in vitro and in vivo.

Methods

Cell Culture

Human non-small cell lung cancer A549 cells were acquired from the American Type Culture Collection. A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone) containing 10% fetal bovine serum (FBS) (HyClone) in the presence of 100 IU/mL penicillin (Sigma) and 100 μg/mL streptomycin (Sigma). HUVECs were isolated from human umbilical cord veins by collagenase treatment as reported previously [22] and grown in DMEM medium, supplemented with 10 % FBS. All of these cell lines were incubated at 37℃ in a humidified atmosphere of 5 % CO_2.

Cell Transfection

Selective targeting of TRPV3 and HIF-1α was performed by specific siRNAs. The siRNAs and a negative control siRNA (NCsiRNA) were synthesized commercially (GenePharma Co. Ltd. Shanghai, China). Transfection of siRNA (20 nM) was performed with X-tremeGENE siRNA transfection reagent (Roche, Penzberg, Germany). A549 cells were seeded in 6-well culture plates. When the cells reached about 30%-50% confluence, they were transfected with TRPV3 siRNA, HIF-1αsiRNA or NCsiRNA. After 24 h of treatment, proteins were extracted from cells and assessed by western blotting.

Quantification of VEGF (ELISA)

The VEGF level in the conditioned media from cultured cells was measured by ELISA assay kit (Raybio, Norcross, GA, USA). The procedure was conducted according to the manufacturer's instructions.

Measurement of $[Ca^{2+}]i$

Intracellular Ca^{2+} distribution was measured with calcium fluorescent probes Fluo 3/ AM (acetoxymethyl ester form, Molecular Probes, Beijing, China) under laser confocal microscope (Olympus, Tokyo, Japan) as described previously [12].

Western Blotting

Protein sample was extracted from the cultured cells or xenografts in nude mice using lysis buffer (Thermo Fisher Scientific,Rockford, IL, USA). The supernatant was then collected after centrifugation at 13,000 rpm and 4 °C for 15 min. Protein concentration was measured using the Bradford method. Equal amounts of protein were separated on SDS-polyacrylamide gels and then were transferred to a PVDF membrane. The membranes were incubated with a primary antibody at 4 °C overnight. After washes the membranes were incubated at 37 °C for 2 h with the appropriate secondary antibody. The immunoreaction was visualized using ECL (Thermo Fisher Scientific).

The primary antibodies used were anti-TRPV3 (Alomone Labs, Jerusa-lem, Israel), anti-HIF-1α (Abcam, Cambridge, MA, USA), anti-phosphorylated CaMKП (p-CaMKП), anti-CaMKП, anti-PCNA, anti-CyclinA, anti-CyclinD1, anti-CyclinE, anti-P27 (Cell Signaling Technology, Boston, MA, USA), anti-VEGF, anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Conditioned medium

A549 cells were cultured in DMEM with 10% FBS until confluence of cells. Then, the old medium was removed, and A549 cells were washed with serum-free DMEM three times. The cells were cultured in serum-free DMEM and treated with RuR 20μM, TRPV3 siRNA, NC siRNA for 24 h, respectively, while the control group with nothing. Cell supernatant of four groups was collected as conditioned medium, including control group, NC group, RuR group, siTRPV3 group. Conditioned medium was centrifuged for 5 min at 1000 rpm and the supernatants were collected and stored at -20˚C until use.

Cell Proliferation Assay

MTT (3-[4, 5-dimethylthylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assays were performed to examine HUVEC proliferation. HUVECs (1×10^3) were cultured in 96-well plates in 100μl DMEM with 10% FBS. After 24 h the medium were removed and four groups conditioned medium were added to each well (100μl/well), respectively. After 48 hours incubation, cells growth was measured by MTT (Sigma). The absorbance of each well was read at 490 nm on a spectrophotometrically.

HUVEC Tube Formation Assay

Human umbilical vein endothelial cells (HUVECs) among different conditioned medium were cultured in a 96-well plate coated with Matrigel (BD Pharmingen, San Diego, CA) at 37 °C. After 12 h, endothelial cell tube formation was visualized under a light microscope. The number of formed capillary tubes was counted and scanned in five fields.

Wound Healing Assay

HUVECs (3×10^5 /well) were seeded in DMEM with 10% FBS in 6-well plates for 6 h to permit cell adhesion. Subsequently, the cell monolayer was scratched in the middle of the well with a standard 200μl pipette tip and washed with PBS. Cells were then treated with conditioned medium. At the different time points, the monolayer scratches were captured using a microscope (Olympus, Tokyo, Japan).

Cell Cycle Analysis

HUVECs were treated with conditioned medium for 48 h. Cells were then harvested and washed twice with PBS. The cells fixed in 70% ethanol for 24 h and then stained with PI solution at 37°C for 30 min. DNA content was analyzed by flow cytometry (FACSAriaI, BD Biosciences, Franklin Lakes, NJ, USA).

Xenograft Experiments in Vivo

Four-week-old male BALB/c nude mice (15-20g, n=24) were purchased from Animal Research Center, Harbin Medical University (Harbin, China) and kept in animal care facilities in a pathogen-free environment. A549 cells (1×10^7) in PBS 100 μl were subcutaneously injected into the right flank of each nude mice. When the diameter of tumors was between 5 and 6 mm, total of 24 mice were randomly divided into four groups, (1) control group: tumors were injected with normal saline 50 μl, (2) NC group: tumors were injected with liquid mixture (NC siRNA 20μl + transfection reagent 20μl + saline 10 μl), (3) RuR group: tumors were injected with TRPV calcium channel blocker ruthenium red (RuR, 20μM) 50 μl and (4) siTRPV3 group: tumors were injected with liquid mixture (TRPV3 siRNA 20μl + transfection reagent 20μl + saline 10 μl). Each group of tumors was pretreated once every 2 days, and tumor volume were measured every 2 days. The tumor volume (mm^3) = $(width)^2 \times length/2$. After 2 weeks, the nude

mice were euthanized by carbon dioxide. The Animal Experimental Ethics Committee of Third affiliated hospital, Guizhou Medical University approved all animal and animal protocols.

Immunohistochemistry (IHC)

Four-micron sections were cut from xenograft tissues and the slides were dewaxed in xylene and rehydrated through a series of graded alcohol. High-temperature antigen retrieval was performed in a citrate buffer for 10 min in a microwave oven and the slides were immersed in 3% H_2O_2 to quench endogenous peroxidase after cooling to room temperature. The sections were treated with normal serum to reduce non-specific binding and then incubated with primary anti-CD34 antibody (1:50 Abcam, Cambridge, MA, USA) overnight at 4 °C. Certain slides were incubated in PBS as a negative control. Secondary antibody was from a SP reagent kit (Zhongshan Biotechnology Company, Beijing, China). The tissue sections were further incubated with streptavidin-horseradish peroxidase complex for 15 min. The sections were stained by diaminobenzidine (DAB) and counterstained with hematoxylin.

Statistical Analysis

Statistical analyses were performed using SPSS 17.0. Date were presented as mean ± standard deviation (SD). The statistical difference of data between groups was analyzed by Student's t-test. Differences were considered significant when $p < 0.05$.

Results

Inhibition of TRPV3 affects the expression of angiogenesis related proteins in A549 cells

To investigate the relationship between TRPV3 and angiogenesis, a TRPV calcium channel blocker RuR and siRNA techniques were used to block and knockdown TRPV3 expression in A549 cells. Intracellular calcium concentration ($[Ca^{2+}]_i$) was detected by immunofluorescence and laser scanning confocal microscope to test the activity of TRPV3 protein. The results showed that $[Ca^{2+}]_i$ of A549 cells was decreased significantly by RuR and TRPV3-siRNA (Fig. 1A). Then the VEGF secretion level was detected in the media of A549 cells by ELISA. We found that inhibition of TRPV3 reduced the VEGF secretion of A549 cells (Fig. 1B). And the protein expression of VEGF and HIF-1α in A549 cells was tested by Western blot. The results showed that TRPV3 depletion decreased the expression of VEGF and HIF-1α (Fig. 1C).

To confirm HIF-1α is a main regulator of VEGF expression, A549 cells were treated with HIF-1α siRNA and inhibitor, KC7F2, and were detected the protein levels of VEGF. The results indicated that HIF-1α-specific

siRNA and KC7F2 reduced protein expression levels of HIF-1α by Western blot (Fig. 1D). And as shown in Figure 1E and F, HIF-1α depletion decreased the VEGF secretion level and expression.

Depletion of the TRPV3 inhibits HUVECs proliferation induced by conditioned medium

Angiogenesis demands the proliferation of vascular endothelial cells. We accomplished MTT assay to evaluate the effects of TRPV3 on proliferation of endothelial cells. The results demonstrated that the HUVEC proliferation induced by the conditioned medium of A549 cells treatment with RuR and TRPV3 siRNA was inhibited compared to that of the control and NC siRNA groups (Fig. 2A). And cell cycle analysis detected by flow cytometry showed that HUVECs cell cycle arrest at the G1/S boundary when treatment with RuR and TRPV3 siRNA A549 conditioned medium (Fig. 2B). As show in Figure 3C, Western blot analysis revealed that the protein levels of PCNA, CyclinA, CyclinD1, CyclinE decreased and P27 increased in HUVECs with conditioned medium of A549 cells treatment with RuR and TRPV3 siRNA.

Depletion of the TRPV3 inhibits HUVECs tube formation and migration induced by conditioned medium

Capillary tube formation of HUVECs is the key step in angiogenesis. HUVECs in four group different conditioned medium respectively were cultured on Matrigel for 12 h. As seen in Fig. 3A, the number of complete tubes induced by the A549 conditioned medium with RuR and TRPV3 siRNA was significantly reduced. To investigate whether TRPV3 affects the migration of HUVECs, scratch wound healing assay was performed. Depletion of TRPV3 in A549 cells inhibited HUVECs migration (Fig. 3B).

Inhibition of TRPV3 suppresses in vivo angiogenic and tumorigenic abilities in A549 cells

To explore whether the TRPV3 expression affects tumor growth and angiogenesis in vivo, we performed a mouse tumor xenograft model with A549 cells to expound the effects of TRPV3 on tumor cell growth. A549 cells in PBS were subcutaneously injected into the right flank of nude mice. When the diameter of tumors was between 5 and 6 mm, tumors were injected with normal saline, RuR, TRPV3 siRNA, or NC siRNA. The gross images of tumors are shown in Fig. 4A. RuR and TRPV3 siRNA treatment reduced both the volume of xenograft tumors (Fig. 4B). In addition, immunohistochemical staining with anti-CD34 was used to confirm the effect of TRPV3 on xenograft tumor angiogenesis (Fig. 4C). Microvessel density (MVD) of $CD34^+$ cells decreased significantly in both RuR and siTRPV3 groups (Fig. 4D). HIF-1α, VEGF

and p-CaMKΠ expression levels in the xenograft tumors of RuR and siTRPV3 groups was reduced by Western blot (Fig. 4E, 4F).

Disscussion

In the present study, we demonstrate that calcium channel protein TRPV3 has significant pro-angiogenic properties in vitro and in vivo. First, we reported that TRPV3 promoted secretion of VEGF and expression of HIF-1α and VEGF proteins in A549 cells. Second, we indicated that TRPV3 contributed to HUVEC proliferation, capillary tube formation and migration. Finally, through xenograft experiments, we demonstrated that inhibition of TRPV3 suppresses tumor growth and angiogenesis in vivo.

Intracellular calcium concentration is considered as an important regulator of many various cell functions and directly involved in signal transduction of VEGF. Higher proliferation and migration rates of ECs is primarily attributed to increased Ca^{2+} influx through store-dependent TRP members, such as TRPC1 and TRPC4 [23]. And TRPC3 and TRPC6, DAG-gated TRP members, contributes to infiltration and migration change of ECs [24]. Redox state-sensitive, TRPM2, TRPM6 and TRPM7, are most potentially involved in all those above processes. The secretion of angiogenic factors is strengthened as well by increasing Ca^{2+} influx via store-dependent TRPs [25]. To investigate if TRPV3 plays a role in tumor angiogenesis, we depleted the TRPV3 in A549 cells. We found that $[Ca^{2+}]_i$ of A549 cells was decreased significantly the secretion and expression of pro-angiogenic factor VEGF were reduced. On the other hand, nude mice experiment in vivo was used, knockdown or inhibit TRPV3 suppresses proliferation and angiogenesis of transplanted lung cancer cells. MVD of $CD34^+$ cells in RuR and siTRPV3 groups was obviously decreased compared with control and NC groups. The expression of VEGF and p-CaMKΠ in xenograft tumors tissue were reduced in RuR and siTRPV3 groups. CaMKII is an extensively considered effector of Ca^{2+}/CaM signaling and has been reported to control many cellular processes [26]. TRPs activation allow extracellular calcium entry and the induction of protein kinases CaMKΠ activation [27]. These results above indicated that TRPV3 contributes to increase Ca^{2+} influx, it plays an important role in NSCLC angiogenesis.

VEGF is indeed important in the angiogenesis cascade [28]. HIF-1α is important in the process of angiogenesis, promoting the expression of VEGF [17]. Some studies have investigated the role of HIF-1α and VEGF in various solid tumors, such as gastric and colorectal cancer [29]. Our results provided evidence that the expression of VEGF was significantly decreased after HIF-1α blocking or knockdown in A549 cells. And depletion of the TRPV3 in A549 cells inhibited the expression of HIF-1α. The expression of HIF-1α in xenograft tumors tissue was reduced in RuR and siTRPV3 groups. These results showed that TRPV3 channel could promote the angiogenesis of lung cancer cells through HIF-1α-VEGF signaling pathway. Du et al. argued that the secretion of VEGF by tumor cells is found to potentially have important roles in promoting the proliferation of HUVECs [30].

Vascular endothelial cells are the basic structural units of blood vessels, whose proliferation, migration and capillary tube formation are the essential part of the angiogenesis response [31]. Our study indicated

that TRPV3 had significant promoting effect on proliferation, migration and tube formation of HUVECs. Depletion of TRPV3 in A549 cells inhibited HUVECs tube formation and migration. TRPV3 knockdown or blocking in A549 cells slowed down the proliferation of HUVECs, decreased their accumulation in the S phase of the cell cycle，lowered the expression of PCNA, CyclinA, CyclinD1, CyclinE and increased the expression of P27. PCNA is a marker of cell proliferation and a substance essential for DNA synthesis of eukaryotic cells [32]. CyclinA is essential for cells through the S phase [33]. CyclinE and cyclinD1 are required for progression via G1/S transition [34, 35]. P27 contributes to blocking the cell cycle progression through G1 to S phase [36]. These results provide a new molecular mechanism that A549 cells induced cell cycle progression of HUVECs is mediated by the TRPV3 channel protein.

Conclusions

In summary, these data showing that high TRPV3 calcium channel protein expression was closely associated with tumor size, high HIF-1a expression, high VEGF secretion and promoted NSCLC angiogenesis. NSCLC A549 cells induces HUVECs proliferation and migration through the TRPV3-HIF-1α-VEGF pathway. Targeting TRPV3 calcium channel protein by novel approaches would be useful for reversing NSCLC angiogenesis.

Abbreviations

TRP: Transient receptor potential; TRPV3: Transient receptor potential vanilloid-3; NSCLC: Non-small cell lung cancer; $[Ca^{2+}]i$: Intracellular calcium concentration; HIF-1α: Hypoxia-inducible factors-1α; VEGF: Vascular endothelial growth factor; EC: Endothelial cell; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; MTT: 3-[4, 5-dimethylthylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide; HUVEC: Human Umbilical Vein Endothelial Cells; siRNA: Small interfering ribonucleic acid; siNC: Negative control siRNA; IHC: Immunohistochemistry; RuR: Ruthenium Red; PCNA: Proliferating Cell Nuclear Antigen; MVD: Microvessel density; CaMKII: Calcium/calmodulin-dependent kinase II; p-CaMKII: Phospho-CaMKII

Declarations

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Availability of data and materials

All data generated and analyzed during this study are included in this published article.

Author Contributions

YC and HJ conceived and designed the experiments; XL performed the experiments; XL and HL analysed the data; HL and ZL contributed reagents/materials tools; XL wrote the paper; DY conducted the Western blot; HJ, ZL and TW conducted other experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This article does not contain any studies with human participants performed by any of the authors. All procedures performed in studies involving animals were in accordance with the ethical standards of Animal Experimental Ethics Committee of Third affiliated hospital of Guizhou Medical University.

Consent for publication

Not applicable.

Competing interests

All the authors declared that there is no conflict of interest in this work.

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Figures

Fig. 1

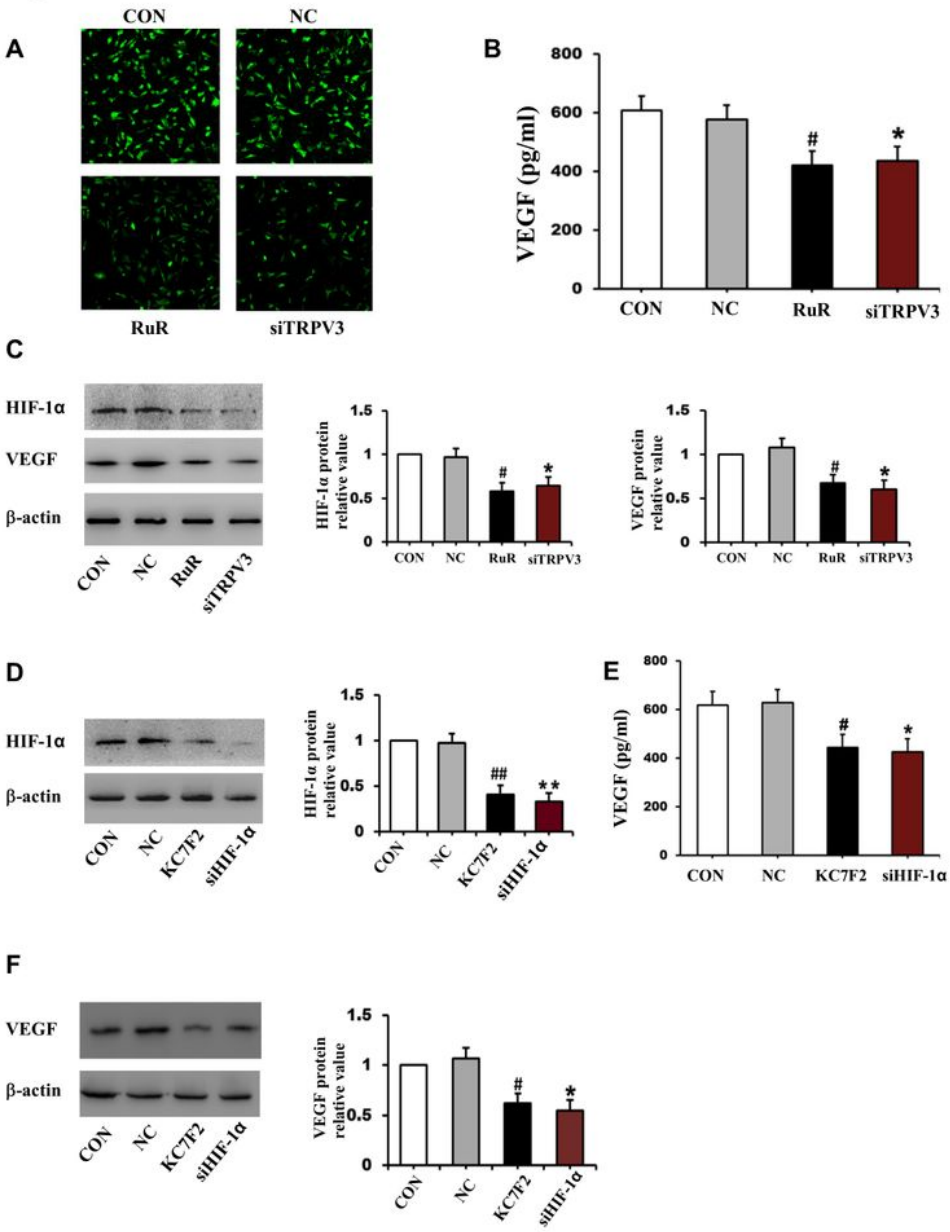

Figure 1

Inhibition of TRPV3 affects the expression of HIF-1α and VEGF in A549 cells. Fluorescent intensity in [Ca2+]i was recorded by laser scanning confocal microscope in different treatments (400□) (A); VEGF levels was examined in A549 cells by ELISA (B); Western blot assay for HIF-1α and VEGF expression in A549 cells (C); A549 cells were treated with HIF-1α-specific siRNA or KC7F2 (40μM) for 24 h. Western blot assay for HIF-1α expression in A549 cells (D); VEGF secretion level and expression were analyzed in A549

cells treated with KC7F2 or HIF-1α-specific siRNA by ELISA (E) and Western blot assay (F). All data were expressed as mean ± SD of triplicates. # P < 0.05 compared with control group; ## P < 0.01 compared with control group; * P < 0.05 compared with NC group; ** P < 0.01 compared with NC group.

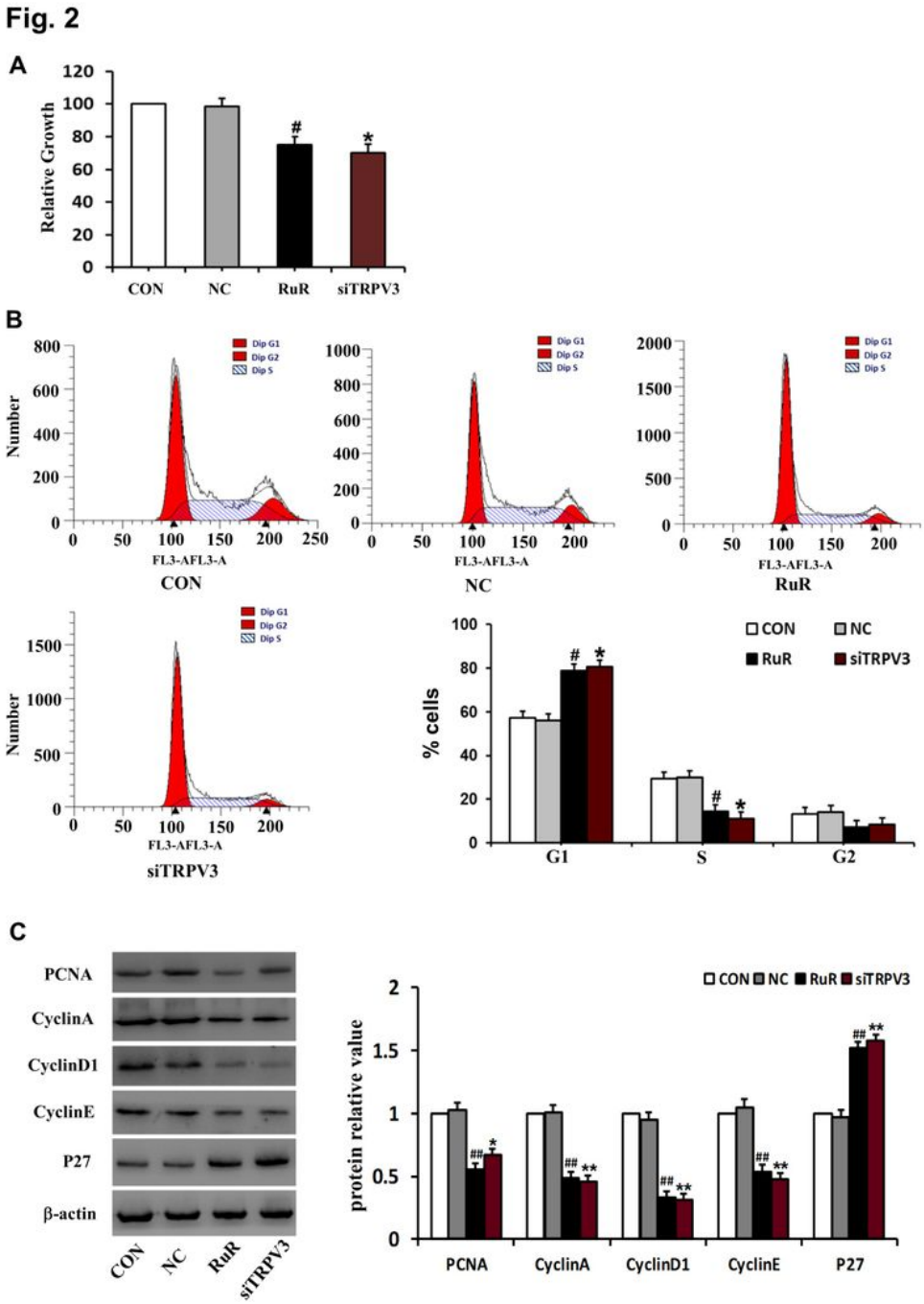

Figure 2

Inhibition of TRPV3 inhibits HUVECs proliferation induced by conditioned medium. HUVECs growth rate was analyzed by MTT (A); Cell cycle phase analysis detected by flow cytometry (B); Western blot analysis

of PCNA and cell cycle related protein levels were changed in HUVECs with conditioned medium of A549 cells treatment with RuR and TRPV3 siRNA (C). All data were expressed as mean ± SD of triplicates. # P < 0.05 compared with control group; ## P < 0.01 compared with control group; * P < 0.05 compared with NC group; ** P < 0.01 compared with NC group.

Fig. 3

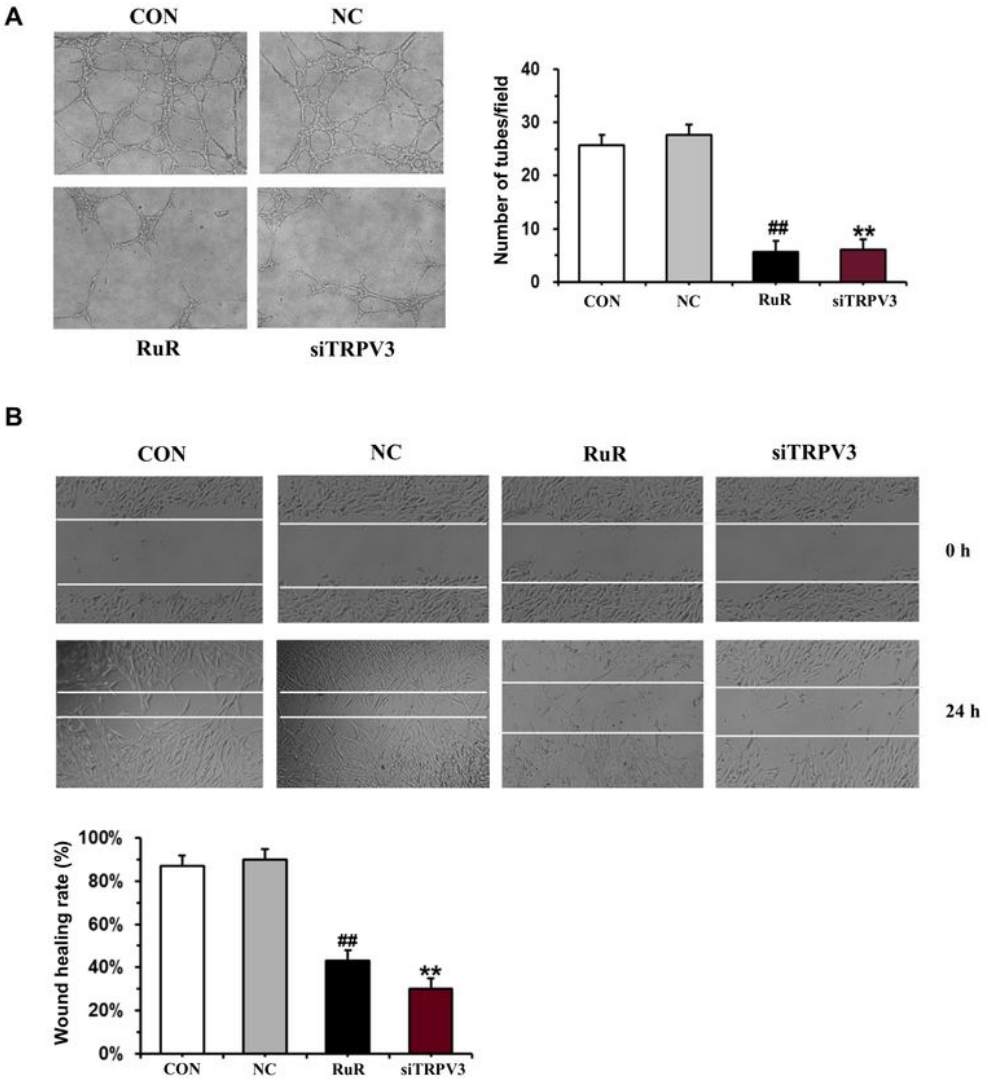

Figure 3

Inhibition of TRPV3 inhibits tube formation ability and migration of HUVECs induced by conditioned medium. Number of tubules decreased in RuR and TRPV3 siRNA groups (100□) (A). HUVEC migration was examined by wound healing assay (B). All data were expressed as mean SD of triplicates. # P < 0.05 compared with control group; ## P < 0.01 compared with control group; * P < 0.05 compared with NC group; ** P < 0.01 compared with NC group.

Fig. 4

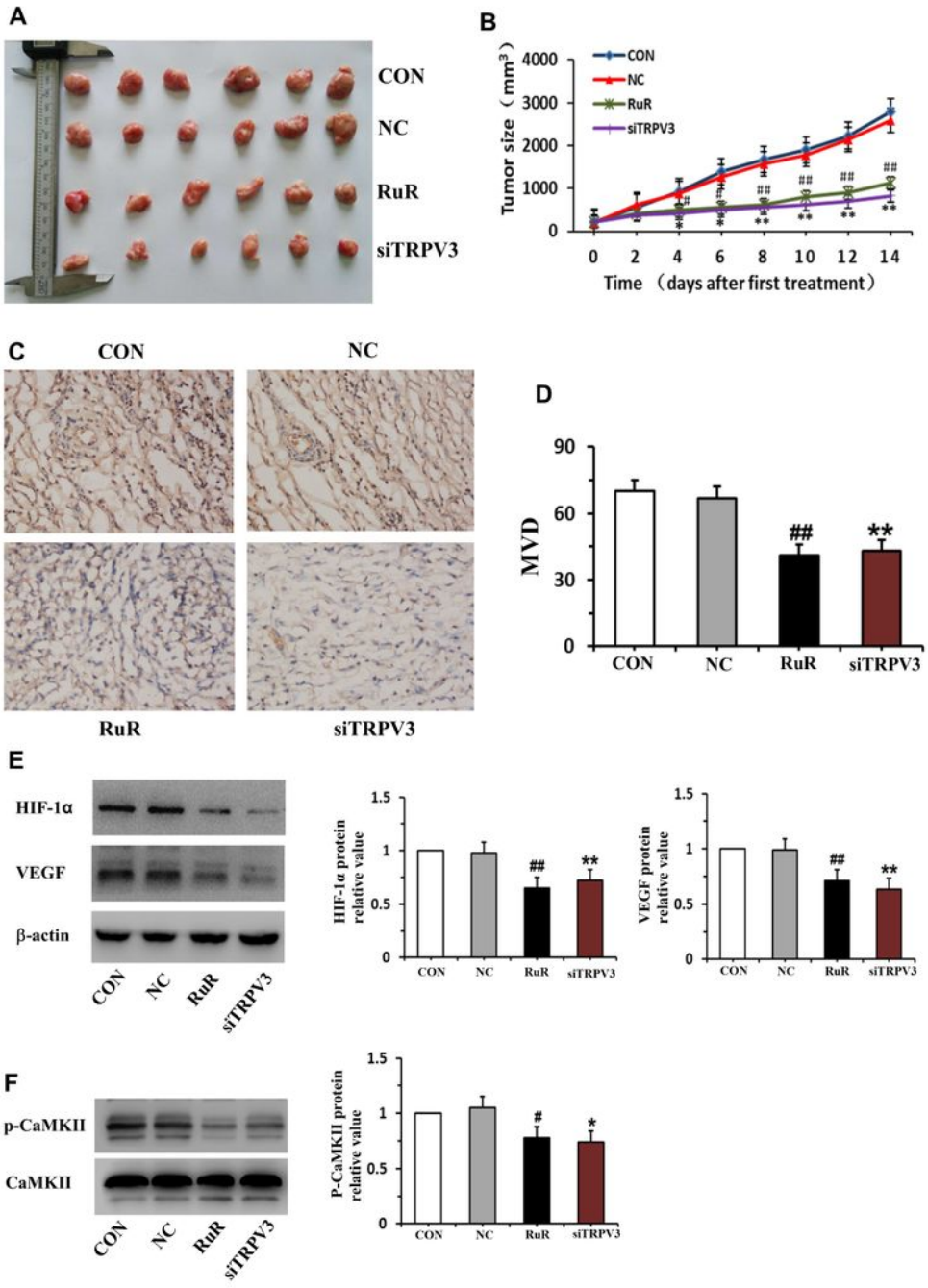

Figure 4

Inhibition of TRPV3 affects tumor growth and angiogenesis in vivo. The gross images of tumors were shown in different groups (A); The volume of xenograft tumors was compared with the controls (B); Immunohistochemical staining for CD34 showed in four groups (200□) (C); Microvessel density (MVD) of CD34+ cells showed a marked decrease in RuR and siTRPV3 groups (D); HIF-1α, VEGF (E) and p-CaMKП (F) protein expression levels in the xenograft tumors were detected by Western blot. All data were expressed as mean SD of triplicates. # P < 0.05 compared with control group; ## P < 0.01 compared with control group; * P < 0.05 compared with NC group; ** P < 0.01 compared with NC group.

Supplementary Files

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