ABBS

Original Article

The influence of YS-1 on the Dll4-Notch1 signaling pathway

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In this study, we investigated the role and molecular mechanism of p43 and YS-1 (recombinant human p43 protein) in Dll4-Notch1 signaling pathway. Active, small interfering RNA and recombinant plasmid targeting of p43 protein were used to infect human umbilical vein endothelial cells (HUVECs). Three-dimensional sprouting model, endothelial cell migration assay, and sprouting and tube formation assay were used to deduce the function of p43 and YS-1 in angiogenesis. Semi-quantitative reverse transcription-polymerase chain reaction and western blot analysis were performed to detect the efficiency of p43 in Dll4-Notch1 signaling in HUVECs. It was found that silencing and overexpression of p43 could upregulate Dll4-Notch and stimulate angiogenesis. p43 plays a complex role in angiogenesis. When the concentration is under 100 nM, it promotes angiogenesis; instead, when the concentration is over 100 nM, it inhibits angiogenesis. In this study, we found that the expression level of p43 was under 60 nM. However, recombinant human p43 protein, YS-1, inhibited endothelial cell sprouting, and 500 μg/ml of YS-1 attenuated the activation of Dll4-Notch1 signaling. These results suggested that YS-1 could directly inhibit angiogenesis through Dll4-Notch1 signal transduction pathway, while p43 plays a modulating role in this signaling pathway.

Keywords   YS-1; p43; Dll4-Notch1 signaling pathway; angiogenesis

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Introduction

The Notch signaling pathway is an evolutionary conserved, cellular signaling pathway mediated by membrane-tethered, receptor–ligand interactions among adjacent cells [1]. In mammals, the core components of the pathway include five canonical DSL (Delta, Serrate, Lag2) ligands (called Dll1, 3, 4, and Jagged1 and 2), and four single-pass transmembrane receptors (Notch1-4). Receptor–ligand binding induces sequential cleavage of the Notch receptor, the last of which is performed by the γ-secretase complex, releasing the Notch intracellular domain (NICD), which subsequently translocates to the nucleus [1,2]. NICD then interacts with the RBPJ/CSL transcription factor leading to the transcription of Notch target genes, such as members of the HES and Hey families [3].

Vascular endothelial growth factors (VEGFs) are crucial regulators of vascular development during embryogenesis (vasculogenesis), as well as blood-vessel formation (angiogenesis) in adults. The ligand Dll4 is upregulated by VEGF in the angiogenic vasculature [4–7]. The high expression level of Dll4 in filopodia-rich endothelial tip cells, which leads and guides new sprouts, is thought to activate Notch. Previous studies have reported that Dll4-Notch signaling acts as a negative regulator for angiogenic sprouting to restrict tip-cell formation in response to VEGF [5,6]. In addition, Notch signaling may interact with the hypoxia-sensing pathway, and hypoxia-inducible factor 1α (HIF-1α) is recruited as the promoter of the Notch target gene *Hey2* [8].

p43 protein is one of the three auxiliary components invariably associated with nine aminoacyl-tRNA synthetases (ARSs) as a multienzyme complex ubiquitous to all eukaryotic cells from flies to humans [9,10]. p43 protein is also secreted to function in immune cells, and triggers a pro-inflammatory response [11,12]. In addition, p43 shows the potential to interact with the subunit of ATP synthase, which has been previously shown to mediate the anti-angiogenic activity of angiostatin [13,14]. In addition, p43 has been considered as the precursor for the cytokine endothelial monocyte-activating polypeptide II (EMAPII) [15], which directly interacts with the VEGF receptors VEGFR1 and VEGFR2 to decrease the expression of VEGF. Dll4-Notch signaling acts as a negative regulator for sprouting to restrict tip-cell formation in response to VEGF. However, the role of p43 in the Dll4-Notch signaling pathway is unclear.

YS-1, a recombinant human p43 protein, was confirmed to have anti-angiogenesis and anti-tumor effects *in vitro* and

in vivo [16]. YS-1 showed potential anti-tumor properties for primary and metastatic solid tumors. Therefore, it is important to investigate its molecular mechanism of anti-angiogenesis.

The aim of this study was to investigate the effect of p43 and YS-1 in the Dll4-Notch1 pathway and the molecular mechanism. We suggested that it is YS-1 itself but not as the precursor for the cytokine EMAPII that has a modulating effect in the Dll4-Notch signaling pathway.

Materials and Methods

Cell lines and reagents

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment, as described previously [17]. Medium 199 and standard fetal bovine serum (FBS) were obtained from Gibco (Grand Island, USA). Endothelial cell growth supplement (ECGS) and epidermal growth factor (EGF) were purchased from Sigma (St Louis, USA). pcDNA3.1$^+$ containing *p43* human recombinant gene was obtained from Shanghai Generay Biotech (Shanghai, China). p43-interfering RNA (siRNA) was obtained from Shanghai GenePharma (Shanghai, China). Lipofectamine 2000 reagent was obtained from Invitrogen (Shanghai, China). Matrigel was purchased from BD Company (New Jersey, USA). Mouse anti-human DLL4 mAb was obtained from R&D (Minneapolis, USA), rabbit anti-human Notch1 mAb was obtained from Cell Signal (Danvers, USA), mouse anti-human p43 mAb was purchased from Santa Cruz (Santa Cruz, USA), mouse anti-human VEGFR1 and VEGFR2 mAbs were purchased from R&D, and horseradish peroxidase-conjugated anti-mouse IgG was purchased from Sigma. Fibrinogen and thrombin were purchased from Sigma. Aminocaproic acid was purchased from J&K Chemical (Beijing, China), and VEGF165 was purchased from Peprotech (Rocky Hill, USA). YS-1 was a gift from Shanghai Pharm (Shanghai, China).

Cell culture and transfection

The harvested cells were grown in medium 199 containing 20% FBS, 30 μg/ml ECGS, and 10 ng/ml EGF [1]. After three to five passages, HUVECs were collected for use in all experiments. Cells (2×10^4) were seeded in a 24-well plate (Corning, New York, USA), and allowed to adhere for 12 h. After being washed with M199 medium without FBS, 400 μl of fresh medium and 100 μl of a mixture that contained 60 pmol p43 siRNA or 2 μg pcDNA$^+$-p43 plasmid, and 1 μl Lipofectamine 2000 were added to each well. The siRNA transfection HUVEC(−) was directly cultured for 35 h. HUVEC(+) cells were incubated with the pcDNA$^+$-p43 for 42 h. Then the medium was changed with M199 containing 10% FBS and cultivated for 6 h.

Immunoblotting analysis

In order to investigate the alteration of the Dll4-Notch1 protein expression levels by YS-1, p43 siRNA, and pcDNA$^+$-p43, immunoblotting analysis was performed as previously described [18]. Briefly, adherent cells were washed twice with phosphate-buffered saline (PBS), and then lysed in RIPA buffer. The cell lysates were incubated at 4°C for 15 min, and cellular debris were pelleted by centrifugation at 15,000 *g* for 15 min at 4°C. Total protein was quantitated with the BCA protein assay reagent kit (Pierce, Rockford, USA). Samples (40 μg per lane) were separated by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Membranes were probed with murine anti-human monoclonal antibodies: VERFR1/R2 antibodies, p43 antibody, Dll4 antibody, and Notch1 antibody. Horseradish peroxidase-conjugated anti-mouse IgG and horseradish peroxidase-conjugated anti-rabbit IgG were used as secondary antibodies. Immunoreactive proteins on the membrane were visualized by enhanced chemiluminescence western blotting detection reagents (Amersham, Buckinghamshire, UK).

Semi-quantitative reverse transcription-polymerase chain reaction

HUVECs(+), HUVECs(−), and control HUVECs were collected and the total RNA was extracted. Exactly 500 ng of total RNA was used for reverse transcription according to the manufacturer's protocol (TaKaRa, Dalian, China). The primers used for semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) of *β-actin*, *Dll4*, *Notch1*, and *p43* were shown as follows: *β-actin* forward 5′-GAGCGGGAAATCGTGCGTGACATT-3′, reverse 5′-GAAGGTAGTTTCGTGGATGCC-3′; the *DII4* upstream primer sequence was 5′-CCCTGGCAATGTACTTGTGAT-3′, the downstream primer sequence was 5′-TGGTGGGTGCAGTAGTTGAG-3′; the *Notch1* upstream primer sequence was 5′-TGGAGACGGGCAACAGCGAGGAA-3′, the downstream primer sequence was 5′-GCCCGTGCGGTCTGTCTGCTTGTG-3′; the *p43* upstream primer sequence was 5′-CGTCTGGATCTTCGAATTGG-3′, and the downstream primer sequence was 5′-GCATCAAAAGTAATTCTGTC-3′. Semi-quantitative PCR was carried out on an iCycler real-time PCR detection system (PTC-200 PCR; Bio-Rad, Hercules, USA) using SYBR green I as the detection dye. The amount of target gene mRNA relative to internal control was calculated using the ΔCT method: relative expression = $2^{-\Delta CT}$, where ΔCT = CT (target gene) − CT (*β-actin*).

Tube formation assay

Matrigel (50 μl per well) was used to coat 96-well plates and allowed to solidify (37°C, 1 h), prior to seeding with HUVEC, HUVEC(+), and HUVEC(−) (2×10^4 cells per

well) that were then cultured (37°C, 7 h) in M199 medium with 10% FBS. The changes of cell morphology were captured by an inverted phase-contrast microscope (IX 70; Olympus, Japan). The number of the tubes was quantified from five random fields.

Wound healing assay

Wound healing was performed using a 24-well system, using 1.5×10^5 HUVECs per well. Cells were allowed to adhere, and a pin was used to divide the cells along the X-axis. The medium was abandoned, the cells were slightly washed with PBS, then fresh medium containing 1% FBS was added. The migration of cells was observed and photographed under a microscope.

Sprouting angiogenesis

HUVECs were released with trypsin solution, resuspended in M199 with 20% FBS, pH 7.5, at a concentration of 4×10^5 cells/ml, and 0.5 ml of M199 was dispensed into each well of a 48-well tissue culture plate. The lid of the plate was turned upside down, and 0.02 ml of the cell suspension was plated out in the middle of the inner side of the lid. The cell suspension formed into a drop, and the lid was turned over and placed on the plate [19,20]. The plates were incubated at 37°C in a tissue culture incubator for 24 h. Fibrinogen (0.3%) and aminocaproic acid (0.5%) were dissolved in PBS, and 200 μl per well of the mixture was dispensed into the 48-well plate. The lid of the plate with hanging drops was carefully lifted. The cell 'clump' was attached to the tip of a syringe needle, and transferred into the mixture. A solution of 20 μl of thrombin (0.1 U/ml) was added, and the plate was incubated for 30 min at 37°C. About 0.3 ml of M199/FBS was added into the mixture in the presence of YS-1 (500, 250, and 125 μg/ml) or 1 μM DAPT as the positive control in each well, and the plate was returned to the incubator for 48 h.

Enzyme-linked immunosorbent assay

VEGF expression levels in HUVECs that were influenced by YS-1 and pcDNA3.1+-p43, were measured using a VEGF ELISA kit (4A Biotech; Shanghai ExCell Biology, Inc., Shanghai, China) according to the manufacturer's protocol.

SDS−PAGE electrophoresis and dot blot

HUVECs (1×10^4) were seeded in 96-well plates. When the cells reached 80% confluent, the medium was replaced with fresh, serum-free medium in the absence or presence of YS-1 (500, 250, and 125 μg/ml), and the medium was collected at 10, 30, and 60 min, respectively. Samples were divided into two groups. About one-fourth volume of SDS loading buffer was added to one of the groups, and then heated at 95°C for 10 min. The samples were separated by

10% SDS−PAGE electrophoresis, and gel was stained with Coomassie brilliant blue. The other group was directly dropped into a nitrocellulose membrane in an equivalent volume, respectively. Membranes were probed with murine anti-human p43 monoclonal antibody.

Statistical analysis

All data represent at least three, independent experiments, and are expressed as the mean ± SD. For statistical analysis, Student's t-test was used, as appropriate.

Results

Effects of p43 silencing on Dll4-Notch1 signaling pathway and endothelial cell sprouting

To analyze whether p43 has an influence on angiogenesis and to investigate its molecular mechanism, p43 siRNA was used to infect HUVECs. As shown in **Fig. 1**, Dll4-Notch1 mRNA and protein levels were upregulated in HUVEC(−) cells. The sprouting length and migration of cells have been stimulated that were determined by the HUVEC(−) three-dimensional culture model and migration assay, the same change trend as tube formation ability (**Fig. 2**). However, the expression level of Notch target gene, *Hey2*, was decreased in the HUVEC(−) cells. We speculated that the p43 silencing blocked Notch target gene transcription.

Effects of p43 overexpression on Dll4-Notch1 signaling pathway and endothelial cell sprouting

HUVEC(+) cells transfected with pcDNA3.1+-p43 showed the excessive activity on angiopoiesis. As shown in **Figs. 3** and **4**, the protein levels of Dll4 and NICD were increased, the same change trend as the Dll4-Notch1 mRNA level. The sprouting length of HUVEC(+) cells was stimulated 1.7-fold growth when compared with control. HUVEC(+) formed more 1.5 folds tube-like structures than the control.

p43 protein overexpression level

p43 plays a complex role in angiogenesis. When the concentration of p43 is under 100 nM, it will promote angiogenesis [21]. The previous results showed that p43 silencing or overexpression displayed excessive activity on angiopoiesis. Both could stimulate sprouting length, tube formation ability, and migration of HUVECs. So we next used blot assay to identify the overexpression level of p43. Fitting the curve with the concentration of p43 and grayscale, and using the formula as follows: $y = ae^{bx}$ ($a = 3.086E-0004$, $b = 3.293E-001$) showed that the overexpression level of p43 was about 60 nM, which was in accordance with previous report. This was the reason why p43 silencing and overexpression both promoted angiogenesis.
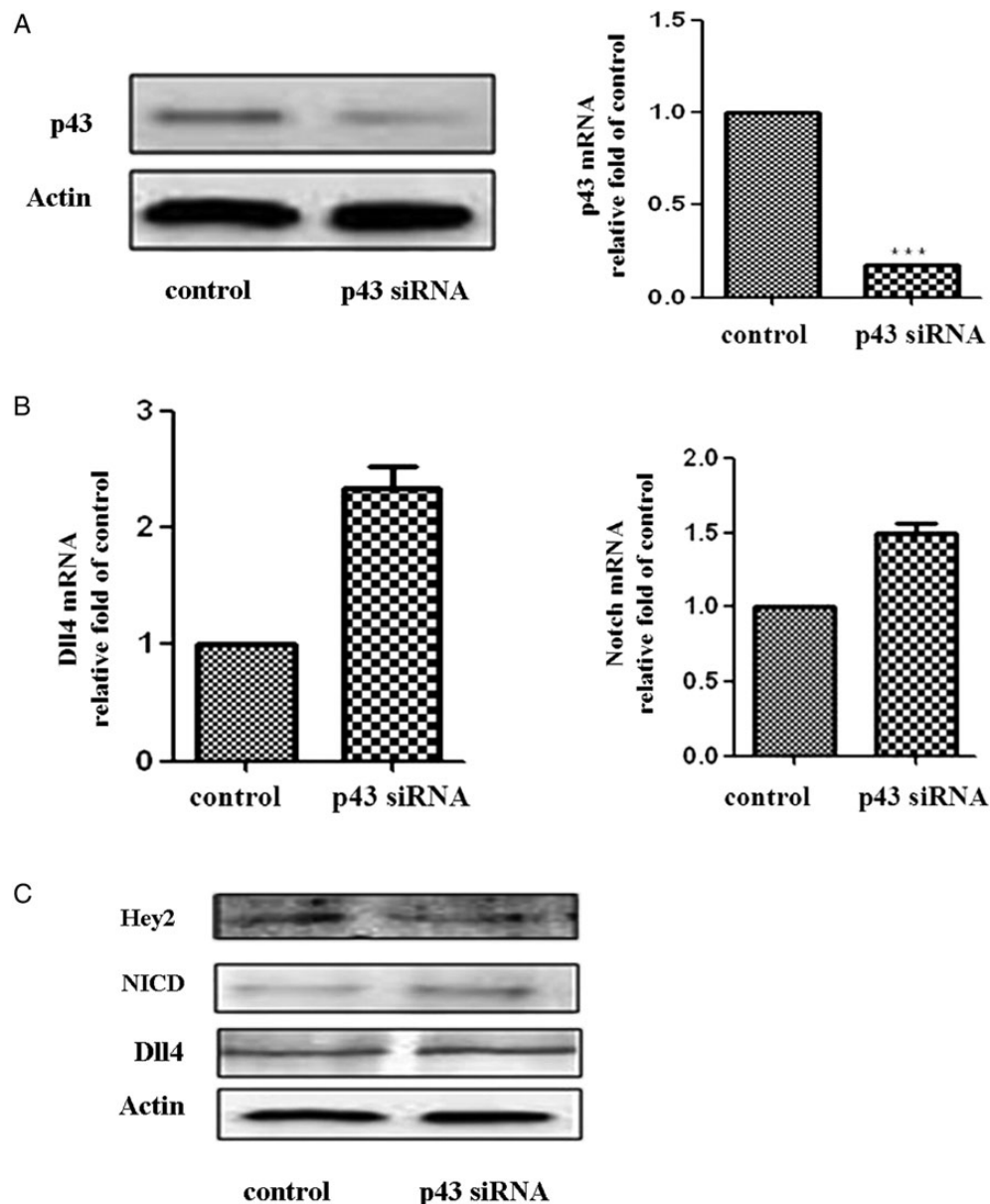
Figure 1. p43 siRNA infected the HUVECs (A) Expression levels of p43 protein and mRNA were determined by western blot analysis and semi-quantitative RT-PCR. (B) Notch1 and Dll4 mRNA levels were investigated by qRT-PCR. (C) The efficiency of the Dll4-Notch1 signaling in the HUVECs was determined by western blot analysis. Results are expressed as the mean $\pm$ SD. **$P < 0.001$.

The effect of YS-1 on HUVECs sprouting

The established *in vitro* model included a three-dimensional HUVECs sprouting model with 10 ng/ml VEGF and different concentrations of YS-1. As shown in **Fig. 5**, 500 μg/ml YS-1 and 1 μM DAPT (48 h) reduced the HUVECs cumulative sprout length growth from 111.13 to 78.63 and 73.08 μm, respectively. These findings suggested that YS-1 could inhibit the growth of HUVECs sprouting and angiogenesis. p43 silencing and overexpression could promote angiogenesis and influence DLL4-Notch signaling pathway, then the effect of YS-1 on VEGF-stimulated Dll4-Notch1 signaling pathway in normoxia and hypoxia was studied.

The effect of YS-1 on VEGF-stimulated Dll4-Notch1 signaling pathway in normoxia and hypoxic

As VEGF plays an important role in angiogenesis, VEGF expression level in HUVECs treated with YS-1 was examined. Dll4 induced by VEGF could activate Notch signaling, and the negative feedback would reduce the protein level of VEGFR2. Notch signaling may interact with the hypoxia-sensing pathway. And HIF-1α was one of the promoters of *Hey2*, and activated the Notch signaling pathway. We then studied the effect of YS-1 on VEGF-stimulated Dll4-Notch1 signaling pathway in normoxia and hypoxia. As shown in **Fig. 6**, YS-1 and pcDNA3.1^{+}-p43 had no remarkable influence on VEGF secretion in HUVECs. The level of Dll4 was

significantly increased after treatment with 10 ng/ml of VEGF. YS-1 (500 μg/ml) and 1 μM DAPT significantly attenuated VEGF-stimulated (1 h) upregulation of Dll4-Notch1 expression, and increased the level of VEGFR2. The level of Dll4 was also significantly increased after 1 h of hypoxia, and treatment with 500 μg/ml YS-1 and 1 μM DAPT significantly attenuated hypoxic upregulation of Dll4-Notch1. So, we made a conclusion that YS-1 could inhibit VEGF and hypoxia-induced Dll4-Notch1 expression and suggested that YS-1 could inhibit angiogenesis by down-regulating Dll4-Notch1 signaling pathway.

YS-1 protein's zymolysis

It is reported that EMAPII could control the angiopoiesis signaling pathway induced by VEGF. For example, it could interfere the combination of VEGF with VEGFR1/2 through inhibiting phosphorylation of VEGFR1/2. p43 is the precursor of the cytokine EMAPII. So, it is important to confirm that it is YS-1, and not cytokine EMAPII, could inhibit angiogenesis through Dll4-Notch signaling pathway. The molecular weight of EMAPII is 22 kDa. SDS−PAGE electrophoresis was carried out to observe EMAPII release within 1 h, and the dot blot assay was performed to analyze the zymolysis of YS-1. As shown in **Fig. 7**, there was no EMAPII lysed fragment using three concentrations of YS-1, although zymolysis had taken place in 1 h. This finding suggested that it is YS-1 not EMAPII could inhibit angiogenesis through Dll4-Notch signaling pathway.

Discussion

ARSs are housekeeping enzymes essential for protein synthesis [21]. There are nine different ARSs and three additional factors: p43, p38, and p18 in mammals. Within this
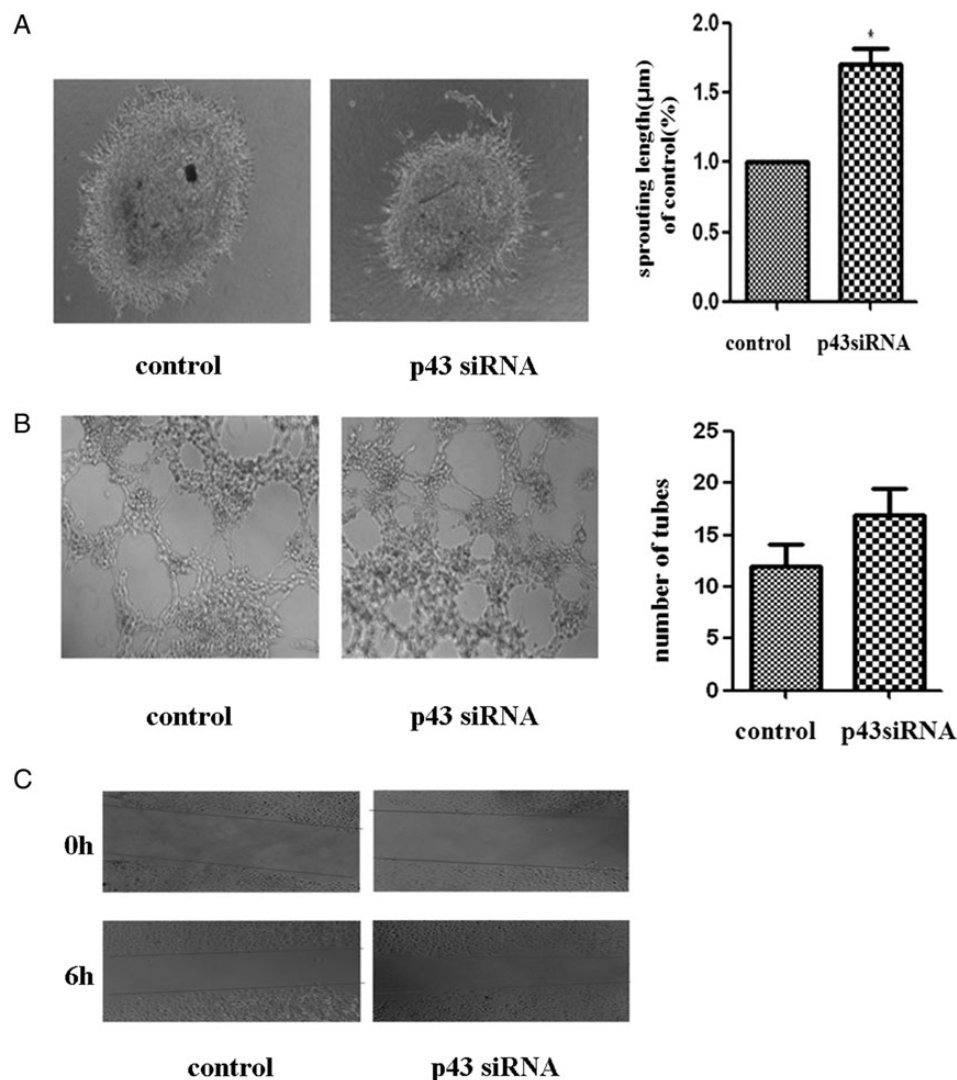
Figure 2. Effect of p43 siRNA on endothelial cell sprouting (×100) (A) Representative images of sprouting. Bar graph shows the results of quantitative analysis of the length of HUVEC sprouting. (B) Representative images of tube formation. Bar graph shows the results of quantitative analysis of HUVEC tube formation. (C) Effect of p43 siRNA on endothelial cell migration (×100). Results are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ vs. control.
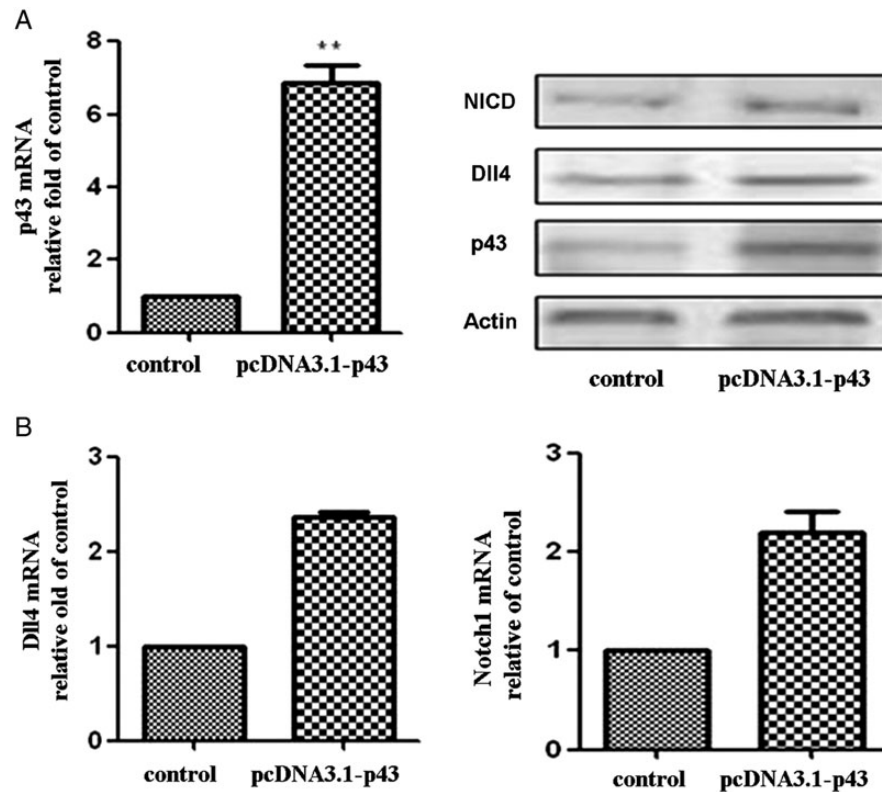
Figure 3. pcDNA⁺-p43 infected the HUVECs (A) Expression levels of p43 mRNA and protein were determined by semi-quantitative RT-PCR and western blot analysis, respectively. The efficiency of the Dll4-Notch1 signaling in HUVECs was determined by western blot analysis. (B) *Notch1* and *Dll4* mRNA levels were investigated by qRT-PCR. Results are expressed as the mean ± SD from three independent experiments. **$P < 0.01$ vs. control.
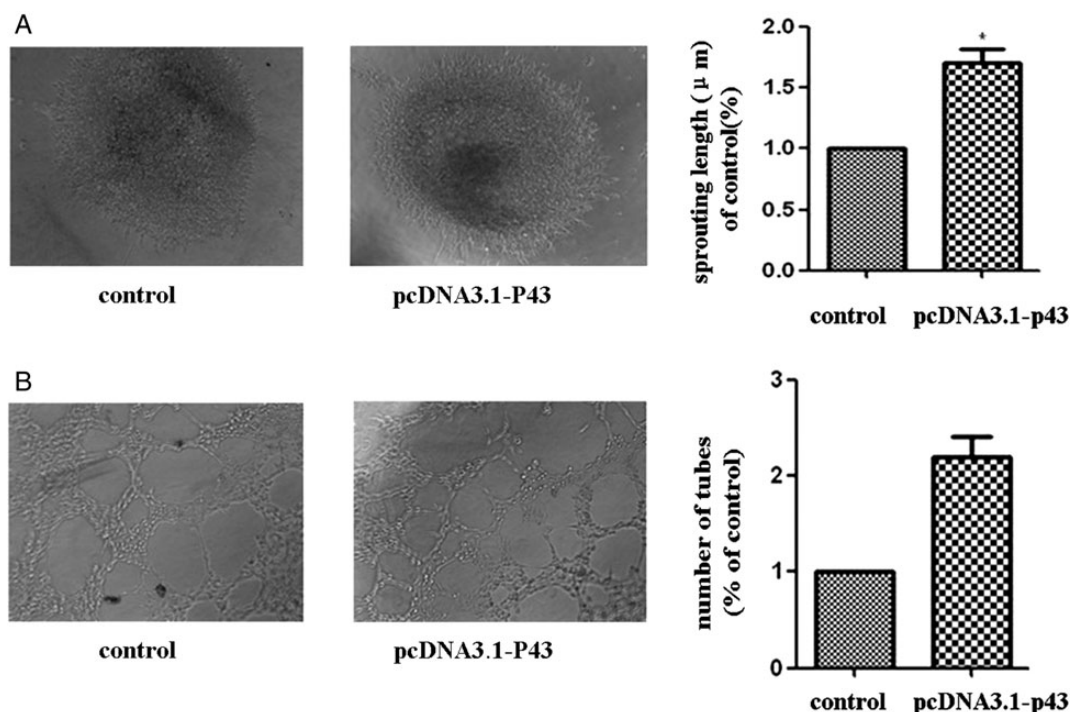

Figure 4. Effect of pcDNA3.1⁺-p43 recombinant plasmid on endothelial cell (A) Effect of pcDNA3.1⁺-p43 recombinant plasmid on endothelial cell sprouting ($\times 100$). Bar graph shows results of quantitative analysis of length of HUVEC sprouting. (B) Effect of pcDNA3.1⁺-p43 recombinant plasmid on tube formation in HUVECs ($\times 100$). Bar graph shows results of quantitative analysis of HUVEC tube formation. Results are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ vs. control, **$P < 0.01$ vs. control.

complex, p43 interacts with arginyl-tRNA synthetase, which is critical for enzyme activity and stability. p43 also plays a complex role in angiogenesis. Although p43 induces the migration of endothelial cells at low concentration, it suppresses angiogenesis by blocking the proliferation and triggering apoptosis of endothelial cells at high concentrations [22]. But its molecule mechanism remains unclear.

In this study, p43 siRNA was used to interfere with HUVECs, which increased NICD level and encouraged angiopoiesis. NICD is the active form of Notch signaling [23,24]. But the expression of its target protein, Hey2, was decreased in the HUVEC(−) cells. Based on these, it was speculated that the p43 silencing blocks Notch target gene transcription and causes the decrease of Hey2 expression. Hey2 is related to heart function and vascular smooth muscle cell phenotype [25]. Therefore, the influence of p43 on vascular function needs to be further explored. In the meantime, pcDNA3.1$^+$-p43 was chosen to transfect HUVECs, and the results revealed that upregulated p43 activates Dll4-Notch1 signaling pathway without raising the secretion of VEGF, which causes angiopoiesis.

YS-1 protein (500 μg/ml) could inhibit the growth of HUVECs sprouting and angiogenesis, and show excessive activity on anti-angiopoiesis through blocking the Dll4-Notch1 signaling pathway that was activated by VEGF and hypoxia. The dual effect of p43 on angiopoiesis was verified.

In addition, p43 has been considered as the precursor for the cytokine EMAPII [26], and it was reported that EMAPII could control the angiopoiesis signaling pathway. But SDS−PAGE electrophoresis showed that there was no EMAPII lysed fragments although zymolysis had taken place in 1 h. Hence, it is YS-1 not EMAPII that could inhibit angiogenesis through the Dll4-Notch1 signaling pathway.

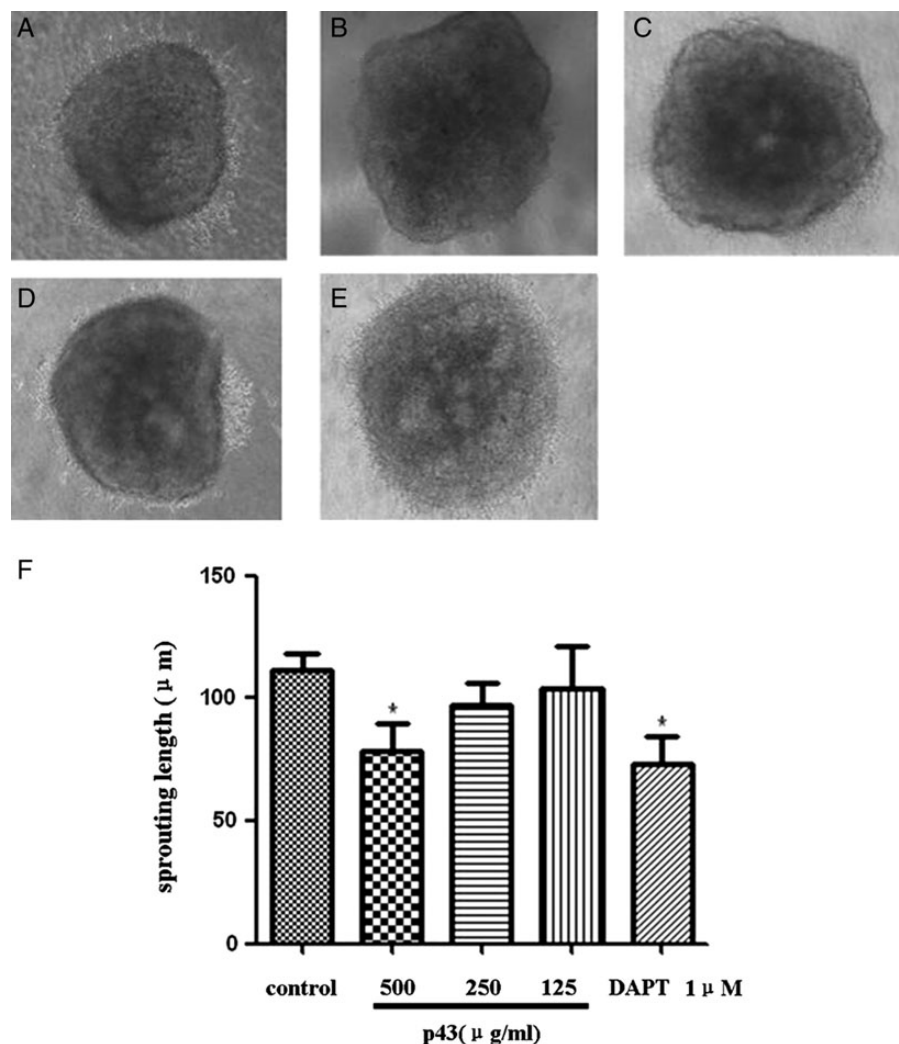Notch signaling plays an oncogenic role in a majority of hematological and solid tumors, including breast cancer

Figure 5. Effect of YS-1 on VEGF-induced HUVEC sprouting after 72 h (A) VEGF-stimulated only. (B) VEGF + 500 μg/ml YS-1. (C) VEGF + 250 μg/ml YS-1. (D) VEGF + 125 μg/ml YS-1. (E) VEGF-stimulated + 1 μM DAPT. (F) Bar graph shows the results of quantitative analysis of length of HUVEC sprouting. Results are expressed as the mean ± SD from three independent experiments. *$P < 0.05$.
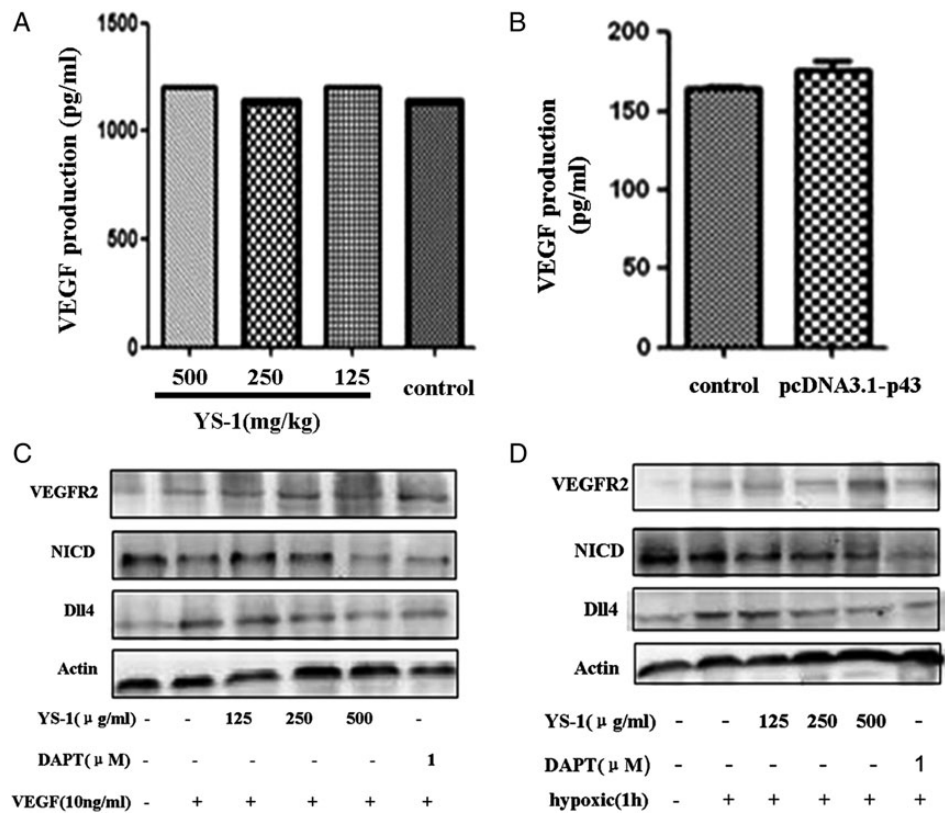
Figure 6. Effect of YS-1 on Dll4-Notch1 signaling pathways (A) VEGF expression in HUVECS. (B) The effects of pcDNA3.1+-p43 on VEGF expression. (C) Effect of YS-1 on VEGF-stimulated activation of Dll4-Notch1 signaling pathways in normoxia. (D) Effect of YS-1 on Dll4-Notch1 signaling pathways in hypoxia.
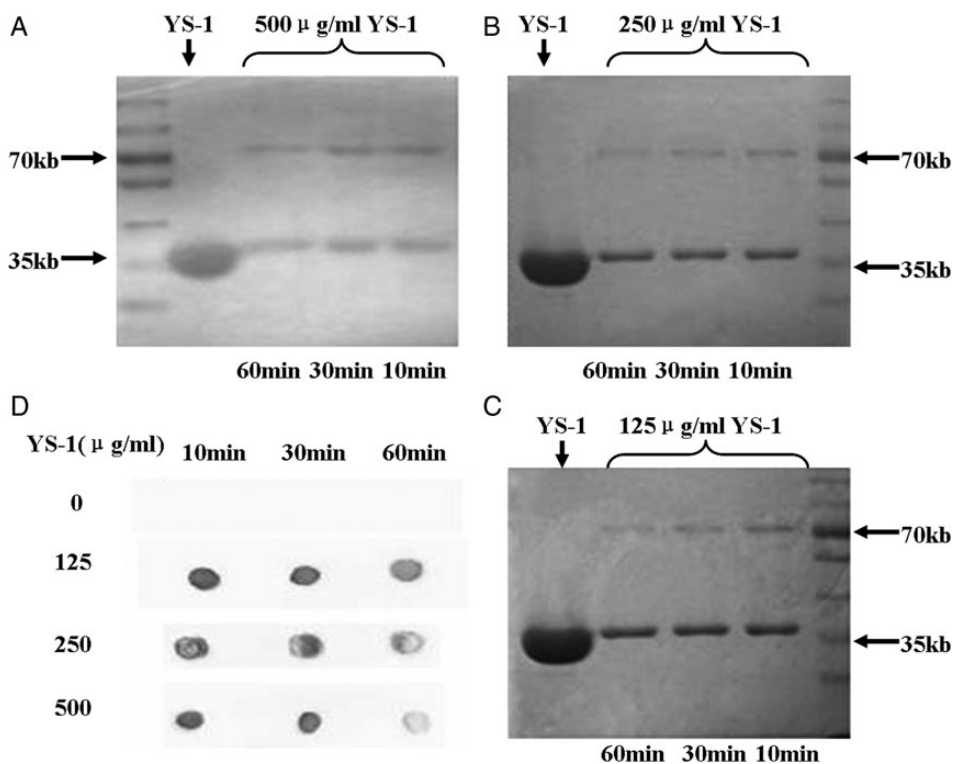

Figure 7. Examination of YS-1 released fragments HUVECs were treated with the indicated concentrations of YS-1 for 1 h. The released fragments of YS-1 were separated by SDS−PAGE and examined by Coomassie brilliant blue. (A) 500 μg/ml YS-1 treated. (B) 250 μg/ml. (C) 125 μg/ml. (D) The dot blot assay was used to manifest the enzymolysis of YS-1.

[27]. Furthermore, it is required for angiogenesis during development and possibly in tumor angiogenesis [28]. Inhibition of Notch signaling pathway may therefore represent a feasible approach for the treatment for cancers. As demonstrated above, YS-1 has a modulating effect on the Dll4-Notch signaling pathway. Although the functional mechanism requires further study, the response of the Dll4-Notch pathway to p43 protein expression should be taken into consideration during tumor angiogenesis therapy.

Funding

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