Vascular Pharmacology 113 (2019) 20–26

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

# Protective effect of rapamycin on endothelial-to-mesenchymal transition in HUVECs through the Notch signaling pathway

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ARTICLE INFO

*Keywords:*
Endothelial-to-mesenchymal transition
Rapamycin
Endothelial cell
Notch signaling pathway

ABSTRACT

Neointima hyperplasia is one of the predominant features of cardiovascular diseases such as atherosclerosis, and is also responsible for the restenosis of vascular surgery including arteriovenous fistula and stent implantation. Endothelial-to-mesenchymal transition (EndMT) contributes to neointima hyperplasia by activation of the Notch or TGF-β signaling pathway. Rapamycin has been utilized as anti-restenosis drug due to its anti-proliferative activity. However, its effects on the EndMT have not been investigated yet. Thus, we examined the biological effects of rapamycin on the EndMT and its potential mechanisms. We showed that rapamycin significantly reversed TGF-β1 stimulated EndMT by upregulating endothelial marker CD31 expression and downregulating mesenchymal marker SMA-α expression in human umbilical vein endothelial cells (HUVECs). Rapamycin also inhibited TGF-β1 induced expression of the Notch signaling pathway components expression, such as Notch-1, Jagged-1, RBP-jκ and Hes-5. Among the different Notch receptors and ligands, Jagged-1/Notch-1 cascade was most remarkably blocked by rapamycin. Finally, consistently with the results from Notch inhibitor DAPT treatment, rapamycin suppressed the migration of HUVECs in vitro. Together, these findings indicate that rapamycin may function as an effective inhibitor of the EndMT in HUVECs by suppressing targeting the Notch signaling pathway.

## 1. Introduction

Neointima hyperplasia is one of the predominant features of cardiovascular disease such as atherosclerosis, and is also responsible for the restenosis of vascular surgerys including arteriovenous fistula and stent implantation. One of the characteristics of neointima hyperplasia is vascular smooth muscle cell (VSMC) proliferation and migration [1]. Recent studies reported that endothelial cell (EC) also plays a very important role in neointima hyperplasia [2]. The damage of EC or the disruption of the endothelium triggers neointima hyperplasia. Furthermore, EC can contribute to neointima hyperplasia as a source of VSMC through the endothelial-mesenchymal transition (EndMT) during vascular injury [3].

The EndMT is a specific form of the epithelial-to-mesenchymal transition (EMT). ECs can undergo the EndMT to lose their endothelial specific markers, gain mesenchymal markers, lose cell-cell junctions, and acquire invasive and migratory properties [4,5]. The EndMT has been demonstrated to be involved in many vascular diseases such as atherosclerosis [6] and regulated cell phenotype change during vein graft remodeling [7]. Our previous studies also demonstrated that the EndMT is involved in neointima hyperplasia of arteriovenous fistula [8].

Rapamycin, also known as sirolimus or AY-22989, is an immunosuppressant that specifically inhibits the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that regulates cell survival, growth, proliferation, aging and migration [9]. The anti-proliferative property of rapamycin has been widely known, it blocks VSMC proliferation and migration and is used clinically on drug-eluting stents to inhibit restenosis through preventing neointima hyperplasia [10]. However, very little is known about the role of rapamycin regulating the EndMT in neointima hyperplasia.

The evolutionarily conserved Notch signaling pathway is significant in determining cell fate and regulating cell proliferation, apoptosis and differentiation [11,12]. It was originally identified in Drosophila, in which a mutant allele gives rise to a notched wing [13]. Mammals express four Notch transmembrane receptors (Notch-1, Notch-2, Notch-3 and Notch-4) and five typical transmembrane ligands (Delta-like 1 (Dll-1), Delta-like 3 (Dll-3) and Delta-like 4 (Dll-4), Jagged-1 and Jagged-2). Notch signaling plays a key role in the development of cardiovascular system, as well as in the stability and remodeling of the vessel wall [11,12]. Activation of Notch signaling in ECs can induce the EndMT and contribute to neointima hyperplasia after vascular injury [2,14].

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https://doi.org/10.1016/j.vph.2018.10.004
Received 29 January 2018; Received in revised form 10 July 2018; Accepted 14 October 2018
Available online 16 October 2018
1537-1891/ © 2018 Published by Elsevier Inc.

Here we examined whether rapamycin plays a protective role in the EndMT process. Our results identified that rapamycin can inhibit the TGF-β1 induced EndMT in human umbilical vein endothelial cells (HUVECs) through blocking Notch signal activation. These data extend our understanding of the molecular mechanisms of the EndMT and will guide the development of intervention methods to prevent the EndMT and alleviate neointima hyperplasia.

## 2. Methods and materials

### 2.1. Reagents and antibodies

Fetal bovine serum (FBS) and RPMI 1640 medium were from Invitrogen (Carlsbad,CA,USA). Recombinant human TGF-β1 and the Notch signaling pathway inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-Lalanyl]-*S*-phenylglycine t-butyl ester) were purchased from Sigma-Aldrich (St. Louis, MO,USA). Rapamycin was purchased from Beyotime Company (Shanghai, China).

Antibodies against Jagged-1, SMA-α, Notch-1 and Hes-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas antibodies against N1ICD and RBP-Jκ were from Millipore (Burlington, MA, USA). The FSP-1 antibody was from DAKO (Carpinteria, CA, USA). Antibodies against CD31 and VE-cadherin were from Bioworld Technology, Inc. (St. Louis Park, MN, USA). The GAPDH antibody was from Sangon Biotech (Shanghai, China). Antibodies against Vimentin were from ZSGB-BIO (Beijing, China). Horseradish peroxidase-conjugated secondary antibody IgG, anti-rabbit, anti-mouse, anti-goat, DyLight 488 goat anti-rabbit IgG (H + L) and DyLight 594 goat anti-rabbit IgG (H + L) were from Beyotime.

### 2.2. Cell culture

HUVECs were subcultured in basal 1640 RPMI medium (Hyclone Technologies, Logan, UT, USA) supplemented in 10% FBS at 37 °C in a humidified 5% $CO_2$ atmosphere with the medium replaced every 48 h. For starvation, ECs were incubated in basal 1640 medium. For experiments, cells was pretreated with rapamycin [15] or Notch inhibitor DAPT (10 μM) for 1 h before TGF-β1 (10 ng/ml) treatment [8].

### 2.3. Real-time reverse transcription PCR

Total RNA was isolated using TRIzol reagent and one microgram of total RNA per sample was then reverse-transcribed into cDNA using iScript TM Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA) according the manufacturer's protocol. Real-time PCR (RT-PCR) was performed with SYBR Green Master Mix (Bio-Rad Laboratories) in a StepOne PCR amplifier (Bio-Rad Laboratories). Untreated cells were used as the reference sample and GAPDH was used as the endogenous control. The primers (Sangon Biotech) used for amplification are listed in Supplemental Table 1. The assay was performed in triplicate, and each experiment was repeated at least three times.

### 2.4. Western blots

HUVECs were grown under identical conditions as described previously. Cells extracts were prepared in RIPA buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate). Protein concentration was determined using a Bradford protein assay kit (Bio-Rad Laboratories). About 30 μg protein was separated by sodium dodecyl sulfate-polyacrylamid gel electrophoresis. After being transferred to a nitrocellulose membranes (Bio-Rad Laboratories), membranes were blocked in blocking buffer at room temperature for 2 h, then incubated overnight at 4 °C with primary antibody, followed by horseradish peroxidase-conjugated secondary antibody IgG, (anti-mouse, anti-rabbit, or anti-goat) for 2 h at room temperature. GAPDH was used as a loading control. Signals were visualized by an enhanced chemiluminescence system (Thermo Fisher Scientific, MA, USA) and detected using an imaging system (Bio-Rad Laboratories).

### 2.5. Immunofluorescence staining

HUVECs were plated on coverslips and treated as described previously. Then, the cells were fixed with 4% paraformaldehyde for 10 min, sequentially permeabilized with 0.1% Triton X-100 for 5 min. After being blocked with blocking buffer for 30 min, cells were incubated with primary antibodies against Notch-1 (1:100) or N1ICD (1:100) overnight at 4 °C, and then incubated with DyLight 488 or DyLight 594. Nuclear DNA was stained with DAPI (4′, 6-diamidino-2-phenylindole) (Boster Biological Technology, Pleasanton, CA,USA).

### 2.6. Wound-healing assay

HUVECs inoculated in 6-well plates were wounded with a 200-μl tip, washed with PBS, and incubated. At different times, photomicrographs were taken to record the migration of cells toward the open area.

### 2.7. Transwell assay

For the Transwell assay, $2 \times 10^4$ cells were seeded onto the cell insert with an 8-μm pore (BD Biosciences, USA) and incubated in normal RPMI 1640 medium with 2% FBS. Cells was treated with or without rapamycin for 1 h before TGF-β1 treatment. Then cells were allowed to migrate for 48 h; non-migrated cells were gently removed with cotton swabs and migrated cells were fixed with methanol and stained with crystal violet. The filters were washed with distilled water and images were obtained under an inverted microscope (Leica, Germany).

### 2.8. Endothelium permeability assay

HUVECs treated as described were kept in 25 mM HEPES (4-[2-hydroxyethyl]-1- piperazineethanesulfonic acid)-RPMI 1640 (pH 7.4) for 15 min. The HEPES-RPMI 1640 was then removed from the top of the well; 100 μl FITC-labeled dextran (12,000 molecular weight; 1 mg/ml in 25 mM HEPES-RPMI 1640) was added to the top of each well. Aliquots (50 μl) were removed from the bottom well at the times specified for each experiment and collected in white 96-well plates. FITC-labeled dextran was measured in a fluorescent spectrophotometer (LS 50B; PerkinElmer, USA) using 480 and 530 nm as the excitation and emission wavelengths, respectively.

### 2.9. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical significance was determined by the Student's *t*-test and one-way ANOVA followed by Newman-Keuls post hoc tests. All statistical analyses were calculated by SPSS 13.0 (IBM, New York, USA). A $p$ value $\leq 0.05$ was considered significant. The results were representative of at least three independent experiments. Calculations were performed using Origin (Originlab, USA) and GraphPad Prism software (San Diego, CA).

## 3. Results

### 3.1. TGF-β1 induced endothelial mesenchymal transition of HUVECs

As TGF-β has been extensively acknowledged to induce the EndMT process [4,5], we used TGF-β1 to establish an EndMT model in HUVECs in vitro. When undergoing the EndMT, ECs lose their endothelial specific markers, such as endothelial markers cluster of differentiation 31

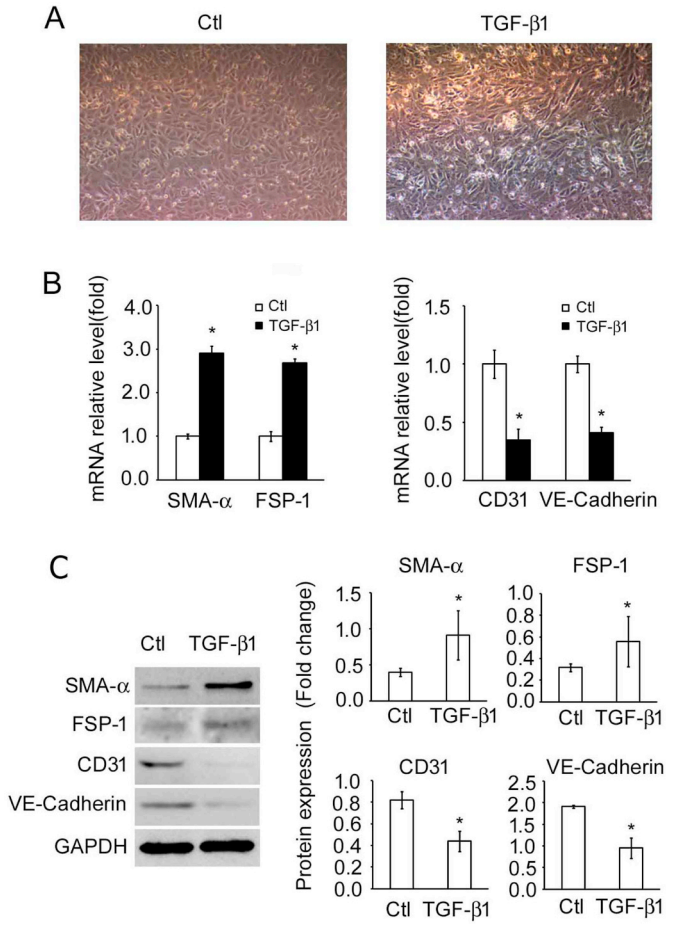


**Fig. 1.** Transforming growth factor beta (TGF-β1) induces Endothelial-mesenchymal transition (EndMT). A. Human umbilical vein endothelial cells (HUVECs) showed remarkable morphological changes after TGF-β1 treated for 48 h. Representative pictures are presented. B. The mRNA levels of the mesenchymal markers fibroblast specific protein-1 (FSP-1), α-smooth muscle actin (SMA-α) and the endothelial cell markers cluster of differentiation 31 (CD31), vascular endothelial cadherin (VE-cadherin) were detected by RT-PCR. GAPDH was used as internal control. C. The protein levels of mesenchymal markers and endothelial cell markers were detected by western blots. Ctl, control; TGF-β1, TGF-β1 treatment; All the figures are from one representative out of more than three similar experiments. * $p < 0.05$ vs. Ctl.

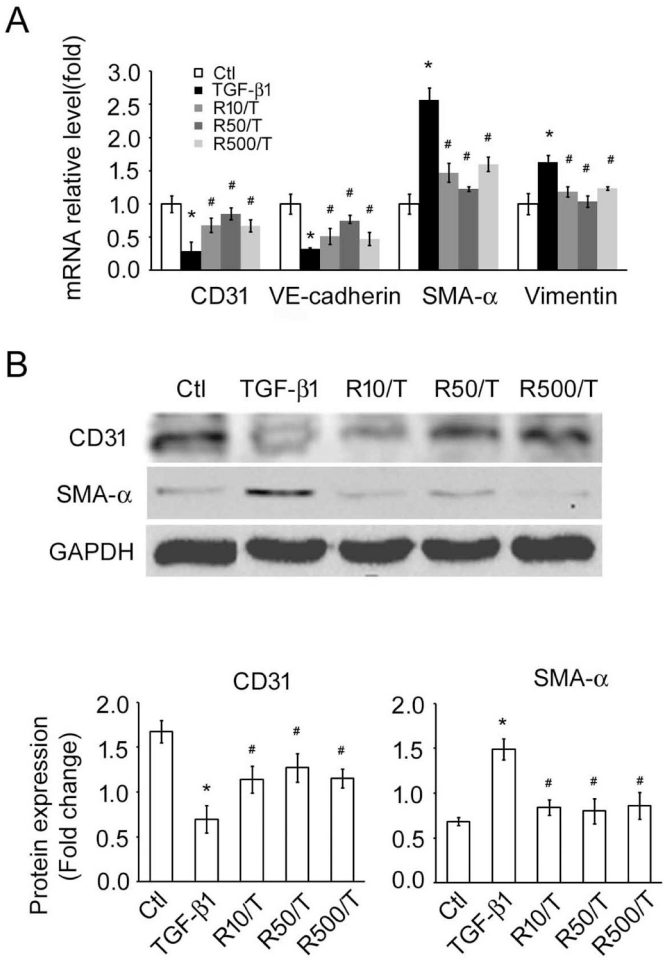


**Fig. 2.** Rapamycin reverses TGF-β1 induced EndMT related molecules expression in HUVECs in an dose-dependent manner. A. The mRNA levels of the endothelial cell markers CD31, VE-cadherin, and the mesenchymal markers SMA-α and vimentin were detected by RT-PCR. GAPDH was used as internal control. B. The protein levels of CD31 and SMA-α were detected by western blots. Ctl, control; TGF-β1, TGF-β1 treatment; R10/T, R50/T or R500/T, cells were pretreated with rapamycin at the dose of 10 nM, 50 nM or 500 nM for 1 h respectively before TGF-β1 treatment. All the figures are from one representative out of more than three similar experiments. * $p < 0.05$ vs. Ctl; # $p < 0.05$ vs. TGF-β1.

(CD31) and vascular endothelial cadherin (VE-cadherin), and gain mesenchymal markers, such as fibroblast specific protein-1 (FSP-1) and smooth muscle actin-α (SMA-α), the phenotype of EC is altered from a typical cobblestone-like to a fibroblast-like appearance [4,5]. It is obvious that TGF-β1 treatment for 48 h led to striking morphological changes of ECs (Fig. 1A). Furthermore, RT-PCR and western bot analysis showed that the expression of VE-cadherin and CD31 were reduced while FSP-1 and SMA-α, were enhanced in TGF-β1 treated HUVECs compared with control cells (Fig. 1B,C). These results confirmed that TGF-β1 induced the EndMT of HUVECs.

### 3.2. Rapamycin prevents TGF-β1 induced EndMT of HUVECs

To evaluate the effect of rapamycin on the EndMT, ECs were pretreated with rapamycin 1 h before incubated with TGF-β1. The concentration of rapamycin used was based on previous studies [15]. TGF-β1 significantly downregulated the mRNA expression of CD31 and VE-cadherin, while upregulated the expression of SMA-α and vimentin. Such expression pattern was reversed by rapamycin in a dose-depend manner (Fig. 2A). The protein level examined by western blots also confirmed that rapamycin has the same effect on SMA-α and CD31 expression (Fig. 2B). These results revealed that rapamycin can block the EndMT induced by TGF-β1. Noticeably, the maximum effect of rapamycin on the EndMT related genes expression was observed at 50 nM treatment group (all $p < 0.05$), but the concentration of rapamycin at 10 nM was effective enough to reverse TGF-β1 regulated such genes expression (all $p < 0.05$). Therefore in all subsequent experiments, the effect of rapamycin on HUVECs behaviors or functions was based on the concentration at 10 nM.

### 3.3. Rapamycin blocks TGF-β1 induced Notch signaling activation

We have previously reported that in chronic kidney disease background, the increased TGF-β1 activates the Notch signaling pathway in ECs and leads to ECs express mesenchymal markers [8]. To evaluate whether rapamycin affects Notch activation in ECs, the expression of the Notch signaling components was detected.

Our results presented that TGF-β1 stimulation upregulated the expression of the Notch ligand Jagged-1, the receptor Notch-1, N1ICD (the cleaved and activated form of Notch-1), the transcription factor

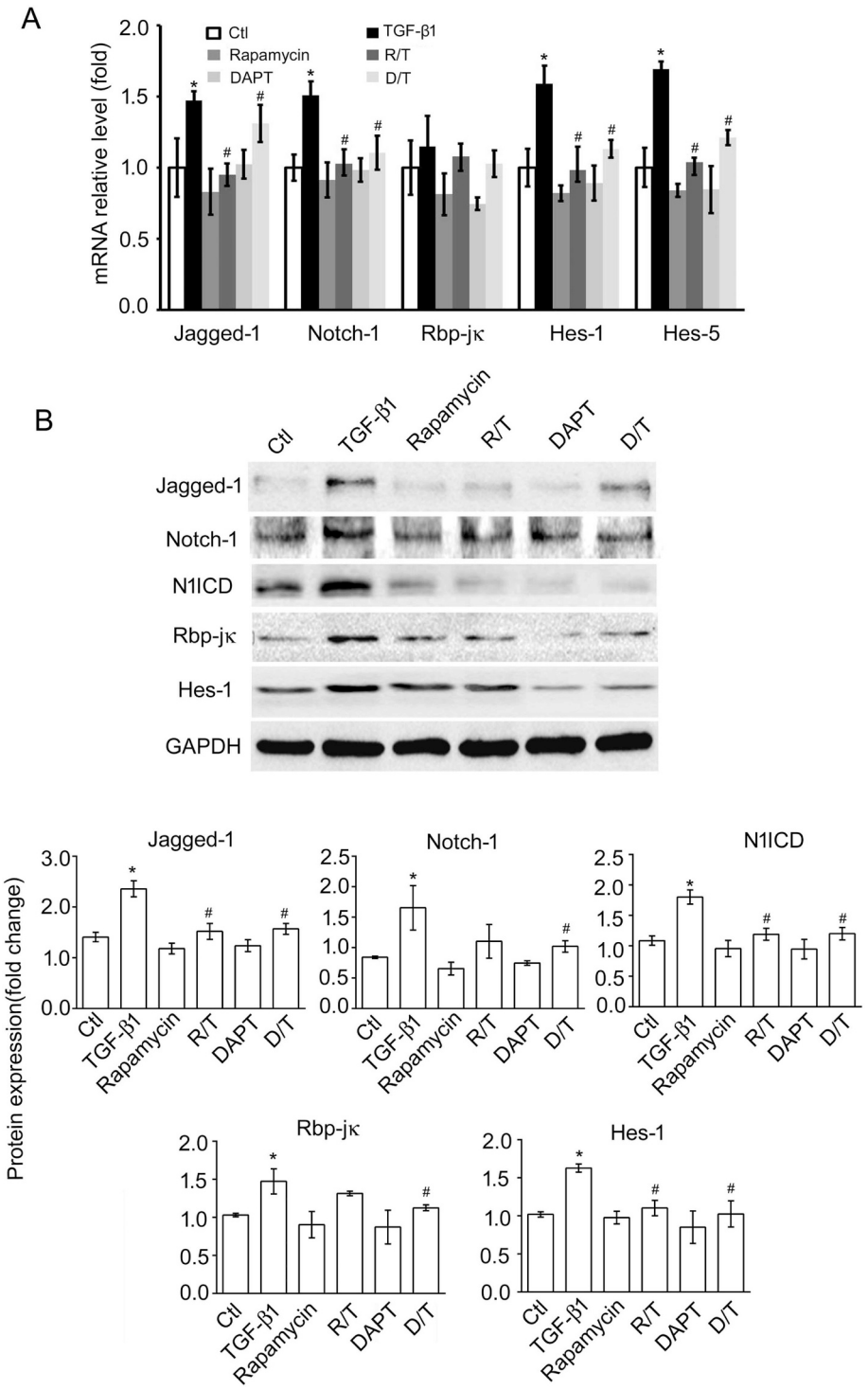


**Fig. 3.** Rapamycin blocks Notch activation induced by TGF-β1 in HUVECs. A.The mRNA levels of Notch signaling molecules Jagged-1, Notch-1, Rbp-jκ, Hes-1 and Hes-5 were detected by RT-PCR. GAPDH was used as internal control. B. The protein levels of Jagged-1, Notch-1, Notch-1 intracellular domain (N1ICD), Rbp-jκ and Hes-1 were detected by western blots. Ctl, control; TGF-β1, TGF-β1 treatment; Rapamycin, rapamycin treatment; R/T, cells were treated with rapamycin for 1 h before TGF-β1 treatment; DAPT, DAPT treatment; D/T, cells were treated with DAPT for 1 h before TGF-β1 treatment. All the figures are from one representative out of more than three similar experiments. * $p < 0.05$ vs. Ctl, # $p < 0.05$ vs. TGF-β1.

Rbp-jκ (also known as CSL for CBF1/Su(H)/Lag-1) and the Notch target genes Hes-1 and Hes-5 in HUVECs (all $p < 0.05$), the Notch signaling inhibitor DAPT effectively blocked the Notch signaling activation. Meanwhile, we observed that rapamycin treatment has the same effect as DAPT (Fig. 3A,B), suggesting that rapamycin significantly inhibited TGF-β1 induced Notch signaling activation. Likewise, immunofluorescent staining showed that in TGF-β1 treated cells, there was an increase in the level of the Notch-1 and N1ICD protein. This effect was abolished by rapamycin or DAPT (Fig. 4).

We also examined the expression of other Notch ligands and receptors. Our results showed that the mRNA transcription of most Notch receptors and ligands (all $p < 0.05$) was increased in HUVECs treated by TGF-β1, however, only the expression of Jagged-1, Jagged-2 and Notch-1 could be significantly reversed by rapamycin (Supplementary Fig. 1).

### *3.4. Rapamycin suppresses TGF-β1 induced EndMT in HUVECs through the Notch signaling inhibition*

To further assess whether the inhibitory effect of rapamycin on the EndMT was dependent on Notch signaling, we examined the change in expression of mesenchymal marker and endothelial marker in HUVECs

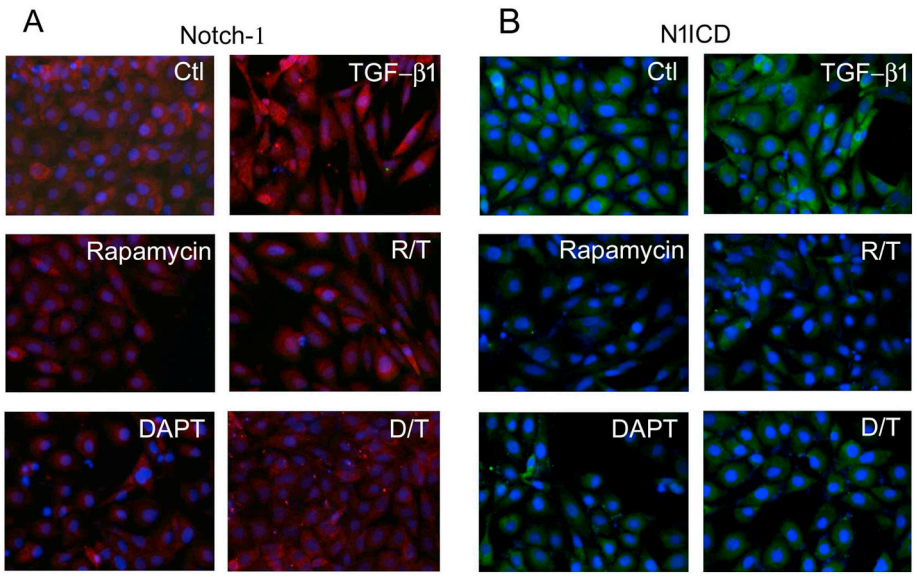


**Fig. 4.** TGF-β1 induced Notch-1 and activated Notch-1 expression in HUVECs were inhibited by rapamycin or DAPT. A. Immunofluorescence staining of Notch-1 (red). B. Immunofluorescence staining of N1ICD (green). Nuclear DNA was stained with DAPI (blue). Representative pictures are presented. Ctl, control; TGF-β1, TGF-β1 treatment; Rapamycin, rapamycin treatment; R/T, cells were treated with rapamycin for 1 h before TGF-β1 treatment; DAPT, DAPT treatment; D/T, cells were treated with DAPT for 1 h before TGF-β1 treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

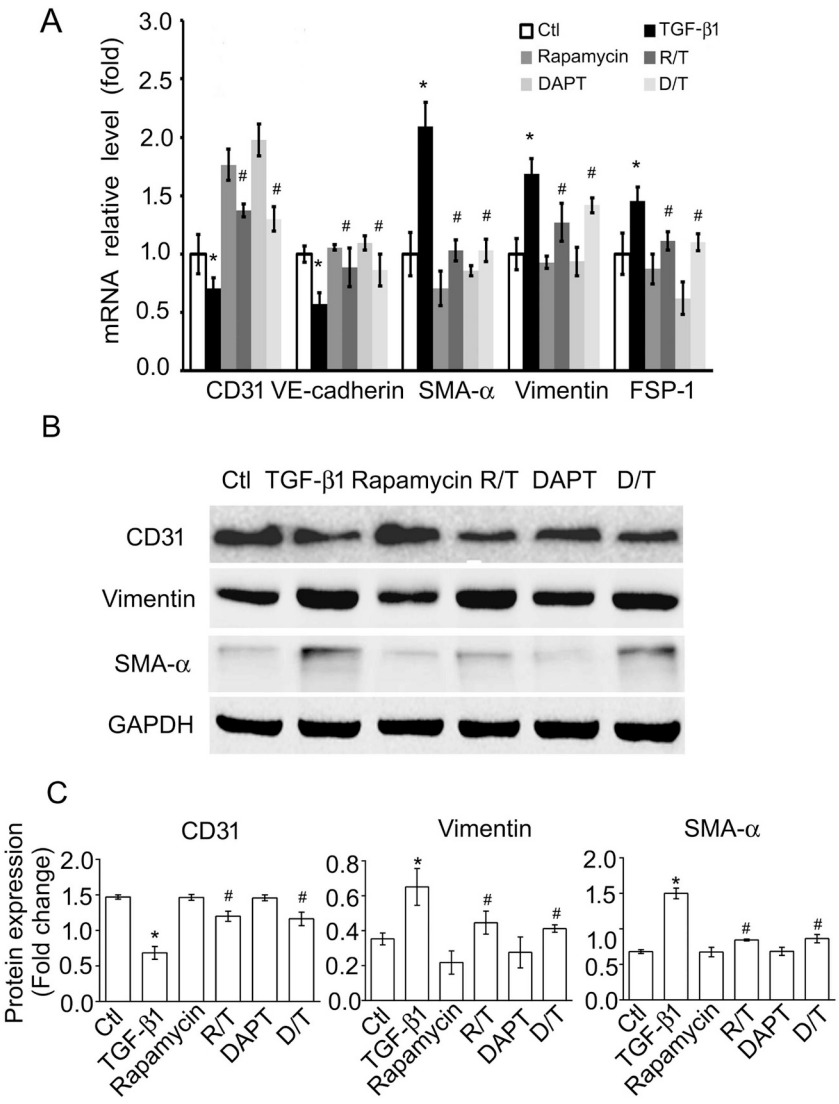


**Fig. 5.** Rapamycin blocks EndMT related molecules expression in HUVECs through Notch signaling inhibition. A. The mRNA levels of endothelial cell markers CD31, VE-cadherin, mesenchymal markers SMA-α, Vimentin and FSP-1 were detected by RT-PCR. GAPDH was used as internal control. B. The protein levels of CD31, SMA-α and Vimentin were detected by western blots. Ctl, control; TGF-β1, TGF-β1 treatment; Rapamycin, rapamycin treatment; R/T, cells were treated with rapamycin for 1 h before TGF-β1 treatment; DAPT, DAPT treatment; D/T, cells were treated with DAPT for 1 h before TGF-β1 treatment. All the figures are from one representative out of more than three similar experiments. * $p < 0.05$ vs. Ctl, # $p < 0.05$ vs. TGF-β1.

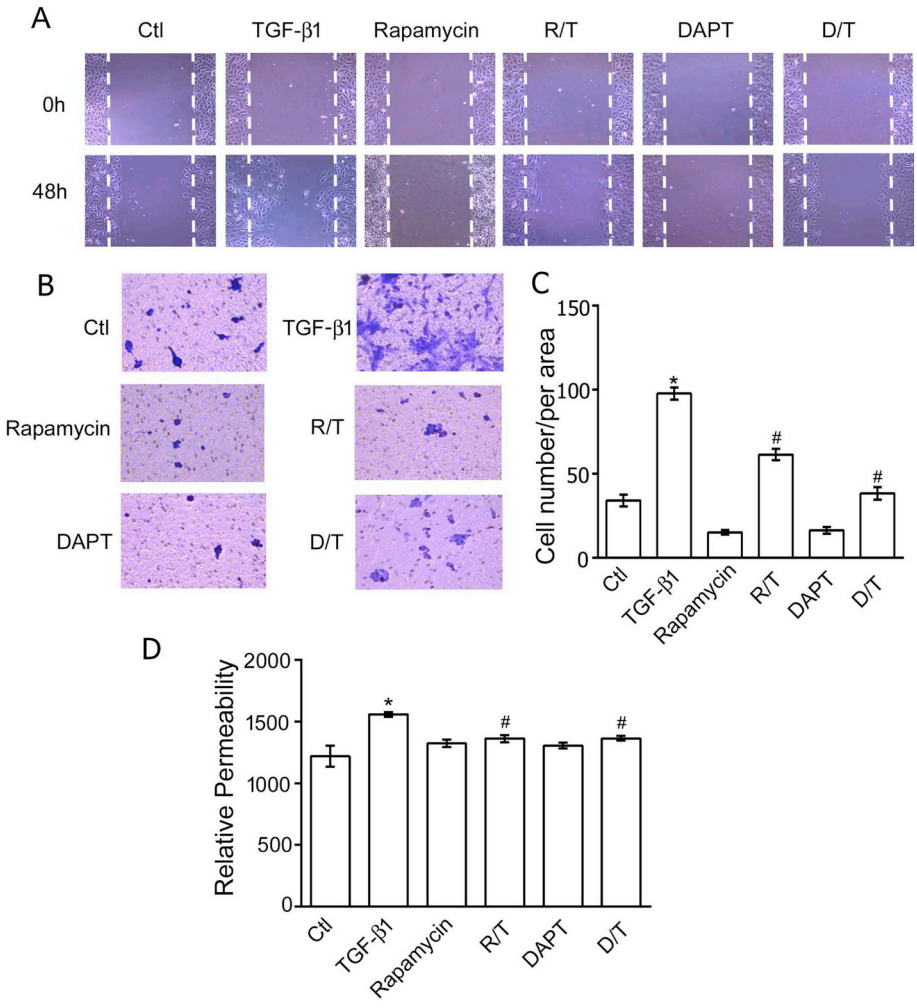


**Fig. 6.** Rapamycin inhibits the migration of HUVECs. A. Wound healing assay was performed to analyze the migration of HUVECs with different treatment. B. Transwell assay was used to test the migration ablility of HUVECs. C. Quantification of data in B. D. Permeability assay was performed to analyze the barrier function of HUVECs. Cells were seeded onto the cell insert with an 8-μm pore, after desired treatment, FITC-labeled dextrans were added, and their accumulation in the lower chamber was measured after 120 min using a fluorescent spectrophotometer. Ctl, control; TGF-β1, TGF-β1 treatment; Rapamycin, rapamycin treatment; R/T, cells were pretreated with rapamycin for 1 h and then treated with TGF-β1; DAPT, DAPT treatment; D/T, cells were pretreated with DAPT for 1 h and then treated with TGF-β1. All the figures are from one representative out of more than three similar experiments. * $p < 0.05$ vs. Ctl; # $p < 0.05$ vs. TGF-β1.

after rapamycin or DAPT treatment. As shown in Fig. 5, TGF-β1 upregulated the expression of Vimentin and SMA-α, but downregulated the expression of CD31. The effect were both reversed by rapamycin or DAPT treatment. These results showed that DAPT, similarly to rapamycin, attenuated TGF-β1-induced EndMT of HUVECs, further suggesting that rapamycin suppressed the EndMT through the Notch signaling inhibition.

One of the characteristics of the EndMT is cells loss cell-cell adhesion and acquire enhanced migratory phenotype. Wound-healing assay and Transwell assay were performed to detect the migration ability of ECs. Wound-healing assay showed that TGF-β1 treatment significantly induced ECs migration, which was attenuated by DAPT or rapamycin (Fig. 6A). The consistent results were also observed in Transwell assay (all $p < 0.05$) (Fig. 6B,C). These results indicated us that rapamycin suppresses the migration ability of EC. Similarly, after TGF-β1 treatment, EC monolayers exhibited an increase in dextran flux. This response was significantly suppressed in ECs pretreated with DAPT or rapamycin. These results indicated that rapamycin plays a protective role in EC barrier dysfunction. Together, these results suggested that the blockage of the Notch signaling pathway by rapamycin reverse the process of the EndMT.

## 4. Discussion

The healing response of vascular walls occurs following mechanical injury, such as balloon angioplasty, endovascular stent implantation and bypass graft, which leads to the development of neointima hyperplasia. The neointima hyperplasia is responsible for restenosis, limiting the success of such vascular surgeries. Although neointimal cells mainly arised through the migration and proliferation of smooth muscle cells from the media, however, ECs as a source of smooth muscle-like cells through the EndMT has gradually been realized [2,3,16]. Here, we investigated the role of rapamycin in the EndMT and identified its protective mechanism.

The EndMT is a critical physiological process of embryonic cardiac development [4,5], it also occurs postnatally as a pathological process and contributes to various diseases, including fibrosis [17], cancer progression [18], and neointima hyperplasia [8]. TGF-β is a well-known multifunctional growth factor regulating the EndMT [4,5]. Patients with coronary atherosclerosis [6] or murine cell lineage-tracing models [7] presented activation of the TGF-β1 signaling, which contributes to the EndMT and leads to neointima hyperplasia. The apoptotic ECs can also induce the EndMT through secreting TGF-β1, resulting in neointima hyperplasia in aortic allograft during transplant arteriosclerosis and allograft function loss [19]. Our previous study reported that increased TGF-β1 during uremia induced ECs expression of mesenchymal markers and barrier dysfunction, leading to accelerated neointima hyperplasia in arteriovenous fistula of chronic kidney mice [8]. Furthermore, TGF-β1 is involved in the wound healing process and plays a significant role in the formation of restenotic lesion after percutaneous transluminal coronary angioplasty (PTCA) or stenting [20]. Thus, we utilized TGF-β1 to induce the EndMT in HUVECs in vitro. Our results confirmed that TGF-β1 induced ECs to express mesenchymal markers and gain mesenchymal phenotypes.

One of the widely accepted way to reduce restenosis is rapamycin-eluting stent, rapamycin can inhibit neointima hyperplasia through

blocking VSMC proliferation and migration [9,10]. Moreover, rapamycin also can inhibit EC proliferation and migration in angiogenesis [19]. Others found rapamycin inhibit EC migration in vitro through an increase of cyclin-dependent kinase inhibitor $p27^{kip1}$ protein levels, resulting in an inhibition of Rho A activation [15]. Our studies confirmed rapamycin not only inhibits EC migration, but also blocks the EndMT induced by TGF-β1 in a dose dependent manner. However, the antimigratory properties of rapamycin prevent stent endothelialization, the delayed re-endothelialization will contribute to neointima hyperplasia and result in thrombosis in the healing of an artery following vascular injury [21]. These findings indicated that rapamycin may have different effect on the development of neointima hyperplasia through regulating the behavior of different cells. Considering the complexity of the development of neointima hyperplasia, targeted therapy can have better therapeutic effect. Based on the previous studies, the mechanism of rapamycin regulating the EndMT need to be further clarified.

The Notch signaling pathway has been well-documented to be involved in the EndMT and contribute to neointima hyperplasia [2]. Activation of Notch signaling is initiated by the Notch receptors recognizing its ligands, leading to the transmembrane Notch receptor cleaved by a disintegrin and metalloproteinases (ADAM) proteases. Then the following second proteolytic cleavage is executed by protease γ-secretase, giving rise to the activated form Notch intracellular domain (NICD). NICD translocates into the nucleus and binds with transcription factor RBP-jκ, upregulates the expression of target genes, and then regulates the activity of cells [2,12]. It has been reported that DAPT, a γ-secretase inhibitor, can block the Notch signaling pathway and reverse the transformed ECs induced by TGF-β into a normal phenotype [20]. We also reported that the blockade of the Notch pathway by DAPT or by RBP-jκ knockout suppresses neointima formation in mice [8]. We checked whether rapamycin regulates the EndMT through the Notch signaling pathway, and we found that rapamycin has the similar effect as DAPT to attenuate the EndMT through preventing the Notch signaling activation.

Except Dll-3, the other four Notch receptors and four Notch ligands are all expressed in mammalian ECs [22,23]. Our results showed that the most robust increase in mRNAs was from Jagged-1 and Notch-1 versus other Notch receptors and their ligands (Supplemental Fig. 1). As Notch-1 is the primary functional Notch receptor during developmental angiogenesis [24], therefore, we focused on Jagged-1/Notch-1 cascade to analyze the effect of rapamycin on the EndMT. Our results showed that rapamycin inhibited the expression of Notch-1, Jagged-1 and the Notch signaling target gene Hes1. However, as we mentioned before, different combination of Notch receptors and their ligands can play different roles in EC behavior [2], more studies of Notch receptors or their ligands will be needed to clarify whether they affect the EndMT.

In conclusion, we have demonstrated that rapamycin attenuates the TGF-β1-induced EndMT through blocking the Notch signaling pathway in HUVECs. Ours studies revealed the underlying mechanisms of EndMT regulated by rapamycin, these results suggested that strategies that blocking the Notch signaling by rapamycin or similar drugs can reverse the EndMT, which may improve the the EndMT related stenosis or vascular diseases. Endothelial specific inhibition of Notch signaling will be necessary through local administration of pharmacological or biologic interventions.

## Disclosures

None.

## Acknowledgments

This work is supported by grants from the National Nature Science Foundation of China (No.81470963 and 81472400) and Natural Science Foundation Project of ChongQing, China (No. cstc 2014jcyjA10098).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2018.10.004.

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