

Original Article

Jagged-1/Notch3 signaling transduction pathway is involved in apelin-13-induced vascular smooth muscle cells proliferation

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The apelin/apelin receptor (APJ, apelin–angiotensin receptor-like 1) system is a newly orphanized G protein-coupled receptor system. Both apelin and APJ that are important regulatory factors are expressed in the cardiovascular system. Our previous studies demonstrated that apelin-13 significantly stimulated vascular smooth muscle cell (VSMC) proliferation. In this paper, our data suggested that the Jagged-1/Notch3 signaling transduction pathway is involved in apelin-13-induced VSMC proliferation by promoting the expression of Cyclin D1. Results indicated that apelin-13 stimulates the proliferation of VSMC and the expression of Jagged-1 and Notch3 in concentration- and time-dependent manners. The increased expression of Jagged-1 and Notch3 induced by apelin-13 could be abolished by extracellular signal-regulated protein kinase (ERK) blockade. PD98059 (ERK inhibitor) can inhibit the activation of Jagged-1/Notch3 induced by apelin-13. Down-regulation of Notch3 using small interfering RNA inhibits the expression of Cyclin D1 and prevents apelin-13-induced VSMC proliferation. In conclusion, Jagged-1/Notch3 signaling transduction pathway is involved in VSMC proliferation induced by apelin-13.

Keywords G protein-coupled receptor; cell proliferation; vascular smooth muscle cell; Jagged-1/Notch3

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Introduction

Apelin is the endogenous ligand of the orphan G protein-coupled receptor (APJ, apelin–angiotensin receptor-like 1). This novel peptidic signaling pathway is widely represented in the heart and vasculature, and is emerging as an important regulator of cardiovascular homeostasis [1]. Functionally,

apelin can stimulate cell proliferation and is an angiogenic factor for retinal endothelial cells [2]. It can also promote thymidine incorporation into the DNA of Chinese hamster ovary cells expressing the APJ receptor and is a new mitogenic peptide for endothelial cells [3]. Moreover, apelin was reported to stimulate gastric cell proliferation and human intropic osteoblast growth [4].

Our previous studies demonstrated that apelin-13 significantly stimulated the proliferation of vascular smooth muscle cells (VSMCs) and increased cell cycle progression mediated by phospho-extracellular signal-regulated protein kinase (ERK) 1/2 expression, which was probably related to Cyclin D1 and Cyclin E [5–7]. We found that apelin-13 promoted angiogenesis and significantly increased Jagged-1 and Notch3 expression in ischemic hearts [8]. Jagged-1/Notch3 plays a very important role in regulating smooth muscle cell (SMC) differentiation and proliferation, which has been proven in the studies of pulmonary arterial hypertension. Pulmonary arterial hypertension is characterized by SMC proliferation in small pulmonary arteries. Li *et al.* [9] showed that overexpression of Notch3 in small pulmonary artery SMCs was characteristic in human pulmonary hypertension. Mice with homozygous deletion of Notch3 did not develop pulmonary hypertension in response to hypoxic insult, and administration with Notch3 inhibitor DAPT could successfully cure mice pulmonary hypertension. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is caused by a mutation of *Notch3* gene. Pathological features of CADASIL include a dramatic reduction of brain VSMCs. The proliferation rate of VSMCs from CADASIL mutation carriers (R133C) was significantly lower than those from healthy controls [10].

However, the functional role of the Jagged-1/Notch3 signaling pathway in VSMC proliferation induced by apelin-13

is poorly determined. Our previous studies demonstrated that apelin-13 promoted the proliferation of VSMCs [5], promoted angiogenesis, and increased Jagged-1 and Notch3 expression in ischemic hearts [8]. Thus, we hypothesized that the Jagged-1/Notch3 pathway may be involved in VSMC proliferation induced by apelin-13. In the present study, we showed that apelin-13 stimulates the proliferation of VSMC and promotes the expression of Jagged-1 and Notch3. Down-regulation of Notch3 prevents apelin-13-induced VSMC proliferation by inhibiting the expression of Cyclin D1. We provide the evidence that the effects of apelin-13 on VSMC proliferation appear to be, at least in part, mediated by Jagged-1/Notch3 signaling pathway through the ERK1/2 pathway.

Materials and Methods

Reagents

The synthetic apelin-13 peptide (pGlu-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe) was purchased from Phoenix Biotech (Phoenix, USA) and rehydrated as a stock solution in phosphate-buffered saline (PBS) before use. PD98059 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St Louis, USA). Bicinchoninic acid protein assay kit was purchased from Hyclone (Logan, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Biosciences (Billerica, USA). Antibodies to Jagged-1, Notch3, Cyclin D1, ERK, and phospho-ERK were all purchased from Cell Signaling (Beverly, USA). β -Actin (I-19) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from Boster Biological Technology (Wuhan, China).

Cell culture

Male C57BL/6J mice (6–8 weeks old) were used for aortic SMC isolation using a standard enzymatic digestion technique. Briefly, aortas were isolated and incubated for 10 min at 37°C in Hank's balanced salt solution containing 315 U/ml collagenase type 2 (Invitrogen, Carlsbad, USA) and 1.25 U/ml elastase (Sigma), penicillin–streptomycin, and amphotericin B. The adventitia was then dissected away and the aorta was incubated for 1 h at 37°C in enzymatic solution. The cell suspension was centrifuged and re-suspended in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), L-glutamine, penicillin–streptomycin, and amphotericin B. VSMCs at passages 3–6 were used for the experiments. The cells were grown to 70%–80% confluence and then rendered quiescent by incubation with DMEM containing 0.5% FBS for 24 h.

Small interfering RNA transfection

In the 6-well tissue culture plates (Corning, New York, USA), 2×10^5 cells were seeded per well. Notch3 small interfering RNA (siRNA) (sc-37136) or non-targeting siRNA control (sc-37007) was transfected into cells using the Tran Messenger Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Western blot analysis

Mouse VSMCs were washed twice with ice-cold PBS and lysed in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer for 10 min on ice. After clarification of the cell lysates by centrifugation at 13,000 g for 15 min, the supernatants were collected. Aliquots containing 30 μ g of protein were electrophoresed in 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked in Tris-buffered saline containing 5% milk and 0.1% Tween-20 for 1 h at room temperature. The membranes were incubated with the primary antibody diluted in blocking solution overnight, and then incubated with HRP-conjugated anti-rabbit IgG as the secondary antibody. Peroxidase activity was detected by enhanced chemiluminescence and analyzed by densitometry using a densitometer and an image.

VSMC proliferation

VSMC proliferation was measured by MTT assay. VSMCs were plated in 96-well plates, and synchronized by incubation in DMEM containing 0.5% FBS for 24 h. Then, the medium was replaced with growth medium (containing 10% FBS). Following the designated incubation interval, 20 μ l of MTT was added to each well and the cells were incubated for 4 h. After removal of the medium, 150 μ l of dimethyl sulfoxide was added and the cells were incubated for 15 min at 37°C. Finally, optical density (OD) values were taken at 570 nm.

VSMC proliferation was also determined by using 5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay kit (Cell Signaling) according to the manufacturer's protocol. In brief, VSMCs were plated in 96-well plates, and synchronized by incubation in DMEM containing 0.5% FBS for 24 h. Then, the medium was replaced with growth medium (containing 10% FBS). Following the designated incubation interval, 10 μ M of BrdU was added to each well and the cells were incubated for 4 h. Finally, OD values were taken at 450 nm.

Statistical analysis

All values are represented as the mean \pm SEM of the indicated number of measurements. The one-way analysis of variance test was used to determine significance, with $P < 0.05$ as the cutoff for statistical significance.

Results

Apelin-13 promotes the expression of p-ERK, Jagged-1, Notch3, and Cyclin D1 in a concentration-dependent manner

Mice VSMCs were incubated with apelin-13 for 4 h at concentrations of 0.5, 1, and 2 μM , respectively. Results from western blot analysis showed that apelin-13 promotes the expression of p-ERK [Fig. 1(A)], Jagged-1 [Fig. 1(B)], Notch3 [Fig. 1(C)], and Cyclin D1 [Fig. 1(D)] in a concentration-dependent manner compared with non-treated cells (0 μM). There was no significant change in the expression of total ERK [Fig. 1(A)].

Apelin-13 increases the expression of p-ERK, Jagged-1, Notch3, and Cyclin D1 in a time-dependent manner

To examine the time effects of apelin-13 on the expression of p-ERK, Jagged-1, Notch3, and Cyclin D1, VSMCs were

incubated with 1 μM of apelin-13 for 0, 1, 2, 4, and 8 h, respectively. The results showed that apelin-13 increases the expression of p-ERK [Fig. 2(A)], Jagged-1 [Fig. 2(B)], Notch3 [Fig. 2(C)], and Cyclin D1 [Fig. 2(D)] in a time-dependent manner.

Up-regulation of p-ERK, Jagged-1, Notch3, and Cyclin D1 induced by apelin-13 is abolished by the ERK inhibitor PD98059

Apelin-13 can promote the expression of p-ERK, Jagged-1, Notch3, and Cyclin D1. Pretreatment with PD98059 (50 μM) for 30 min before treatment with apelin-13 (1 μM) for 4 h, the expression of p-ERK [Fig. 3(A)], Jagged-1 [Fig. 3(B)], Notch3 [Fig. 3(C)], and Cyclin D1 [Fig. 3(D)] induced by apelin-13 was inhibited. Compared with the control cells, apelin-13 promoted cell proliferation. However, when the VSMCs were pretreated with PD98059 (50 μM) for 30 min and then incubated with apelin-13 (1 μM) for 24 h, the results

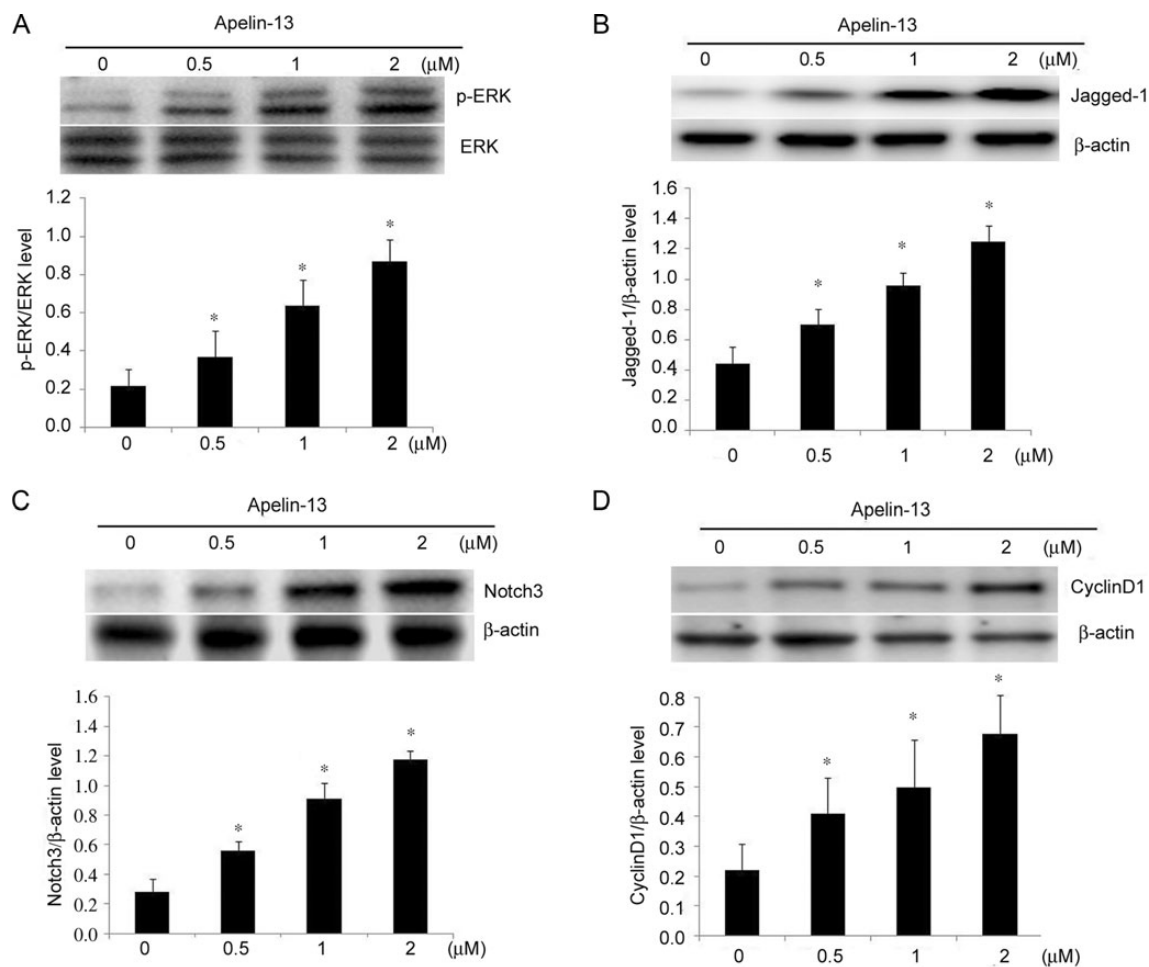


Figure 1 Apelin-13 promotes the expression of p-ERK, Jagged-1, Notch3, and Cyclin D1 in a concentration-dependent manner The VSMCs were treated with 0.5, 1, and 2 μM of apelin-13 for 4 h. The results indicated that apelin-13 increased the expression of p-ERK (A), Jagged-1 (B), Notch3 (C), and Cyclin D1 (D) in a concentration-dependent manner compared with non-treated cells (0 μM). Data were presented as the mean \pm SEM. $n = 4-6$ independent experiments. * $P < 0.05$ vs. non-treated cells.

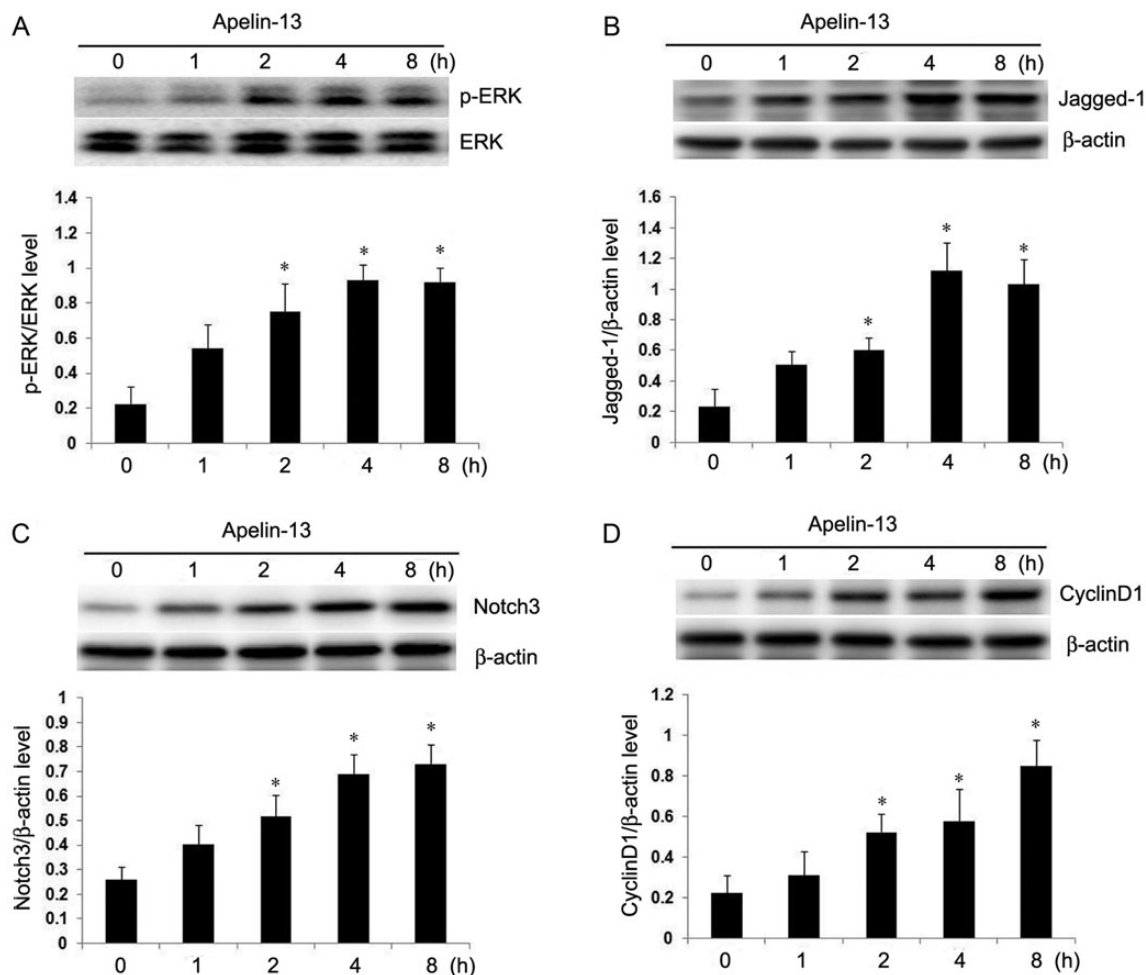


Figure 2 Apelin-13 promotes the expression of p-ERK, Jagged-1, Notch3, and Cyclin D1 in a time-dependent manner The VSMCs were incubated with 1 μ M of apelin-13 for 0, 1, 2, 4, and 8 h. The results showed that apelin-13 increased the expression of p-ERK (A), Jagged-1 (B), Notch3 (C), and Cyclin D1 (D) in a time-dependent manner. Data were presented as the mean \pm SEM. $n = 4-6$ independent experiments. $*P < 0.05$ vs. non-treated cells (0 μ M).

showed that PD98059 could inhibit VSMC proliferation induced by apelin-13 [Fig. 3(E,F)].

siRNA-Notch3 impairs apelin-13-induced VSMC proliferation by inhibiting the expression of Cyclin D1

siRNA-Notch3 (targeting Notch3) was transiently transfected into VSMCs and incubated for 24 h. The results from western blot analysis indicated that the expression of Notch3 protein [Fig. 4(A)] was markedly down-regulated both in control and in apelin-13 (1 μ M)-treated cells, while control siRNA had no such effect. After siRNA-Notch3 were transfected into cells for 24 h and then apelin-13 (1 μ M) was treated for 4 h, we found that siRNA-Notch3 reversed the expression level of Cyclin D1 induced by apelin-13; while control siRNA had no effect on Cyclin D1 [Fig. 4(B)]. Cells transfected with siRNA-Notch3 for 24 h were then treated with apelin-13 (1 μ M) for another 24 h. Compared with the control cells, apelin-13 can stimulate VSMC proliferation,

and siRNA-Notch3 inhibits apelin-13-induced VSMC proliferation [Fig. 4(C,D)].

Discussion

Apelin, the endogenous ligand for APJ receptor, was isolated from bovine stomach extracts and was reported to show some sequence similarities with angiotensin II [11]. *Apelin* mRNA encodes a 77-amino acid (aa) preproprotein that is proteolytically cleaved to yield bioactive peptides 36, 17, and 13 aa in size. Each of these peptides contains the C-terminal region of the precursor protein and the bioactivity is thought to reside in the terminal 13 aa fragment. The sequence of the 13 aa peptide is 100% conserved between frogs and humans, suggesting that a critical function has been evolutionarily conserved [12]. In preclinical models, apelin causes nitric oxide-dependent vasodilatation, reduces ventricular preload and afterload, and increases cardiac

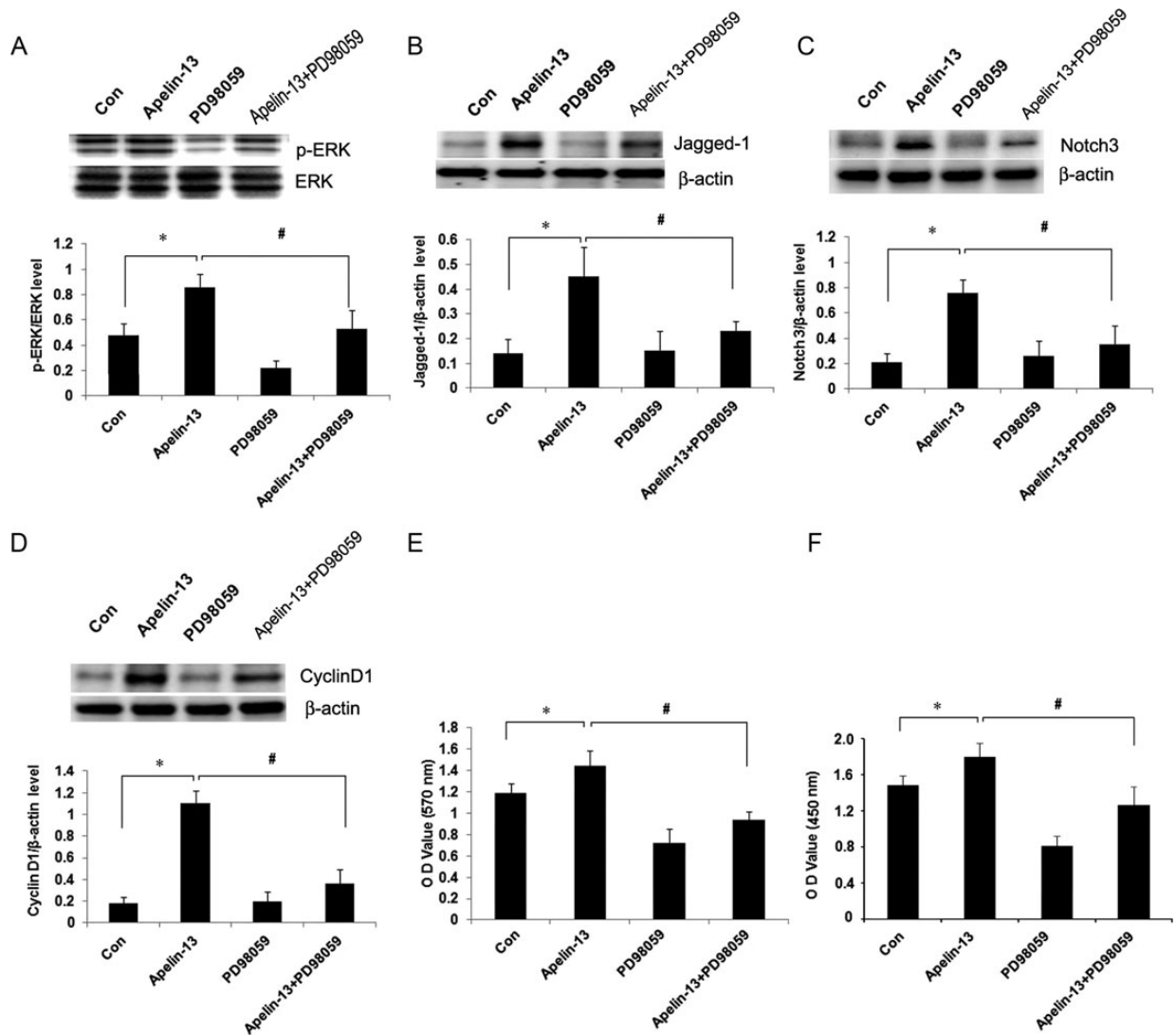


Figure 3 PD98059 reverses apelin-induced up-regulation of p-ERK, Jagged-1, Notch3, and Cyclin D1. Pretreatment with PD98059 (50 μ M) for 30 min then treatment with 1 μ M apelin-13 for 4 h, PD98059 inhibited the expression of p-ERK (A), Jagged-1 (B), Notch3 (C), and Cyclin D1 (D) induced by apelin-13. The cells were cultured with synthetic apelin-13 (1 μ M) for 24 h. MTT and BrdU assays showed that apelin-13 increased VSMC proliferation (E,F) compared with control cells. Data were presented as the mean \pm SEM. $n = 4$ independent experiments. * $P < 0.05$ vs. control (0 μ M) and # $P < 0.05$ vs. apelin-13-treated cells.

contractility in rats with normal and failing hearts [13]. Apelin/APJ signaling also attenuates ischemic myocardial injury and maintains cardiac performance in aging and chronic pressure overload [14]. Apelin has been shown to be involved in the regulation of cardiovascular function, fluid homeostasis, vessel formation, and cell proliferation [15,16].

In this study, the results indicated that apelin-13 can increase the expression of p-ERK, Jagged-1, and Notch3 in time- and dose-dependent manners. These results also suggested that apelin-13 induces the activation of Jagged-1/Notch3 signaling pathway in mouse VSMCs. The Jagged-1/Notch3 signaling pathway is evolutionarily conserved, which plays vital roles in vascular development and homeostasis and influences cell fate by regulating cell growth, apoptosis, and differentiation [17–19]. Tissue

distribution of the Notch proteins varies widely. In mammals, there are four Notch family receptors that have been described. Notch1 and 4 are predominantly endothelial, prominent in both arteries and veins. The expression of Notch2 is typically confined to the pulmonary endothelium, but Notch3 is mainly expressed in adult arterial VSMCs. Notch3 activation is triggered by interactions with ligands of the Delta and Serrate/Jagged families that results in the induction of the expression of Hairy and Enhancer of Split-related basic helix–loop–helix transcription factors [20]. In the cardiovascular system, Notch signaling plays a role in several aspects of vascular development, including vasculogenesis, angiogenesis, differentiation, vascular remodeling, and VSMC maturation, migration, and proliferation [21].

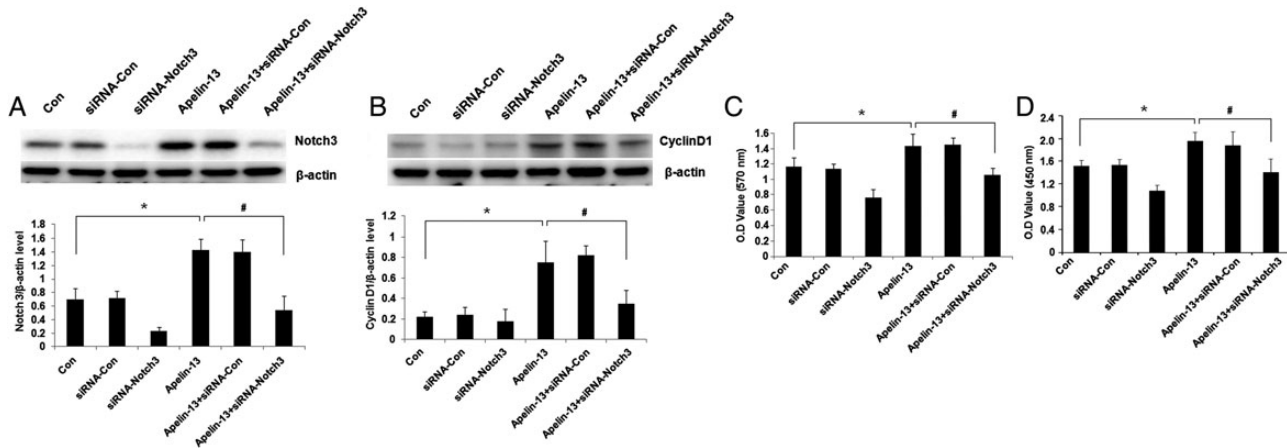


Figure 4 siRNA-Notch3 impairs apelin-13-induced VSMC proliferation by inhibiting the expression of Cyclin D1 siRNA-Notch3 was transiently transfected into the cells for 24 h. (A) The results from western blot analysis indicated that the expression of Notch3 protein was markedly down-regulated both in control and apelin-13-treated cells, while control siRNA had no such effect. Cells transfected with siRNA-Notch3 for 24 h were then treated with apelin-13 (1 μM) for 4 h. (B) Compared with the control cells, apelin-13 up-regulated the expression of Cyclin D1, but siRNA-Notch3 reversed the expression level of Cyclin D1 induced by apelin-13. (C,D) MTT and BrdU results showed that treatment with apelin-13 (1 μM) for 24 h can stimulate VSMC proliferation compared with control cells. After being transfected by siRNA-Notch3 for 24 h, then treated with apelin-13 for another 24 h, siRNA-Notch3 obviously inhibited apelin-13-induced VSMC proliferation. Data were presented as the mean ± SEM. *n* = 4 independent experiments. **P* < 0.05 vs. control (0 μM) and #*P* < 0.05 vs. apelin-13-treated cells.

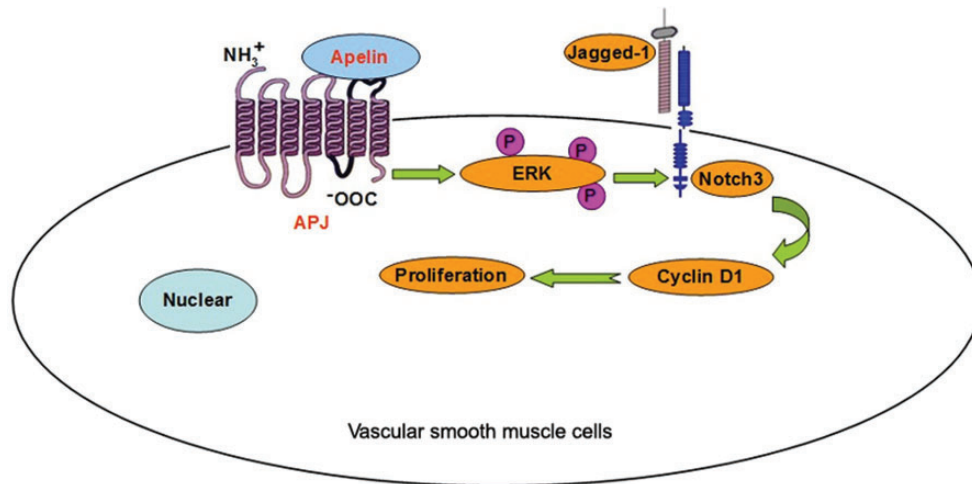


Figure 5 Jagged-1/Notch3 signaling transduction pathway is involved in VSMC proliferation induced by apelin-13 Apelin-13 stimulates the proliferation of VSMCs and promotes the expression of Jagged-1 and Notch3. Apelin-induced up-regulation of Jagged-1 and Notch3 was abolished by ERK blockade. Apelin-13-induced VSMC proliferation appears to be partially mediated by Jagged-1/Notch3 signaling transduction via the ERK1/2 pathway.

We further showed that apelin increased the expression of Jagged-1 and Notch3. These observations further point to the link between Notch and apelin signaling involved in VSMC proliferation. siRNA-Notch3 was transiently transfected into the VSMCs. The expression of Notch3 protein was markedly down-regulated both in control and apelin-13-treated cells, which led to an obvious decrease of the expression of Cyclin D1. These results indicated that inhibition of Jagged-1/Notch3 signaling pathway results in the interruption of apelin signaling transduction.

We also showed that apelin-13 promotes the proliferation of VSMCs. Similarly, it has also been reported that apelin is

capable of stimulating the growth of retinal endothelial cells [9], human umbilical endothelial cells, and human osteoblasts [10], and the proliferation of gastric cells [10]. To further explore the role of Notch3 in apelin-13-induced proliferation of VSMCs, we transiently transfected siRNA-Notch3 into the VSMCs. The results showed that siRNA-Notch3 inhibited obvious VSMC proliferation induced by apelin-13. Therefore, we conclude that apelin-13 stimulates the activation of Jagged-1/Notch3 signal pathway in the VSMCs to promote cell proliferation (Fig. 5).

In summary, some preliminary understanding of the biological functions and novel mechanistic insights of the role

of apelin/APJ signaling in VSMC proliferation have been presented. The effect of apelin-13 on promoting VSMC proliferation has a close relationship with Jagged-1/Notch3 signaling pathway. Apelin-13-induced VSMC proliferation could be inhibited by siRNA-Notch3. Since VSMC proliferation plays a critical role in the pathogenesis of atherosclerosis, it is reasonable to consider the apelin/Jagged-1/Notch3 signaling pathway as a potential therapeutic target. However, the role of Jagged-1/Notch3 signaling pathway in apelin-induced VSMC proliferation *in vivo* will require further investigation, and whether there is any other signaling pathway mediating the activity of apelin 13-induced VSMC proliferation remains to be further explored.

Funding

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