Ageing-exaggerated proliferation of vascular smooth muscle cells is related to attenuation of Jagged1 expression in endothelial cells

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Aims Ageing has been shown to enhance neointima formation due to abnormal growth of vascular smooth muscular cells (VSMC), which is regulated by endothelial functions. The mechanism of how endothelium affects the growth of VSMC in the process remains unclear. We here examined the role of Jagged1, a regulator of cell growth.

Methods and results Male Sprague–Dawley rats at 3 (young) and 22 (old) months of age were subjected to a balloon catheter injury in the thoracic aorta. After 4 weeks, the neointima formation in the injured artery of old rats was more than that of young rats. Compared with the young rats, the increase in Jagged1 expression in the endothelium of old rats after the injury was delayed, weakened, and shortened, suggesting an impaired response of Jagged1 to the injury. In contrast, the increase in the expression of proliferating cell nuclear antigen in the neointima was more significant and maintained longer in old rats than in the young ones. Moreover, the expression of Jagged1 in the cultured arterial endothelial cells (EC) of old animals was less than those of the young ones, which promoted the Platelet-derived growth factor (PDGF)-induced growth and migration of the co-cultured VSMC. Furthermore, suppression of Jagged1 expression by a small interfering RNA in the EC of young rats reduced α-smooth muscle actin and calponin expression and also intensified the PDGF-increased growth and migration of the co-cultured VSMC.

Conclusion Ageing enhanced VSMC proliferation, at least in part, through impairing Jagged1 expression in the EC after vascular injury.

KEYWORDS Ageing; Endothelial cell; Jagged1; Neointima; Smooth muscle cell

1. Introduction

Both experimental and clinical studies have shown that ageing is an independent risk factor for the development of cardiovascular diseases.1 It has been reported that ageing exaggerated neointima formation after arterial injury.2–4 However, the mechanism of how ageing affects the process is undefined. Since the excessive growth of vascular smooth muscular cells (VSMC) is a main pathological process in the neointimal proliferation, it is reasonable to investigate the influence of ageing on the proliferation of VSMC after vascular injury. Some previous studies have shown that aortic VSMC from the aged animals had a greater proliferative capacity than those from the young counterparts,5–7 while others showed the opposite.8 Although the reason for the controversy is unclear, the differences in culture condition and the complexes in the process of regulation in in vivo experiments may account for it.8

Notch signalling plays a role not only in the development of embryonic tissue, but also in the remodelling of the adult tissue after injury. Notch has been shown to dictate cell fate and influence cell proliferation, differentiation, and apoptosis. In humans, Notch 1 through 4 comprises the receptor family, and Jagged1, Jagged2, and Delta1 are among the ligands.9 Recently, Conboy et al.10 have shown that age-dependent Notch decline in the muscular satellite cells results in dysfunctions of the muscle repair, suggesting a potential link between Notch and repair of the aged tissue. Alterations in Notch pathways have also been observed in injured arteries. Linder et al.11 reported that Jagged1 expression in the endothelium, especially in the regions contacting the proliferative VSMC, was up-regulated, suggesting a cell–cell interaction related to Jagged1. However, the role of Jagged1 in age-exacerbated neointimal proliferation after artery injury is still unknown.
Although the senescence of vascular cells contributes to the age-exacerbated neointimal proliferation, reciprocal regulations between the vascular endothelial cells (EC) and the VSMC, two major neighbouring cells constituting the vessel wall, play a critical role in maintaining the normal vascular function and adaptable responses to a variety of stimulations. We therefore hypothesized that the expression of Jagged1 in the EC might regulate VSMC growth, thereby participating in the age-exacerbated neointima formation after vascular injury.

2. Methods

2.1 Animals and balloon expansion injury

Male Sprague–Dawley rats of 3 month (young, n = 20)- or 22 month (old, n = 20)-old (purchased from the Third Military Medical University, Chongqing, China) were randomized into four groups (n = 5 for each group): control group, with sham operation; 7, 14, and 28 days groups, subjected to a thoracic aorta injury for the indicated times. Thoracic aorta injury was performed by expansion of the artery with a balloon catheter as previously described. Denudation of the endothelium was performed and verified properly by an in vivo Evans blue staining. All protocols were approved by guidelines of Fudan University and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.2 Quantitative morphometry and immunohistology

The thoracic aorta was harvested for quantitative morphometry analysis and immunohistochemical staining. Briefly, the artery at harvesting was subjected to a perfusion fixation with 4% paraformaldehyde, followed by en bloc excision. After fixation with 4% paraformaldehyde overnight, the middle one-third of the descending thoracic aorta was cut into two segments at ~5-mm length of each. The specimens were embedded in paraffin, cross-sectioned, and stained with haematoxylin–eosin (HE). Measurements of neointimal and medial areas were obtained from each specimen using computerized morphometry system as described elsewhere. Four sections were measured and averaged for each artery. The degree of neointima formation was expressed as both the absolute area of neointima and the ratio of the neointimal area to the medial area (N/M). The expression of Jagged1 and proliferating cell nuclear antigen (PCNA) was evaluated with immunohistochemical stains using an anti-Jagged1 (Santa Cruz, CA, USA) or PCNA (Zymed, South San Francisco, USA) antibody, respectively, as previously described.

2.3 Cell culture

EC and VSMC were isolated from rat aorta and cultured as previously described (Supplementary material online, Table S1). The EC of passage 1 were used to examine the expression of Jagged1. The EC and the VSMC of passage 3–5 were used in other experiments. More than 95% of the EC and the VSMC in the culture were positive as stained by anti von Willebrand factor, specifically expressed in the EC (sc-59810, Santa Cruz), and anti α-smooth muscle actin (α-SM-actin), specifically expressed in VSMC (sc-53142, Santa Cruz), antibodies, respectively (Supplementary material online, Tables 2 and 3).

2.4 Transfection of a siRNA

RNA interference technique was used to knock down the expression of Jagged1 in the EC. A small interfering RNA (siRNA) of Jagged1 (sc-61881) and its scramble RNA (sc-36869) were obtained from Santa Cruz Biotechnology. After preparation with the siRNA transfection reagent (sc-29528), the RNA or vehicle was transfected into the cultured EC (at 50% confluence) according the manufacturer’s instructions. To confirm the effect of siRNA on the expression of Jagged1, some of the cells after transfection for 48 h were subjected to a western blot analysis using an anti-Jagged1 antibody (sc-6011, Santa Cruz).

2.5 Co-culture of EC with VSMC

An in vitro co-culture model, in which EC and VSMC were, respectively, seeded in the lower and the upper chamber, was employed to investigate the effects of EC on the VSMC with ageing. Briefly, EC were inoculated in 6-well plates (Corning, New York, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Logan, UT) with 10% fetal bovine serum (FBS, Hyclone), some of which were transfected with the siRNA of Jagged1 or the scramble RNA. When growing to 90% of confluence, culture medium was changed with the serum-free DMEM. VSMC were then inoculated on the transwell insert membranes (Corning, with pores of 0.4 μm in diameter) at a density of 10^5 cells/ml. Twenty-four hours later, the transwell inserts with the VSMC were inserted into the culture dishes of the EC which were cultured in the lower chamber of the dishes. The medium in the two chambers could communicate through the pores of the insert membranes. Platelet-derived growth factor (PDGF, Peprotech, EC, London, UK) was then added to the lower chamber at final concentration of 10 ng/ml. After 24 h of the incubation, VSMC in the upper chamber were harvested for further experiments.

2.6 Migration assay

VSMC migration assay in the co-culture system was performed as previously described with some modifications. In brief, VSMC, grown on the transwell insert membranes (with pores of 8 μm in diameter) in the 24-well co-culture system (Corning) with EC were transfected with the Jagged1 siRNA or the scramble one, were incubated with PDGF (10 ng/ml) or vehicle for 6 h. The insert was then removed and non-migrated VSMC were scraped off the membrane. The VSMC migrated to the lower surface of the membrane were stained with haematoxylin, counted in five randomly selected optical fields (~400) and averaged. Data were expressed as the number of cells per field.

2.7 DNA synthesis

Proliferative ability of the VSMC in the co-culture system was determined with a DNA synthesis assay by assessing the incorporation of [3H] thymidine ([3H]-TdR, Atomic Nucleus Research Institute, Shanghai, China) into the cells according to the manufacturer’s instructions. After 24 h of incubation with 1 ng/ml PDGF-B (37 kBq/ml), the co-cultured VSMC were harvested and the total radioactivities were measured by a liquid scintillation spectrometer (Beckman, USA). Data were expressed as the counts per min per well (cpm/well).

2.8 Quantification of growth factors in the culture medium of EC

The levels of nitric oxide (NO), PDGF-B, interleukin-1 alpha (IL-1 α), and bradykinin in the culture medium of EC were measured to determine the release of these factors by EC. Briefly, after 90% confluence, the EC were cultured with the serum-free DMEM for 24 h and the supernatants were then collected. NO levels were determined by measuring the accumulation of its stable degradation products, nitrite and nitrate, using the Griess method as described elsewhere. Data were expressed as nmol of nitrate/ml of culture medium (nmol/ml). The levels of PDGF-B (sensitivity: 7.7 pg/ml, R&D Systems, Minneapolis, MN, USA), IL-1 α (Sensitivity: 3.0 pg/ml, BioSource, Camarillo, CA, USA), and bradykinin (sensitivity: 10.0 pg/ml, Rapidbio, Columbia, CA, USA) were determined using the enzyme-linked immunosorbent assay according to the manufacturer’s instructions. Data were expressed by pg/ml.
2.9 Western blot
The expressions of Jagged1, PCNA, α-SM-actin, and calponin were analyzed by western blotting. Briefly, total cell proteins was separated by the electrophoresis on the SDS-polyacrylamide gel and transferred to the PVDF membrane (Roche, Basel, Switzerland). The membrane was immunoblotted with the antibodies against Jagged1, PCNA, α-SM-actin, or calponin (sc-16604, Santa Cruz), respectively, at 4 °C overnight. The immunoreactivity was detected using the enhanced chemiluminescence reaction system (Amersham Pharmacia Biotech, NJ, USA) according to the manufacturer’s directions. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The expression of each protein was quantified, respectively, by scanning densitometry and normalized by GAPDH. Data were expressed as relative optical density value.

2.10 Fluorescence immunocytochemistry and fluorescence-activated cell sorter analysis
Fluorescence immunocytochemistry and fluorescence-activated cell sorter (FACS) were used to determine the Jagged1 expression in the cultured EC from rat aorta. Briefly, after 60 to ~70% of confluence, EC were fixed with 4% paraformaldehyde, incubated with the anti-rat Jagged1 antibody or with phosphate-buffered saline solution as a negative control for 1 h, and then with the Cy3 conjugated antibody (Sigma, St. Louis, MO, USA) for 30 min. The staining of the cells was analyzed with a fluorescence microscope (Leica DMIRB, German) or a flow cytometry (FACS Calibur, Becton Dickinson, USA).

2.11 Statistical analysis
Data were expressed as mean ± SEM. Differences were determined either by unpaired Student’s t-test or by one-way ANOVA followed by post-hoc test to compare the difference between two groups. Values of P < 0.05 were considered significant.

3. Results

3.1 Up-regulation of Jagged1 expression in the endothelium after arterial injury was delayed and impaired in old rats
We first confirmed the neointima formation in the thoracic aorta after a balloon injury in the old and the young rats. At 28 days after the injury, the neointima was more in the vessels from older animals than from their younger counterparts (neointimal area: 0.22 ± 0.01 vs. 0.16 ± 0.01 mm², n = 5, P < 0.01; N/M: 0.35 ± 0.01 vs. 0.28 ± 0.01, n = 5, P < 0.01).

We then examined the expression of Jagged1 in the endothelium of aorta. The expression of Jagged1 in the uninjured endothelium was similarly lower between the young and the old rats. However, in responding to the balloon injury, Jagged1 expression in the endothelium was dramatically up-regulated on day 7, lowered on day 14 and returned to basal levels by day 28 in the young rats, whereas the change of Jagged1 expression was quite different in the old rats. In the old rats, the increase in Jagged1 expression after the injury was only weakly observed on day 14 and quickly disappeared by day 28, suggesting a delayed and impaired response of Jagged1 expression to the endothelium injury (Figure 1A and B).

3.2 PCNA expression in the neointima was more increased in old rats
PCNA is a marker of DNA synthesis, which might be increased during formation of the neointima. We therefore examined the protein expression of PCNA in the neointima of aorta (Figure 1A and C). In the artery without injury, PCNA expression was nearly undetectable in both the young and the old rats. After the injury, however, in the old rats, PCNA expression was significantly increased on day 7 with a peak on day 14, and the increase was maintained nearly by day 28, whereas in the young rats the increase in PCNA expression at each time point was much less than that in the old ones, and completely disappeared by day 28. These results suggested an enhanced up-regulation of PCNA expression responding to the endothelial injury in the old rats, which was in contrast to the responses of Jagged1 expression.

3.3 The expression of Jagged1 in the old EC was down-regulated which promoted PDGF-induced proliferation and migration of the VSMC
Morphology of the cultured EC from the artery of old rats was not different from that of the young rats. However, the expression of Jagged1 protein in the EC, which was specifically located at the cell membrane, was much lower in old rats than in the young ones (46.55 ± 3.65 vs. 85.43 ± 2.33, n = 3, P < 0.05) (Figure 2).

VSMC growth is regulated not only by cytokines, but also by the adjacent EC.13 It has been reported that vaso-active substances and cytokines including NO, PDGF, IL-1, and bradykinin significantly influence the growth of VSMC.21–23 We therefore investigated whether the release of these growth factors by EC might be altered with ageing. At basal condition without the stimulation, levels of NO, PDGF-B, IL-1α, and bradykinin in culture medium of the EC were similar between the young and the old rats (Table 1), suggesting that the release of these growth factors from EC was not changed with ageing.

We then investigated whether the regulation of VSMC by EC might be altered with ageing using the co-culture system. At basal condition without the stimulation, the proliferative ability characterized by a DNA synthesis assay was similar between the VSMC co-cultured with the young and the old EC (9822 ± 499 vs. 9600 ± 343 cpm/well, n = 5, P > 0.05). When exposed to PDGF (10 ng/ml for 24 h), however, the VSMC co-cultured with the old EC exhibited a higher proliferation level than those with the young EC (26438 ± 831 vs. 16698 ± 929 cpm/well, n = 5, P < 0.05) (Figure 3A).

We also tested the effect of co-cultured EC on the migration of VSMC. Consisting with the data of VSMC proliferation, the migration of VSMC co-cultured alone with the young or the old EC for 24 h was similarly less (5 ± 1 vs. 7 ± 2 cells/field, n = 5, P > 0.05). After addition of PDGF to the cells for 6 h, the migration of co-cultured VSMC was increased. However, the increase in the VSMC co-cultured with the old EC was larger than that with the young ones (32 ± 2 vs. 18 ± 2 cells/field, n = 5, P < 0.05) (Figure 3B).

3.4 Suppression of Jagged1 in EC accelerated PDGF-induced proliferation and migration of VSMC
To confirm the role of Jagged1, we also investigated the effects of EC with a knocking down of Jagged1 on the
proliferation and migration of VSMC using the EC of young rats and the co-culture system. Western blotting verified that the expression of Jagged1 in the EC was specifically and significantly suppressed by the transfection with a cognate Jagged1 siRNA duplex compared with a mock transfection or the transfection with a scramble sequence (Figure 4).

Consistent with previous data, PDGF significantly increased H\(^3\)-TdR incorporation into the VSMC co-cultured with the young EC (16442 ± 807 vs. 10500 ± 537 cpm/well, n = 5, P < 0.01). In the VSMC co-cultured with the EC transfected with a siRNA of Jagged1, H\(^3\)-TdR incorporation after incubation with PDGF was further increased (23074 ± 1209 vs. 16442 ± 807 cpm/well, n = 5, P < 0.05). However, transfection of a scramble RNA into the EC did not intensify the PDGF-induced increase in H\(^3\)-TdR incorporation into the VSMC (16955 ± 1111 vs. 16442 ± 807 cpm/well, n = 5, P > 0.05) (Figure 5A).

Also, the expression of PCNA protein in the co-cultured VSMC, another marker for cell proliferation, was up-regulated by the stimulation with PDGF (0.51 ± 0.02 vs. 0.26 ± 0.02, n = 5, P < 0.01). Transfection with the Jagged1 siRNA but not with the scramble one into the EC further accelerated the PDGF-induced increase in PCNA protein expression (0.61 ± 0.01 vs. 0.51 ± 0.02, n = 5, P < 0.05; 0.50 ± 0.01 vs. 0.51 ± 0.02, n = 5, P > 0.05, respectively) (Figure 5B and C).

Migration of the co-cultured VSMC after suppression of Jagged1 by the siRNA in the EC was also examined.

Figure 1 Time course of Jagged1 and PCNA expression in the neointima after the arterial injury in the old and young rats. The old (22 months) and the young (3 months) rats were subjected to a thoracic aorta balloon catheter injury. At the 0, 7, 14, and 28 days after the injury, the artery was fixed, embedded, sectioned and stained by HE, and immunohistological method using anti-Jagged1 and PCNA antibodies. (A) Representative photomicrographs of HE, Jagged1 and PCNA staining are shown. Jagged1 (open arrow) and PCNA (closed arrow) were detected in endothelium and neointima, respectively. (B and C) Quantitative analysis of Jagged1 and PCNA expressions. Jagged1 and PCNA positive cells were counted in whole section. Data are expressed as the mean ± SEM (n = 5, *P < 0.05 vs. the old rats at 14 days; **P < 0.01 vs. the young rats at 7 days.)
of VSMC.  

We finally examined the effects of knocking down \( \alpha \)-SM-actin and calponin protein expression in the co-cultured VSMC. As expected, PDGF significantly reduced the protein expression of \( \alpha \)-SM-actin and calponin in the co-cultured VSMC. Although transfection of a scramble RNA into the EC accelerated the PDGF-induced migration of the co-cultured VSMC (27 ± 2 vs. 15 ± 2 cells/field, \( n = 5, P < 0.05 \); 16 ± 1 vs. 15 ± 2 cells/field, \( n = 5, P > 0.05 \), respectively) (Figure 5D and E).

### 3.5 Knocking down of Jagged1 in the EC promoted the reduction of expression of phenotype proteins in the co-cultured VSMC

The levels of expression of phenotype proteins, such as \( \alpha \)-SM-actin and calponin which are highly expressed in the contractile VSMC but lowly in the proliferative ones, can indicate proliferation capacities and phenotype transition of VSMC. We finally examined the effects of knocking down of Jagged1 in the EC on the expression of \( \alpha \)-SM-actin and calponin in the co-cultured VSMC. As expected, PDGF significantly reduced the protein expression of \( \alpha \)-SM-actin and calponin (0.49 ± 0.03 vs. 0.74 ± 0.01, \( n = 3, P < 0.05 \); 0.43 ± 0.02 vs. 0.61 ± 0.02, \( n = 3, P < 0.05 \), respectively). Although transfection of a scramble RNA into the EC did not influence the PDGF-reduced \( \alpha \)-SM-actin and calponin protein expression in the co-cultured VSMC (0.50 ± 0.03 vs. 0.49 ± 0.03, \( n = 3, P > 0.05 \); 0.46 ± 0.02 vs. 0.43 ± 0.02, \( n = 3, P > 0.05 \), respectively), introduction of a Jagged1 siRNA into the EC aggravated the PDGF-induced reduction of \( \alpha \)-SM-actin and calponin protein expression in the co-cultured VSMC (0.25 ± 0.04 vs. 0.49 ± 0.03, \( n = 3, P < 0.05 \); 0.21 ± 0.01 vs. 0.43 ± 0.02, \( n = 3, P < 0.05 \), respectively) (Figure 6).

### 4. Discussion

Although it has been known that the neointimal hyperplasia after percutaneous coronary intervention (PCI) might be exaggerated in the elderly, the underlying mechanisms of how ageing affects the process are poorly understood. We show in the present study that increases in Jagged1 expressions in the young EC replicated cellular events of the VSMC co-cultured with the old EC, suggesting that ageing exerts effects on the dysfunctions of endothelium and the neointima formation, at least in part, through impairing the expression of Jagged1. The senescence of vascular cells has been shown to contribute to the age-exacerbated neointimal proliferation. In the present study, we demonstrated that the abnormal regulation of VSMC by the EC is also involved in the mechanism of age-related vascular events. 

Neointimal proliferation is a major contributor to certain cardiovascular diseases including primary atherosclerosis, in-stent restenosis, and allograft vasculopathy, which have been indicated to associate with a steeply increased morbidity in the elderly. Consistent with previous reports, we observed an aggravated neointima in the ageing rats than in the young ones after vascular injury. The abnormal VSMC overgrowth has been regarded as the common pathogenesis of vasoproliferative disorders. Some studies showed that injured vessels or cultured VSMC of the ageing animals exhibited an increased proliferative ability in response to mitogen. However, some other studies could not observe the similar results, and even indicated an age-dependent reduction of the VSMC proliferation in older animals. Although the reason for the controversy is unclear, the differences in culture condition and the complexes in the regulation process of proliferation in in vivo experiments may account for it.
Notch pathway, a conserved fundamental mechanism regulating cell fate, is significantly involved in the responses of the vasculatures to the injury and growth stimulations. Its dysfunction may cause cardiovascular disorders.\(^{25}\) We observed that Jagged1 expression in the endothelium after the artery injury was quite different between the young and the old rats. The response of Jagged1 expression to the injury was significantly delayed and lowered in the old rats, suggesting that the impairment of Jagged1 up-regulation plays a critical role in the ageing-associated dysfunction of the endothelium regeneration after the vascular injury. Besides, the PCNA expression in the neointima was significantly higher and maintained longer in old rats than in the young ones, indicating an opposite pattern of the expression to that of Jagged1. These data suggest a potential link between Jagged1 in the endothelium and PCNA in the neointima during the development of neointimal proliferation in the elderly.

Abnormal proliferation and migration of VSMC, which is the major cause for neointima formation after arterial injury, may be affected by growth factors and their adjacent EC.\(^{13,21–23}\) The present study using co-culture system showed that neither the young nor the old EC alone could influence the proliferation and migration of the co-cultured VSMC. However, when exposed to PDGF, a proliferation stimulator to the VSMC after vascular injury, the increase in the proliferation and migration was more in the VSMC co-cultured with the old EC, which expressed a low level of Jagged1, than in those with the young EC. Since the primarily isolated EC are in proliferative state in the culture condition, the evidence that the old EC expressed a low level of Jagged1 is consistent with the in vivo data that the expression of Jagged1 in the endothelium of old rats was impaired after arterial injury. Moreover, the levels of the classical growth factors including NO, PDGF-B, IL-1\(\alpha\), and bradykinin in the supernatants were similar between the old and the young EC, indicating that the release of these growth factors by EC is not altered with ageing. These data collectively suggest that ageing-related abnormal growth of VSMC might be induced by a down-regulated expression of Jagged1 in the old EC.

Jagged1, a ligand for Notch receptors, can activate Notch1 signalling.\(^{26,27}\) It has been shown that Jagged1/Notch signalling can regulate the growth of VSMC and EC.\(^{27–30}\) However, it remains unknown whether it influences the EC–VSMC interaction. To confirm the role of Jagged1 in the EC–VSMC interaction, we suppressed Jagged1 expression in the young EC by a siRNA, and found that the suppression promoted PDGF-induced proliferation and migration of the co-cultured VSMC, further suggesting that enhanced VSMC proliferation after arterial injury in the elderly is related to the impairment of Jagged1/Notch pathway in the EC. Interactions between the EC and the VSMC are fundamental in maintaining the normal vascular functions and the adaptable response to the stimulations. Previous studies by Brown et al.\(^{31}\) have shown that EC regulate the phenotype of VSMC through the activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway in the VSMC. However, the specified mechanism remains unknown. It has been known that the activation of the Notch pathway in EC induces
differentiation-associated growth arrest, in part through the activation of the Notch pathway by either stimulation of cell surface Notch receptors with cross-linked soluble delta-like 4 (sDll4)/Jagged1 (sJag1) or the constitutive expression of the Notch1 intracellular domain (NIC). More recent studies by Liu et al.\textsuperscript{30} have reported that the inhibitory effect of Notch1 signalling on the EC proliferation was mediated in part by the negative regulation of Notch1 signalling on both the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways. Their study underlines the crosstalk between the Notch1 signalling and the MAPK and PI3K/Akt pathways. In the present study, Jagged1 was poorly

Figure 5  Enhancement of PDGF-induced VSMC proliferation and migration by co-culture with the EC transfected with a siRNA of Jagged1. EC were transfected with a siRNA of Jagged1 (sirNA) or a scramble RNA (scRNA), and subjected to the co-culture with VSMC as described in Methods. (A) Quantification of H\textsuperscript{3}-TdT incorporation in the VSMC after 24 h of PDGF (10 ng/ml) incubation. (B and C) Representative photogram (B) and quantification (C) of PCNA expression from the western blot analysis after addition of PDGF (10 ng/ml for 24 h) to the VSMC. (D and E) Representative photographs (D) and quantification (E) of VSMC migration after incubation with PDGF (10 ng/ml for 6 h). Data were expressed as the mean ± SEM (n = 5).

Figure 6  Jagged1-knocking down in the co-cultured EC further decreased the expression of α-SM-actin and calponin proteins in the VSMC stimulated with PDGF. (A) Representative photographs from western blotting. The expression of GAPDH served as a loading control. (B) Quantification of α-SM-actin and calponin protein expression. Data were expressed as fold of the GAPDH (mean ± SEM, n = 3).
expressed in the old EC but abundantly in the young EC, and the EC with down-regulated Jagged1 promoted PDGF-induced VSMC proliferation. Although we did not examine the mechanism of the effects of Jagged1, it can be inferred that the regulation of EC on the VSMC via Notch1/Jagged1 signalling involves the PI3K/Akt pathways in the VSMC. It should be elucidated in the future study.

VSMC originate from the mesoderm and bone marrow progenitors. In contrast to the skeletal and cardiac muscle cell lineages, where cellular differentiation is functionally coupled to irreversible exit from the cell cycle, VSMC retain the capacity to proliferate and modulate their phenotype during postnatal development. A variety of phenotype molecules including α-SM-actin and h1-calponin have been used as the markers of the VSMC at differentiation-maturation state. These proteins are highly expressed in the contractile phenotypic VSMC, but lowly in the synthetic phenotypic VSMC. The switching of VSMC from the contractile type to the synthetic state is a critical step of VSMC proliferation. In the present study, knocking down of the Jagged1 expression in the EC was sufficient and necessary to induce PDGF-reduced α-SM-actin expression in the VSMC, leading to the increase in the VSMC proliferation and migration.

Although the in vitro cell co-culture model could not completely represent the in vivo state, our findings can illustrate that Jagged1 losing in the EC might prevent Notch/CSL activation and subsequently down-regulate α-SM-actin expression in the VSMC, leading to the increase in the VSMC proliferation and migration. The ultimate remodelling results were mainly determined by the fine balance between the endothelial regeneration and the VSMC proliferation. Although drug eluting stents can inhibit VSMC proliferation, the inhibition is non-selective and can also disturb the endothelial repair. Our data suggest that enhancement of Jagged1 expression during regeneration of the endothelium could be used as a method to maintain the contractile phenotype of VSMC, thereby inhibiting the VSMC overgrowth and neointimal proliferation in the elderly after the arterial injury including PCI.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**Conflict of interest**

none declared.

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**References**


