Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;49:985-997
DOI: 10.1159/000493229
Published online: 5 September, 2018
Accepted: 27 August, 2018

Original Paper

Integrin β3 Mediates the Endothelial-to-Mesenchymal Transition via the Notch Pathway

Weisen Wang[a,b] Zhi Wang[a] Dingyuan Tian[a,b] Xi Zeng[a] Yangdong Liu[c] Qining Fu[c] Anlin Liang[d] Yi Zhang[a] Qiangguo Gao[a] Jizhong Cheng[e] Yun Wang[a]

[a]Department of Cell Biology, College of Basic Medical Sciences, Army Medical University (Third Military Medical University), Chongqing, [b]Cadet Battalion, College of Basic Medical Sciences, Army Medical University (Third Military Medical University) Chongqing, [c]Department of Vascular Surgery, First Affiliated Hospital of Chongqing Medical University, Chongqing, [d]Department of Orthopaedics, First Affiliated Hospital of Chongqing Medical University, Chongqing, China, [e]Department of Medicine, Baylor College of Medicine, Houston, USA

Key Words
Integrin β3 • endothelial cells • endothelial-to-mesenchymal transition • Notch signaling pathway • neointimal hyperplasia

Abstract
Background/Aims: Neointimal hyperplasia is responsible for stenosis, which requires corrective vascular surgery, and is also a major morphological feature of many cardiovascular diseases. This hyperplasia involves the endothelial-to-mesenchymal transition (EndMT). We investigated whether integrin β3 can modulate the EndMT, as well as its underlying mechanism. ***Methods:*** Integrin β3 was overexpressed or knocked down in human umbilical vein endothelial cells (HUVECs). The expression of endothelial markers and mesenchymal markers was determined by real-time reverse transcription PCR (RT-PCR), immunofluorescence staining, and western blot analysis. Notch signaling pathway components were detected by real-time RT-PCR and western blot analysis. Cell mobility was evaluated by wound-healing, Transwell, and spreading assays. Fibroblast-specific protein 1 (FSP-1) promoter activity was determined by luciferase assay. ***Results:*** Transforming growth factor (TGF)-β1 treatment or integrin β3 overexpression significantly promoted the EndMT by downregulating VE-cadherin and CD31 and upregulating smooth muscle actin α and FSP-1 in HUVECs, and by enhancing cell migration. Knockdown of integrin β3 reversed these effects. Notch signaling was activated after TGF-β1 treatment of HUVECs. Knockdown of integrin β3 suppressed TGF-β1-induced Notch activation and expression of the Notch downstream target FSP-1. ***Conclusion:*** Integrin β3 may promote the EndMT in HUVECs through activation of the Notch signaling pathway.

W. Wang and Z. Wang contributed equally to this work.

Yun Wang
and Jizhong Cheng

Department of Cell Biology, College of Basic Medical Sciences, Army Medical University (Third Military Medical University), Chongqing 400038 (China)
E-Mail yunwang@tmmu.edu.cn; jizhongc@bcm.edu

Introduction

Neointimal hyperplasia is responsible for stenosis, which requires corrective vascular surgery such as arteriovenous fistula surgery and stent angioplasty, and is also a major morphological feature of many cardiovascular diseases such as atherosclerosis and hypertension [1]. The initiating event in neointimal hyperplasia is endothelial cell (EC) damage. Disruption of the overlying layer of the endothelium exposes the underlying vascular smooth muscle cells (VSMCs) to circulating blood elements, which then activate a cascade of events culminating in neointimal hyperplasia [2]. Neointimal lesions mainly comprise smooth muscle-like cells. The endothelium is a source of smooth muscle-like cells and contributes to neointimal hyperplasia through the endothelial-to-mesenchymal transition (EndMT) [1, 3]. However, little is known about the mechanism regulating this process.

The EndMT is a specific form of the epithelial-to-mesenchymal transformation (EMT) [4]. During the EndMT, ECs lose their endothelial-specific markers, gain mesenchymal markers, lose cell–cell junctions, and acquire invasive and migratory properties. The EndMT is not only critical to embryonic cardiac development [5], but is also involved in the pathogenesis of many diseases, including neointimal hyperplasia [6], fibrosis [7], and cancer [8]. Our previous studies reported that the ECs of the neointima of arteriovenous fistulas (AVFs) in uremic mice or end-stage renal disease (ESRD) patients expressed mesenchymal markers [6]. Others studies have identified that the EndMT occurs in myoendothelial cells in human atherosclerotic plaques. After being exposed to a disturbed flow, ECs undergo the EndMT, which contributes to neointimal hyperplasia and induces their atherogenic differentiation [3]. Atherosclerotic ApoE$^{(-/-)}$ mice exhibit extensive development of the EndMT and increased neointimal formation. Furthermore, patients with coronary atherosclerosis show activation of transforming growth factor β (TGF-β) signaling and the development of the EndMT in atherosclerotic plaques [9].

The integrin β3 family consists of αIIbβ3 and αvβ3. The former is primarily expressed on platelets, whereas the latter is found in ECs and selective inflammatory cells. Our previous report showed that integrin β3 is expressed in both circulating endothelial progenitor cells and in platelets, influencing EC regeneration and blocking neointimal formation in arteriovenous grafts in mice [10]. However, whether integrin β3 influences the EndMT in neointimal hyperplasia remains unknown.

Our aim was to identify the role of integrin β3 in the process of the EndMT. We assessed the expression of endothelial and mesenchymal markers and determined how these changes are regulated in ECs. Our results showed that integrin β3 can induce the EndMT by activating the Notch pathway. These findings will guide the development of interventional methods to prevent the EndMT and alleviate neointimal hyperplasia.

Materials and Methods

Reagents and antibodies

Fetal bovine serum (FBS) and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA). Recombinant human TGF-β1 and Xt-199 were purchased from Sigma-Aldrich (St. Louis, MO).

Antibodies against Jagged-1 and smooth muscle actin (SMA)-α (rabbit) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), whereas antibodies against N1ICD and RBP-Jκ were from Millipore (Burlington, MA). The FSP-1 antibody was from DAKO (Carpinteria, CA). Antibodies against CD31, integrin β3, and VE-cadherin (mouse) were from Bioworld Technology, Inc. (St. Louis Park, MN). The GAPDH antibody was from Sangon Biotech (Shanghai, China). Antibodies against vimentin were from ZSGB-BIO (Beijing, China). Horseradish peroxidase-conjugated secondary antibody IgGs (anti-rabbit, anti-mouse, and anti-goat) were from Beyotime (Shanghai, China). DyLight 488 goat anti-rabbit IgG (H+L) and DyLight 594 goat anti-mouse IgG (H+L) were from Invitrogen.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were subcultured in basal RPMI 1640 medium (Hyclone Technologies, Logan, UT) supplemented with 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 medium.

Human AVF samples

Samples of AVFs from six hemodialysis patients ranging in age from 31 to 86 years were studied. In each case, samples of failed AVFs were collected during the creation of a new AVF with the approval of the Army Medical University Review Board.

Luciferase plasmid construction and transfection

To determine FSP-1 promoter activity, up to 2.1 kb upstream from the transcription start site of the FSP-1 promoter was cloned into a luciferase vector. The Xho I restriction enzyme recognition sites (underlined) were added at the 5′ ends of upstream primers and Hind III restriction enzyme recognition sites (underlined) were added at the 5′ ends of downstream primers (5′-CTC GAG TGA GCC CCC ACG CCT CACT-3′, 5′-AAG CTT GAG AGG GGA AGA ATG GGG-3′). The PCR fragments were cloned into a firefly luciferase vector, pGL4.1-Luc (Promega, Madison, WI). Using Lipofectamine 2000, HUVECs were transfected with plasmids and incubated in serum-free media for 24 h before being treated with TGF-β1 (10 ng/ml) for a further 24 h. pRL-TK was co-transfected into the cells and the level of firefly luciferase activity was normalized to Renilla luciferase activity measured by the dual luciferase system (Promega).

Integrin β3 small interfering RNA design and lentivirus transfection

Integrin β3 small interfering RNA (siRNA) lentivirus was used to examine the function of integrin β3. A third generation of the self-inactivating lentiviral vector pLV-EGFP, which contains eGFP (enhanced green fluorescence protein) and puromycin dual reporter genes, was purchased from Cyagen Inc. (Guangzhou, China). The following siRNAs were designed to target integrin β3: siRNA a, 5′-GATGCAGTGAATTGTACCTAT-3′; siRNA b, 5′-CCACGTCTACCTTCACCAATA-3′; and siRNA c, 5′-GATGACTGTGTCGTCAGATTC-3′. A scrambled sequence (5′-CCTAAGGTTAAGTCGCCCTCG-3′) was used as a negative control (Scr) lentivirus. The sequences were cloned into the pLV-EGFP (Cyagen Inc.) to generate the lentiviral vectors.

HUVECs were inoculated in a 12-well culture plate (1×10^5 cells/well). Subsequently, lentivirus and polybrene (8 μg/ml) were added to each well. The transfection efficiency was checked by GFP and cells were selected with puromycin (1 μg/ml). Stable cell lines were established after a 1-week selection. The expression of relevant proteins was examined by western blot analysis.

Plasmids and transfection

The pcDNA3.1-beta3 plasmid, which contains human full-length integrin β3 cDNA, was purchased from Addgene (Cambridge, MA). HUVECs were inoculated in a 6-well culture plate (2×10^6 cells/well), then cells were transfected with 2 μg plasmid pcDNA3.1-beta3 or pcDNA3.1 using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h transfection, cells were incubated in basal RPMI 1640 medium (Hyclone Technologies) supplemented with 10% FBS for 48 h and the expression of integrin β3 was measured by western blot.

Real-time reverse transcription PCR

Total RNA was isolated using TRIzol reagent (Promega). One microgram of total RNA per sample was then reverse-transcribed into cDNA in a 20-μl volume including 20 U AMV (Takara Bio, Inc., Kusatsu, Japan) according to the manufacturer's protocol. Real-time reverse transcription PCR (RT-PCR) amplification was performed with SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) in a StepOne PCR amplifier (Bio-Rad Laboratories). Untreated cells were used as the reference sample and GAPDH was used as the endogenous control.

Table 1. Primers used for real-time RT-PCR

Primer	Forward	Reverse
Integrin β3	5′-AGACACTCCCACTTGGCATC-3′	5′-TCCTCAGGAAAGGTCCAATG-3′
VE-cadherin	5′-ATCCATTGTGTGCAAGCCACA-3′	5′-CCGTGGTGTTATGTCCTTGTC-3′
SMA-α	5′-CAAGTGATCACCATCGGAAATG-3′	5′-GACTCCATCCCGATGAAGGA-3′
FSP-1	5′-GGTGTCCACCTTCCACAAGT-3′	5′-GCTGTCCAAGTTGCTCATCA-3′
Hes-5	5′-ACATCCTGGAGATGGCTGTC-3′	5′-TAGTCCTGGTGCAGGCTCTT-3′
TGF-β1	5′-GCTCCACGGAGAAGAACTGCT-3′	5′-CTGCTCCACCTTGGGCTTGC-3′
GAPDH	5′-CGACCACTTTGTCAAGCTCA-3′	5′-AGGGGTCTACATGGCAACTG-3′

The primers used for the amplification reaction are summarized in Table 1. Gene expression levels were normalized with GAPDH, and data were analyzed with StepOne software (Applied Biosystems, Foster City, CA). The assay was performed in triplicate, and each experiment was repeated at least three times.

Western blot analysis

After the corresponding treatment, cell 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 by BCA Reagent (Thermo Fisher Scientific, Waltham, MA). About 30 μg protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After being transferred to a nitrocellulose membrane (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. Signals were visualized by an enhanced chemiluminescence system (Thermo Fisher Scientific). GAPDH antibody was used as a loading control. The protein bands were visualized using an imaging system (Bio-Rad Laboratories).

Immunofluorescence

Cells were plated on serum-coated coverslips and incubated for the indicated time at 37°C. Thereafter, the cells were fixed and permeabilized by sequential incubation in 4% paraformaldehyde/phosphate-buffered saline (PBS) and 0.5% Triton X-100/PBS for 10 and 5 min, respectively. The actin cytoskeleton was visualized with Alexa 488-conjugated phalloidin (Invitrogen). For the detection of other molecules, after being blocked with blocking buffer (Beyotime), samples were incubated with primary antibody to VE-cadherin (1:100), integrin β3 (1:100), or SMA-α (1:300) and then incubated with DyLight 594 or DyLight 488. Nuclear DNA was labeled with DAPI (4′, 6-diamidino-2-phenylindole) (Boster Biological Technology, Pleasanton, CA).

Wound-healing assay

Cells 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 HUVECs toward the open area.

Transwell assay

For the Transwell assay, 2×10^4 cells were seeded onto the cell insert with an 8-μm pore and incubated in normal RMPI 1640 medium with 2% FBS. Xt-199 was added immediately after cell seeding to the cell insert and the lower chamber. Cells were allowed to migrate for 24 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 (Olympus, Hamburg, Germany). Cell numbers were determined and statistically analyzed.

Statistical analysis

Data are expressed as mean ± standard error. Results were analyzed using a *t*-test when the results from two groups were compared or using two-way analysis of variance when data from over three groups were studied. A p-value < 0.05 was considered statistically significant.

Results

Integrin β3 expression in AVFs

Failed AVFs from patients showed severe neointima hyperplasia (Fig. 1A). SMA-α was detected in the neointima (Fig. 1B). Our previous studies confirmed that SMA-α was also expressed in the endothelial layer of AVFs with severe neointima hyperplasia [6]. These results showed that the ECs of AVFs from ESRD patients express the mesenchymal cell marker, SMA-α. We also reported that integrin β3 expression was located in the endothelium and neointima of arteriovenous grafts from patients [10]. Double staining of SMA-α and integrin β3 in AVFs from mice and patients confirmed that SMA-α and integrin β3 were located in the endothelium and the neointima (Fig. 1C and D). There was increased integrin β3 and TGF-β1

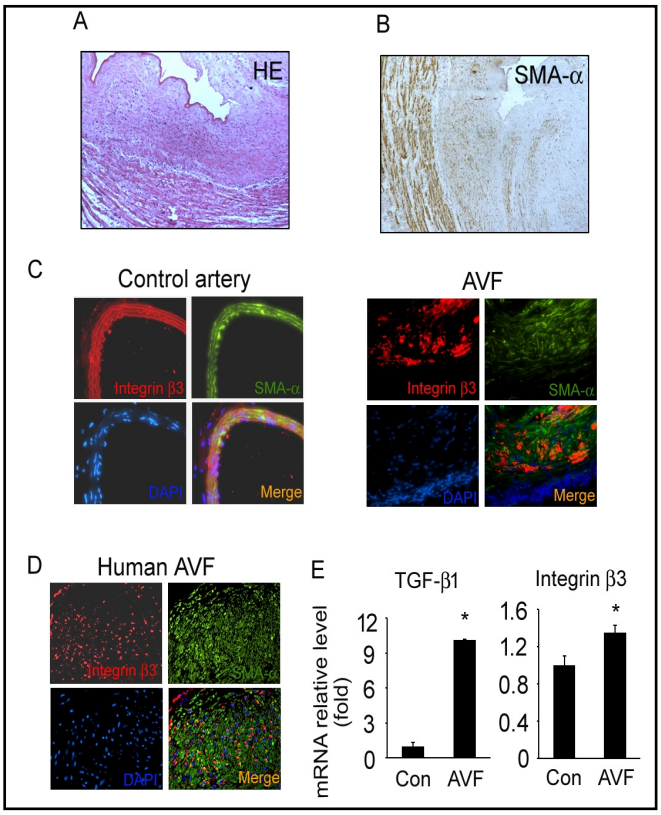

Fig. 1. Integrin β3 expression in AVFs. (A) Hematoxylin and eosin staining and (B) SMA-α expression detected by immunostaining in AVFs of patients (100× amplification). (C) Double immunofluorescence staining of integrin β3 (red) and SMA-α (green) was performed in control artery and 1-month-old mouse AVFs (400× amplification). (D) Double immunofluorescence staining of integrin β3 (red) and SMA-α (green) in AVFs of patients (200× amplification). Representative AVFs are shown. (E) Integrin β3 and TGF-β1 expression in control arteries and in venous anastomoses of AVFs were detected by real-time RT-PCR analysis. n = 3. *p<0.05.

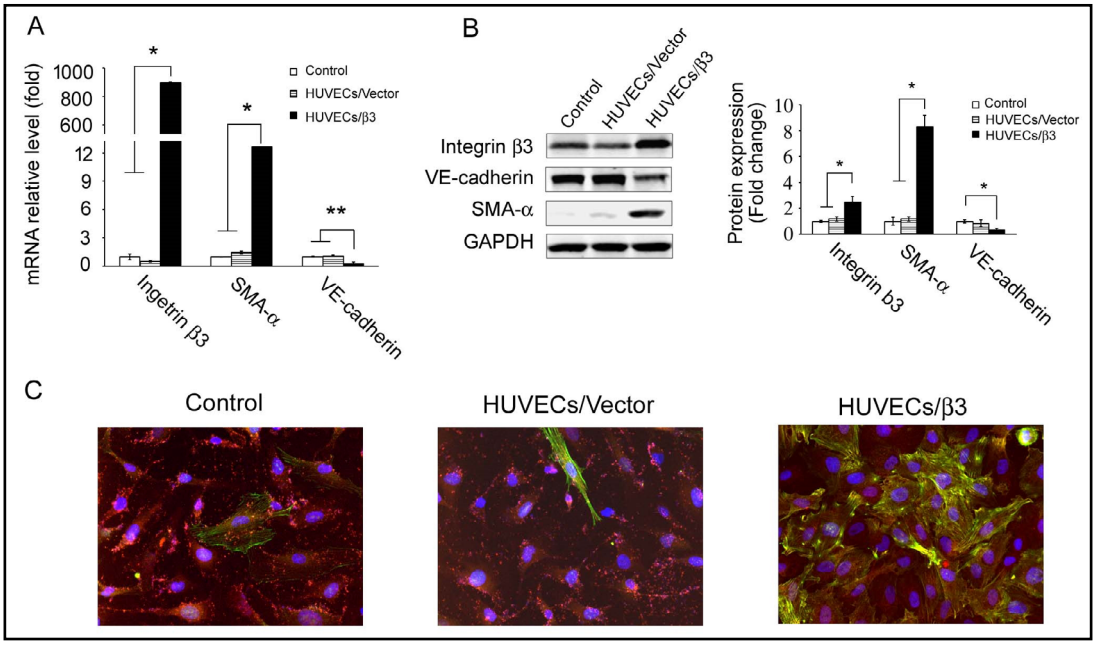

Fig. 2. Overexpression of integrin β3 induces the EndMT in HUVECs. (A) Real-time RT-PCR analysis and (B) western blot analysis of endothelial markers and mesenchymal markers in HUVECs. GAPDH was used as an internal control. n = 3. *p<0.05; **p<0.01. (C) Immunofluorescence staining of VE-cadherin (red) and SMA-α (green) in control HUVECs or HUVECs overexpressing integrin β3. Representative pictures are shown. Control, HUVECs; HUVECs/Vector, HUVECs transfected with pcDNA3.1; HUVECs/β3, HUVECs transfected with pcDNA3.1-beta3.

expression in the venous anastomoses of the AVFs versus control arteries (Fig. 1E). Because TGF-β1 induces EMT and EndMT [4, 11], these results indicate that increased expression of integrin β3 could be related to EndMT.

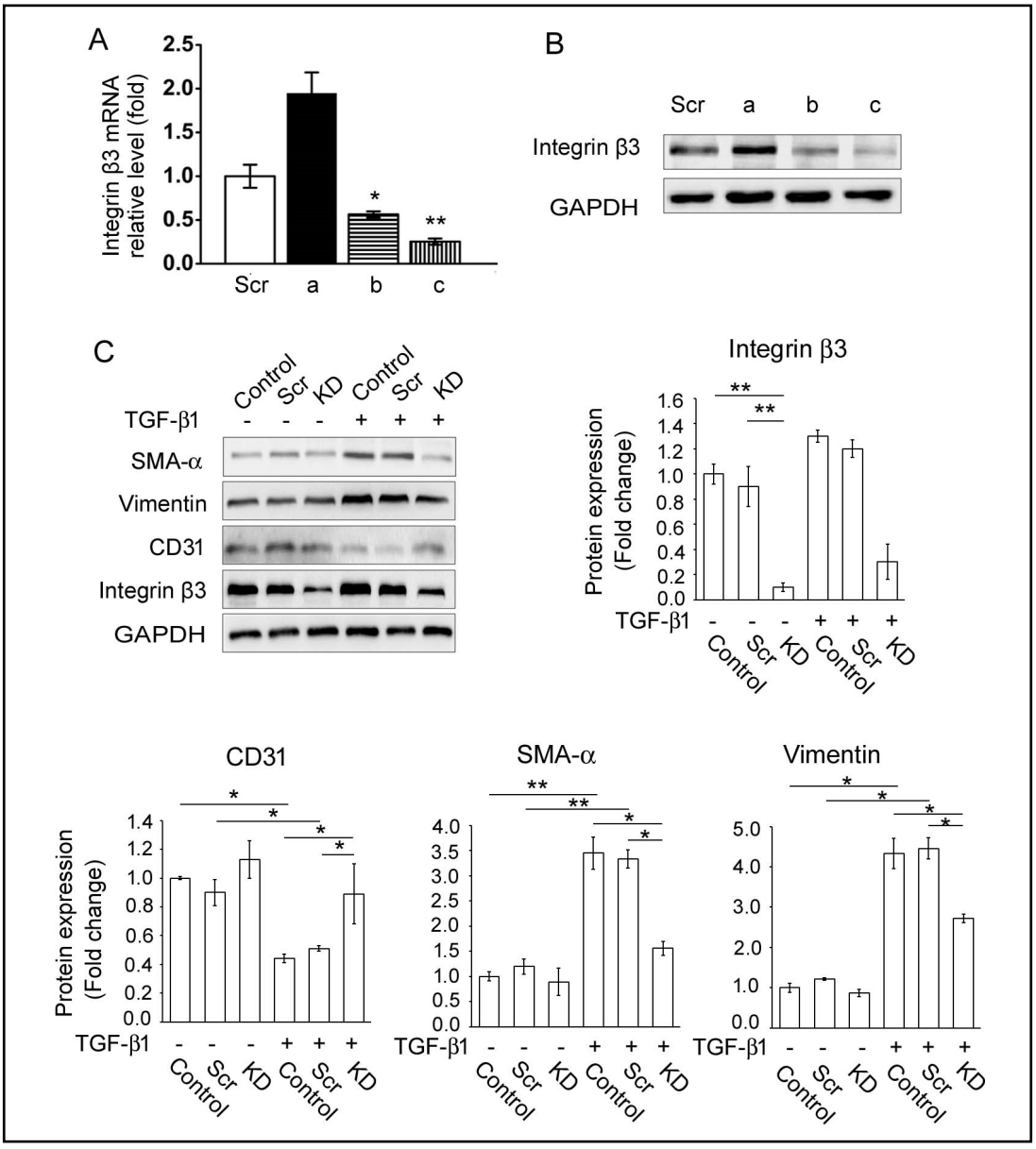

Fig. 3. Integrin β3 knockdown in HUVECs suppresses the EndMT. The mRNA transcription and protein expression levels of integrin β3 level were detected by real-time RT-PCR (A) and western blot analysis (B), respectively. Control, HUVECs; Scr, HUVECs transfected with scrambled siRNA; a, b, and c indicates HUVECs transfected with integrin β3 siRNA(a), integrin β3 siRNA(b), and integrin β3 siRNA(c), respectively. (C) The expression of mesenchymal markers in HUVECs was detected by western blot. GAPDH was used as an internal control. KD indicates HUVECs transfected with integrin β3 siRNA(c). All figures are representative of more than three similar experiments. n = 3. *p<0.05; **p<0.01.

Integrin β3 regulates the EndMT in HUVECs

When undergoing the EndMT, ECs lose their endothelial-specific markers, such as VE-cadherin and CD31, and gain mesenchymal markers, such as SMA-α and FSP-1 [12, 13]. To examine whether integrin β3 affects the EndMT in HUVECs, we examined the change in the expression of the two biomarkers in HUVECs. After transfection of HUVECs with plasmids containing full-length human integrin β3 cDNA, integrin β3 overexpression was confirmed

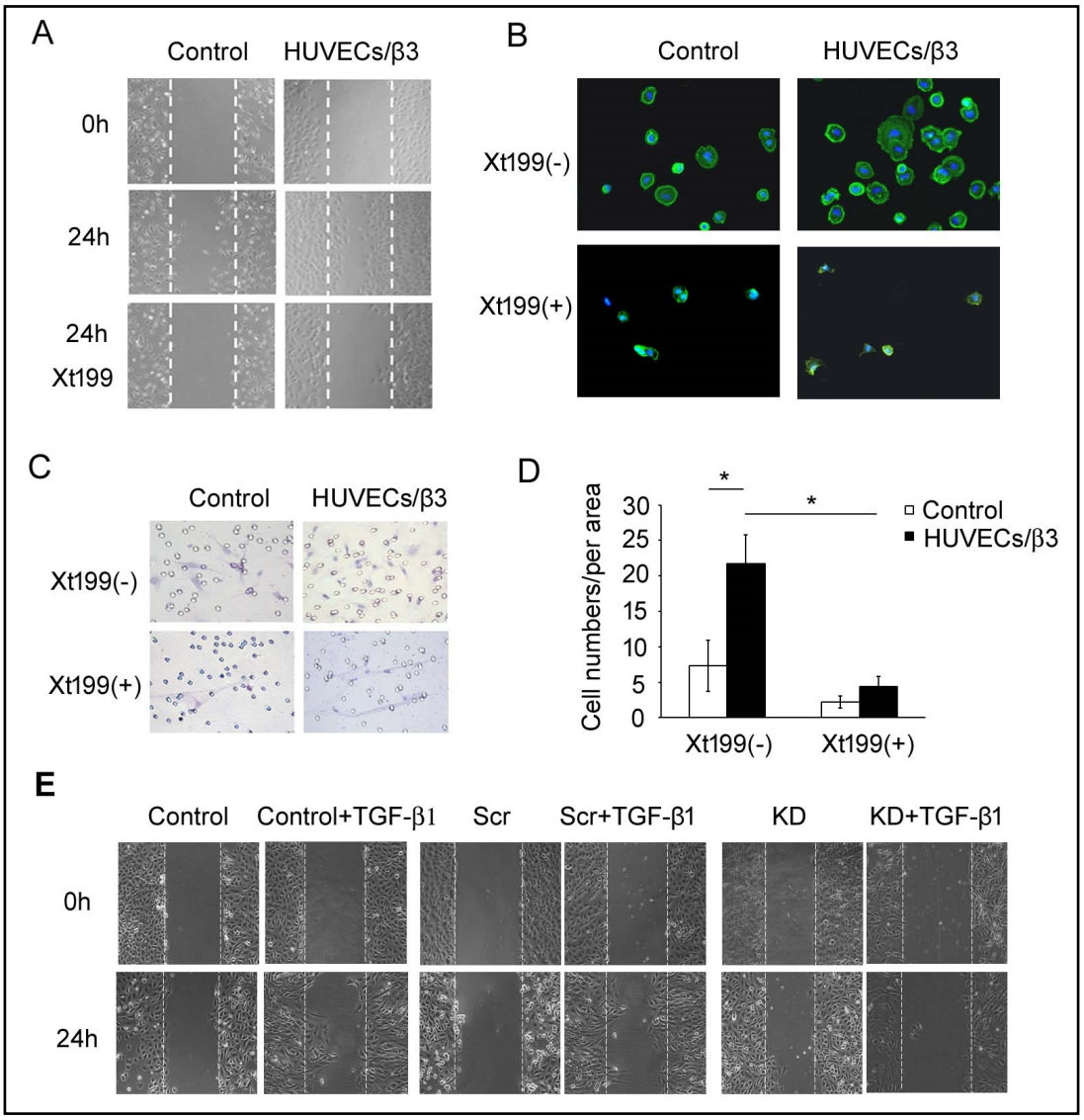

Fig. 4. Integrin β3 promotes HUVEC migration and integrin β3 knockdown inhibits the cell migration induced by TGF-β1. (A) Wound-healing assays of HUVECs showed that overexpression of integrin β3 in HUVECs induced cell migration 24 h after being scratched; this migration was blocked by the integrin β3 antagonist Xt199. (B) Spreading assay of HUVECs. Cells were stained at 4 h with FITC-phalloidin to visualize F-actin (green) and DAPI to indicate nuclei (blue). (C) Transwell assays of HUVECs showed that overexpression of integrin β3 in HUVECs induced cell migration, which was also blocked by Xt199. (D) Statistical analysis of the Transwell assays. (E) Wound-healing assays showed that TGF-β1 induced HUVEC migration, whereas knockdown of integrin β3 inhibited TGF-β1-induced cell migration. Representative figures are shown from three repeated experiments. Control, HUVECs; HUVECs/β3, HUVECs transfected with pcDNA3.1-beta3; Xt199, cells treated with Xt199; Scr, HUVECs transfected with scrambled siRNA; KD, HUVECs transfected with integrin β3 siRNA(c); TGF-β1, cells treated with TGF-β1. *p<0.05.

by real-time RT-PCR and western blot (Fig. 2A and B). HUVECs transfected with vector were used as a control. In addition, we observed that VE-cadherin was reduced while SMA-α was increased in integrin β3-overexpressing HUVECs compared with vector-transfected control cells (Fig. 2A and B). Double staining of VE-cadherin and SMA-α further verified that the overexpression of integrin β3 induced the EndMT of HUVECs (Fig. 2C).

Knockdown of integrin β3 in ECs suppresses the EndMT

To evaluate the effect of integrin β3 on the EndMT, we used lentivirus-mediated siRNA to knockdown integrin β3 gene expression in HUVECs. The knockdown efficiency of integrin β3 was verified by real-time RT-PCR (Fig. 3A) and western blot (Fig. 3B). The expression of integrin β3 gene was significantly decreased in HUVECs infected with integrin β3 siRNA (b and c) when compared with cells infected with scrambled siRNA. Because integrin β3 siRNA (c) more efficiently reduced integrin β3 expression than (a) and (b), integrin β3 siRNA (c)-infected HUVECs were used in subsequent experiments. TGF-β can induce integrin β3 expression and the EMT [14]. Therefore, HUVECs were treated with TGF-β1. As shown in Fig. 3C, TGF-β1 treatment significantly enhanced the expression levels of integrin β3, SMA-α, and vimentin in control and Scr-transfected HUVECs, whereas the expression of V–-E-cadherin and CD31 was downregulated. After silencing of integrin β3 gene expression in HUVECs, the endothelial markers were increased and mesenchymal markers were reduced (Fig. 3C). These results indicate that overexpression of integrin β3 promotes the EndMT and that decreased expression of integrin β3 inhibits this process.

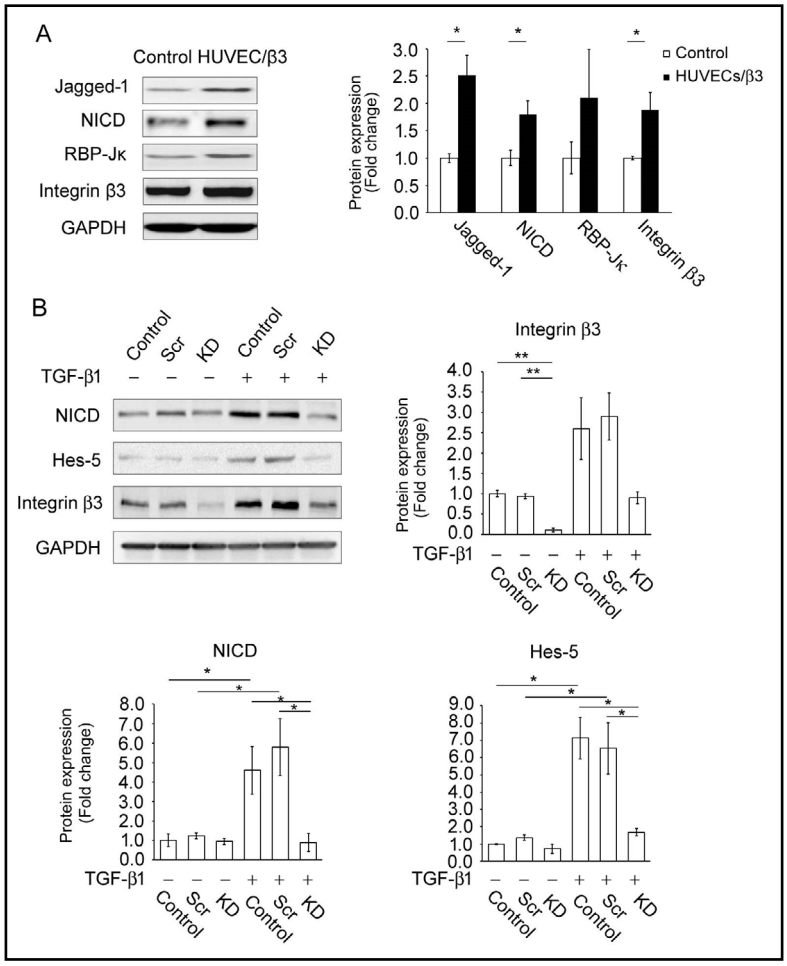

Fig. 5. Integrin β3 activates the Notch signaling pathway. (A) Western blot analysis confirmed increase Notch-1 cleavage in integrin β3-overexpressing HUVECs. n = 3. *p<0.05. (B) After integrin β3 knockdown, the Notch signaling pathway was suppressed in HUVECs. GAPDH was used as an internal control. All figures are representative of more than three similar experiments. Control, HUVECs; HUVECs/β3, HUVECs transfected with pcDNA3.1-beta3; Scr, HUVECs transfected with scrambled siRNA; KD, HUVECs transfected with integrin β3 siRNA (c). n = 3. *p<0.05; **p<0.01.

Integrin β3 promotes HUVEC migration and adhesion

ECs change their phenotype through activation of the EndMT program, which enhances cell migration [4]. A wound-healing assay showed that overexpression of integrin β3 in HUVECs or TGF-β1 treatment induced cell migration, which was attenuated by the integrin β3 antagonist Xt199 or by integrin β3 knockdown (Fig. 4A and E). A Transwell assay (Fig. 4C and D) confirmed that the number of migrated cells in the integrin β3-overexpressing group was dramatically increased compared with normal HUVECs ($p < 0.05$). Cells were incubated on serum-coated coverslips and the cell boundary was visualized with phalloidin staining. As shown in Fig. 4B, HUVECs overexpressing integrin β3 exhibited a well-spread, dish-like morphology compared with control after 30 min plating. Xt-199 suppressed the spreading induced by integrin β3.

Integrin β3 activates the Notch signaling pathway

Notch signaling regulates cell fate determination and differentiation [15], and activated Notch-1 or -4 can induce the EndMT in cultured ECs [16]. We checked whether the Notch signaling pathway is activated by integrin β3. Notch signaling is initiated by an interaction between Notch receptors and their ligands; the receptor is then cleaved by γ-secretase and the Notch intracellular domain (NICD) is released, which translocates into the nucleus

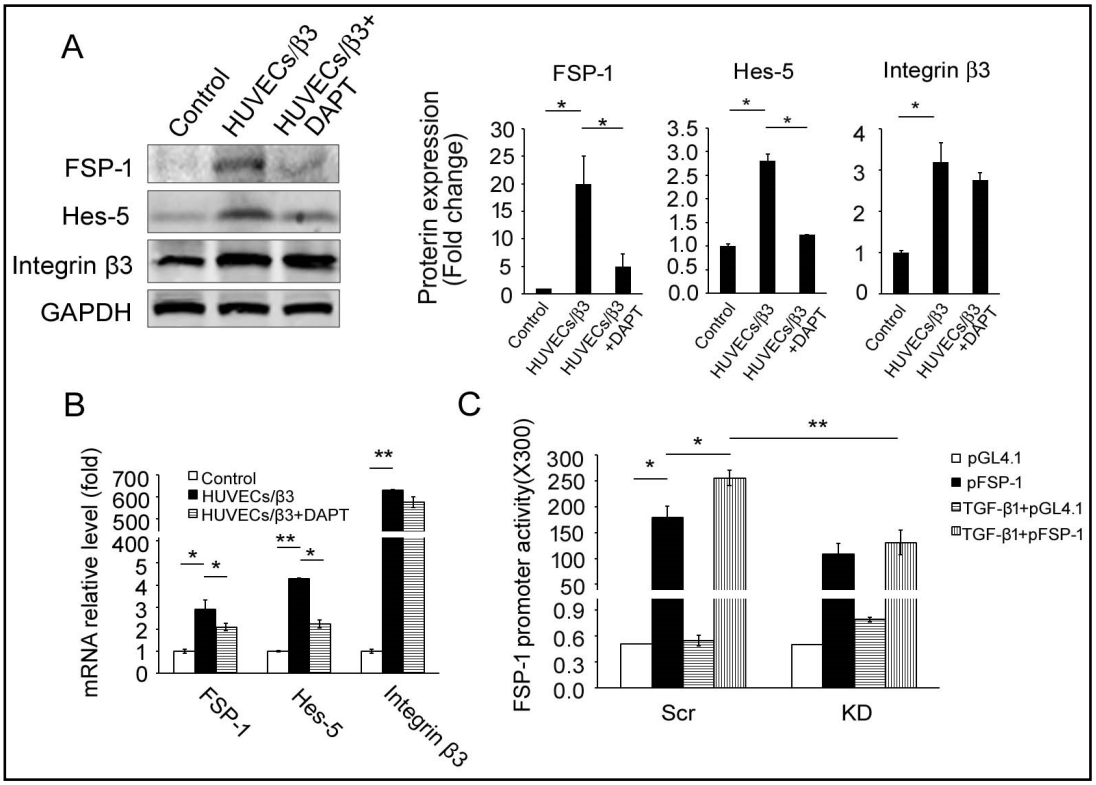

Fig. 6. Overexpression of integrin β3 enhances FSP-1 expression in HUVECs. (A) Western blot analysis and (B) real-time RT-PCR revealed that the expression of FSP-1 and Hes-5 was increased in integrin β3-overexpressing HUVECs compared with control HUVECs. GAPDH was used as an internal control. All figures are representative of more than three similar experiments. Control, HUVECs; HUVECs/β3, HUVECs transfected with plasmid-inserted full-length human integrin β3; DAPT, cells treated with DAPT. n = 3. *p<0.05; **p<0.01. (B) HUVECs were transfected with pGL4.10 vector or pFSP-1 and stimulated with or without TGF-β1 before analysis of luciferase activity. All experiments were performed more than three times. Scr, HUVECs transfected with scrambled siRNA; KD, HUVECs transfected with integrin β3 siRNA(c); pGL4.10 and pFSP-1 indicate cells transfected with pGL4.10 and pFSP-1, respectively; TGF-β1, cells treated with TGF-β1. n = 3. *p<0.05; **p<0.01.

and associates with the DNA-binding protein recombinant binding protein-Jκ (RBP-Jκ) plus coactivators and triggers the expression of target genes. Our results showed that the expression of the Notch ligand Jagged-1 and NICD, the cleaved and activated form of the Notch-1 receptor, was increased in integrin β3-overexpressing HUVECs; TGF-β1 also upregulated NICD expression (Fig. 5B). However, integrin β3 knockdown inhibited this effect. The expression of the transcription factor RBP-Jκ was not influenced in integrin β3-overexpressing HUVECs, although we detected expression of the Notch target gene Hes-5 in integrin β3 knockdown HUVECs and in TGF-β1-treated HUVECs. The results showed that integrin β3 knockdown significantly inhibited TGF-β1-induced Notch signaling activation.

Integrin β3 regulates FSP-1 expression through the Notch signaling pathway

To examine whether integrin β3-activated Notch signaling affects the EndMT in HUVECs, the expression of FSP-1 and the Notch target genes Hes-5 was detected after the cells were treated with the Notch signaling inhibitor DAPT to block Notch activation. Real-time RT-PCR and western blot showed that there was an increase in the level of Hes-5 and FSP-1 in integrin β3-overexpresing HUVECs. However, the upregulation of Hes-5 was inhibited after DAPT treatment, suggesting that Notch pathway activation was blocked. Although the expression of integrin β3 was not influenced by DAPT, the expression of FSP-1 was downregulated after DAPT treatment (Fig. 6). These results suggested that overexpression of integrin β3 activates Notch signaling and increases FSP-1 expression and that inhibition of Notch signaling can suppress the process.

There are RBP-Jκ binding sites in the FSP-1 promoter in mouse smooth muscle cells [17]. To explore whether integrin β3 influences the expression of FSP-1 through activation of Notch signaling, we created a luciferase-reporter construct containing the region 2100 bp upstream of the transcription start site of human FSP-1 promoter. In the absence of FSP-1 promoter sequences, there was no luciferase activity detected with or without TGF-β1 stimulation. However, integrin β3 knockdown suppressed TGF-β1-induced FSP-1 promoter activities by ~70% versus control HUVECs. Taken together, these results indicate that integrin β3 increases FSP-1 expression by activating Notch signaling.

Discussion

Neointimal formation is an exaggerated healing process that occurs in the vessel wall after injury. ECs play vital roles in the process of neointimal hyperplasia. The damage to ECs initiates the development of neointimal hyperplasia and endothelial regeneration occurs to restore the protective barrier at the end of the neointimal hyperplasia [18]. In addition, VSMC proliferation and migration are common characteristics of neointimal hyperplasia; ECs also contribute to neointimal hyperplasia as a source of smooth muscle-like cells [1]. We and others have previously shown that ECs can acquire a fibro-proliferative mesenchymal phenotype through the EndMT under pathological conditions [3, 6]. Our present data showed that TGF-β1-induced integrin β3 expression can modulate the EndMT by promoting mesenchymal marker expression in a Notch-dependent manner.

Integrins are transmembrane heterodimeric glycoprotein receptors consisting of α and β subunits that are crucial for modulating fundamental cell processes and functions including cell adhesion, proliferation, migration, and survival. Circulating endothelial progenitor cells deficient in integrin β3 show impaired adhesion activity toward exposed subendothelium, fail to differentiate into mature ECs, and promote neointimal formation at arteriovenous graft sites [10]. However, integrin αvβ3 arginine-glycine-aspartic acid (RGD) antagonists have been used to reduce neointimal hyperplasia, mainly by blocking VSMC accumulation [19]. Here, the severe neointima hyperplasia of patients showed significant integrin β3 expression (Fig. 1). Our *in vitro* experiments showed that HUVECs overexpressing integrin β3 exhibited smooth muscle-like cell phenotypes (Fig. 2 and 3). These results suggested that integrin β3 in different types of cells may regulate cell behavior differently and that clinical

strategies targeting specific cells should be designed to maximize the curative effects.

Integrin family members are involved in the EMT and the EndMT and contribute to cancer metastasis [20] and fibrosis [21]. Recent work showed that RGD antagonists that interact with integrin αvβ3 could revert the TGF-β1-induced EndMT in endothelial precursor cells [22]. These previous studies indicated that integrin β3 may play an important role in endothelial transdifferentiation. We found that AVF patients had high integrin β3 and TGF-β1 expression at areas of anastomosis (Fig. 1). TGF-β1 itself is a growth factor and a potent inducer of the EndMT. Not only TGF-β1, but also overexpression of integrin β3 in HUVECs promoted the EndMT. Because TGF-β1 induces integrin β3 expression and knockdown of integrin β3 inhibits the TGF-β1-induced EndMT (Fig. 2–4), this evidence reveals that TGF-β1 possibly mediates the EndMT by regulating integrin β3 expression.

As a major regulator of cell phenotype, Notch is involved in the process of the EndMT. 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], Dll-3, Dll-4, Jagged-1, and Jagged-2). As mentioned, different combinations of Notch receptors and their ligands can play different roles in EC behavior [1]. Noseda et al. [16] provided the first evidence that Notch activation in ECs results in morphological, phenotypic, and functional changes and induces the EndMT. Because Notch-1 is the primary functional Notch receptor during developmental angiogenesis [23], we assessed Notch-1 activation to analyze the mechanism of integrin β3 in the EndMT. Notch1 activation is clearly observed in TGF-β1-treated HUVECs and in integrin β3-overexpressing HUVECs through upregulation of Notch-1 cleavage and target gene Hes-5 expression (Fig. 5 and 6). In addition, the Notch inhibitor DAPT inhibited Hes-5 expression but had no effect on integrin β3 expression (Fig. 6). Thus, these results proved that integrin β3 is an upstream regulator of the Notch signaling pathway. Because integrin regulates cell adhesion and migration, the signal transduction of the Notch signaling pathway is limited between cells that are in contact with each other. Our data showed that overexpressed integrin β3 induced the EndMT through activation of the Notch signaling pathway. Elevated Notch signaling upregulates mesenchymal marker expression in ECs and leads to endothelial barrier dysfunction in AVFs [6]. Our findings explained the mechanism of neointimal formation in vascular disease regulated by integrin β3. FSP-1 (S100A4, also known as metastasin) is a member of the S100 family of calcium-binding proteins. It was isolated from mouse mammary adenocarcinoma cells, and its mRNA is detectable in cells of the bone marrow, spleen, and thymus and in lymphocytes [24]. FSP-1 regulates cellular motility through a direct interaction with myosin-IIA [25]; FSP-1 is also associated with cancer cell metastasis [20], including the invasive growth of mouse ECs [26] or neurons [27]. FSP-1 can play a role in vascular remodeling. We previously identified that secreted FSP-1 influences the proliferation of VSMCs in arteriovenous grafts [28] and that knockout of FSP-1 suppresses VSMC migration and ameliorates neointimal formation [17]. According to our data, overexpression of integrin β3 promotes the expression of FSP-1 in HUVECs by activating the Notch pathway and inducing transcription factor binding to the FSP-1 promoter (Fig. 6). Increased expression of FSP-1 in ECs leads to EC barrier dysfunction, the EndMT, and cell migration.

Conclusion

We found that the TGF-β1-induced expression of integrin β3 promotes the EndMT. During this process, integrin β3 induces FSP-1 promoter activity and enhances FSP-1 expression via activation of the Notch signaling pathway. In this way, ECs acquire smooth muscle-like cell pheonotypes and enhanced cell migration ability. Our findings identify a novel vascular protective mechanism of integrin and suggest that inhibition of the EndMT might provide a novel therapeutic strategy to suppress neointimal hyperplasia in vascular disease.

Acknowledgements

This research was supported by grants from the National Nature Science Foundation of China (No. 81470963 and 81472400), Foundation Project of Chongqing Education Commission (No. KJ1600208), Foundation of Health and Family Planning Commission of Chongqing (No. 2016ZDXM004), Natural Science Foundation Project of ChongQing (No. cstc2014jcyjA10098) and American Heart Association grants (No. 15GRNT25700209) and NIH (DK095867).

Disclosure Statement

The authors declare to have no competing interests.

References

1 Tian DY, Jin XR, Zeng X, Wang Y: Notch signaling in endothelial cells: Is it the therapeutic target for vascular neointimal hyperplasia? Int J Mol Sci 2017;18.
2 Brahmbhatt A, Remuzzi A, Franzoni M, Misra S: The molecular mechanisms of hemodialysis vascular access failure. Kidney Int 2016;89:303-316.
3 Moonen JR, Lee ES, Schmidt M, Maleszewska M, Koerts JA, Brouwer LA, van Kooten TG, van Luyn MJ, Zeebregts CJ, Krenning G, Harmsen MC: Endothelial-to-mesenchymal transition contributes to fibro-proliferative vascular disease and is modulated by fluid shear stress. Cardiovasc Res 2015;108:377-386.
4 Kalluri R, Weinberg RA: The basics of epithelial-mesenchymal transition. J Clin Invest 2009;119:1420-1428.
5 Armstrong EJ, Bischoff J: Heart valve development: Endothelial cell signaling and differentiation. Circ Res 2004;95:459-470.
6 Wang Y, Liang A, Luo J, Liang M, Han G, Mitch WE, Cheng J: Blocking notch in endothelial cells prevents arteriovenous fistula failure despite ckd. J Am Soc Nephrol 2014;25:773-783.
7 Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, Chandraker A, Yuan X, Pu WT, Roberts AB, Neilson EG, Sayegh MH, Izumo S, Kalluri R: Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 2007;13:952-961.
8 Potenta S, Zeisberg E, Kalluri R: The role of endothelial-to-mesenchymal transition in cancer progression. Br J Cancer 2008;99:1375-1379.
9 Chen PY, Qin L, Baeyens N, Li G, Afolabi T, Budatha M, Tellides G, Schwartz MA, Simons M: Endothelial-to-mesenchymal transition drives atherosclerosis progression. J Clin Invest 2015;125:4514-4528.
10 Liang M, Wang Y, Liang A, Dong JF, Du J, Cheng J: Impaired integrin beta3 delays endothelial cell regeneration and contributes to arteriovenous graft failure in mice. Arterioscler Thromb Vasc Biol 2015;35:607-615.
11 Xiang Y, Zhang Y, Tang Y, Li Q: Malat1 modulates tgf-beta1-induced endothelial-to-mesenchymal transition through downregulation of mir-145. Cell Physiol Biochem 2017;42:357-372.
12 Kovacic JC, Mercader N, Torres M, Boehm M, Fuster V: Epithelial-to-mesenchymal and endothelial-to-mesenchymal transition: From cardiovascular development to disease. Circulation 2012;125:1795-1808.
13 Lin F, Wang N, Zhang TC: The role of endothelial-mesenchymal transition in development and pathological process. IUBMB Life 2012;64:717-723.
14 Xu J, Lamouille S, Derynck R: Tgf-beta-induced epithelial to mesenchymal transition. Cell Res 2009;19:156-172.
15 Gama-Norton L, Ferrando E, Ruiz-Herguido C, Liu Z, Guiu J, Islam AB, Lee SU, Yan M, Guidos CJ, Lopez-Bigas N, Maeda T, Espinosa L, Kopan R, Bigas A: Notch signal strength controls cell fate in the haemogenic endothelium. Nat Commun 2015;6:8510.
16 Noseda M, McLean G, Niessen K, Chang L, Pollet I, Montpetit R, Shahidi R, Dorovini-Zis K, Li L, Beckstead B, Durand RE, Hoodless PA, Karsan A: Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. Circ Res 2004;94:910-917.

17 Liang M, Wang Y, Liang A, Mitch WE, Roy-Chaudhury P, Han G, Cheng J: Migration of smooth muscle cells from the arterial anastomosis of arteriovenous fistulas requires notch activation to form neointima. Kidney Int 2015;88:490-502.
18 Ahanchi SS, Tsihlis ND, Kibbe MR: The role of nitric oxide in the pathophysiology of intimal hyperplasia. J Vasc Surg 2007;45 Suppl A:A64-73.
19 Kokubo T, Uchida H, Choi ET: Integrin alpha(v)beta(3) as a target in the prevention of neointimal hyperplasia. J Vasc Surg 2007;45 Suppl A:A33-38.
20 Chen M, Sinha M, Luxon BA, Bresnick AR, O'Connor KL: Integrin alpha6beta4 controls the expression of genes associated with cell motility, invasion, and metastasis, including s100a4/metastasin. J Biol Chem 2009;284:1484-1494.
21 Shi S, Srivastava SP, Kanasaki M, He J, Kitada M, Nagai T, Nitta K, Takagi S, Kanasaki K, Koya D: Interactions of dpp-4 and integrin beta1 influences endothelial-to-mesenchymal transition. Kidney Int 2015;88:479-489.
22 Bianchini F, Peppicelli S, Fabbrizzi P, Biagioni A, Mazzanti B, Menchi G, Calorini L, Pupi A, Trabocchi A: Triazole rgd antagonist reverts tgfbeta1-induced endothelial-to-mesenchymal transition in endothelial precursor cells. Mol Cell Biochem 2017;424:99-110.
23 Krebs LT, Starling C, Chervonsky AV, Gridley T: Notch1 activation in mice causes arteriovenous malformations phenocopied by ephrinb2 and ephb4 mutants. Genesis 2010;48:146-150.
24 Ebralidze A, Tulchinsky E, Grigorian M, Afanasyeva A, Senin V, Revazova E, Lukanidin E: Isolation and characterization of a gene specifically expressed in different metastatic cells and whose deduced gene product has a high degree of homology to a ca2+-binding protein family. Genes Dev 1989;3:1086-1093.
25 Li ZH, Bresnick AR: The s100a4 metastasis factor regulates cellular motility via a direct interaction with myosin-iia. Cancer Res 2006;66:5173-5180.
26 Schmidt-Hansen B, Ornas D, Grigorian M, Klingelhofer J, Tulchinsky E, Lukanidin E, Ambartsumian N: Extracellular s100a4(mts1) stimulates invasive growth of mouse endothelial cells and modulates mmp-13 matrix metalloproteinase activity. Oncogene 2004;23:5487-5495.
27 Kiryushko D, Novitskaya V, Soroka V, Klingelhofer J, Lukanidin E, Berezin V, Bock E: Molecular mechanisms of ca(2+) signaling in neurons induced by the s100a4 protein. Mol Cell Biol 2006;26:3625-3638.
28 Cheng J, Wang Y, Liang A, Jia L, Du J: Fsp-1 silencing in bone marrow cells suppresses neointima formation in vein graft. Circ Res 2012;110:230-240.