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Original Article

EphB4 signaling maintains the contractile phenotype of adult venous smooth muscle cells

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Abstract: Background: Autologous vein grafting remains the gold standard for surgical bypass grafts. However, vein bypasses still have significant incidence of failure. Ephrin type-B receptor 4 (EphB4), the embryonic venous determinant, may modulate vein graft adaptation. Although EphB4 is expressed in venous endothelial and smooth muscle cells (SMCs), it is not known whether EphB4 is functional in human SMCs. Materials and methods: Human adult venous SMCs were obtained from the inferior vena cava of an adult human liver donor. Primary SMCs were stimulated with EphrinB2/Fc or transfected with an EphB4-expression vector (GV219-EphB4). Expression of SMC phenotype markers, migration, and proliferation were evaluated. Results: Activation of EphB4 with EphrinB2/Fc increased the mRNA and protein expression of the venous SMC contractile markers alpha actin, calponin-1, SM22, and MYH11, while decreasing the expression of the synthetic marker osteopontin. EphrinB2/Fc treatment inhibited SMC migration, but not proliferation. In addition, overexpression of EphB4 increased mRNA expression of SMC contractile markers, while decreasing expression of the apoptosis marker caspase-9. Conclusions: EphB4 was present and functional in adult human venous SMCs. Stimulation of EphB4 increased expression of contractile SMC phenotypic markers and decreased SMC migration *in vitro*, functioning to retain the contractile phenotype of SMCs. EphB4 activation, therefore, recapitulates changes observed during vein graft adaptation to the arterial environment *in vivo*. EphB4 represents a new strategy to inhibit neointimal hyperplasia during vein graft adaption.

Keywords: Vein graft, human, EphB4, smooth muscle cell

Introduction

Cardiovascular disease remains a significant source of mortality and morbidity in modern society, with cardiac disease being the leading cause of death worldwide [1]. Atherosclerosis is a systemic disease that contributes to both coronary disease and peripheral arterial disease. Although endovascular therapy is frequently performed for atherosclerotic complications, surgical bypass remains a popular therapeutic choice for advanced disease.

The use of an autologous vein continues to be the gold standard for vascular grafting due to its superior patency and reduced number of infections compared to prosthetic grafts [2]. A distinct property of vein grafts is the venous adaptive response to the arterial environment after the surgical procedure. Venous adaptative remodeling is likely to be responsible for the superior performance of vein grafts compared with prosthetic grafts [3]. Vein graft adaptation, commonly known as "arterialization", is characterized by venous wall thickening, as well as increased venous diameter. Failure of a grafted vein to adapt may account for 20%-50% of bypass failures [3].

Ephrin type-B receptor 4 (EphB4), a member of the trans-membrane receptor tyrosine kinase family, is a determinant of venous fate during embryonic development and is also expressed in adult veins [4]. We previously demonstrated

that EphB4 is functional in adult human veins [5], EphB4 stimulation inhibits neointimal thickening of vein grafts [6], and EphB4 promotes venous adaptation in the adult arterial environment [7]. Furthermore, EphB4 is more highly expressed in the venous endothelium compared to media [7]. Therefore, it is not surprising that there are more studies examining the function of endothelial EphB4 compared to medial EphB4 [7-9].

EphB4 signaling mediates endothelial cell migration, proliferation [8, 9], and nitric oxide synthase phosphorylation [10]. EphB4 also induces endothelial secretion of smooth muscle cell (SMC) mitogens and chemoattractants [8]. These data suggest that SMC function may be regulated by EphB4 signaling. However, the role of EphB4 in SMCs is not well understood. Adult SMCs are highly specialized and differentiated cells yet retain plasticity in response to various environmental cues [11, 12]. Under normal physiological conditions, SMCs within the walls of adult blood vessels have a contractile phenotype: extremely low rates of proliferation and protein synthesis and expression of a particular repertoire of contractile proteins [13]. However, after injury or in the presence of atherosclerotic disease, SMCs change their phenotype to a highly "synthetic" phenotype that is characterized by increased proliferation and migration as well as markedly decreased expression of contractile proteins. Furthermore, the pathophysiological mechanisms of vein graft intimal hyperplasia are considered to involve this SMC phenotypic switch [14]. As EphB4 is expressed on SMCs [15], we hypothesized that EphB4 mediates phenotypic changes of SMCs. Here, we utilized a rare opportunity to obtain freshly isolated human adult venous SMCs to determine if EphB4 is functional in these cells.

Materials and methods

Antibodies and reagents

Mouse recombinant EphrinB2/Fc Chimera was purchased from R&D Systems (Minneapolis, MN). Alpha-smooth muscle actin (#19245), EphB4 (#14960) and Calponin-1 (#17819) antibody were purchased from Cell Signaling Technology (Boston, MA). GAPDH antibody was purchased from Abclonal Technology (Wuhan, China). All antibodies and reagents were used according to the manufacturers' instructions.

Primary venous SMC isolation and culture

Isolation of human venous SMCs was performed as previously described [16]. Human inferior vena cava tissue was harvested from the liver from a healthy 46-year-old male donor who was killed in a car accident. The liver was harvested for transplant surgery by perfusion with hypertonic citrate adenine solution followed by storage in University of Wisconsin cold storage solution on ice. A redundant 2-cm segment of the inferior vena cava was harvested. Informed consent was obtained from the family and permission for the study was granted by the Administrative Review Board of the Xiangya Second Hospital (Changsha, China).

To harvest SMCs from the vena cava tissue, the intima and adventitia layers were scraped off under sterile conditions and the remaining tissue was cut into approximately 1-mm^3 sections. These tissue pieces were placed flat on the bottom of a culture dish and allowed to dry for 15 min. SMC culture media (ScienCell; Los Angeles, CA) was then slowly added to cover the adherent sections. Venous SMCs migrated out of the tissue pieces after 5-7 days in culture. Migrated cells were trypsinized, cultured, and used for experiments in passages 5-8.

SMCs were cultured in SMC culture media supplemented with 2% fetal bovine serum, 1% SMC growth supplement (ScienCell; Los Angeles, CA), and 1% penicillin-streptomycin in 5% CO_2 at 37°C. Culture media was changed every 3 days. Venous SMCs were starved with DMEM/F12 (JET Biofil; Guangzhou, China) containing 0.5% fetal bovine serum for 12-24 h before experiments.

Quantitative PCR

Total RNA was isolated from cells using RNAprep Pure Cell/Bacteria Kit (TIANGEN, Beijing, China). For quantification of total RNA, each sample was measured using a Nanodrop 2000c (Thermo Scientific, Waltham, MA) and RNA quality was confirmed by measuring the 260/280 nm ratio. Reverse transcription was performed using SuperScript III First-Strand Synthesis Supermix (Thermo Scientific, Waltham, MA), according to the manufacturer's

Table 1. Primers used for gene expression analysis by real-time PCR

Gene	Forward Primers, 5'-3'	Reverse Primers, 5'-3'
ACTA	AAAAGACAGCTACGTGGGTGA	GCCATGTTCTATCGGGTACTTC
CNN	CTGTCAGCCGAGGTTAAGAAC	GAGGCCGTCCATGAAGTTGTT
TAGLN	AGTGCAGTCCAAAATCGAGAAG	CTTGCTCAGAATCACGCCAT
MYH11	AGATGGTTCTGAGGAGGAAACG	AAAACTGTAGAAAGTTGCTTATTCACT
EphB4	GTCTGACTTTGGCCTTTCCC	TGACATCACCTCCCACATCA
OPN	TTGCAGTGATTTTGCTTTTGC	GCCACAGCATCTGGGTATT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

instructions. Real-time quantitative PCR was performed using SYBR Green SuperReal PreMix Plus (TIANGEN, Beijing, China). Specific gene sequences were amplified for 40 cycles using a Real-Time PCR Detection system (Roche, Indianapolis, IN). Primer efficiencies were determined by melting curve analysis. All samples were normalized to GAPDH. Primers are listed in **Table 1**.

Western blotting

Cells were harvested with extraction buffer (RIPA Buffer) including protease inhibitors and then centrifuged (13,500 rpm) for 10 min. Equal amounts of protein from each treatment group were loaded for SDS-PAGE and transferred onto nitrocellulose membranes followed by western blot analysis. Membranes were probed with antibodies as described above. Protein was detected using an ECL detection reagent (abs920-2 Absin, Shanghai, China) and a film processor SRX-101A. Western blot densitometry results were quantitated with Image J software.

Cell migration-scratch assay

SMCs were seeded in 6-well plates until cells were 70% confluent. The cellular monolayer was scratched with a sterile 1000-μl pipette tip and then washed in PBS three times. EphrinB2/Fc (2 μg/ml) was added to the treatment group and then incubated with culture media for 24 h. Cells were stained with gentian violet (Solarbio, Life Science, Beijing, China) and photographed. Migrated cells were counted under a light microscope.

Cell migration-transwell assay

SMCs were trypsinized and centrifuged (13,500 rpm); 5×10^4 cells/200 μl were added to the upper chamber of a 24-well Transwell chamber (Chemicon; Corning, Billerica, MA). DMEM/F12 containing 10% FBS (600 μl) was added to the lower chamber. EphrinB2/Fc (2 μg/ml) was added to the upper chambers of the treatment groups. Following incubation for 24 h, the cells on the interior and lower surface of the upper chamber were fixed in 4% paraformaldehyde for 15 min and then stained at room temperature for 20 min with gentian violet. Cells on the interior of the inserts were removed using a cotton-tipped swab, rinsed with three times in PBS, imaged, and counted under a light microscope using 200× magnification.

Cell proliferation-CCK8 assay

Venous SMCs (1×10^4/100 μl) were seeded in 96-well plates and incubated for 24 h. Each treatment group was performed in replicates of 6 wells. EphrinB2/Fc (2 μg/ml) or media alone was added; cells were incubated for an additional 24 h. CCK8 medium (MedChem Express, China) 10 μl was added to each well for 2 hours. The absorbance of each sample was measured at 450 nm using a spectrophotometer (Perkin Elmer, Ltd., Männedorf, Switzerland).

Transfection with an EphB4-expressing vector

SMCs were seeded into 6-well plates and cultured in media without antibiotics. At 70% confluence, the SMCs were transfected with either GV219-EphB4 or GV219-control plasmid (Genechem Co., Ltd., Shanghai, China) using lipofectamine 2000 reagent (Thermo Scientific, Waltham, MA). After 12 h, the SMCs were cultured in SMC media for an additional 24 h prior to analysis. Lipid/plasmid DNA complexes were prepared at a ratio of 3 μl to 1.5 μg, respectively, per well.

Statistics

Statistical significance was determined using the t-test or analysis of variance (ANOVA) with appropriate post-hoc testing between groups. All tests were 2-tailed and a P-value ≤ 0.05 was considered statistically significant (Prism 6.0, Graphpad Software, San Diego, CA).

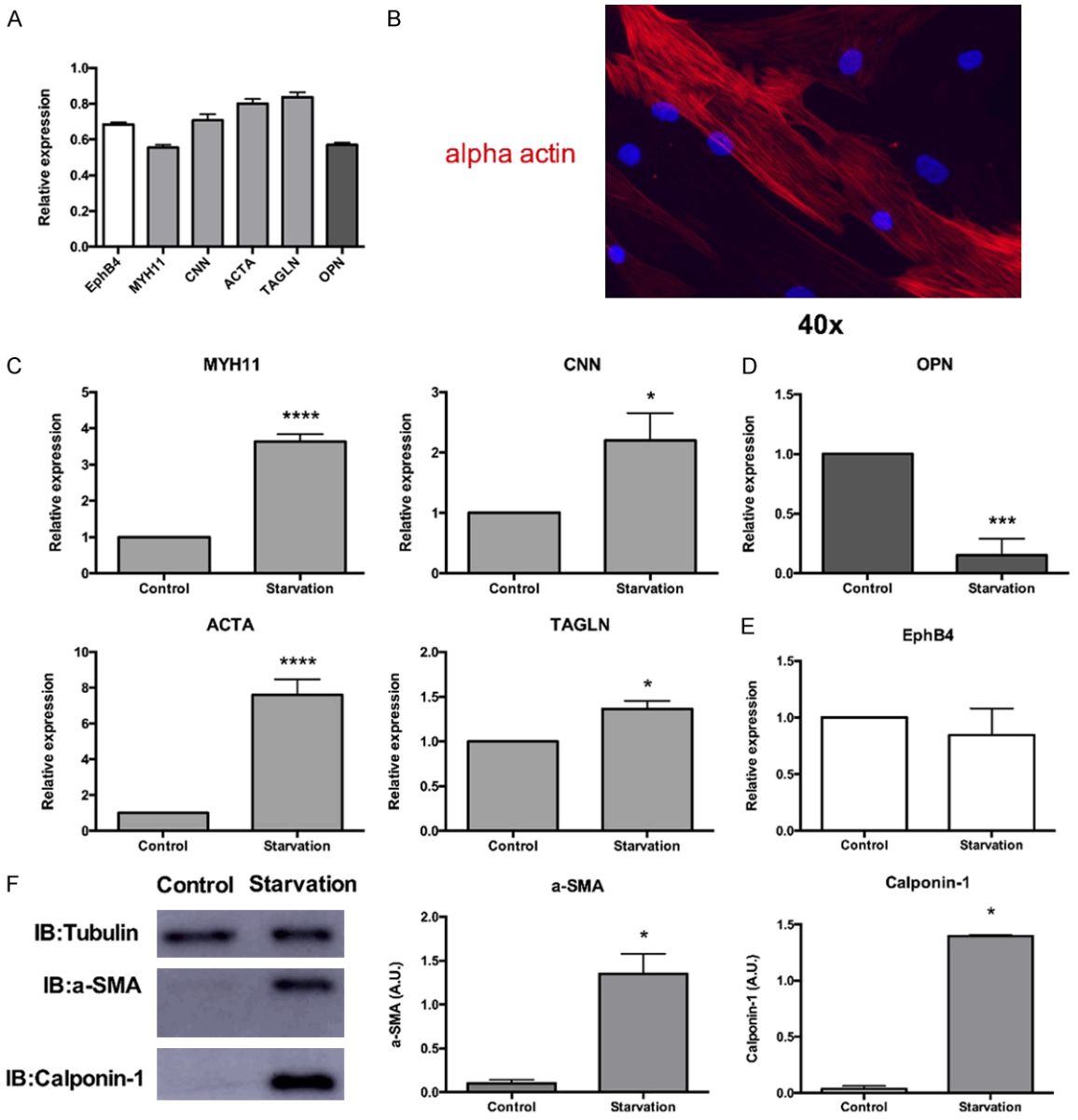

Figure 1. Baseline contractile and synthetic marker expression in human venous smooth muscle cells (SMCs). (A) Bar graph shows detection of RNA transcripts for EphB4 as well as contractile and synthetic SMC markers (n=5). (B) Immunofluorescence shows expression of alpha-SMA (red) and DAPI (blue) in human venous SMCs. Following 2 weeks of serum starvation, (C) expression of MYH11, CNN, ACTA and TAGLN were increased, (D) osteopontin decreased, and (E) EphB4 was not significantly altered (n=3). (F) Representative western blots with bar graphs representing densitometry of alpha-SMA and calponin-1 expression after starvation for 2 weeks (n=2). *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

Results

Human venous SMC-marker expression

To determine if EphB4 is functional in human venous SMCs, primary human venous SMCs were obtained from the inferior vena cava of a human adult liver. The baseline expression of several venous smooth muscle cell markers was examined using quantitative PCR. EphB4 was detectable in SMCs, as were markers of the contractile phenotype [myosin heavy chain 11 (MYH11), calponin (CNN), alpha smooth muscle actin (ACTA), and transgelin (TAGLN)] and the synthetic phenotype (osteopontin; **Figure 1A**). Immunofluorescence confirmed the presence of α-SMA in these cells (**Figure 1B**).

To determine if the expression of these markers could be altered in tissue culture, we reduced the serum concentration in the cell culture medium. Low serum concentrations have been shown to induce a quiescent cell phenotype [17]. Serum starvation increased the RNA expression of contractile markers (**Figure 1C**) and decreased expression of the synthetic marker osteopontin (**Figure 1D**) without inducing any change in EphB4 expression (**Figure 1E**). Serum starvation also increased the protein expression of the contractile markers α-SMA and calponin-1 (**Figure 1F**). These results show that human venous SMCs express the synthetic phenotype after isolation and culture, but retain the ability to express the contractile phenotype.

Activation of EphB4 increases expression of SMC contractile markers

Since human venous SMCs expressed EphB4, we next sought to determine if these receptors were functional. Cells were stimulated with soluble EphrinB2/Fc, a ligand that dimerizes and activates EphB4 [7]. Activation of EphB4 with EphrinB2/Fc increased mRNA expression of MYH11, CNN, ACTA and TAGLN as early as 6 h after treatment (**Figure 2A**) and decreased mRNA expression of osteopontin (**Figure 2B**). EphB4 activation also increased α-SMA and calponin-1 protein expression after 72 h (**Figure 2C**). These data demonstrate that EphB4 activation can alter expression of SMC phenotype markers, suggesting that EphB4 is functional in human venous SMCs.

Activation of EphB4 inhibits SMC migration but not proliferation

Since Ephrin-B2/Fc altered the SMC phenotype, we examined the ability of EphB4 activation to directly alter SMC function, including cell migration and proliferation. EphB4 activation decreased SMC migration, both in a scratch assay (**Figure 3A**) and in a transwell migration assay (**Figure 3B**). However, EphB4 activation had no effect on SMC proliferation (**Figure 3C**). These data demonstrate that human venous SMCs express functional EphB4.

Overexpression of EphB4 increases expression of SMC contractile markers

To determine if these cells could be transfected with an expression vector and if EphB4 overexpression could alter SMC phenotype, the EphB4 plasmid expression vector GV219-EphB4 was used to transfect the venous SMCs. GV219-control was used as a control. EphB4 protein and mRNA expression were both significantly increased compared to the GV219-control group (**Figure 4A**, **4B**). Overexpression of EphB4 decreased caspase 9 mRNA expression (**Figure 4C**) and increased the expression of MYH11, CNN, ACTA and TAGLN compared to the control group (**Figure 4D**). These data show that human venous SMCs can be transfected successfully and overexpression of EphB4 can alter SMC phenotype.

Discussion

In this study, freshly isolated human adult venous smooth muscle cells expressed the venous determinant EphB4. Activation of EphB4 signaling increased the expression of SMC contractile markers and decreased the expression of a synthetic marker. In addition, activation of EphB4 inhibited SMC migration but not proliferation. These data demonstrate that human adult venous SMCs have functional EphB4.

Mechanisms that control SMC phenotypic plasticity have been examined in cultured cells and include regulation by platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), nitric oxide, reactive oxygen species, matrix metalloproteinases, and shear stress [10]. Although changes in expression and function of EphB4, the embryonic determinant of venous identity [18], are associated with altered vessel function [5, 19], the role of EphB4 in SMC phenotypic modulation is not well understood. Our data show that EphB4 signaling activation and increased EphB4 expression augments the expression of contractile proteins in adult venous SMCs; these proteins are required for contractile function [13]. Additionally, EphB4 inhibits SMC migration and reduces caspase-9 expression, which may indicate a decreased apoptotic rate.

The effects of EphB4 in contractile SMCs reflected the normal and physiological conditions of adult mature SMCs. In this study, freshly isolated, healthy, adult human venous SMCs from the inferior vena cava were examined. Use of these primary cells is unusual and has not been previously reported. In addition, the function of vascular SMCs is frequently examined in

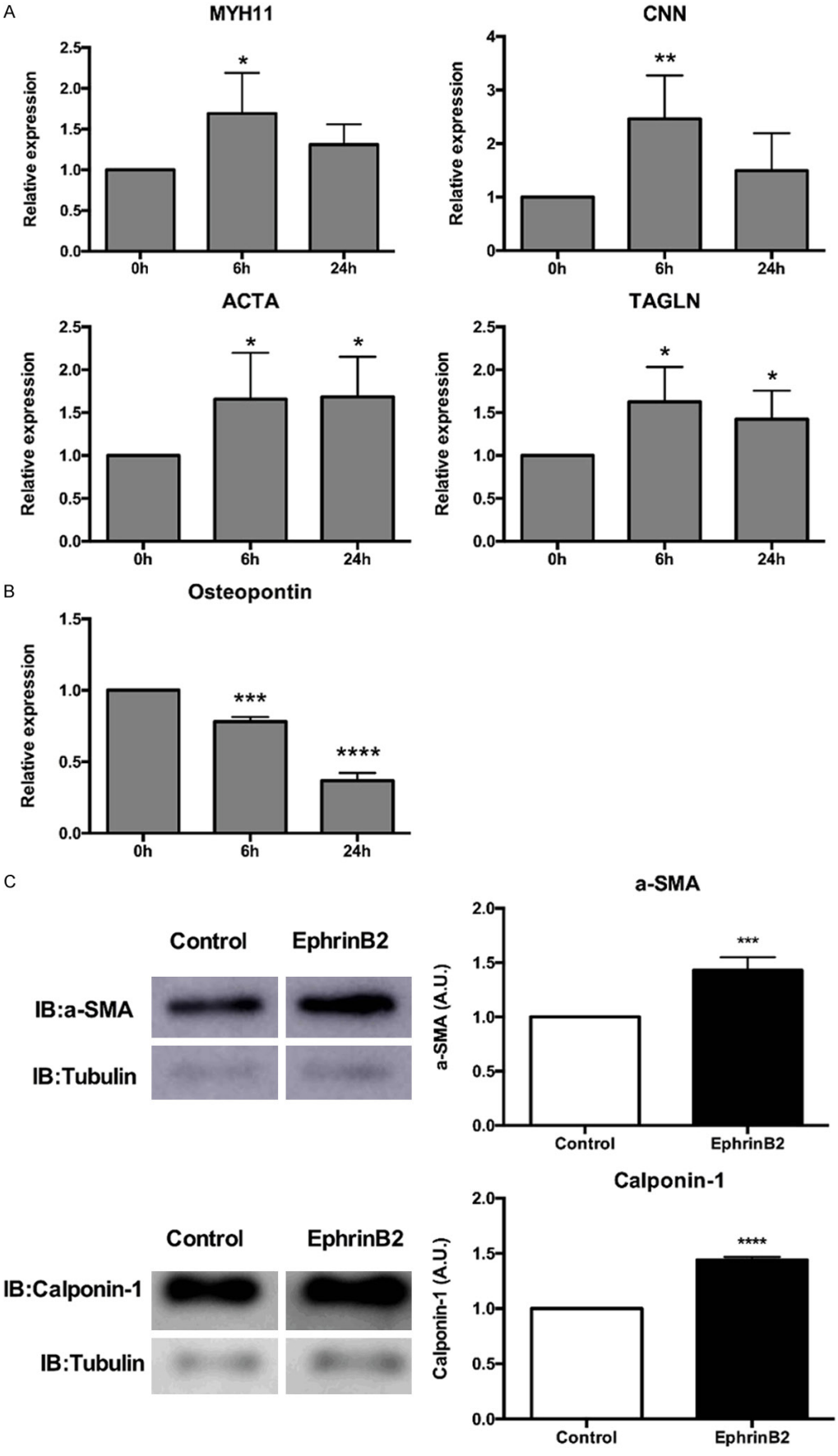
A
MYH11
Relative expression
0h
6h
24h
CNN
ACTA
TAGLN
B
Osteopontin
C
Control
EphrinB2
IB:a-SMA
IB:Tubulin
a-SMA
a-SMA (A.U.)
IB:Calponin-1
Calponin-1
Calponin-1 (A.U.)

Figure 2. Effects of EphrinB2/Fc on the expressions of SMC phenotypic markers. A. Quantitative PCR shows mRNA expressions of MYH11, CNN, ACTA and TAGLN before and after EphrinB2/Fc treatment for 6 h or 24 h (n=4-7). B. Expression of osteopontin before and after EphrinB2/Fc treatment for 6 h or 24 h (n=3). C. Representative western blots and bar graphs show densitometry of alpha-SMA and calponin-1 expression. Cells were treated with EphrinB2/Fc for 72 h that was replaced every 24 h. Control had no EphrinB2/Fc treatment (n=3). *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

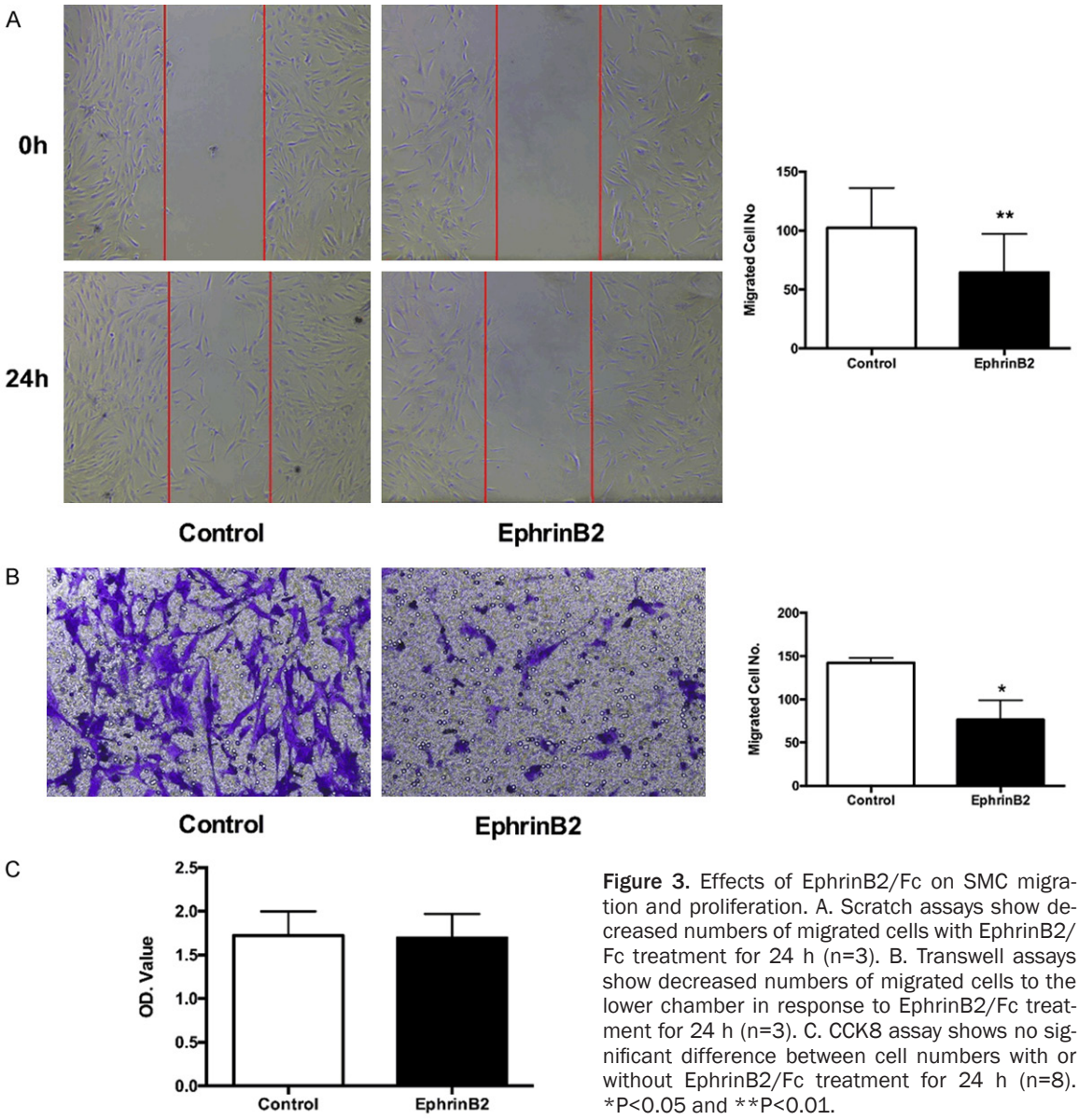

Figure 3. Effects of EphrinB2/Fc on SMC migration and proliferation. A. Scratch assays show decreased numbers of migrated cells with EphrinB2/Fc treatment for 24 h (n=3). B. Transwell assays show decreased numbers of migrated cells to the lower chamber in response to EphrinB2/Fc treatment for 24 h (n=3). C. CCK8 assay shows no significant difference between cell numbers with or without EphrinB2/Fc treatment for 24 h (n=8). *P<0.05 and **P<0.01.

arterial cells [8, 17, 20, 21]. Furthermore, EphB4 has differential effects in venous endothelial cells and SMCs. Ephrin-B2/Fc stimulates proliferation and migration in endothelial cells [9, 22], but inhibited venous SMC migration in the current study.

The effect of EphB4 activation in isolated cells *in vitro* may not reflect their function *in vivo*, in which SMCs are in contact with endothelial cells, other SMCs, and potentially other types of cells including fibroblasts and inflammatory cells. Therefore, dissection of the effect of EphB4 on SMCs may require analysis of cross-talk between these cell types using three-dimensional co-culture models [23].

The change in SMCs from a contractile to a synthetic phenotype may play a role in vein graft adaptation. Our data suggest that EphB4, via

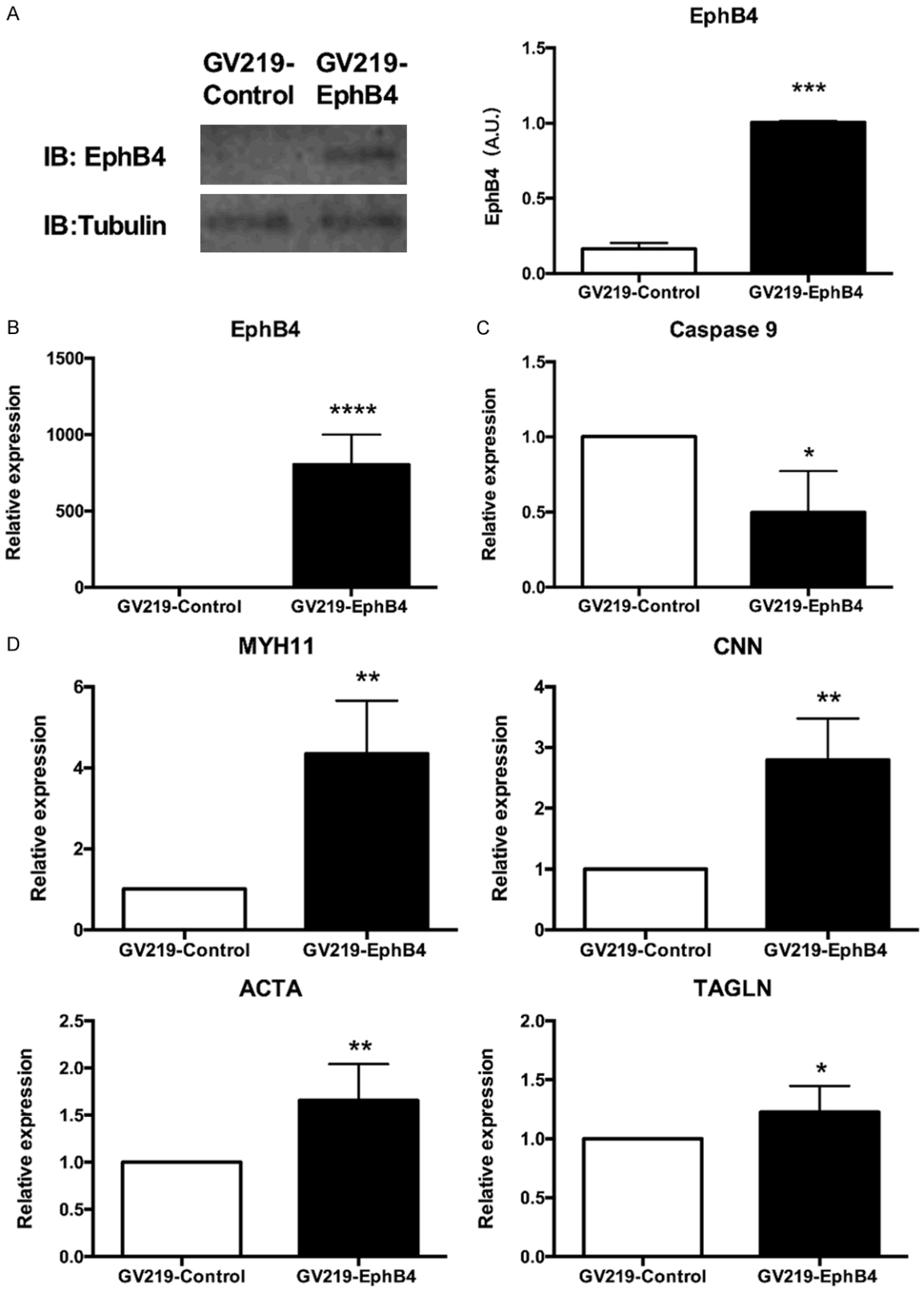

Figure 4. Expression of SMC phenotypic markers after overexpression of EphB4. (A) Representative western blots and bar graph showing densitometry of EphB4 after transfection of SMCs with control plasmid or GV219-EphB4 plasmid (n=3). (B-D) Relative mRNA expression of EphB4 (n=5) (B), caspase-9 (n=3) (C), and SMC contractile markers (MYH11, CNN, ACTA and TAGLN) (n=5) (D), after transfection of SMCs with GV219-control plasmid or GV219-EphB4 plasmid. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

activation of signaling and/or increased expression, may regulate this phenotypic shift. Importantly, these data show the importance of venous SMC function for successful vein graft adaptation to the arterial environment. Therefore, stimulation of EphB4 during vein graft adaptation may represent a novel strategy to inhibit intimal hyperplasia and improve vein graft patency.

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Disclosure of conflict of interest

None.

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