

# GPR30 decreases with vascular aging and promotes vascular smooth muscle cells maintaining differentiated phenotype and suppressing migration via activation of ERK1/2

Fang Huang<sup>1,2</sup>  
Jianguo Yin<sup>2</sup>  
Keyu Li<sup>1</sup>  
Ying Li<sup>1</sup>  
Heng Qi<sup>1</sup>  
Li Fang<sup>1</sup>  
Cong Yuan<sup>1</sup>  
Weiwei Liu<sup>1</sup>  
Min Wang<sup>2</sup>  
Xiangping Li<sup>2</sup>

<sup>1</sup>Department of Cardiology, The First Hospital of Changsha, <sup>2</sup>Department of Cardiology, The Second XiangYa Hospital of Central South University, Changsha, Hunan Province, People's Republic of China

**Abstract:** Estrogen receptors, including classic nuclear receptors ER $\alpha$ , ER $\beta$ , and membrane receptor GPR30, are expressed in vascular tissues and exert protective actions in vascular diseases. But the expression pattern and functional roles of GPR30 in vascular smooth muscle cells (VSMCs) remain unclear. In this study, we found that ER $\alpha$ , ER $\beta$ , and GPR30 were decreased with VSMCs passaging in vitro or growing in vivo and activation of GPR30 promoted ER $\alpha$  expression. Then, we validated that activation of GPR30 significantly decreased migratory capability of VSMCs and suppressed ER $\alpha$ , whereas PDGF-BB (20 ng/mL) treatment caused increase of migration. And activation of GPR30 led to reduction of osteopontin and cellular retinol binding protein 1, enhancement of calponin and 3F8, and upregulation of total and phosphorylated ERK1/2 expression in VSMCs knocked down by GPR30, ER $\alpha$ , and ER $\beta$  or treated with PDGF-BB. These data suggest that GPR30 promotes VSMCs reducing migration and maintaining differentiated phenotype via activation of ERK1/2 pathway. Our findings provide novel mechanisms of GPR30 protection of VSMCs as well as a new target for prevention of vascular aging.

**Keywords:** vascular smooth muscle cell, estrogen receptor, G protein-coupled receptor 30, phenotype, migration

## Introduction

The vascular smooth muscle cells (VSMCs) are highly specified cells in adults, possessing the contraction and adjustment of the blood vessel tension, maintain the blood pressure, and show a contractile phenotype with relatively low proliferative and migratory activity.<sup>1</sup> However, in the development of age-related diseases, such as hypertension and atherosclerosis, the functions and properties of VSMCs will be affected by the pathological factors and manifest a synthetic phenotype, highly proliferative and migratory activity, contributing to arterial stiffening and thickening, calcification and plaque formation, and increasing the risk of cardiovascular events.<sup>2-4</sup> Therefore, exploring the factors that play a critical role in controlling phenotype transformation and migration of VSMCs is helpful and necessary for development of novel therapeutic strategies for vascular diseases.

Estrogen can inhibit the endogenous aging of skin and reverse vascular calcification induced in vitro.<sup>5,6</sup> The protective effects of estrogen on endothelium and vascular smooth muscle are dependent on its binding to the estrogen receptor.<sup>7</sup> The loss

Correspondence: Xiangping Li  
Department of Cardiology, The Second XiangYa Hospital of Central South University, 139 Renmin Road, Changsha 410011, Hunan Province, People's Republic of China  
Tel +86 731 139 748 7901  
Email lixp@medmail.com.cn



of estrogen receptor will contribute to vascular aging and development of cardiovascular disease.<sup>8–10</sup> Estrogen receptor ER $\alpha$ , ER $\beta$ , and GPR30 (a transmembrane estrogen-binding protein) have been identified in blood vessels of human and animal models.<sup>11,12</sup> Experimental evidence suggests beneficial effects of estrogen on the vasculature, involving activation of ER $\alpha$ - and ER $\beta$ -mediated genomic effects in endothelial cells and VSMCs via the related signaling pathways.<sup>13,14</sup> Estrogen is able to affect the functional genes regulating vascular tone as well as responding to vascular injury and atherosclerosis. Estrogen increases the expression of genes for vasodilatory enzymes, such as prostacyclin synthase and nitric oxide synthase. GPR30-mediated nongenomic effects are rapid responses and typically involve regulation of membrane bound and cytoplasmic regulatory proteins.<sup>15</sup> In some cell types, ER $\alpha$  and ER $\beta$  correlate with caveolae and other signaling molecules to trigger GPR30-mediated second messengers and intracellular pathways, including MAPK and PI3K/Akt, and activation of ion channel fluxes.<sup>16</sup> Although studies have shown that estrogen receptors (ERs) play a protective role in vascular aging, the expression pattern, interrelationship, and action mechanisms of GPR30, ER $\alpha$ , and ER $\beta$  in the protection of VSMCs from aging remain unclear.

In this study, we firstly determined the expression profile of GPR30, ER $\alpha$ , and ER $\beta$  in VSMCs and arterial tissues, and then investigated the effects of GPR30 on the expression of ER $\alpha$  and ER $\beta$  in VSMCs, the migration and phenotypic transformation of VSMCs, and the underlying mechanisms. Our findings highlight that GPR30 plays a crucial role in ERs-mediated protective actions in VSMCs.

## Materials and methods

### Cell culture

Ethical approval was sought by the ethical committee of the First Hospital of Changsha. Primary VSMCs of rat carotid artery were purchased from PriCells (RAT-CELL-0009, Wuhan, People's Republic of China), cultured in VSMCs basal medium (MED-0003, PriCells), and supplemented with special additives (SUP-003, PriCells), according to the manufacturer's instructions, and incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells between passages 2 and 8 were used in the study.

### Quantitative real-time reverse transcription polymerase chain reaction

Total RNA was extracted from VSMCs using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions, and then the RNA was reverse transcribed using the PrimeScript RT Master Mix Perfect Real

Time kit (TaKaRa, Dalian, People's Republic of China) to obtain the cDNA. Using the cDNA as the template, a real-time PCR assay was performed using the following pairs of primers: ER $\alpha$  forward, 5'-CGCTTTTGAACCAGCAGG-3' and ER $\alpha$  reverse, 5'-TTCCCAGAGGCTTTGGTGTG-3'; ER $\beta$  forward, 5'-ATCTGTCCAGCCACGAATCA-3' and ER $\beta$  reverse, 5'-ATTAGCACCTCCATCCAGCA-3'; GPR30 forward, 5'-AGCTCAGGCTGTATGTGGCG-3' and GPR30 reverse, 5'-TGCTCCGTGCTGTCTGGTAT-3'; and  $\beta$ -actin forward, 5'-AGGCCCTCTGAACCCTAAG-3' and  $\beta$ -actin reverse, 5'-CCAGAGGCATACAGGGACAAC-3'. The 20  $\mu$ L real-time PCR reaction included 0.5  $\mu$ L of cDNA template, 0.25  $\mu$ L of Primer F, 0.25  $\mu$ L of Primer R, 10  $\mu$ L of RNase-free dH<sub>2</sub>O, and 8  $\mu$ L of 2.5 $\times$  RealMasterMix (SYBR Green I). The reaction conditions included a predenaturation step at 95°C for 10 seconds, and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. After the reaction, the data were subjected to statistical analysis.

### Short hairpin RNA expression plasmids and stable transfection

The shRNA expression vectors against ER $\alpha$ , ER $\beta$ , and GPR30 were prepared by cloning of double-stranded oligonucleotides into the BamHI and HindIII sites in the pRNAT-U6.1/Neo vector (Thermo Fisher Scientific, Waltham, MA, USA). The sequences of shRNA against ER $\alpha$ , ER $\beta$ , and GPR30 were 5'-AAGTCGATTCCGCATGATGAA-3', 5'-AAGTTCTAGCGAGCAGGTACA-3', and 5'-CGAGCAGTATTACGATATC-3', respectively. All of the constructs were confirmed by DNA sequencing. The VSMCs were transfected with lentivirus packaged pRNATU6.1-ER $\alpha$ -shRNA, -ER $\beta$ -shRNA, or GPR30-shRNA vector. The stable suppression of ER $\alpha$ , ER $\beta$ , or/and GPR30 in VSMCs was identified by Western blotting and quantitative real-time RT-PCR (qRT-PCR) assays. And the stable transfectants were selected and used for the following studies.

### Western blotting

An amount of 1 $\times$ 10<sup>7</sup> VSMCs treated as indicated was lysed in cell lysate, and then centrifuged at 12,000 $\times$  g for 20 minutes at 4°C. The supernatant was collected and denatured. Proteins were separated in 10% SDS-PAGE (dodecyl sulfate, sodium salt polyacrylamide gel electrophoresis) and blotted onto polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was treated with Tris Buffered Saline Tween containing 50 g/L skimmed milk at room temperature for 4 hours, followed by incubation with the primary antibodies anti-GPR30 (1:250, Abcam, Cambridge, MA, USA), anti-ER $\alpha$  (1:200, Abcam), ER $\beta$  (1:1,000, Abcam), anti-OPN

(1:1,000, Abcam), anti-CRBP1 (1:200, Santa Cruz Biotechnology Inc., Dallas, TX, USA), anti-calponin (1:20,000, Abcam), anti-3F8 (1:500, Santa), Collage I/II (1:1,000, Abcam), anti-fibronectin (1:1,000, Abcam), anti-PI3K (1:500, Abcam), p-PI3K (1:500 dilution, Abcam), ERK1/2 (1:1,000, Abcam), p-ERK1/2 (1:500, Abcam), PKA (1:1,000, Abcam), p-PKA (1:2,500 dilution, Abcam), and anti- $\beta$ -actin (1:2,500 dilution, ABZOOM BIOLABS Inc, Dallas, TX, USA), respectively, at 37°C for 1 hour. Membranes were rinsed and incubated for 1 hour with the correspondent peroxidase-conjugated secondary antibodies. Chemiluminescent detection was performed with the ECL kit (Pierce Chemical, Rockford, IL, USA). The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of  $\beta$ -actin.

### Immunocytochemistry analysis

Animal experiments were performed in strict accordance with the guide for the care and use of laboratory animals of Central South University. The rats were maintained under specific pathogen-free conditions in the Institute of Zoology, Xianya Hospital of Central South University. Six female rats were randomly divided into two groups and raised to 2 months and 18 months, respectively. Then, the rats were euthanized and the carotid artery tissues were excised and embedded in paraffin. The expressions of ER $\alpha$ , ER $\beta$ , and GPR30 in rat carotid artery tissues of 2- and 18-month-old rats were determined by immunocytochemistry. The root of the aorta was obtained from rat and fixed in 4% paraformaldehyde overnight. Tissue specimens were then cut at 5  $\mu$ m thickness, and a standard immunostaining procedure was performed using antibodies against ER $\alpha$ , ER $\beta$ , and GPR30. The mean optical density value ( $D$ ) and area ( $A$ ) of brown particles in three visual fields of each section were calculated by the Leica Q550 image analysis system (Leica Microsystems, Wetzlar, Germany). The expression levels of target molecules in tissues were evaluated using the formula: integral density =  $D \times A$ .

### Cell migratory capacity analysis

VSMCs of passage 2 treated with G-1 (1 nM, Tocris Bioscience, Bristol, UK) for 48 hours or PDGF-BB (10 ng/mL) for 24 hours as indicated were plated at a final concentration of  $2 \times 10^3$  cells per well in 96-well plates. The migratory capacity of cells was evaluated by transwell assay. In brief, migration experiments were performed in polycarbonate transwell inserts (8  $\mu$ m pores, Corning Incorporated, Corning, NY, USA). Cells ( $1 \times 10^6$ ) in 200  $\mu$ L culture medium were seeded in the upper chamber and cultured at 37°C for

6 hours. And the migrating cells in the bottom of the chamber were fixed and stained with crystal violet and photographed. The crystal violet was then eluted and the eluent of each group was measured by an ELISA reader (ELX-800 type, BioTek, Highland Park, FL, USA) to determine the optical density at 570 nm (OD570).

### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation from at least three separate experiments. Statistical analysis was carried out using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). The difference between two groups was analyzed by the Student's  $t$ -test. A value of  $P < 0.05$  was considered as statistically significant.

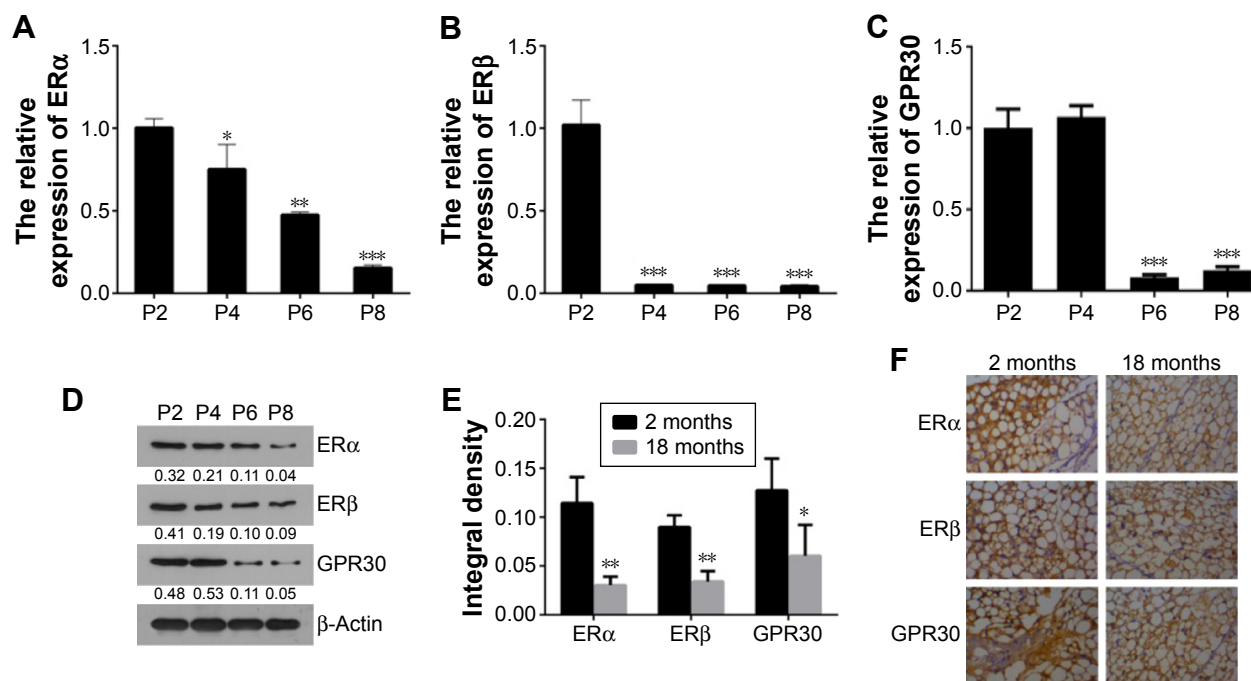
## Results

### ER $\alpha$ , ER $\beta$ , and GPR30 decrease in VSMCs with cells passaging or rat aging

To investigate whether the expression levels of ERs alter with VSMCs passaging in vitro or growing in vivo, the relative expression of ER $\alpha$ , ER $\beta$ , and GPR30 was compared among VSMCs of passages 2, 4, 6, 8, or between rat carotid artery tissues of 2- and 18-month-old rats. The quantitative RT-PCR and Western blot results showed that the ER $\alpha$ , ER $\beta$ , and GPR30 mRNAs and proteins were decreased with VSMCs passaging from P2 to P8 (Figure 1A–D). Then, we further found that the ER $\alpha$ , ER $\beta$ , and GPR30 proteins were more expressed in carotid artery tissues of 2-month-old rats compared with the 18-month-old rats (Figure 1E and F). These results demonstrate that estrogen receptors ER $\alpha$ , ER $\beta$ , and GPR30 were downregulated in VSMCs along with its passaging in vitro or growing in vivo.

### GPR30 has promotion actions on ER $\alpha$ expression but no clear effects on ER $\beta$

To explore the influences of GPR30 on ER $\alpha$  and ER $\beta$  expression, the VSMCs were cotransfected with ER $\alpha$  + ER $\beta$  shRNAs or ER $\alpha$  + ER $\beta$  + GPR30 shRNAs. The quantitative RT-PCR and Western blot assays confirmed that the expression levels of ER $\alpha$ , ER $\beta$  mRNAs, or proteins were significantly suppressed in VSMCs cotransfected with ER $\alpha$  + ER $\beta$  shRNAs (Figure 2A–C). We then found that the ER $\alpha$  was less expressed in VSMCs cotransfected with ER $\alpha$  + ER $\beta$  + GPR30 shRNAs compared with ER $\alpha$  + ER $\beta$  shRNA group. Moreover, activation of GPR30 using its selective agonists G-1 could significantly enhance ER $\alpha$  mRNA levels in VSMCs cotransfected with ER $\alpha$  + ER $\beta$  + GPR30 shRNAs, even notably more than its expression in VSMCs cotransfected with ER $\alpha$  + ER $\beta$  shRNAs. However, neither



**Figure 1** ERα, ERβ, and GPR30 were decreased in VSMCs with cells passing or rat aging.

**Notes:** The relative expression of ERα (A), ERβ (B), and GPR30 (C) mRNAs in VSMCs of passages 2, 4, 6, and 8 was determined using qRT-PCR. (D) The representative images of Western blot analysis. (E) The integral density (brown particles) of ERα, ERβ, and GPR30 in tissues of rat carotid artery from 2- to 18-month-old rats. (F) The representative images of immunocytochemistry analysis, magnification 400×. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 versus P2 group or 2 months group.

**Abbreviation:** VSMCs, vascular smooth muscle cells.

inhibition nor activation of GPR30 had clear influence on ERβ (Figure 2D–F). These results indicate that activation of GPR30 may promote ERα expression, but it has no clear influence on the expression of ERβ.

## Activation of GPR30 promotes VSMCs maintaining differentiated phenotype and reducing migration

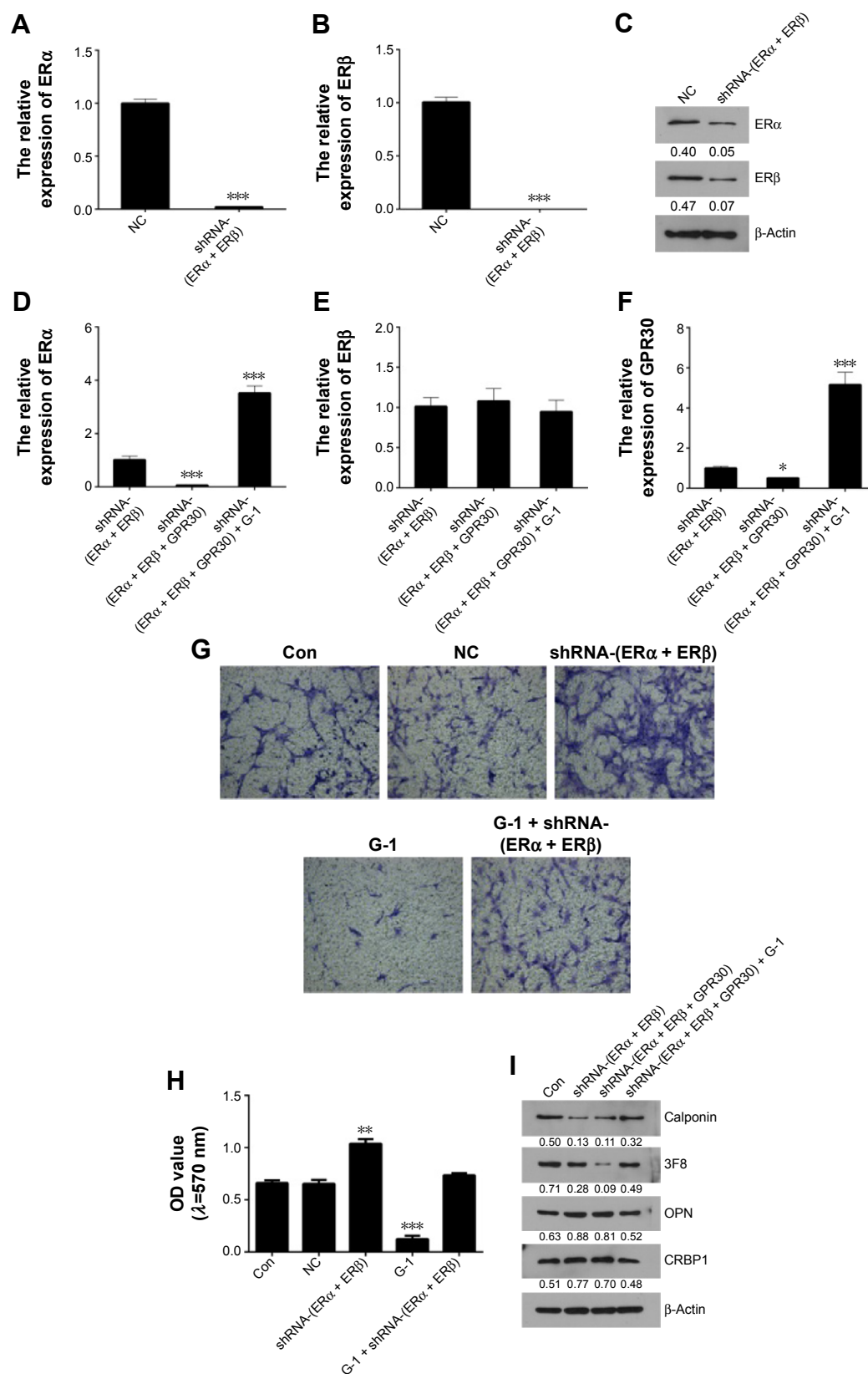
To elucidate the functional roles of GPR30 in VSMCs, the migration and phenotypic markers in VSMCs of passage 2 were determined using transwell assays and Western blot analysis. It was shown that inhibition of ERα and ERβ increased migration of VSMCs. However, activation of GPR30 using its selective agonists G-1 could decrease migration of VSMCs and force the enhanced migratory capability of VSMCs induced by inhibition of ERα and ERβ return to normal levels (Figure 2G and H). It suggests that ERα, ERβ, and GPR30 may play an inhibitory role in regulation of migratory capability in VSMCs. Then, the Western blot results illustrated that inhibition of ERα, ERβ, and/or GPR30 reduced the expression of differentiated phenotype markers (calponin and 3F8) and enhanced the expression of synthetic phenotype markers (OPN and CRBP1) in VSMCs, but activation of GPR30 in these VSMCs could partly resume the reduced expression of differentiated phenotypic markers

(Figure 2I). It indicates that ERα, ERβ, and GPR30 may contribute to VSMCs maintaining a differentiated phenotype.

## GPR30 protection of VSMCs involving activation of ERK1/2

To reveal whether ERK1/2 pathway participates in GPR30 regulating the phenotype of VSMCs, the VSMCs of passage 2 were treated with PDGF-BB or G-1 and the expression levels of phenotype markers were determined. As shown in Figure 3, PDGF-BB (20 ng/mL) induced reduced calponin and 3F8 and enhanced OPN, CRBP1, collagen I/II, and fibronectin in VSMCs in a time-dependent manner. It confirms that PDGF-BB can promote the conversion of VSMCs from a contractile phenotype into a synthetic phenotype and stimulate production of vascular fibrosis- and calcification-related factors in VSMCs. It is shown that PDGF-BB-induced reduced calponin and 3F8 expression, enhanced OPN and CRBP1 expression, and elevated migratory capability in VSMCs could be partly reversed by activation of GPR30 using G-1. We then found that activation of GPR30 could also partly recover PDGF-BB-induced decreased total and phosphorylated ERK1/2 expression in VSMCs. These data indicate that GPR30 protection of VSMCs from suffering pathological changes induced by PDGF-BB is possibly involved in upregulation of expression and activity of ERK1/2.

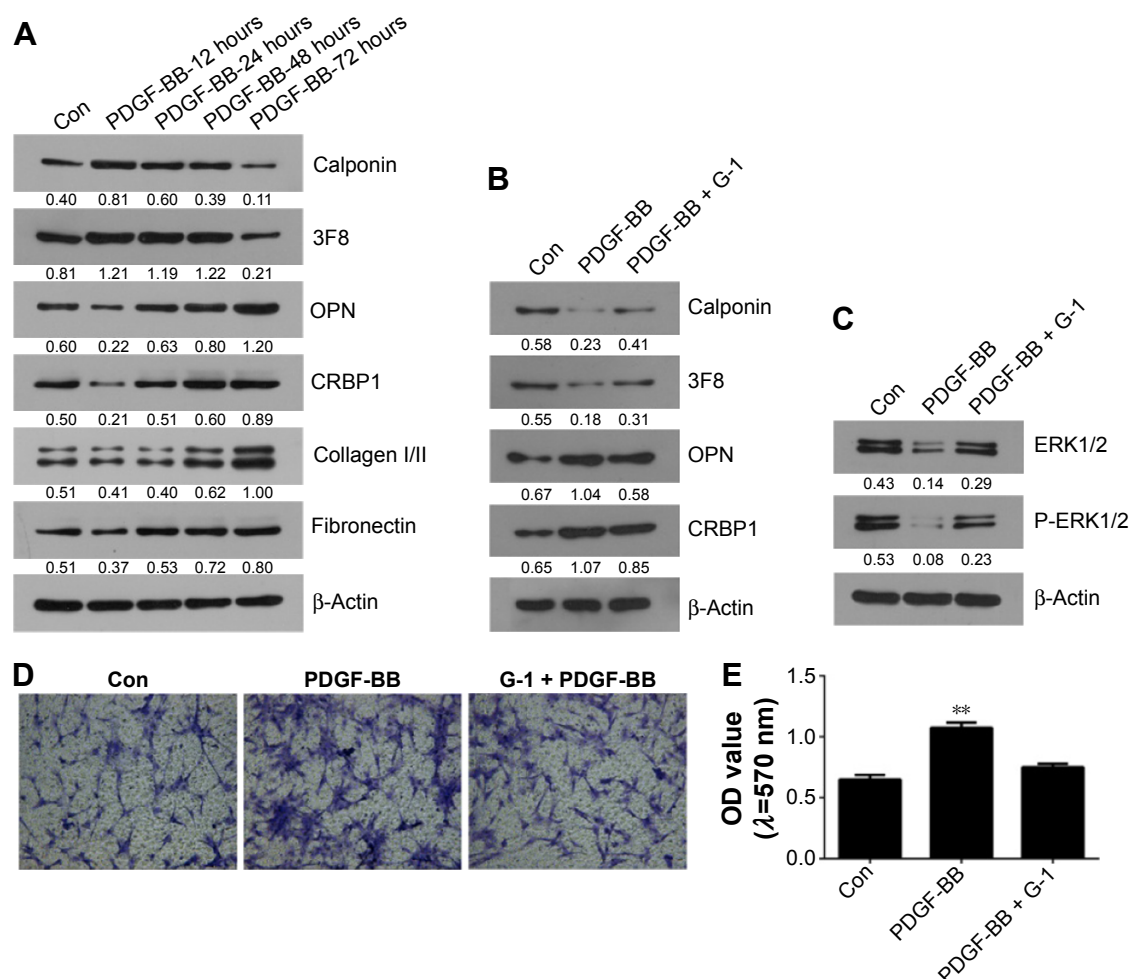




**Figure 2** Effects of GPR30 on the expression of ER $\alpha$  and ER $\beta$  and the migration in VSMCs.

**Notes:** The mRNAs (**A** and **B**) and protein (**C**) of ER $\alpha$  and ER $\beta$  were significantly knocked down in VSMCs cotransfected with ER $\alpha$  + ER $\beta$  shRNAs. The relative expression of ER $\alpha$  (**D**), ER $\beta$  (**E**), and GPR30 (**F**) in VSMCs cotransfected with ER $\alpha$  + ER $\beta$  + GPR30 shRNAs and treated with G-1 was determined with qRT-PCR. (**G** and **H**) The migratory capacity of VSMCs was determined using transwell assay, magnification 100 $\times$ . The expression of calponin, 3F8, OPN, and CRBP1 in VSMCs was determined using Western blot analysis (**I**). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus negative control (NC) or ER $\alpha$  + ER $\beta$  shRNA group.

**Abbreviations:** VSMCs, vascular smooth muscle cells; OD, optical density.



**Figure 3** The protective actions of GPR30 on VSMCs.

**Notes:** (A) PDGF-BB (20 ng/mL) reduced calponin and 3F8 and enhanced OPN, CRBP1, collagen I/II, and fibronectin in VSMCs at 12, 24, 48, and 72 hours. Activation of GPR30 using G-1 partly recovered PDGF-BB altering the expression of OPN, CRBP1, calponin, 3F8 (B), ERK1/2 (C) expression, and migratory capacity in VSMCs (D and E), magnification 100×. \*\* $P < 0.01$  versus control (Con).

**Abbreviations:** OD, optical density; VSMCs, vascular smooth muscle cells.

## Discussion

Estrogen receptors play an important regulatory role in the reproductive, cardiovascular, nervous, immune system, and so on. ERs can be divided into the classic nuclear receptors, including  $ER\alpha$  and  $ER\beta$ , which induce a genomic effect by transcription of specific target genes, and the membrane receptor GPR30, which mediates a rapid nongenomic effect by a second messenger system.<sup>17</sup> GPR30 is located in the surface of cell membrane, apparatus reticulare, and endoplasmic reticulum.<sup>18</sup> It is widely distributed in human heart, lung, liver, ovary, breast, brain tissues, and so on and expressed in carotid, thoracic, mesenteric, and pulmonary arterial vessels.<sup>19,20</sup> Epidemiological investigations and experimental studies have revealed that estrogen has protective effects on vascular system against aging via binding of ERs. The alteration of ERs with age in VSMCs may affect its response to estrogen.<sup>21</sup> It is reported that GPR30 expression was

significantly decreased in the aorta of aged female Lewis rats and adult male rats, causing reduced vasorelaxation to estradiol and G-1.<sup>8</sup> Furthermore, this decline has nothing to do with estrogen reduction. Therefore, it is necessary to reveal the expression alternation of GPR30 in vascular tissues, including VSMCs with aging. In the present study, we found that the expression levels of  $ER\alpha$ ,  $ER\beta$ , and GPR30 were decreased with VSMCs passing from passages 2 to 8 and less expressed in carotid artery tissues of 18-month-old rats than the 12-month-old rats. These results demonstrate that estrogen receptor  $ER\alpha$ ,  $ER\beta$ , and GPR30 may diminish in VSMCs along with its passaging in vitro or growing in vivo, implying that estrogen receptors may decline with vascular aging.

To reveal the effects of GPR30 on  $ER\alpha$  or  $ER\beta$  expression in VSMCs, we firstly established a cell model of VSMCs in which  $ER\alpha$  and  $ER\beta$  were effectively knocked down.

Then we discovered that further knockdown of GPR30 in this cell model would lead to less ER $\alpha$  expression. Accordingly, activation of GPR30 using its selective agonists G-1 caused enhancement of ER $\alpha$  expression. But neither inhibition nor activation of GPR30 had a clear influence on ER $\beta$ . Studies indicate that a potential crosstalk or cooperation may exist among GPR30, ER $\alpha$ , aryl hydrocarbon receptor, and ErbB family signaling pathways.<sup>22,23</sup> GPR30 may function in conjunction with ER $\alpha$  to assemble a signal complex essential for rapid estrogen signaling.<sup>16</sup> Our results suggest that activation of GPR30 signaling pathway may promote ER $\alpha$  expression, possibly resulting in accentuation of the estrogen-induced ER $\alpha$  signaling and effects in VSMCs.

The alteration of VSMC features represents a critical event in the pathobiology of arterial wall since it contributes to the vascular remodeling and decline of function with aging and favors the progression of atherosclerosis.<sup>24</sup> ERs are possibly involved in regulation of phenotypic transformation and migration in VSMCs.<sup>25–27</sup> Our results confirmed that activation of GPR30 could partly resume the loss of ER $\alpha$ - and ER $\beta$ -mediated increase of migration in VSMCs. Inhibition of ER $\alpha$ , ER $\beta$ , and/or GPR30 reduced calponin and 3F8 and enhanced OPN and CRBP1 expression in VSMCs; however, activation of GPR30 partly recovered the differentiated phenotypic markers expressed in VSMCs. Then, we further validated that activation of GPR30 could inhibit PDGF-BB promotion of VSMCs migration and dedifferentiation. The potential metabolism is involved in upregulation of expression and activity of ERK1/2. Our results suggest that GPR30 plays a suppressor in regulation of migratory capability of VSMCs and contributes to VSMCs maintaining a differentiated phenotype via activation of ERK1/2 pathway. GPR30 activation may cause the dissociation of G $\alpha$ -GTPase from the heterotrimeric G $\alpha\beta\gamma$  complex. Dissociated G $\beta\gamma$  subunit will activate membrane associated matrix metalloproteinases with subsequent transient activation of mitogen-activated protein kinase ERK-1/2.<sup>28,29</sup> And G $\alpha$ -GTPase may act on membrane-associated adenylyl cyclase, generating the second messenger cAMP, which in turn inhibits ERK-1/2 activity via a cAMP-dependent protein kinase mechanism.<sup>30</sup> In addition, in some breast cancer cells, silencing of GPR30 function had no effects on estradiol-induced cAMP elevation and ERK activation. Although GPR30 has been identified in the vasculature, its action mechanisms need further investigation.

In conclusion, we found that ER $\alpha$ , ER $\beta$ , and GPR30 were decreased with VSMCs passaging in vitro or growing in vivo, and activation of GPR30 promoted ER $\alpha$  expression.

The results confirm that GPR30 promotes VSMCs maintaining differentiated phenotype and reducing migration through activation of ERK1/2 pathway. Our findings provide novel mechanisms of GPR30 protection of VSMCs as well as a target for prevention of vascular aging.

## Disclosure

The authors report no conflicts of interest in this work.

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