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Osteopontin is required for angiotensin II-induced migration of vascular smooth muscle cells

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Migration and proliferation of vascular smooth muscle cells (VSMCs) play a prominent role in the development of atherosclerotic plaques and restenosis lesions. Angiotensin II (Ang-II) is typically associated with excessive proliferation and migration of VSMCs and vascular remodeling. High levels of osteopontin (OPN) mRNA and protein were reported in human atherosclerotic plaque from the aorta, carotid and coronary arteries. However whether OPN plays a role in VSMCs migration induced by Ang-II is unknown. Here we show that, in primary cultured rat VSMCs, Ang-II exhibits chemotactic effect on cultured VSMCs and induces OPN expression dose-dependently. With a lentiviral shRNA specifically targeting OPN and transwell migration assay, we find that blockade of OPN with shRNA inhibits Ang-II-induced MMP9 upregulation and VSMCs migration. Our results demonstrated that OPN is required for Ang-II to induce VSMCs migration and suggested OPN as a potential target in preventing atherosclerotic development.

1. Introduction

Vascular remodeling diseases, including atherosclerosis, restenosis following reconstructive vascular operation and hypertension, continue to be the leading cause of morbidity and mortality in the Western World. Atherosclerosis is a progressive inflammatory disease that ultimately leads to formation of advanced or complicated focal lesions which develop subsequently to a series of specific cellular and molecular responses including enhanced vascular smooth muscle cells (VSMCs) proliferation and migration (Chaulet et al. 2001). Several studies suggested that migration and proliferation of VSMCs in the vascular tunica media as a pathological basis for vascular remodeling (Li et al 2000; Schwartz et al. 2002). VSMCs proliferation is an important feature in experimental arterial injury models, a few proliferating VSMCs have been detected in human primary or secondary atherosclerotic plaques (Gordon et al. 1990; Pickering et al. 1993), which suggested that the prominent role of the migration process in the development of atherosclerotic plaques and restenosis lesions.

Proliferation and migration of VSMCs into the sub-intimal space play an important role in intimal thickening in atherosclerosis and restenosis and influences the long-term patency of the venous graft (Jia et al. 2006). Initiation and progression of the atherosclerotic plaque and restenotic vessel involve complex patterns of interaction between the cells of the arterial wall, in which cytokines, chemokines, and growth factors are known to play a critical role (Kwak et al. 2002; Jia et al. 2007). The relationships between growth factors and the extracellular matrix (ECM) in the vascular remodeling process are inseparable. Therefore, careful attention to the reciprocal relationship between growth factors and the ECM should lead to an enhanced understanding of the molecular mechanisms underlying vascular remodeling.

Angiotensin II (Ang-II) is a potent growth factor and plays a significant role in mediating VSMC migration and proliferation (Daemen et al. 1991) and contributes to the pathogenesis of atherosclerosis and restenosis (Mehta and Griendling 2007; Higuchi 2007; Zheng et al. 2006). OPN is a functionally important protein in the ECM, which is believed to contribute to vascular remodeling after injury (Ashkar et al. 2000; Missihoun et al. 2009; Wu et al. 2007). Recent studies suggest that Ang-II is a potent upregulator of OPN expression (Abe et al. 2008). However, the relationship of Ang-II and OPN in the cell migration of VSMC is still poorly understood.

The present study was undertaken to investigate the role of OPN in Ang-II induced vascular remodeling, with special emphasis on Ang-II induced migration of VSMCs. We constructed an OPN specific lentivirus shRNA vector and successfully knockdown OPN expression in rat VSMCs. Further results showed that Ang-II can induce significant migration of wild type VSMCs and the Ang-II induced migration was significant suppressed when knockdown the expression of OPN. The results show that OPN mediates the Ang-II induced migration of VSMCs.

2. Investigations and results

2.1. Screening for shRNAs with high interference efficiency

To investigate OPN function in the VSMCs, we designed three shRNA targeting sites in the open reading frame of rat OPN mRNA. As shown in Fig. 1A, the nucleotides with red color are the targeting sequences, and the nucleotides with blue color indicate the linker region. The DNA oligo encoding these shRNA fragments were cloned into the lentivirus vectors pLKO.1 and verified by DNA sequencing. The shRNA lentiviruses were packaged with 293T cells and VSMCs were infected with the

A

Oligo name	Sequence (5'–3')
OPN1-F	CCGGAAGATGATAGGTATCTGAAATCTCGAGATTTCAGATACCTATCATCTTTTTTG
OPN1-R	AATTCAAAAAAAGATGATAGGTATCTGAAATCTCGAGATTTCAGATACCTATCATCTT
OPN2-F	CCGGAAGGATGAACCAAGCGTGGAACTCGAGTTCCACGCTTGGTTCATCCTTTTTTTG
OPN2-R	AATTCAAAAAAAGGATGAACCAAGCGTGGAACTCGAGTTCCACGCTTGGTTCATCCTT
OPN3-F	CCGGAAGCTAGTCCTAGACCCTAAGCTCGAGCTTAGGGTCTAGGACTAGCTTTTTTTG
OPN3-R	AATTCAAAAAAAGCTAGTCCTAGACCCTAAGCTCGAGCTTAGGGTCTAGGACTAGCTT

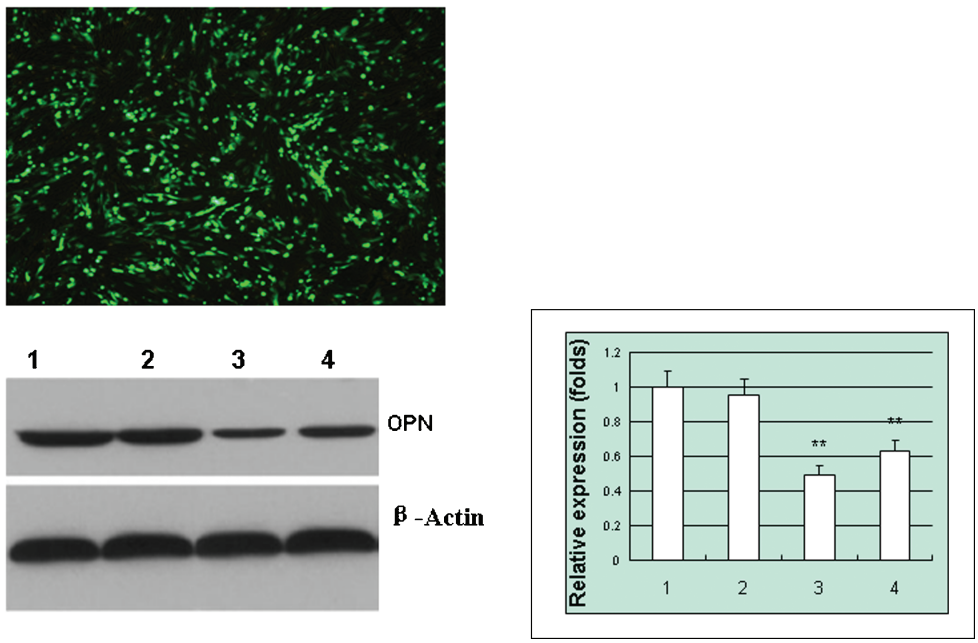

Fig. 1: Construction and screening of lentivirus vectors expressing shRNA targeting rat OPN. A. The shRNA oligo sequences designed. B. Representative infection efficiency of VSMCs by the lentivirus expressing green fluorescence protein. C. The OPN shRNA lentiviruses knockdown efficiency in the VSMCs. OPN protein expression was checked by western blot. Left panel, representative developed western blot images; right panel, quantitation of three independent experiments. (1, Control virus; 2, OPN-shRNA-1; 3, OPN-shRNA-2; 4, OPN-shRNA-3). ** $p < 0.01$, compared with mock infection group

lentiviruses. We use a recombinant virus expressing green fluorescence protein as an indicator of infection efficiency, and found rat primary cultured VSMCs are quite infectable at high efficiency by the virus we prepared (Fig. 1B). Among the infections with the viruses targeting the OPN, the shRNA-2 knockdown the expression of OPN significantly in VSMCs, determined by western blot with OPN antibody (Fig. 1C, D). So we used this shRNA-2 virus for further functional studies.

To eliminate the possible off-target by the shRNA, we constructed a scramble control shRNA according the composition of shRNA-2, which possesses highest efficiency when knockdown OPN. With this set of shRNA, we checked if the OPN shRNA can knockdown the mRNA expression of OPN specifically in VSMCs. As RT-PCR and q-PCR results shown in Fig. 2A, B, 72 h after infection by viruses, OPN shRNA virus infection leads to 80% knockdown of endogenous OPN mRNA expression, while no obvious changes were observed in the scrambled control shRNA virus when compared to mock infection. Since protein is the molecule to perform gene function, so we further tested the protein levels in the same samples, and the results showed that 72 h infection with OPN shRNA leads to 76% knockdown of endogenous OPN protein expression, however, no such effect was observed in the control virus infected VSMCs or the mock infected group (Fig. 2C).

2.2. Ang-II dose-dependently up regulates OPN protein levels in VSMCs

Vascular remodeling is caused by excessive proliferation and migration of VSMCs, which are the most characteristic pathological features in restenosis. As previous studies have reported, both Ang-II and OPN were involved in vascular remodeling and VSMCs migration. However, their relationship in these process are largely unknown. We use primary cultured rat VSMCs as *in vitro* cell model to study their relationships. We first look at OPN expression upon Ang-II treatment in VSMCs. The western blot result showed that the expression level of endogenous OPN in rat VSMCs is relatively low. The expression level of OPN is robustly increased when the VSMCs were stimulated with Ang-II. Impressively the induction of OPN expression is dose-dependent (Fig. 3). This results are consistent with studies in other VSMCs derivate from different species and suggested the function and regulatory mechanism may conserved across species.

2.3. Knockdown OPN decreases Ang-II-induced upregulation of MMP9

MMP9, integrin, E-cadherin, and TIMP-1 are proteins known to be critical for cell migration. We further tested whether the expression levels of these molecules are regulated upon Ang-II treatment and knockdown of OPN. The results showed that MMP9, integrin, and TIMP-1 displayed significant response to the Ang-II treatment, but the expression of E-cadherin did not. When cells treated with the OPN shRNA, the upregulation of MMP9 by Ang-II was abolished. However, OPN shRNA did not inhibit the integrin and TIMP-1 upregulation by Ang-II (Fig. 4). As a control, the scramble shRNA does not affect the expression of these proteins in the context of Ang-II treatment, confirming the specificity of the shRNA. Our results indicate that OPN is specifically required for Ang-II-induced MMP9 expression and suggested that they may regulate cell migration in same pathway.

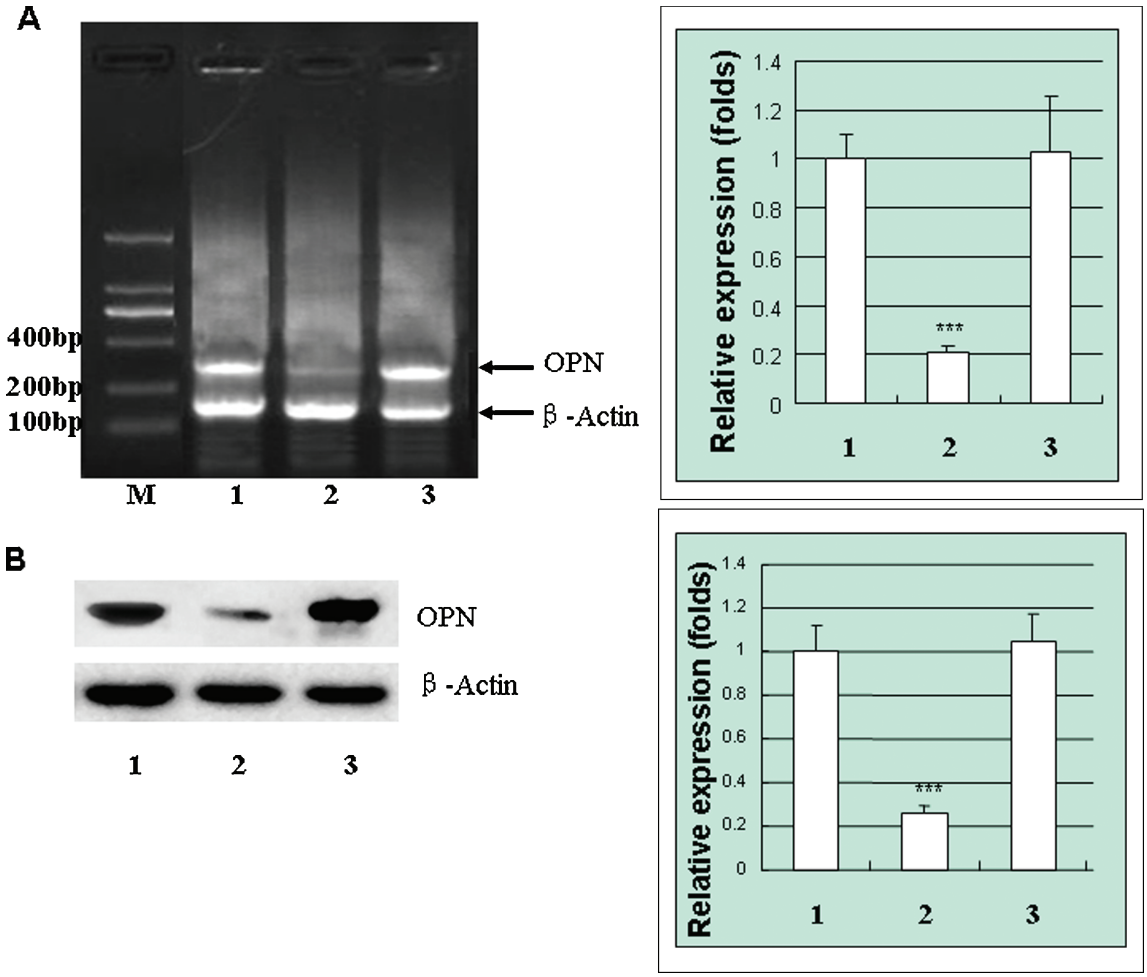

Fig. 2: OPN shRNA-2 knockdown OPN expression specifically in VSMCs. A. Left panel, Representative agarose gel of the RT-PCR products for detection of OPN mRNA expression. mRNA was made from cells at 72 h after shRNA-2 virus infection. The gene expression level was calculated as the folds of mock infection group, β-actin was used as internal control. Right panel, Statistics analysis of the agarose gel. M, DNA marker, 1, Mock infection, 2, OPN shRNA-2 virus infection, 3, scrambled shRNA-2 virus infection. B. OPN shRNA-2 knockdown the protein expression of OPN in VSMCs. Detection of OPN protein expression by western blot was conducted 72 h after virus infection. β-actin serves as loading control. 1, Mock infection, 2, OPN shRNA-2 virus infection, 3, scrambled shRNA-2 virus infection. Right panel, Statistics analysis of the Western blot. ***$p < 0.001$, compared with mock infection group

2.4. Knockdown OPN expression inhibited Ang-II induced migration of VSMCs

Our results showed that OPN is upregulated by Ang-II and required for Ang-II-induced MMP9 expression. This suggested that OPN could function in Ang-II induces cell migration of VSMCs. To prove this hypothesis, we used lentivirus to express OPN shRNA in rat VSMCs and determined the cell migration with transwell analysis. 72 hours after virus infection, a time point at which OPN suppression has been demonstrated previously (Fig. 2 and 3), cells were harvested for transwell assay. We found that 100 nM Ang-II treatment significantly increased VSMCs migration by more than three fold. However, this stimulation of migration by Ang-II was almost totally inhibited by

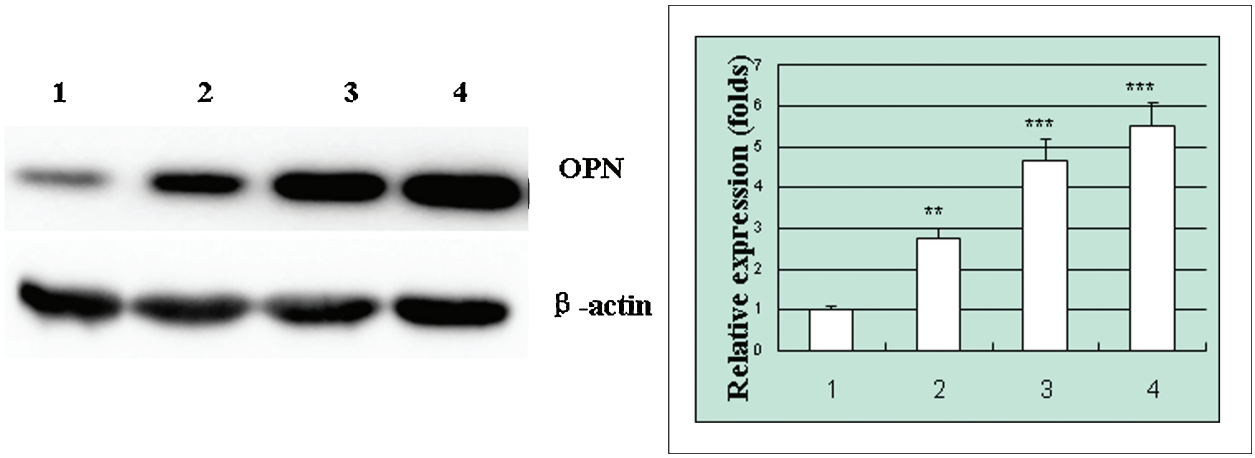

Fig. 3: Ang-II upregulates OPN protein expression in VSMCs. Rat VSMCs were treated with different dosages of Ang-II for 24 h, and the cells were harvested for OPN expression analysis by western blot, β-actin was used as loading control. 1, Vehicle control; 2: Ang-II 1 nM; 3, Ang-II 10 nM; 4, Ang-II 100 nM. A. Representative western blot analysis. B. Statistics analysis of three independent experiments. **$p < 0.01$; ***$p < 0.001$, compared with vehicle control

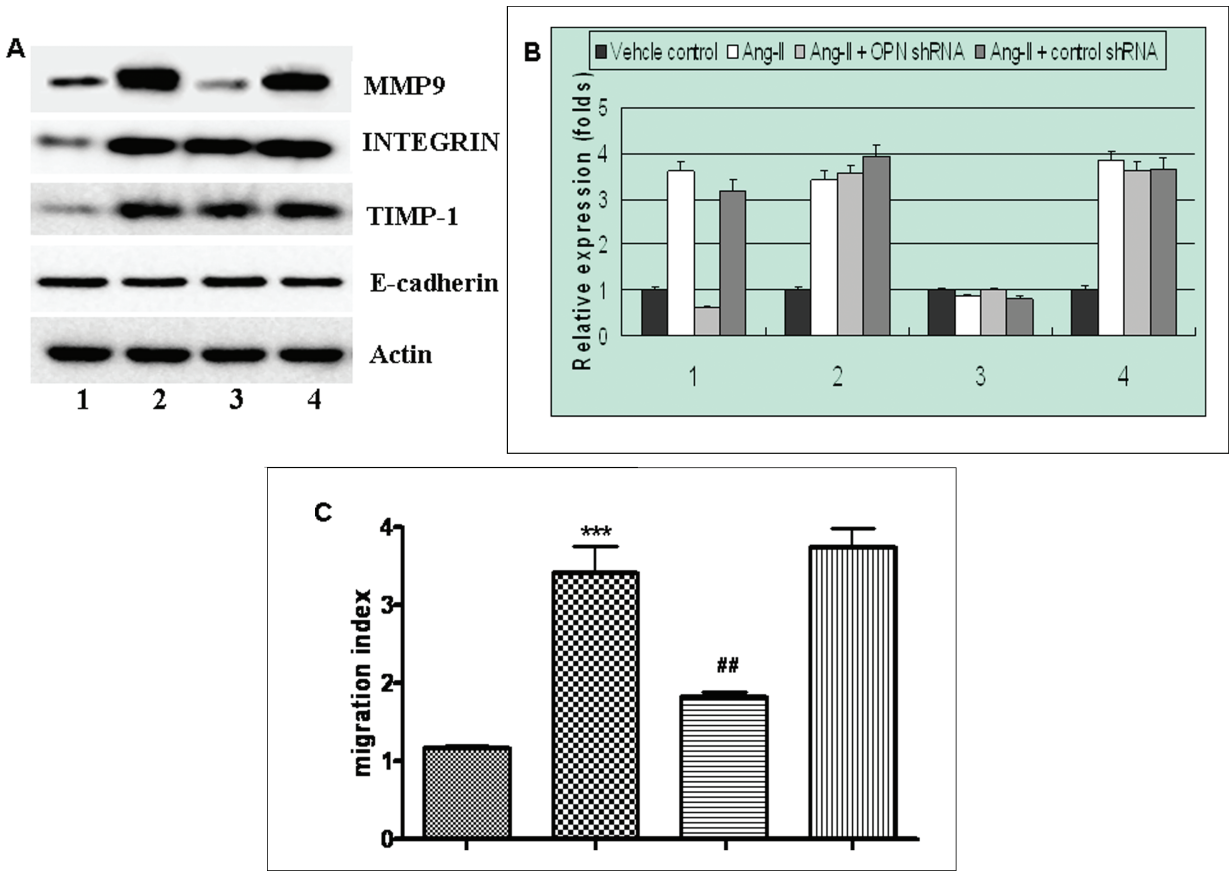

Fig. 4: Knockdown of OPN inhibited Ang-II-induced migration of VSMCs. A Knockdown OPN decreases Ang-II-induced upregulation of MMP9. VSMCs infected with OPN shRNA virus or control virus for 72 h, cells were treated with 100 nM Ang-II for 24 h, then the cells were harvested for western blot analysis, β-actin was used as loading control. B. Statistics of three independent western blot analysis.1, MMP9, 2, INTEGRIN, 3, TIMP1, 4, E-cadherin. C. Cell migration ability analysis by transwell assay. 1, Vehicle control; 2, Ang-II; 3, Ang-II + OPN shRNA; 4, Ang-II + control shRNA. ***$p < 0.001$, ##$p < 0.01$, compared with vehicle control

OPN shRNA but not the control shRNA (Fig. 5). These results demonstrated definitively that OPN is required for Ang-II to induce VSMCs migration.

3. Discussion

Migration of VSMCs into sub-intimal space plays an important role in intimal thickening in atherosclerosis and restenosis and influences the long-term patency of the venous graft (Jia 2006). Previous studies have reported that both Ang-II and OPN are involved in VSMCs migration (Chaulet et al. 2001; Daemen et al. 1991; Wu et al. 2007) while their relationships in the process keep largely unclear. Our present work confirmed that Ang-II treatment can induce expression of OPN in rat VSMCs and provided clear-cut evidence for the first time that OPN plays a critical role in Ang-II-induced cell migration of VSMCs.

Ang-II is typically involved *in* hypertension *via* vasoconstriction and stimulation of aldosterone release. It is also associated with vascular remodeling, inflammation, and oxidative stress (Schiffrin and Touyz 2004). Vascular remodeling is among the most characteristic pathological features in restenosis and is caused by excessive proliferation and migration of VSMCs (Bevan et al. 1976; Slepian et al. 1996). The Ang-II executives its function through the angiotensin type 1 receptor–mediated signaling. Signaling activation through this receptor activates several pathways, including G proteins-coupled receptor (tyrosine and non-receptor–tyrosine kinases) activation; MAP kinases activation, PKC and NAD(P)H oxidase activation and ROS generation (Mehta et al. 2007). The activation of these pathways results in various physiological and pathological effects, such as growth and migration of VSMCs (Huang et al. 2003). Besides these signaling pathways activation by Ang-II, what could be a direct target of Ang-II? The activation of the MAP kinase family ERK1/2 and JNK via Src activation, for example, is important for Ang-II-induced migration of VSMCs (Kyaw et al. 2004). Our data showed that Ang-II unregulates OPN expression in primary cultured rat VSMCs dose-dependently, and OPN is a molecule required for Ang-II-induced VSMCs migration. Our results are consistent with previous studies (Shanahan et al. 1993) and suggest that the regulatory mechanism could be conserved in mouse, human and rat.

OPN is a secreted cell-binding phosphoprotein, with functions in a number of different organs and pathological states. In normal tissue, OPN is mainly found in bone and epithelial surfaces. During pathological processes, OPN can be produced by a variety of cell types, including endothelial cells, T cells, certain tumor cells, macrophages and VSMCs (Liaw et al. 1995; Giachelli et al. 1993). High levels of OPN mRNA and protein were reported in human atherosclerotic plaque from the aorta, carotid and coronary arteries (Kwon et al. 2000; Abe et al. 2008) and it has been implicated in development of these diseases (Shanahan et al. 1994; O'Brien et al. 1995). Recently, OPN was found to be an important factors in adhesion and chemokine of VSMCs, and could interact with integrin, a multiple function molecule in adhesion and chemokine. OPN has even been suggested to act as a cytokine mediating tissue repair and inflammation (O'Regan and Berman 2000). In this study □ we employed RNA interference (RNAi), which is a highly specific and effective tool in silencing gene (Okamura et al. 2008; Rossi 2008; Xiang et al. 2006), to explore the OPN function in VSMCs migration. The results demonstrated that OPN could regulate the expression of migration markers MMP9 and is required for Ang-II to induce VSMCs migration.

In summary, our data demonstrated that OPN mediated the Ang-II induced VSMCs migration. Extending the current studies to animal models will further improve our understanding of roles and mechanisms for OPN and Ang-II in VSMCs migration and related diseases development and may provide a basis for the prevention and treatment of these diseases.

4. Experimental

4.1. Cell culture

The study was approved by the Ethics Committee of the Nanjing First Hospital. Rat VSMCs were prepared from thoracic aortas of Wistar rats as previously described (Chaulet et al. 2001). Cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen), 150 mmol/L HEPES, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies) at 37 °C in a humidified atmosphere of 5% CO2. Cells were identified by the immunocytochemical method with antibody against α-smooth muscle actin. VSMCs from passages 3 to 5 were used throughout the study.

4.2. Total RNA isolation and quantitative RT-PCR analysis

Total RNA was extracted from the cultured cells using Trizol reagent (Invitrogen). The purity and quantity of the RNA preparation were determined by measuring the optical densities at 260 and 280 nm. To estimate the mRNA levels of OPN and actin, quantitative RT-PCR was performed using the LightCycler system (Roche Diagnostics). Total RNA was reversely transcribed with random hexamers using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV). The reaction was processed as follows: incubation of samples at 25 °C for 10 min and then at 42 °C for 60 min, followed by 99 °C for 5 min and cooling to 4 °C for 5 min. The quantitative PCR was performed with the LightCycler instrument using LightCycler FastStart DNA Master SYBR Green I, LightCycler-Primer Set, Opn, sense: 5′-TGAAGCCTGACCCATCTC-3′, anti-sense: 5′-CGTAAGCCAAGCTATCACC-3′; Actin, sense: 5′-GAGGGAAATCGTGCGTGAC-3′, anti-sense: 5′-GCATCGGAACCGC TCATT-3′. PCR conditions: 94□ for 4 min; 94□ for 1 min, annealing at 52□ (Opn), or 58□ (Actin) for 30 s, 72□ for 30 s, 35 cycles; 72□ for 10 min. Products were resolved by 1% agarose gel, and bands were visualized by ethidium bromide staining. Densitometric analysis of bands was performed using BioImaging Systems (UVP, CA, USA). The expression of mRNA levels were measured as the ratio of each mRNA and the β-actin mRNA.

4.3. RNA interference for knockdown of rat OPN in VSMCs

RNAi-mediated knockdown of rat OPN was performed previously described (Yin et al. 2009). The targeted sequence resides within the open reading frame of the rat OPN gene (accession: M99252). Three shRNAs targeting the open reading frame of the rat OPN gene (accession: M99252) were designed using Ambion siRNA finder (http://www.ambion.com/techlib/misc/siRNA_finder.html). The DNA encoding the shRNA for rat OPN were of sequences: sense: 5′-CCGGAAGGATGAACCAAGCGTGGAACTCGAGTTCCACGCTTGGT TCATCCTTTTTTTG-3′, antisense: 5′-AATTCAAAAAAAGGATGAA CCAAGCGTGGAACTCGAGTTCCACGCTTGGTTCATCCTT-3′. The synthesized DNA encoding the shRNA were cloned into the pLKO.1 lentivirus vector by digested with Age I and EcoRI. The lentivirus was packaged according to the standard protocol (Stewart et al. 2003). The knockdown efficiency of OPN expression in the VSMCs by OPN shRNA lentiviruses was checked by RT-PCR and western blot.

4.4. Migration assay

Cell migration was performed with the Transwell (Costar) system, which allows cells to migrate through a 8-mm pore size polycarbonate membrane. Briefly, cells were trypsinized, washed, and resuspended in serum-free DMEM. This suspension was added to the upper chamber of Transwell system. The lower chamber was filled with serum-free DMEM containing Ang-II in appropriate concentrations or not containing Ang-II. For checkerboard analysis, appropriate concentrations of Ang-II were added in the lower, upper, or both chambers. After a 6-hour stimulation by Ang-II, filters were removed, and cells remaining on the upper surface of the membrane (i.e., that had not migrated through the filter) were removed with a cotton swab. Then, membranes were washed with PBS, and cells present beneath the membrane were fixed with cold methanol for 15 min and stained with Hemalun. Cells were counted in 10 high-power microscope fields. Analysis was performed on 3 wells for each condition, and each experiment was repeated 3 times.

4.5. Western blot analysis

VSMCs were lysed directly in the dish at 4 °C for 15 min with RIPA buffer (50 mmol/L Tris-HCl [pH 7.5], 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing phosphatase and protease inhibitors (1 mmol/L Na_3VO_4, Sigma-Aldrich; 1 mmol/L AEBSF, Interchim). Cell debris was eliminated by a 2-minute centrifugation at 10000 g. The protein concentration of the cell lysate was determined by the microBCA method (Pierce). Proteins were separated by SDS-PAGE on a 10% acrylamide gel under reducing conditions and blotted onto polyvinylidine difluoride membrane (Millipore). Membranes were probed with primary antibody, and then with an anti-mouse IgG-peroxidase conjugate (Amersham Pharmacia). Signals were visualized by chemoluminescence with an enzyme-linked chemiluminescence kit (Amersham Pharmacia). The homogeneity of sample loading was checked by probing with anti–β-actin antibody (Santa Cruz). The monoclonal antibody OPN (Santa Cruz) was used for OPN detection.

4.6. Statistics

ANOVA and unpaired Student's t test were performed for statistical analysis. Probability values of $P < 0.05$ were considered statistically significant. Data are expressed as mean ± SD.

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