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Original Article

Transcription factor Mef2C regulates the migration of vascular smooth muscle cells

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Abstract: Accumulating evidence demonstrates that abnormal migration of vascular smooth muscle cells (VSMCs) plays an important role in the pathophysiology of many vascular diseases. Our study aimed to investigate the effect of the transcription factor, Mef2C, on the migration of primary VSMCs. Cell migration was measured using the transwell assay method. The mRNA and protein expression of the indicated genes was determined by real time PCR and western blotting, respectively. Results showed that VEGF promoted Mef2C expression in VSMCs, which was suppressed by the p38 MAPK inhibitor. Moreover, Mef2C up-regulation had no obvious effect on VSMC migration, but Mef2C overexpression alone or combined with a p38 MAPK inhibitor inhibited VEGF-activated VSMC migration. Furthermore, hypoxic treatment suppressed the VEGF-induced expression of Mef2C and repressed the migration of VSMCs overexpressing Mef2C, which was suppressed by a MAPK signaling inhibitor. In addition, VEGF treatment increased the expression of myocardin, SMA, and SM22, which was repressed by addition of MAPK inhibitor under both normoxic and hypoxic conditions. Our study demonstrates that Mef2C regulates VSMC migration and that Mef2C may be a potential therapeutic target for cardiovascular diseases.

Keywords: Mef2C, migration, VSMC, VEGF

Introduction

A growing body of literature suggests that deregulated proliferation and migration of vascular smooth muscle cells (VSMCs) play important roles in the pathogenesis of many vascular diseases such as atherosclerosis and restenosis after angioplasty [1, 2]. In healthy blood vessels, VSMCs reside within the media and remain quiescent. In response to atherogenic factors, VSMCs proliferate and migrate from the media to the intima, which subsequently induces intimal hyperplasia and vascular stenosis [3]. Both *in vitro* and *in vivo* studies indicated that inhibition of VSMC proliferation and migration can alleviate intimal hyperplasia [4, 5]. Although the molecular mechanism underlying vascular diseases is not fully elucidated, hypoxia is considered one of the principal contributors to the proliferation and migration of VSMCs [6, 7].

The transcription factor Mef2C is a member of the myocyte-enhancer factor 2 family, including four vertebrate MEF2 genes, *MEF2A*, *MEF2B*, *MEF2C*, and *MEF2D* [8, 9]. It presents homology with a MCM1, Agamous, Deficiens, serum response factor and an adjacent motif known as the MEF2 domain [10, 11]. Increasing evidence shows that Mef2C is critically important in cell growth and migration in the cardiovascular system [12, 13]. In endothelial cells, Mef2C is specifically induced by VEGF-A, and thus, is implicated in the control of angiogenesis. In addition, hypoxic treatment reduces the VEGF-A-mediated up-regulation of Mef2C in endothelial cells [14]. However, the regulatory role of Mef2C in VSMCs has not yet been elucidated. Herein, we investigated the role of Mef2C in VSMCs both under normoxic and hypoxic conditions.

Materials and methods

Cell culture

VSMCs were isolated from thoracic aorta explants of Sprague-Dawley (SD) rats. When the cells formed a confluent monolayer, they were passaged and cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in humidified atmosphere of 95% air and 5% CO_2 at 37°C.

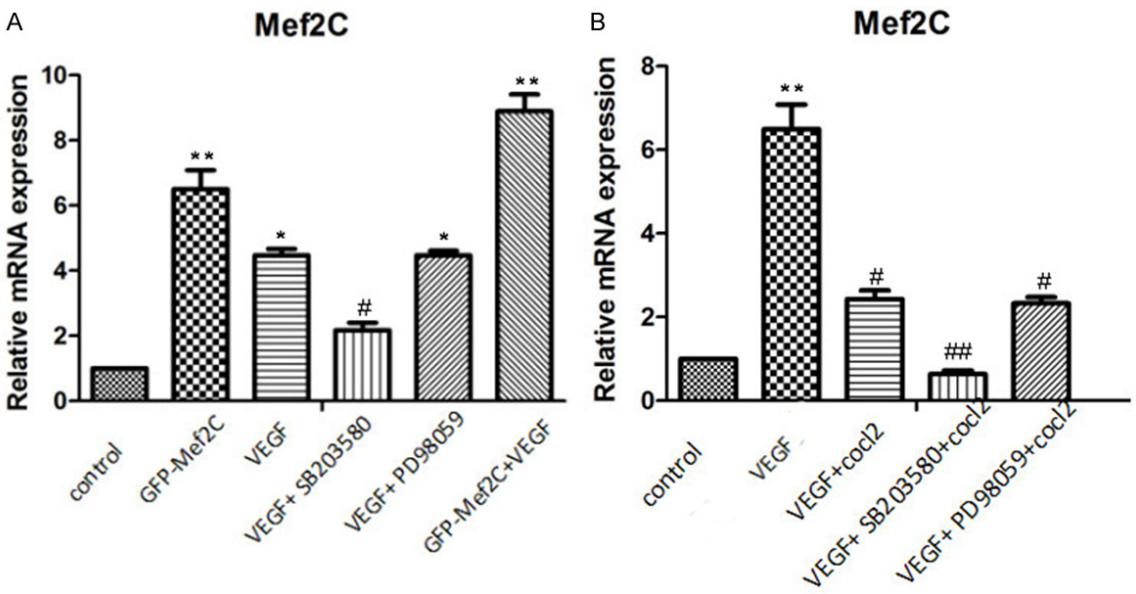


Figure 1. Mef2C expression in VSMCs under normoxic or hypoxic conditions. VSMCs were transfected with a Mef2C-encoding plasmid or treated with VEGF alone or in combination with SB203580 and PD98059. Mef2C mRNA levels in VSMCs under normoxic (A) or hypoxic (B) conditions were determined by real time PCR. *P<0.05, **P<0.01, compared to control; #P<0.05, ##P<0.01, compared to VEGF.

Cell migration assay

VSMCs (5×10^4 cells/mL) were plated in the upper chamber in DMEM, while DMEM containing the indicated drugs was placed in the chamber below. Cells that migrated to the underside of the filters were fixed with methanol and stained with hematoxylin after incubation for 24 h at 37°C. Finally, the migrating cells were counted from five high-power fields (×400) per well.

Real-time PCR

The mRNA levels of each gene were measured by using real time PCR. The primers were as follows: GAPDH-F: TGTGAACGGATTTGGCCGTA; GAPDH-R: GATGGTGATGGGTTTCCCGT; Mef2C-F: AATGGCCAATCCCGCCG; Mef2C-R: AAGTGTCTGTGACTGCGAGG; SMA-F: ACCATCGGGAATGAACGCTT; SMA-R: CTGTCAGCAATGCCTGGGTA; SM22-F: TGGCGTGATTCTGAGCAAGT; SM22-R: GTTCTTGGTCACGGCCAAAC.

Values were normalized to the *GAPDH* mRNA level used as an internal control and measured by using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Protein expression was determined by western blotting. Briefly, cells were lysed, and protein samples were electrophoresed through a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. The membrane was incubated with the myocardin (1:1000) antibody (Abcam, USA) overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, protein bands were detected with an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA).

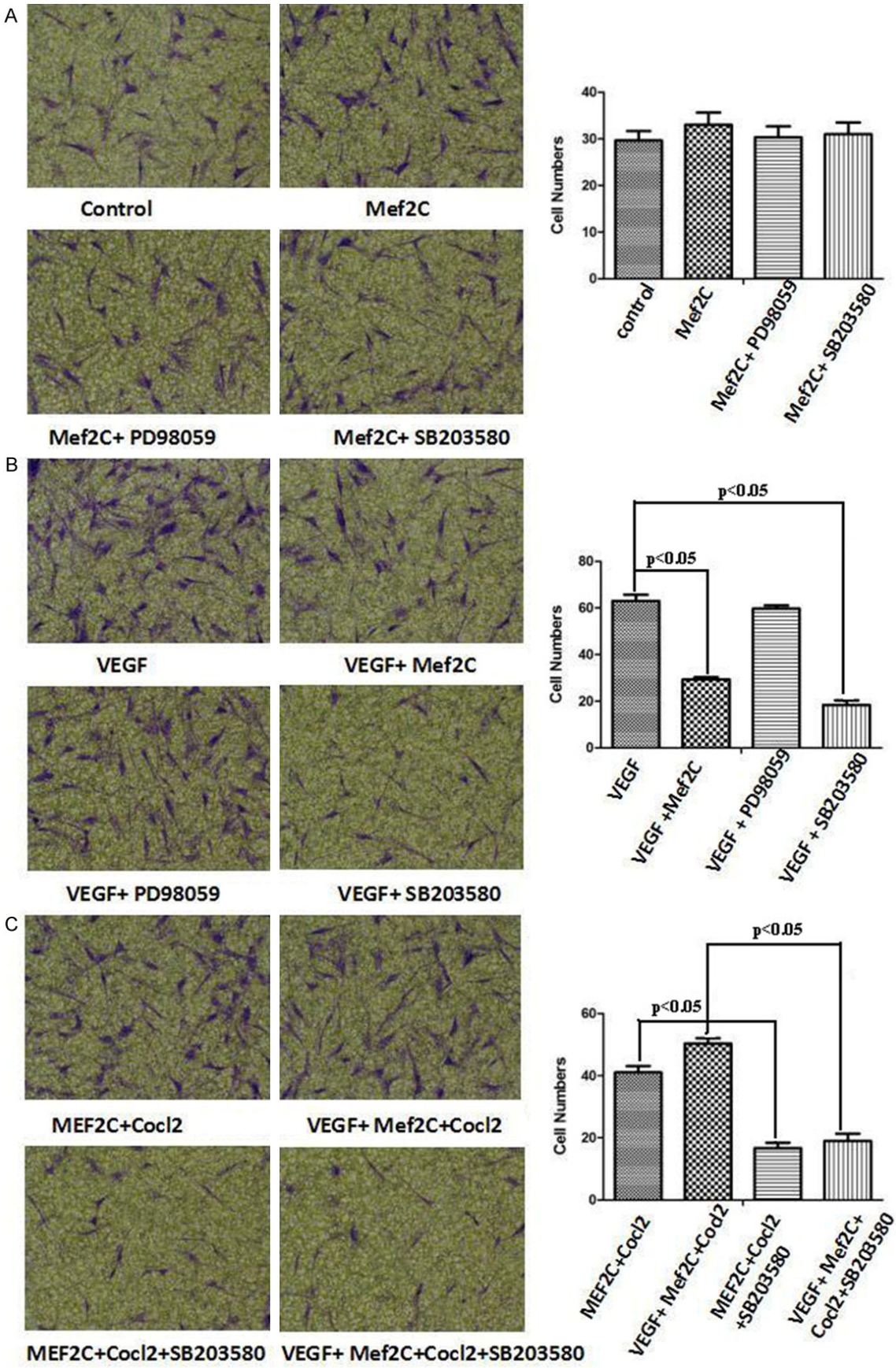
Statistical analysis

Data are expressed as the means ± SD. The SPSS software (SPSS, Chicago, IL, USA) was used to perform the statistical analyses. Differences between multiple groups were analyzed by one-way ANOVA. P<0.05 was considered statistically significant.

Results

Mef2C expression levels in VSMCs under normoxic or hypoxic conditions

In order to explore the role of Mef2C in VSMCs, efficient up-regulation of Mef2C was validated by real time PCR (**Figure 1A**). In addition, VSMCs were treated with VEGF, and Mef2C


A
Control
Mef2C
Mef2C+ PD98059
Mef2C+ SB203580
Cell Numbers
40
30
20
10
0
control
Mef2C
Mef2C+ PD98059
Mef2C+ SB203580
B
VEGF
VEGF+ Mef2C
VEGF+ PD98059
VEGF+ SB203580
p<0.05
p<0.05
Cell Numbers
80
60
40
20
0
VEGF
VEGF +Mef2C
VEGF + PD98059
VEGF + SB203580
C
MEF2C+Cocl2
VEGF+ Mef2C+Cocl2
MEF2C+Cocl2+SB203580
VEGF+ Mef2C+Cocl2+SB203580
p<0.05
p<0.05
Cell Numbers
60
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MEF2C+Cocl2
VEGF+ Mef2C+Cod2
MEF2C+Cocl2 +SB203580
VEGF+ Mef2C+ Cocl2+SB203580

Figure 2. Effects of Mef2C overexpression on VSMC migration. Transwell assay was used to measure the migratory ability of VSMCs transfected with a Mef2C-encoding plasmid with or without SB203580 and PD98059 (A). The migration of VSMCs was measured under normoxic (B) or hypoxic (C) conditions.

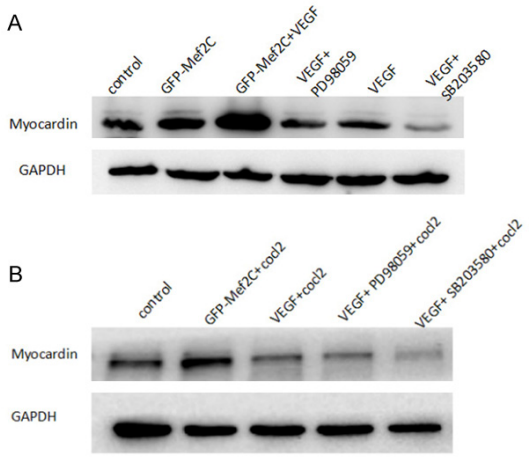

Figure 3. Myocardin expression in VSMCs under normoxic or hypoxic conditions. VSMCs were transfected with a Mef2C-encoding plasmid or treated with VEGF alone or in combination with SB203580 and PD98059. Mef2C protein expression in VSMCs under normoxic (A) or hypoxic (B) conditions was determined by western blotting.

expression was determined by real time PCR. VEGF treatment increased Mef2C expression in VSMCs. Moreover, treatment of VSMCs with a p38 MAPK inhibitor, SB203580, suppressed the VEGF-induced expression of Mef2C (**Figure 1A**). Meanwhile, the MEK inhibitor, PD98059, had no inhibitory effect on Mef2C expression in VSMCs in the presence of VEGF.

Next, we measured Mef2C expression in VSMCs under hypoxic conditions. $CoCL_2$ treatment suppressed the VEGF-mediated expression of Mef2C (**Figure 1B**). Moreover, addition of SB203580 further repressed Mef2C expression in VSMCs.

Effects of Mef2C overexpression on VSMC migration under normoxic or hypoxic conditions

First, we determined the role of Mef2C in VSMC migration using the transwell assay. Ectopic expression of Mef2C alone or combined with SB203580 or PD98059, had no obvious effect on VSMC migration (**Figure 2A**). However, up-regulation of Mef2C alone or combined with SB203580 inhibited VSMC migration in the presence of VEGF under normoxic conditions (**Figure 2B**). In addition, hypoxic treatment promoted the migration of VSMCs overexpressing Mef2C, which was suppressed by addition of SB203580 (**Figure 2C**).

Expression of Mef2C downstream genes in VSMCs under normoxic or hypoxic conditions

We examined the expression of myocardin, a Mef2C downstream gene, by western blotting. Results showed that addition of VEGF alone or combined with Mef2C up-regulation promoted the expression of myocardin in VSMCs (**Figure 3A**). Moreover, addition of SB203580, a p38 MAPK inhibitor, suppressed the induced expression of myocardin in VSMCs (**Figure 3A**).

In addition, we detected the expression of myocardin in VSMCs under hypoxic conditions. Data showed that $CoCL_2$ treatment inhibited VEGF-activated expression of myocardin (**Figure 3B**). Furthermore, combined treatment with SB203580 further inhibited the expression of myocardin in VSMCs (**Figure 3B**).

We also examined several other Mef2C downstream genes such as *SMA* and *SM22*. Real time PCR showed that VEGF treatment increased the expression of *SMA* (**Figure 4A**) and *SM22* (**Figure 4B**), which was repressed by addition of SB203580 both under normoxic and hypoxic conditions.

Discussion

The proliferation of VSMCs plays a central role in the pathogenesis of vascular diseases such as atherosclerosis, hypertension, and restenosis after angioplasty. The mechanisms underlying VSMC proliferation in these vascular diseases remain unclear. Herein, we assessed the expression profile of Mef2C and its downstream genes in VSMCs under normoxic or hypoxic conditions.

The transcription factor MEF2 family, composed of four members, is involved in the control of muscle development via protein-protein interactions with other transcription factors. MEF2s are widely expressed in many tissues and organs where they control the activation

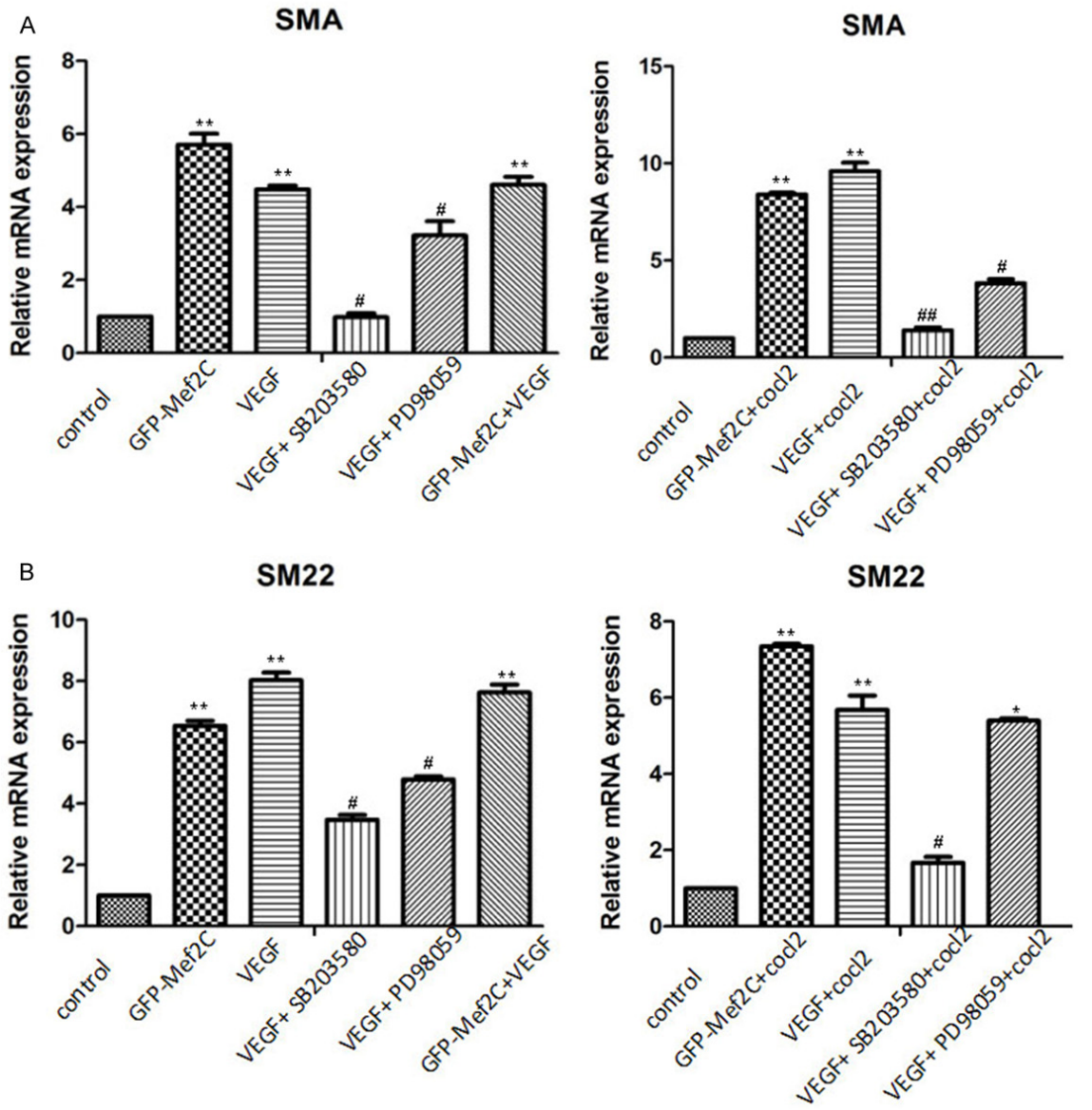

Figure 4. Expression of Mef2C downstream genes in VSMCs. VSMCs were transfected with a plasmid encoding Mef2C or treated with VEGF alone or in combination with SB203580 and PFD98059. The mRNA levels of *SMA* (A) and *SM22* (B) in VSMCs under normoxic or hypoxic conditions were determined by real time PCR. *P<0.05, **P<0.01, compared to control; #P<0.05, ##P<0.01, compared to VEGF.

of cellular differentiation processes [15-17]. Genetic studies indicated that Mef2c is required for thick filament formation in nascent muscle fibers, and thus, plays a critical role in myofiber maturation [18]. In addition, MEF2C contributes to the regulation of the proliferation and cell cycle of skeletal muscle cells in a phosphorylation-dependent manner [19]. In the cardiovascular system, a number of studies indicated that MEF2C is implicated in cell proliferation, migration, and cellular homeostasis in both endothelial cells and VSMCs and plays important roles in modulating their phenotypes [12-14]. In our study, VEGF increased the expression of Mef2C in VSMCs, which was suppressed by a p38 MAPK inhibitor. Moreover, transfection with a Mef2C-encoding plasmid alone or combined with SB203580 or PD98059 had no obvious effect on VSMC migration. However, up-regulation of Mef2C alone or combined with a p38 MAPK inhibitor inhibited VSMC migration in VEGF-treated cells, suggesting that the MAPK pathway may be involved in these cellular events.

Tissue hypoxia is an essential feature of chronic inflammatory diseases. In the cardiovascular system, when arterial wall thickens and the blood-diffusion capacity is low in atherosclerotic lesions, hypoxia plays a key role in the development of atherosclerosis [20-22]. The cellular effects of hypoxia are primarily mediated by the hypoxia-inducible transcription factor-1 (HIF-1) [22]. We found that hypoxic treatment repressed the VEGF-induced expression of Mef2C, and p38 MAPK inhibitor further inhibited Mef2C expression under hypoxic conditions. Moreover, hypoxic treatment promoted the migration of VSMCs overexpressing Mef2C, which was suppressed by a MAPK signaling inhibitor.

Furthermore, we examined the expression of Mef2C downstream genes, including myocardin, *SMA*, and *SM22*. The myogenic coactivator myocardin is specifically expressed in contractile smooth and cardiac muscle tissues in adulthood [23, 24]. Previous studies indicated that myocardin negatively regulates VSMC proliferation and migration [14]. In our study, VEGF treatment increased the expression of several Mef2C downstream genes, myocardin, *SMA*, and *SM22*. In addition, SB203580 administration repressed the activated expression of such genes induced by VEGF both under normoxic and hypoxic conditions.

Taken together, in the presence of VEGF, Mef2C up-regulation inhibited VSMC migration and promoted the expression of contractile phenotype-related genes, including myocardin, *SMA*, and *SM22*. Our study demonstrates that the transcription factor Mef2C negatively regulates VSMC migration via the MAPK signaling pathway. Therefore, Mef2C may serve as a potential therapeutic target for the treatment of cardiovascular diseases.

Disclosure of conflict of interest

None.

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