BMP-2 overexpression augments vascular smooth muscle cell motility by upregulating myosin Va via Erk signaling

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BMP-2 Overexpression Augments Vascular Smooth Muscle Cell Motility by Upregulating Myosin Va via Erk Signaling

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Background. The disruption of physiologic vascular smooth muscle cell (VSMC) migration initiates atherosclerosis development. The biochemical mechanisms leading to dysfunctional VSMC motility remain unknown. Recently, cytokine BMP-2 has been implicated in various vascular physiologic and pathologic processes. However, whether BMP-2 has any effect upon VSMC motility, or by what manner, has never been investigated.

Methods. VSMCs were adenovirally transfected to genetically overexpress BMP-2. VSMC motility was detected by modified Boyden chamber assay, confocal time-lapse video assay, and a colony wounding assay. Gene chip array and RT-PCR were employed to identify genes potentially regulated by BMP-2. Western blot and real-time PCR detected the expression of myosin Va and the phosphorylation of extracellular signal-regulated kinases 1/2 (Erk1/2). Immunofluorescence analysis revealed myosin Va expression locale. Intracellular Ca$^{2+}$ oscillations were recorded. Results. VSMC migration was augmented in VSMCs overexpressing BMP-2 in a dose-dependent manner. siRNA-mediated knockdown of myosin Va inhibited VSMC motility. Both myosin Va mRNA and protein expression significantly increased after BMP-2 administration and were inhibited by Erk1/2 inhibitor U0126. BMP-2 induced Ca$^{2+}$ oscillations, generated largely by a “cytosolic oscillator”. Conclusion. BMP-2 significantly increased VSMCs migration and myosin Va expression, via the Erk signaling pathway and intracellular Ca$^{2+}$ oscillations. We provide additional insight into the pathophysiology of atherosclerosis, and inhibition of BMP-2-induced myosin Va expression may represent a potential therapeutic strategy.

1. Introduction

Recent studies demonstrate that BMP-2, a cytokine of the transforming growth factor-β superfamily, plays an important role in both physiological and pathophysiological vascular development [1, 2]. Genetically manipulated BMP-2 deficient mice die between days 7 and 10 of life from cardiac defects prior to bone formation, suggesting the significant cardiovascular importance of BMP-2 [3]. Vascular smooth muscle cells (VSMCs) are a significant source of BMP-2 [4]. VSMC migration from the vascular media to the intima is pivotal in atherosclerosis, playing a central role in the genesis of atherosclerotic plaques and restenotic lesions [5, 6].

VSMC migration is dependent upon cellular motility, driven by cycles of actin polymerization, cellular adhesion, and actin-myosin contraction. Myosins are a large family of structurally diverse actin-dependent molecular motors. All myosins utilize energy from ATP hydrolysis to generate force for unidirectional movement along actin filaments and are regarded as the most essential proteins driving cellular migration [7–9]. The myosin superfamily consists of both conventional and unconventional myosins [10, 11].
Found in various organelles, unconventional myosins are involved in RNA and protein transport, cellular movement, signal transduction, cellular morphology maintenance, and membrane trafficking [12].

The unconventional myosin Va is an actin-based motor protein that transports intracellular cargos and can bundle actin in vitro. The relationship and function of myosin Va pertaining to cytoskeletal aspects, cellular morphology, filopodia motility, and neurite extension have been reported [13–15]. Recently, myosin Va was implicated in human cancer dissemination [16, 17]. However, the function of myosin Va within cardiovascular disease remains unclear. Whether BMP-2 affects VSMCs migration via myosin Va, and if so, by what mechanism, has never been determined. We investigate the role of BMP-2 as a potential regulator of myosin Vain VSMCs and dissect the involved underlying mechanisms.

2. Material and Methods

The study was carried out in accordance with the institutional review board (IRB) approval. The study protocol was approved by the institutional ethics committee and IRB of the Beijing Anzhen Hospital, Affiliate of Capital Medical University.

2.1. Cell Culture. Rat vascular smooth muscle cells were primary-cultured via explant method and grown in RPMI-1640 supplemented with 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37°C in 5% CO2 atmosphere. rVSMCs were utilized for experimentation at passage 4–8 [18].

2.2. Recombinant Ads. ViralpAV.EX1d-CMV constructs containing the rat BMP-2/myc/IRES/EGFP expression were from Cyagen Biosciences Inc. (Guangzhou, China). HEK293 cells were transfected by viruses via lipofectamine. The culture medium supernatant was collected and purified by double cesium chloride gradient ultracentrifugation [19]. The viruses were titrated by plaque assay in HEK293 cells. Physical viral particle concentration (vp/mL) was determined spectrophotometrically by wavelength (260 nm) absorbance [20]. Viruses were stored at ~80°C until use.

2.3. Cell Motility. Cells were plated on 60 mm glass microwell dishes and cultured overnight in RPMI-1640 containing 10% FBS. Cellular movements were monitored by Leica SP5 inverted microscopy. Video images were collected by CCD camera (model 3000; Leica) at 15-minute intervals for 6 hours, digitized, and stored as image stacks via Image J 1.41i software (National Institutes of Health, http://rsb.info.nih.gov/ij/). Image stacks were converted to QuickTime movies. Nuclei positions were tracked to quantify cell motility, and velocities were calculated in μm at 15-minute intervals by the same software [21].

2.4. Boyden Chamber Assay. A 2 × 10^4 aliquot of each cell type was plated onto a 24-well BioCoat Invasion Chamber (BD Biosciences, USA) and cultured for 24 hours. Cells were fixed by methanol and stained by crystal violet. Five cell fields were counted at approximately 40-fold magnification [22].

2.5. Wounding Assay. Cells were seeded in 35 mm culture dishes (density 2 × 10^4 cells per well). An incision was made after 24 hours in the central region of confluence in the culture dish. After an additional 48 hours, the dish was carefully washed to remove detached cells. Fresh medium was added. Cultures were observed at the time of incision and after 48 hours. Phase-contrast microscopy pictures were taken of 6 separate fields of the incised region. The distance between two broad edges of cells was measured and analyzed by Leica LAF software.

2.6. RNA Extraction, cRNA Preparation, and Gene Chip Array. High quality rat RNA from BMP-2 infected VSMCs and control cells were obtained by gel electrophoresis (18S and 28S bands) and absorbance spectroscopy (240–320 nm). Briefly, 8 μg of total RNA was reverse-transcribed by oligo (dT) primer coupled to a T7 RNA polymerase binding site. Biotinylated complementary RNA (cRNA) was then synthesized from the resulting complementary DNA (cDNA) via T7 polymerase. 25 μg of biotinylated cRNA was randomly sheared and hybridized for 16 hours to Affymetrix gene chips.

The Affymetrix microarrays (Arabidopsis ATH1 genome array) contain 22,810 probe sets, representing approximately 80% of the gene sequences on a single array. Labeling and hybridization on the ATH1 microarrays (one sample per chip) were performed according to manufacturer’s instructions (http://www.affymetrix.com/estore/). The probe arrays were scanned and further analyzed with Genespring software (ver 5.0; Silicon Genetics). Normalization per gene and per chip of the log2 values was performed to allow comparison of three independent replicates performed for each experiment set. Genes were considered to be up- or downregulated if the ratio between BMP-2 and control cells was, respectively, greater than 2 or less than 0.5.

2.7. RT-PCR. Gene expression was measured by reverse transcription kit (Promega, WI, USA). Briefly, after the RT of 3 μg of total RNA, cDNA was synthesized. The RT products were subjected to PCR with 2720 thermal cycles (abi) and qRT-PCR via real-time PCR system Fast 7500 (abi) with the primer sets listed in Table I(a). The cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control. The semiquantitative RT-PCR consisted of 30 cycles of 94°C, 57.5°C, and 72°C (each for 30 seconds).

2.8. Western Blot Analysis. Cellular pellets were lysed by RIPA buffer. 30 μg of total protein samples was separated by 10% SDS-PAGE and electrophoretically transferred to PVDF membranes. Membranes were blocked by 5% nonfat milk in TBPS and incubated for 1 hour at room temperature with primary antibody (see Table I(b)). Secondary antibody HRP-IgG was applied for 1 hour. After three additional TBPS washes, signals were detected by enhanced chemiluminescence (Amersham Bioscience).
2.9. Knockdown of Myosin Va, Expression by siRNA. Three specific sequences of small interfering RNA (siRNA) targeting different regions of rat MYO5a mRNA sequence were designed (RiboBio Co., Ltd, China. 124171130126): siRNA-1: 5'-TATATGCTGCACCTGTACC-3'; siRNA-2: 5'-ACCTACGGAACCTGACAT-3'; siRNA-3: 5'-GGCTGAACTAACCAAGGACT-3'.

2.10. Immunofluorescence Analysis. Cells were seeded upon cover slips at the bottom of culture dishes until subconfluence and were then fixed with 5% acetic acid/95% ethanol (v/v) for 20 minutes. To block nonspecific reactions, 5% nonfat milk was added for 30 minutes, and then anti-MYO5a polyclonal antibody (1:250 dilutions in PBS) was administered at room temperature for 1 hour. After washing, anti-goat IgG, FITC fluorescein (1:800) was added for 30 minutes, and then anti-MYO5a polyclonal antibody (1:250 dilutions in PBS) was administered at room temperature for 1 hour. DAPI (1:2000 in PBS; DABCO, Sigma) stained for total nuclei. Imaging was performed by Leica SP5 laser scanning confocal microscope.

2.11. [Ca^{2+}]i Determination Assay. Cells were treated with BMP-2 for 48 hours in dye-free media supplemented with 10% FBS, centrifuged prior to resuspension in calcium-free modified Tyrode buffer (145 mM NaCl, 5.6 mM KCl, 100 μM EGTA, 1.0 mM MgCl2, 10 mM glucose, and 5.0 mM HEPES, pH 7.2), and incubated for an additional 15 minutes. Fluorescence was measured at room temperature via Leica SP5 confocal imaging system. Two-dimensional confocal images were taken at 1.5 second time intervals. Fluo-4 AM (Dojindo, Japan) was excited at wavelength 488 nm, and fluorescence was measured at room temperature via Leica SP5 confocal imaging system. Two-dimensional confocal images were taken at 1.5 second time intervals. Fluo-4 AM (Dojindo, Japan) was excited at wavelength 488 nm, and fluorescence was measured at room temperature via Leica SP5 confocal imaging system. Two-dimensional confocal images were taken at 1.5 second time intervals. Fluo-4 AM (Dojindo, Japan) was excited at wavelength 488 nm, and fluorescence was measured at room temperature via Leica SP5 confocal imaging system. Two-dimensional confocal images were taken at 1.5 second time intervals. Fluo-4 AM (Dojindo, Japan) was excited at wavelength 488 nm, and fluorescence was measured at room temperature via Leica SP5 confocal imaging system. Two-dimensional confocal images were taken at 1.5 second time intervals.
than those infected by vector alone. Swifter VSMC migration resulted in larger spanning stellate "star" formations (Figure 1(b)). The average distance travelled by a moving single cell in a six-hour observation period was confirmed every 15 minutes (BMP-2 travelled 0.24 ± 0.2 μm versus control: 0.12 ± 0.1 μm, Figure 1(c)). Migratory cell mounts of rat VSMCs overexpressing BMP-2 were compared to control cells in a Boyden chamber assay (Figure 1(d)). BMP-2 overexpression increased cellular intensity 130%, compared to vector alone (P < 0.01, Figure 1(e)). The wounding assay demonstrated BMP-2 influenced cellular population movement as well within 48 hours (BMP-2: 93.3 ± 17.8 μm versus control: 43.7 ± 16.7 μm, P < 0.01, Figure 1(f)).

3.2. BMP-2 Increases Motility of Both Unicellular and Multicellular VSMC Populations in Dose-Dependent Manner. VSMCs were treated with varying BMP-2 concentrations (ranging from 50 to 500 ng/mL) via Boyden chamber for
Figure 2: Dose-dependent effect of BMP-2 upon cellular motility. A Boyden chamber assay was performed upon rat VSMCs treated by exogenous BMP-2 concentrations. * Student’s test: P < 0.01 versus control.

48 hours. Figure 2 demonstrates VSMCs exhibit a dose-dependent migratory effect in response to BMP-2 concentrations exceeding 100 ng/mL. Time-lapse video microscopy and wounding assay, respectively, demonstrated unicellular and multicellular VSMC populations responded in dose-dependent manner to BMP-2 (Table 2).

3.3. Identification of Genes Regulated by BMP-2 Overexpression. To gain mechanistic insight concerning BMP-2-mediated VSMC migration facilitation, microarray analyses were performed to determine global gene expression changes in BMP-2 overexpressing VSMCs. In VSMCs overexpressing BMP-2, 554 genes were downregulated, and 437 genes were upregulated. Not surprisingly, genes involved in the BMP signaling pathway (such as Fstl1, Fstl3, Smad1, and Msx1) were among those upregulated during BMP-2 overexpression. Although our gene chip array determined myosin Va was consistently upregulated during BMP-2 overexpression, myosin Vb and Vc were not detected. In addition, expression of smooth muscle alpha-actin, a characterizing marker of the systole phenotype, remained unchanged. The mRNA and protein levels of OPN and MGP, characterizing markers of the diastolic phenotype, were markedly altered (Figures 3(a) and 3(b)).

3.4. BMP-2 Overexpression Increases Myosin Va Expression. Via RT-PCR, we determined adenoviral-mediated BMP-2 overexpression significantly increased myosin Va mRNA sevenfold compared to control (Figure 4(a)), in a dose-dependent manner (Figure 4(b)). We then confirmed BMP-2 stimulation increased myosin Va protein expression by characterizing actin and myosin Va expressing VSMCs by immunofluorescence analysis. Representative photographs in Figure 4(c) display abundantly distributed myosin Va protein binding actin in the cytoplasm of VSMCs overexpressing...
Figure 4: Myosin Va expression regulated by BMP-2. (a) qRT-PCR analysis revealed mRNA upregulation of the myosin Va gene associated with adenoviral-mediated BMP-2 overexpression in rat VSMCs. (b) Myosin Va expression reflects a dose-dependent response to BMP-2 administration. (c) Immunofluorescent assay characterizes actin and myosin Va expression. Myosin Va proteins were abundantly distributed in the cytoplasm of cells binding closed actin in BMP-2 overexpressing VSMCs, compared to control (which manifested only weakly positive signals).

BMP-2, compared to the weakly positive signals detected in control cells. Together, these results strongly support augmented myosin Va expression during BMP-2 overexpression.

3.5. siRNA-Mediated knockdown of VSMC Myosin Va Inhibits Migration. We generated three myosin Va-specific siRNA constructs. Compared to control or other generated siRNA constructs, siRNA-construct-3 (henceforth termed siRNA-3) significantly reduced the expression of both myosin Va mRNA (Figure 5(a)) and protein (Figure 5(b)). siRNA-mediated knockdown of myosin Va decreased its expression in locations known to be detectable by immunofluorescence assay in VSMCs simulated by BMP-2 (Figure 5(c)). We next determined the functional consequence of myosin Va knockdown in the setting of BMP-2 stimulation. VSMCs subjected to myosin Va-knockdown by siRNA-3 migrate significantly slower compared to control or VSMCs subjected to siRNA constructs 1 or 2 (Figures 5(d) and 5(e)). These results suggest myosin Va may have significant role in BMP-2-mediated acceleration of VSMC migration.

3.6. Erk1/2 Modulates Myosin Va Expression by BMP-2. The signaling mechanisms responsible for the effects of BMP-2 overexpression upon VSMC migration are unclear. Software (IPA, Ingenuity Sys) analysis revealed the relationship and interaction between BMP-2, Erk1/2, myosin Va, and actin...
Figure 5: Effects of siRNA-mediated knockdown of myosin Va in rat VSMCs subjected to BMP-2 (250 ng/mL). (a) qRT-PCR analyses, demonstrating varying siRNA-mediated suppression of myosin Va mRNA expression. siRNA3 construct inhibited myosin Va mRNA expression to 0.13-fold of scramble levels. (b) Western blot demonstrating siRNA-3 construct inhibits the most myosin Va of all three generated siRNA constructs. (c) Myosin Va expression, determined by immunofluorescence, inhibited by siRNA-3. (d) Unicellular motility detected by Boyden chamber assay. (e) Multicellular population motility determined by wound assay. Both (d) and (e) demonstrate that siRNA3 significantly inhibits migration compared to scramble control (unicellular motility 144 ± 25 versus 248 ± 20 cells/well, multicellular population 17.5 ± 5 μm versus 45.2 ± 4.8 μm). * Student’s t-test: *P < 0.01 versus control.

Table 2: BMP-2 increased uni- and multicellular population motility.

| Condition                        | Control   | 50 ng/mL | 100 ng/mL | 250 ng/mL | 500 ng/mL |
|----------------------------------|-----------|----------|-----------|-----------|-----------|
| Signal cell movement distance    | 0.112 ± 0.01 | 0.134 ± 0.01 | 0.165 ± 0.02 | 0.202 ± 0.05 | 0.221 ± 0.028 |
| (um/15 min)                      |           |          |           |           |           |
| P value with ctrl                | 0.08      | 0.0021*  | 0.0003*   | 0.00032*  | 0.0007*   |
| Populations movement distance    | 40.5 ± 10.2 | 48.0 ± 12.6 | 56.9 ± 10.3 | 76.7 ± 9.6 | 80.1 ± 13.2 |
| (um/24 h)                        |           |          |           |           |           |
| P value with ctrl                | 0.32      | 0.034*   | 0.0004*   | 0.0007*   |           |

Compared to control group, Student’s t-test: *P < 0.01.
(Figure 6(a)). We investigated the degree of Erk1/2 stimulation in response to BMP-2 doses referenced in previous studies [23]. VSMCs were either treated with exogenous 250 ng/mL BMP-2 for 48 hours or adenovirally transfected to overexpress BMP-2. VSMCs were consequently exposed to Erk1/2 inhibitor U0126. Erk1/2 activation was determined by Western blot. Erk1/2 inhibitor significantly increased BAD protein production (typically downregulated by BMP-2) and significantly decreased Bcl-xl and myosin Va expression (typically upregulated by BMP-2) (Figure 6(b)). qRT-PCR significantly decreased Bcl-xl and myosin Va expression protein production (typically downregulated by BMP-2) and 250 ng/mL BMP-2 for 48 hours or adenovirally transfected.

2. Data analysis and results

2.1. Ca2+ Oscillation

We have demonstrated BMP-2 overexpression stimulates cellular migration. We employed IPA software to analyze the relationships and interactions among BMP-2, Erk1/2, Myosin Va, and Ca2+, but previous reports have not revealed any direct relationship between BMP-2 and myosin Va [30–32]. Erk1/2 inhibition not only blocked BMP-2-mediated down-regulation of Bcl-xl protein production but also significantly decreased expression of both myosin Va and BAD. Myosin Va may mediate the functions of Bcl-xl by promoting islet cell migration and invasion [15]. We have demonstrated BMP-2 modulates myosin Va expression in VSMCs via Erk1/2 but cannot preclude the involvement of other contributory factors regulating the expression of myosin Va, such as gene regulatory elements like promoters and miRNAs. Further work is necessary to fully characterize the myosin Va promoter.

Spontaneous calcium oscillations occur in cells originating from excitable tissues of muscular, neuronal, and embryonic stem origin [33–35]. Calcium is an important second messenger regulating both intracellular and extracellular communications. The amplitude and duration of calcium transients can promote the expression of specific genes. Myosin Va is necessary for localization of IP3 receptors, which may connect with intracellular Ca2+ oscillations in cultured VSMCs [24, 25]. In the current study, we observe the presence of spontaneous [Ca2+]i oscillations in cultured VSMCs, further activated in the presence of BMP-2. The Erk signaling pathway can be stimulated by increased [Ca2+]i oscillations, augmenting Erk1/2 phosphorylation [36, 37], supported by our results (Figure 6). Additional studies specifically defining the precise roles of Ca2+ as a second messenger in this system are ongoing.

3. Discussion

We have made several important observations in the current study. Firstly, we demonstrated BMP-2 overexpression in VSMCs augments both unicellular and multicellular population motility. Secondly, we demonstrated BMP-2 overexpression increases the expression of myosin Va but not myosin Vb or Vc. Finally, we provide mechanistic evidence of ERK1/2 involvement in the modulation of myosin Va expression by BMP-2, with Ca2+ involvement.

Myosin Va directly assembles actin into antiparallel bundles [26, 27] localized at the leading edge of membrane ruffles [28]. A veritable actin-based motor protein, myosin Va, functions as an intracellular vesicle and organelle transporter and delivers cargo critical for maintaining cellular movement, thereby supporting cellular migration. Heretofore, whether myosin Va regulates VSMC migration was unknown. In this study, we demonstrate the abundant distribution of myosin Va binding actin in the cytoplasm of BMP-2 overexpressing VSMCs, compared to control cells. We also demonstrate knockdown of myosin Va inhibits VSMC motility, suggesting myosin Va is requisite for VSMC migration. More detailed analysis is required to elucidate how myosin Va specifically affects cellular motility, but it is plausible myosin Va participates in cell cytoskeleton reorganization, an essential event driving cellular movement [13, 29].

We clearly demonstrate BMP-2 overexpression stimulates cellular migration. We employed IPA software to analyze the relationships and interactions among BMP-2, Erk1/2, Myosin Va, and Ca2+, but previous reports have not revealed any direct relationship between BMP-2 and myosin Va [30–32]. Erk1/2 inhibition not only blocked BMP-2-mediated down-regulation of Bcl-xl protein production but also significantly decreased expression of both myosin Va and BAD. Myosin Va may mediate the functions of Bcl-xl by promoting islet cell migration and invasion [15]. We have demonstrated BMP-2 modulates myosin Va expression in VSMCs via Erk1/2 but cannot preclude the involvement of other contributory factors regulating the expression of myosin Va, such as gene regulatory elements like promoters and miRNAs. Further work is necessary to fully characterize the myosin Va promoter.

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VSMCs can change phenotype in vivo depending upon functional demands and can be contractile, proliferative, migratory, and/or synthetic [38, 39]. We demonstrate that BMP-2 overexpression increased expression of synthetic phenotypic markers OPN and MGP and decreased contractile phenotypic marker SM22α. The present study advances the notion BMP-2 may modulate SMC phenotype towards a synthetic state. Further studies are necessary to determine whether Ca2+ oscillations may have phenotypic implications.

In summary, our study demonstrates BMP-2 enhances VSMC migration via Erk1/2 signaling activation, which regulates myosin Va expression. Inhibition of BMP-2-induced myosin Va expression may represent a potential future therapeutic strategy attenuating atherosclerosis.
Figure 6: BMP-2-mediated regulation of myosin Va may be modulated by Erk signaling with Ca²⁺ involvement. (a) IPA software analysis of the relationships and interactions between BMP-2, Erk1/2, myosin Va, and actin. Although communication between the genes of these proteins has previously been reported, heretofore, a direct interaction between BMP-2 and myosin Va was not clarified. (b) Western demonstrates the presence of key proteins involved with myosin Va and Erk signaling in the lysates of rat VSMCs either adenovirally transected to overexpress BMP-2 or treated with exogenous BMP-2 (250 ng/mL) for 48 hours. (c) qRT-PCR analysis demonstrating myosin Va gene mRNA was upregulated in VSMCs treated with 250 ng/mL BMP-2 for 48 hours and downregulated in the presence of Erk1/2 inhibitor U0126 (dose 5 μM). (d) Migration of VSMCs subjected to identical conditions was detected by Boyden chamber assay (BMP-2 alone: 253±29 versus BMP-2+/U0126+: 176±11). (e) Oscillatory increase of intracellular Ca²⁺ concentration ([Ca²⁺]i) within individual cells of the colony. Chronological image sequence goes from left to right at 1.5-second intervals over 5 minutes. (f) Oscillations exhibited a mean interval of intracellular Ca²⁺ concentration (BMP-2 concentration 250 ng/mL, n = 18). (g) Fluorescence (F/F₀) of individual cells, values represent mean ± SD; *Student’s t-test: P < 0.01).
Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution
Ming Zhang and Min Yang contributed equally to the study.

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