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# BMP-2 enhances the migration and proliferation of hypoxia-induced VSMCs via actin cytoskeleton, CD44 and matrix metalloproteinase linkage

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ABSTRACT

The persistent proliferation of hypoxia-induced vascular smooth muscle cells (VSMCs) in the arterial wall underlie the development of atherosclerosis. However, the mechanism that regulates the behavior of VSMCs, which involve in actin aggregation, and impedes their migration is still elusive. Here, we report that bone morphogenetic protein 2 (BMP-2) leads to enrichment of CD44 and F-actin stress fiber and secretion of matrix metalloproteinases-2 (MMP-2) during hypoxia in vitro and following artificial hypoxia-induced atherosclerosis exacerbation in vivo. To test this hypothesis, fluorescence immunostaining, immune-hybridization and flow cytometry analyses were performed to understand the relationship among BMP-2, CD44 and MMP-2 linkage. The cellular actin cytoskeleton was reduced, and smaller adhesion plaques were formed in hypoxia-induced T/G HA-VSMC cell line, but BMP-2 against disruption of F-actin and increase the motility and migration behaviors of VSMC during hypoxic cultured. Aggregation of F-actin dependents on the interaction between the cell surface integral membrane protein CD44 and Vinculin which enhanced by rBMP-2. This activity of Actin/CD44/ linkage was inhibited by competing with the active site of the CD44 using recombined the hemopexin-like C-terminal domain (PEX) of MMP-2. These results lead to the proliferation and migration of VSMCs were inhibited in response to MMP-2 activity when the cell is in a hypoxic environment. Collectively, our discovery indicates that BMP-2 could enhance migration and proliferation of hypoxia-induced VSMCs via the Actin/CD44/MMP-2 molecular pathway.

## 1. Introduction

It has been observed that actin cytoskeletal organization is disrupted and vinculin adhesion plaques are broken when alternative actin aggregates are formed in hypoxic vascular smooth muscle cells (VSMCs) [1–3]. This reorganization of the actin cytoskeleton leads to the preferential migration of VSMCs to the thrombosis at the fibrous cap [4]. However, the mechanisms connecting the related cellular events remain obscure.

The migration and adhesion of VSMCs rely on the function of the membrane-associated actin cytoskeleton system [5,6]. Over the past decades, numerous studies have access to reveal the aggregation of actin substructures and to approve how they mediate the form of locomotion. The complex interactions between many signaling and

*Abbreviations:* BMP-2, bone morphogenetic protein 2; VMSCs, vascular smooth muscle cells; ECM, extracellular matrix; MMPs, matrix metalloproteinases; PEX, hemopexin-like C-terminal domain; IP3, inositol phosphatidyl 3; CABG, Coronary Artery Bypass Grafting; CCA, Common Cartied Artery; ICA, Internal Cartied Artery; ECA, External Cartied Artery; EC, Endothelial cell

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structural molecules in regulating the assembly have been the delineated [7], which highly associated with stress fibers, adhesion complexes and lamellipodia, respectively [8]. These complementary findings have provided a compelling molecular/structural overview, suggesting that branched F-actin networks might drive translocating lamellipodia and lead to the assembly of adhesion complexes and their associated stress fibers [9]. The challenge of how coordinated interactions between actin subunits regulate cell migration and how they interact with membrane proteins and the matrix components to prompt cell migration motivates further explorations into the molecular links in VSMCs.

Recent findings indicate that cell adhesion molecules cooperate closely with extracellular matrix (ECM)-degrading proteases during cell migration [10,11]. CD44 is a ubiquitous, multi-structural and multifunctional cell surface adhesion molecule that is involved in cell-cell and cell-matrix interactions [12]. It is expressed in actively migrating cells of embryonic tissues [13]. The external domain of CD44 interacts with ECM matrix metalloproteinases (MMPs) to regulate cell locomotion [14]. Activated MMPs promote the malignant behavior of VSMCs during rapid growth and migration by degrading the ECM [11,15]. MMPs could form a complex with CD44 by binding CD44 at the PEX domain, which is a hemopexin-like C-terminal domain of MMPs, to promote the migratory potential of cells [16]. The cytoplasmic tail of CD44 interacts with ERM and/or Ankyrin, both of which bind the F-actin cytoskeleton [17,18]. Additional evidence suggests that CD44 interacts with Rho-kinase [19], cyclins, growth factors [20], inositol phosphatidyl 3(IP3) [21], and the ERK pathway after vascular injury [22]. These findings support a role for CD44 in relaying the signals that facilitate cell motility or growth. It appears that there is a cross-membrane molecular link between the actin cytoskeleton and the ECM proteases through CD44 that plays both structural and signaling roles in the control of cell migration. It would be necessary to investigate the actin/CD44/MMP molecular linkage further, including its role in regulating cell migration activities and the response to drug treatment.

In our previous works, we found that bone morphogenetic protein 2 (BMP-2), a cytokine of the transforming growth factor-β superfamily, augments VSMC migration by upregulating an unconventional actin-based motor protein, myosin, when VSMCs lose their normal functional behaviors [23]. In this study, we established that BMP-2 recombinant protein enhanced differential expression of CD44 and MMP as well as altered actin aggregation through vinculin in hypoxia-induced VSMC. And also increase the proliferation and migration of hypoxia-induced VSMCs in vivo. Importantly, intravascular administration of PEX could block VSMC proliferation, inhibit migration and reduce CD44 levels in VSMCs. It is likely that CD44-mediated molecular linkage is crucial for the potential and sensitivity of VSMCs to PEX. Both the reduction of CD44 expression and the blocking of the interaction between CD44 and MMP-2 by PEX resulted in the interruption of the actin/CD44/MMP molecular linkage and in the consequent inhibition of VSMC proliferation and migration. The proliferation and migration of VSMCs facilitate the development of atherosclerosis. The presented in vivo evidence provides potentially novel therapeutic targets against the response to hypoxia caused by vascular injury.

## 2. Materials and methods

### 2.1. Cells, samples and reagents

Human vascular smooth muscle tissues were obtained from coronary arteries of a 52-year-old male patient who underwent coronary artery bypass grafting (CABG); T/G HA-VSMCs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/L glutamine at 37 °C in 5% CO2 atmosphere or cultured in a hypoxia chamber containing a 0.5% $O_2$ gas mixture supplied by a hypoxia tank (Forma, Thermo, US). All culture reagents were purchased from Invitrogen. Inc. USA. Serum and blood samples were collected from patients who received a hepatectomy and frozen; the patients provided written informed consent. Acquisition and use of these samples were approved by the Ethics Committee of Beijing Anzhen Hospital. All studies involving human samples adhered to the principles of the Declaration of Helsinki, and the animal experiments were performed in accordance with the NIH guidelines (Guide for the care and use of laboratory animals, 2011 version).

### 2.2. Protein isolation and immunoblot analysis

Western blot analyses were performed, and immunoreactive bands were visualized by chemiluminescence detection as usual as the manufacturer's instructions. Briefly, total cell lysates were prepared in RIPA lysis buffer containing 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L EDTA, and one tablet of protease inhibitor cocktail per 10 ml lysis buffer (Roche, Mannheim, Germany). Each 30 μg equal amounts of protein were subjected to electrophoresis on 10% PAGE gels. Transferring to PVDF membrane and blocked by 5% fat-free dried milk, the primary antibodies that were used for the first step of immunoblotting are described in Supplemental Table 1. HRP-conjugated antibodies were used as secondary antibody (1:50000 dilution, Jackson ImmunoResearch, West Grove, PA, USA). Subsequently, the membranes were detected using Immobilon™ Western Chemiluminescent HRP substrate(Millipore, Germany).

### 2.3. Cell proliferation assay

Cell viability was measured using the CellTiter Aqueous One Solution Cell Proliferation Assay kit according to the instructions of the manufacturer (Promega, USA). Briefly, Every $10^4$ cells per well were incubated with the MTS reaction solution for 2 h at 37 °C in 96 well Coring Plate. The absorption at 490 nm was measured by FLUOstar OPTIMA (BMG LABTECH. USA). The results are presented as a percentage of controls.

### 2.4. Migration assay

The invasive capability of cells was determined using a Boyden chamber with 8-μm pores (BD Biosciences) as previously described [24]. $1 \times 10^5$ suspension cells were added to the inner chamber with FBS free medium. And then 500 μl of completed culture medium containing 10% FBS were added to the bottom chamber and incubated 16 h at 37 degrees in 5% atmosphere CO2. After removing the upper cells of the inner chamber with cotton swabs. invaded cells on the bottom surface of the inner chamber were fixed by pure MOTH, stained with crystal violet, and counted. Six random fields of each group were imaged, and the experiments were performed in triplicate.

### 2.5. Quantitative reverse transcription-PCR ( qRT-PCR )

ALP, RUNX2, BGLAP RNA levels were examined by qRT-PCR using M-MLV reverse transcriptase kits (Invitrogen, Carlsbad, CA) as the manufacturer's instructions. Briefly, cDNA was synthesized using a total of 2 μg of RNA by M-MLV reverse transcriptase, and then subjected to qRT-PCR using a real-time PCR system 7500 Fast (Applied Biosystems, Foster City, CA). qRT-PCR analysis of the cDNA (diluted 1:4) was performed 40 cycles (95 °C for 10 s, 60 °C for 40 s, and 72 °C for 10 s) using SYBR select Master Mix(Applied Biosystems, Foster City, CA) and Primers (ALP: F: 5′-ACTGGTACTCAGACAACGAGAT-3′, R: 5′-ACGTCA ATGTCCCTGATGTTATG-3′; RUNX2: F: 5′-TGGTTACTGTCATGGCGG GTA-3′, R: 5′-TCTCAGATCGTTGAACCTTGCTA-3′; BGLAP: F: 5′-CACT CCTCGCCCTATTGGC-3′, R: 5′-CCCTCCTGCTTGGACACAAAG-3′). The result in each sample was normalized to the expression of GAPDH (internal control housekeeping gene) and compared with control samples. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method where