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Construction of a eukaryotic expression vector pEGFP-C1-BMP-2 and its effect on cell migration*

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Abstract: Objective: Bone morphogenetic proteins (BMPs) are known to play an important role in bone and cartilage development. Recent research has shown that BMPs may induce tumorigenesis and promote tumor to spread, but the molecular mechanisms have not been elucidated. The aim of the present study was to investigate the regulatory function of BMP-2 in the migration of COS-7 cells and the underlying mechanisms. Methods: Human *BMP-2* genetic fragment was amplified and introduced into the pEGFP-C1 vector. After being confirmed by *Xho*I and *Bam*HI digestion analyses and DNA sequencing, the recombinant pEGFP-C1-BMP-2 plasmid was transfected into COS-7 cells. The influence of BMP-2 on cell migration and cofilin activity was detected by cell scratch assay and Western blotting. Results: The recombinant pEGFP-C1-BMP-2 was effectively expressed in COS-7 cells. An increased phosphorylation of both LIMK1 and cofilin and an enhancement of cell migration were observed in cells with overexpression of BMP-2. Conclusions: A recombinant pEGFP-C1-BMP-2 vector was successfully constructed and overexpression of BMP-2 regulated the activities of the downstream molecules of the Rho GTPase signaling pathway, which might contribute to the enhancement of cell migration.

Key words: Cell migration, Bone morphogenetic protein-2 (BMP-2), COS-7, Cofilin
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1 Introduction

Bone morphogenetic proteins (BMPs), also known as osteogenetic proteins, were first extracted from bone matrix by Urist (1965) and were named after their ability to induce ectopic new bone formation in rodents (Wozney *et al.*, 1988). Most BMPs are members of the transforming growth factor-beta (TGF-β) superfamily, which is a group of secretory multi-functional proteins playing important regulatory roles in cell growth, differentiation, apoptosis, and morphogenesis in a variety of tissues and organs. Since the TGF-β signaling pathway is also closely related to the molecular events of tumorigenesis and metastasis, the linkage between BMPs and carcinogenesis and tumor progression has gained extensive attention in recent years. However, there have been conflicting reports, with many in vitro and in vivo experimental data suggesting that BMPs promote tumor proliferation and metastasis (Fong *et al.*, 2008; Lai *et al.*, 2008; Kang *et al.*, 2011) and others reporting the opposite, namely that BMPs can sometimes function as tumor suppressors (Singh and Morris, 2010; Thawani *et al.*, 2010).

BMP-2 is an essential member of the BMP family. It is highly expressed in many cancers, such as

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mouth neoplasms, osteosarcoma, and bone tumors, and promotes tumor invasion and metastasis, which is closely related to the process of cell migration (Jin *et al*., 2001). Various studies have demonstrated that BMP-2 signaling is involved in the regulation of cell migration of multiple cell types. For example, Fong *et al*. (2008) showed that BMP-2 increases migration of human chondrosarcoma cells via the phosphoinositide 3-kinase (PI3K)/Akt pathway. Goldstein *et al*. (2005) reported that the migration of neural crest mesenchymal cells relies on the BMP-2 signaling pathway. BMP-2 can also promote the migration of osteoblasts and epithelial cells in vitro (Fiedler *et al*., 2002; Sotobori *et al*., 2006). However, unlike the well-understood molecular mechanism of transcriptional activity of BMPs, the mechanism by which BMP-2 mediates cell migration has not yet been elucidated.

To study the effects of BMP-2 on cell migration and to explore further the molecular mechanism, a eukaryotic expression vector pEGFP-C1-BMP-2 plasmid was constructed and transfected into COS-7 cells by liposomes. The effects of the overexpressed BMP-2 on the migration of COS-7 cells and the underlying molecular mechanism were investigated.

2 Materials and methods

2.1 Materials

The pMD18-T vector containing the full-length sequence of human *BMP-2* was obtained from Sino Biological Inc. (Beijing, China). The plasmid of pEGFP-C1 was gifted by the Central Laboratory of Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, China. COS-7 cell line was preserved in our laboratory. The pMD18-T simple vector and moloney murine leukemia virus reverse transcriptase (M-MLV RTase) complementary DNA (cDNA) synthesis kit were purchased from TaKaRa (Dalian, China). Restriction enzymes, T4 DNA ligase, DNA Ladder, and pre-stained protein marker were from Fermentas (Vilnius, Lithuania). Mini plasmid preparation kits and gel extraction kits were purchased from Axygen (Hangzhou, China). Fetal bovine serum (FBS) was obtained from the Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (China). Lipofectamine™ 2000 and Trizol reagent were from Invitrogen (Carlsbad, USA). BMP-2 antibody and horseradish peroxidase (HRP)-labeled secondary antibody were purchased from Proteintech (Chicago, USA). Antibodies against LIMK1, p-LIMK1, cofilin, and p-cofilin were purchased from Cell Signaling (Danvers, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Good Here Company (Hangzhou, China). Polymerase chain reaction (PCR) primers were synthesized by the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China.

2.2 PCR amplification of human *BMP-2* fragment

According to the sequence of the human *BMP-2* gene, a pair of primers targeting the BMP-2 open reading frame (ORF) containing the sequence coding for a signal peptide was designed. The upstream primer Pl 5′-GATctcgagATGGTGGCCGGGACCC GCTGTCTTC-3′ contained an *Xho*I site, and the downstream primer P2 5′-CCGggatccACGCGACA CCCACAACCCTCCA-3′ contained a *Bam*HI site. The pMD18-T plasmid with *BMP-2* cDNA was used as a template to amplify human *BMP-2* specifically with P1 and P2 primers. The PCR reaction parameters were: pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 68 °C for 1 min for 33 cycles, with a final extension at 68 °C for 10 min. PCR amplification products were subjected to agarose gel electrophoresis. The target band was cut and the *BMP-2* cDNA fragment was extracted from the agarose gel according to the instructions for the gel extraction kit.

2.3 TA cloning of *BMP-2* gene

Recovered *BMP-2* fragment was inserted into pMD18-T simple vector by a reaction at 16 °C for 3 h. The product was used to transform competent *Escherichia coli* DH5α. The positive clones were picked from kanamycin plates and the plasmid was prepared with a mini plasmid preparation kit. After digestion by the restriction enzymes, the products were subjected to agarose gel electrophoresis to identify plasmids with the proper gene inserts.

2.4 Generation of plasmids

Both the recombinant plasmid containing the *BMP-2* gene and the pEGFP-C1 plasmid were subjected to digestion with *Xho*I and *Bam*HI, and the target gene fragments and linear vector fragments

were recovered by gel electrophoresis. After the insert fragment *BMP-2* and pEGFP-C1 were linked, the product was subsequently used to transform competent DH5α. Positive clones were picked for mini plasmid preparation, and identified by double restriction enzyme digestion and sequencing (Fig. 1).

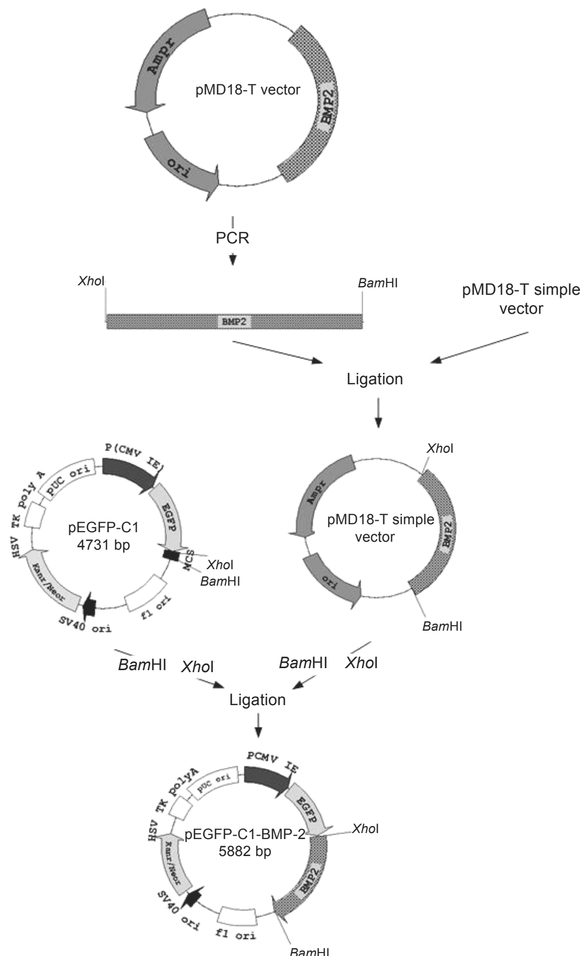

Fig. 1 Schematic map of pEGFP-C1-BMP-2 vector construction

2.5 Cell culture and transfection

COS-7 cells were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin and streptomycin, in an incubator at 37 °C, with 5% CO_2 and saturated humidity. One day before transfection, the cells were divided into three groups (the recombinant plasmid pEGFP-C1-BMP-2 group, an empty vector pEGFP-C1 group, and a blank control group) and were cultured in RPMI 1640 medium free of antibiotic. The next day, when cells reached 90% confluence, the transfection was conducted following the manual of Lipofectamine™ 2000. At 48 h after transfection, the transfection efficiency was measured using a fluorescence microscope, and the cells were subjected to subsequent operations.

2.6 Reverse transcription-polymerase chain reaction (RT-PCR) assay

Total cellular RNA was isolated using Trizol reagent according to the manufacturer's instructions. First-strand cDNA was synthesized from a 0.5 μg sample of total RNA with the M-MLV RTase cDNA synthesis kit. For amplification of *BMP-2* cDNA, the primer sequences were 5′-GCGTTGCTGCTTCCCC AGGT-3′ (sense) and 5′-CCCGGCTGACCCGAGT CCTG-3′ (antisense). In each reaction, RT-PCR of GAPDH was included as an internal control for normalization of the loading. The primer sequences for GAPDH were 5′-TGAAGGTCGGAGTCAACG GATTTGGT-3′ (sense) and 5′-CATGTGGGCCATG AGGTCCACCAC-3′ (antisense). The cDNA was amplified in a 20-μl system under the following conditions: denaturation at 94 °C for 45 s, annealing at 60 °C for 30 s, and extension at 72 °C for 2 min for 30 cycles. Products were electrophoresed in 2% agarose gels (0.02 g/ml) and visualized with ethidium bromide.

2.7 Western blotting analysis

Cells were washed with ice-cold phosphate buffered saline (PBS) 48 h after transfection, and total proteins were extracted using radio immunoprecipitation assay (RIPA) lysis buffer. The protein concentration was measured using the bicinchoninic acid (BCA) method. After denaturation, samples with the same amount of protein were subjected to 10% polyacrylamide electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with BMP-2 antibody (1:2000), LIMK1 (1:1000), p-LIMK1 (1:1000), cofilin (1:1000), p-cofilin (1:1000), or GAPDH (1:2000) diluted in blocking solution, at 4 °C overnight. After incubation with HRP-labeled secondary antibody (1:2000) at room temperature for 2 h, the blots were developed with enhanced chemiluminescence (ECL) reagents and exposed to X-ray film to obtain images. The intensity of each band was quantified using Image J software [National Institutes of Health (NIH), USA] and normalized to values for GAPDH.

2.8 Cell scratch assay

To quantify cell migration, a scratch was made down the center of each well in a 6-well plate using a p1000 pipette tip at 48 h after transfection. Along the scratch line, the cells were washed away and replaced with serum-free culture medium. An inverted phase-contrast microscope (Nikon) was used to take pictures every 12 h for 48 h. The distances between the parallel cell edges were measured at each time point using Image J software. For each well, three different fields along the scratch were analyzed in triplicate. Cell motility was calculated as the percentage of the cell migration distance with respect to the initial scratch distance.

2.9 Statistical analysis

All measurement data are expressed as mean± standard error (SE). One-way analysis of variance (ANOVA) was performed to evaluate the differences between the treatment groups and controls. Statistical significance was defined as $P<0.05$ or $P<0.01$.

3 Results

3.1 Identification of recombinant plasmid

The pMD18-T plasmid containing human *BMP-2* was used as the template for PCR amplification of the *BMP-2* genetic fragment with *Xho*I and *Bam*HI sites. The PCR products were analyzed by 1% agarose gel electrophoresis (0.01 g/ml). A band at approximately 1.2 kb was separated, which was consistent with the expected molecular mass. The recovered fragment from gel electrophoresis was subcloned into a pMD18-T simple vector, which was then digested with *Xho*I and *Bam*HI restriction enzymes, producing two bands of 1.2 kb (target gene) and 2.7 kb (vector DNA fragment), respectively. The *BMP-2* target fragment was recovered and inserted into the pEGFP-C1 plasmid vector. Two bands of 1.2 and 4.7 kb were obtained from the pEGFP-C1-BMP-2 vector after digestion with *Xho*I and *Bam*HI (Fig. 2a). The plasmid was then sent to Shanghai Sangon for sequencing, and the sequencing results matched the expected sequence completely (Fig. 2b).

3.2 Detection of recombinant plasmid expression

At 48 h after transfection, a large number of COS-7 cells with green fluorescence were visible under a fluorescence microscope (Fig. 3a). EGFP was uniformly distributed throughout the cytoplasm, indicating that the recombinant vector was successfully constructed and expressed in COS-7 cells. This was further confirmed by both semi-quantitative RT-PCR and Western blotting detection. The size of the BMP-2 protein was approximately 28 kDa (Fig. 3b). Compared to the low-level expression of endogenous BMP-2 in cells of both the blank control group and the empty vector pEGFP-C1 group, BMP-2 expression in cells transfected with pEGFP-C1-BMP-2 plasmid significantly increased.

3.3 Identification of cell motility by cell scratch assay

After scratch models of the COS-7 cells were prepared, the cells were photographed under an inverted microscope every 12 h, and the cell migration distance of each sample was measured and cell motility was evaluated.

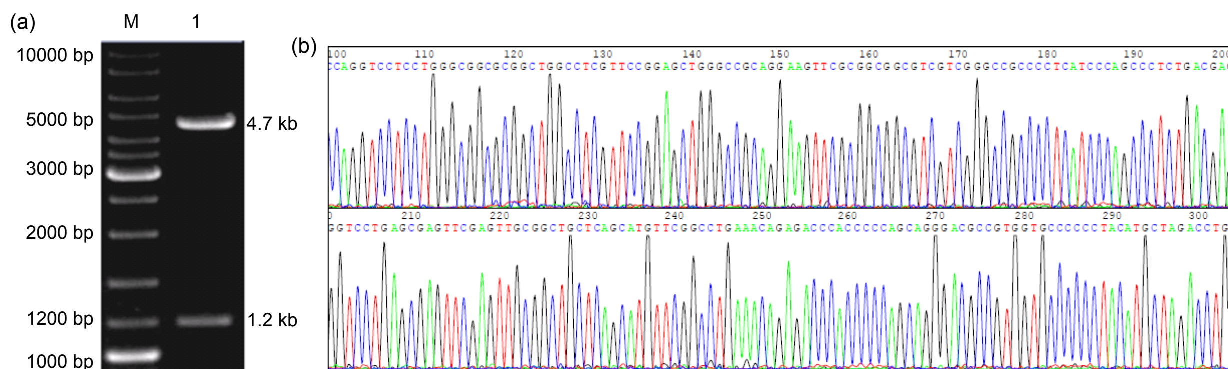

Fig. 2 Construction and identification of the recombinant plasmid pEGFP-C1-BMP-2
(a) Identification of the recombinant plasmid pEGFP-C1-BMP-2 by *Xho*I and *Bam*HI digestion (M: DNA marker; Lane 1: recombinant plasmid digested with *Xho*I and *Bam*HI); (b) Partial sequencing of pEGFP-C1-BMP-2

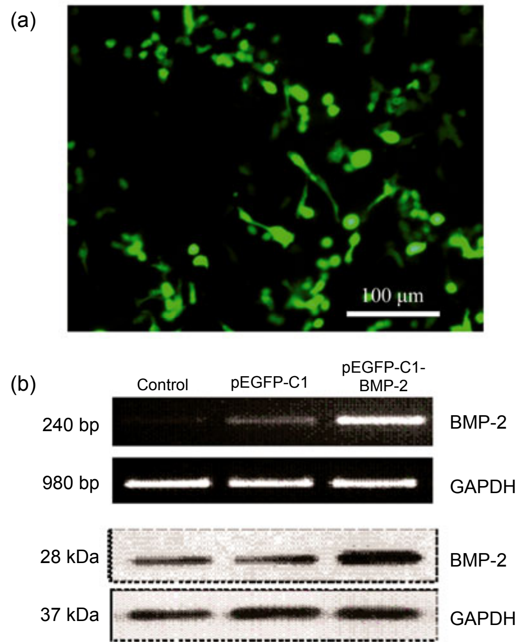

Fig. 3 Expression of recombinant plasmid pEGFP-C1-BMP-2 in COS-7 cells
(a) Expression of EGFP in COS-7 cells at 48 h after transfection with plasmid pEGFP-C1-BMP-2, detected by immunostaining; (b) Expression of BMP-2 in COS-7 cells at 48 h after transfection, detected by semi-quantitative RT-PCR (upper panel) and Western blotting (lower panel)

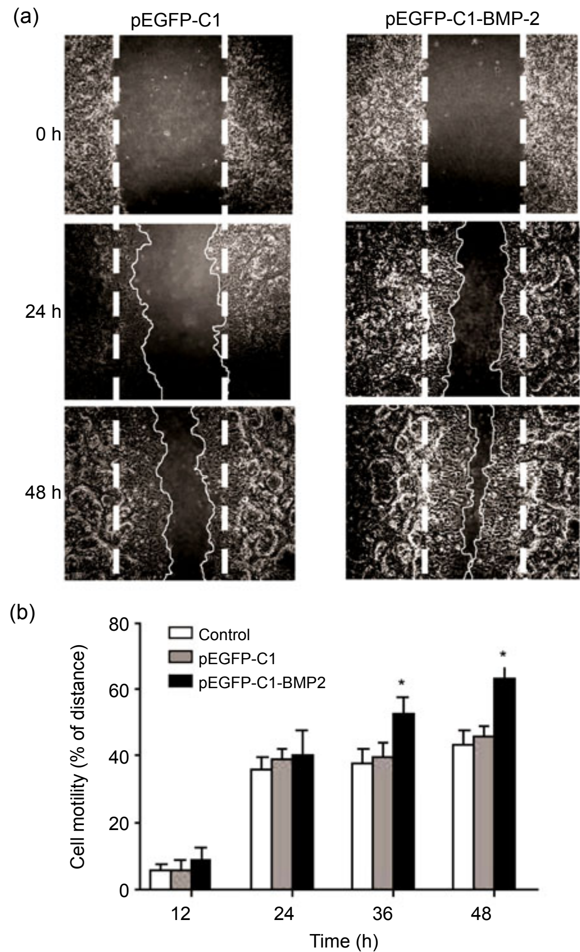

Fig. 4 Cell migration promoted by BMP-2 in COS-7 cells
(a) Image examples of migrated cells. The dotted straight lines indicate the dimensions of the scratch. The solid irregular lines indicate the cell edges. (b) Quantitative measurements of the cell motility. After the scratch was made, cell motility was evaluated every 12 h for 48 h. Increased cell motility was observed at 36 and 48 h in cells transfected with the pEGFP-C1-BMP-2 plasmid. Data are expressed as mean±SE (n=5). $^*P$<0.05, compared with the pEGFP-C1 group (one-way ANOVA)

Compared to the non-transfected control group there was no significant change in the motility of the cells transfected with the pEGFP-C1 empty vector group at each time point. Interestingly, the migration of cells transfected with pEGFP-C1-BMP-2 plasmid was significantly increased at 36 and 48 h compared with the control group and the pEGFP-C1 group (P<0.05), suggesting that overexpression of BMP-2 might promote cell migration (Fig. 4).

3.4 Detection of phosphorylation of cofilin

Western blotting was employed to detect the effect of high-level expression of BMP-2 on cofilin activity and to elucidate the mechanism. Compared to the empty vector and control groups, at 48 h after transfection, the phosphorylation level of LIMK1 significantly increased, while the total protein expression level of LIMK1 did not changed (Fig. 5). Furthermore, the phosphorylation level of the downstream effector of LIMK1, cofilin, an actin depolymerization factor, also significantly increased.

4 Discussion

At least 40 BMPs have been described (Matthews, 2005) and most have been found to be the members of TGF-β superfamily, which can play biological roles by binding to serine/threonine kinase receptors on the plasma membrane. The receptors of BMPs include mainly Type I (BMPRIA, BMPRIB, ActRI) and Type II (BMPRII, ActRIIA, ActRIIB) receptors (Maiti and Singh, 1998). BMP precursors

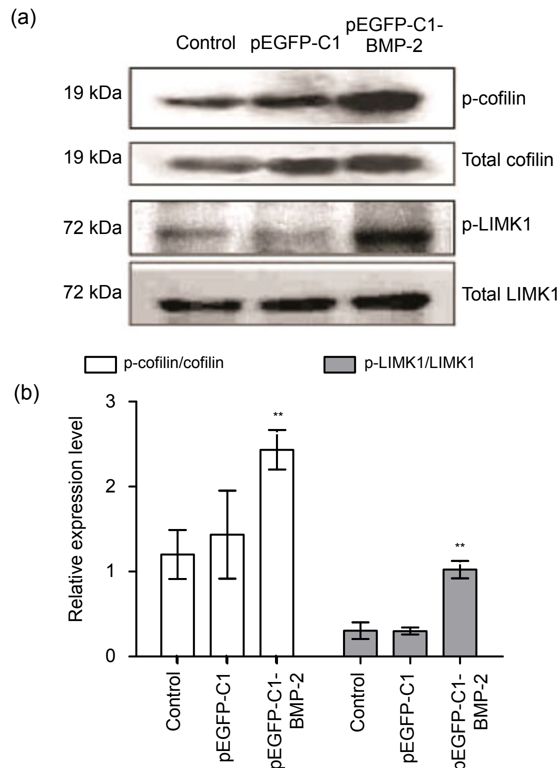

Fig. 5 Activities of downstream signals regulated by overexpression of BMP-2 in COS-7 cells
(a) BMP-2 increased phosphorylation of both LIMK1 and cofilin. Western blotting was performed to detect the phosphorylation levels of LIMK1 and cofilin. Overexpression of BMP-2 led to increased phosphorylation of both proteins. (b) Quantification of phosphorylation level of the proteins. Data are expressed as mean±SE (n=4). $^{**}P<0.01$, significant differences from the corresponding control (one-way ANOVA)

include a signal peptide, a pro-domain, and a carboxyl-terminal region. They do not have any biological functions unless the C-terminals are removed from the precursor proteins by proteolytic enzymes, and dimers are formed. The signal transduction of BMPs is controlled by the interaction with the BMP receptor complex (Sieber *et al*., 2009).

As a subtype of BMP with strong osteogenic ability, BMP-2 plays an important role in the repair process in bone fracture and damage. In recent years, it has also been found to be involved in the generation, development, and metastasis of a variety of tumors. In most previous studies, BMP-2 protein purified from animal bone matrix was used in the experiments (Gamell *et al*., 2011). However, administration of purified BMP-2 has several limitations including its high price and rapid inactivation in in-vivo experiments. Using genetic engineering technology to transfect a target gene into cells so as to produce protein with biological activity has become a good method to study the function of a specific protein (Jiang *et al*., 2008). BMP-2 is a secretory protein, which is either secreted via exocytosis or accumulated in cytoplasm after synthesis is completed. During the translocation of the newly synthesized proteins, the signal peptide is cleaved off by signal peptidases, which induces a conformational change in the protein resulting in a mature form. In this process, the signal peptide at the N-terminal of the protein largely determines the fate of the protein (von Heijne, 1998). In the present study, to generate the BMP-2 eukaryotic expression vector with secretion function, the N-terminal signal peptide sequence was preserved when amplifying the fragment of the human *BMP-2* gene. Since the signal peptide was cleaved during the process of cotranslational translocation, EGFP at the N-terminal of the fusion protein was removed as well, so that the target protein detected by Western blotting was the same size as that of endogenous BMP-2 (28 kDa).

Recently, it was reported that BMP-2 is actively involved in the generation, development, and metastasis of a variety of human cancers and cancer cell lines. A high expression of BMP has been found in prostate cancer (Ye *et al*., 2007), gastric cancer (Kang *et al*., 2011), breast cancer (Bobinac *et al*., 2005), non-small cell lung cancer (Kraunz *et al*., 2005), and other cancer cell lines like prostate cancer C4-2B cells (Wozney *et al*., 1988), breast cancer MDA-MB-231 cells (Arnold *et al*., 1999) and osteosarcoma K7M2 cells (Wozney *et al*., 1988). In-vivo and in-vitro experiments indicate that recombinant human BMP-2 enhances the migration and invasion of A549 cells and H7249 cells, and promotes tumor growth in nude mice (Blanco Calvo *et al*., 2009). Its involvement in tumor invasion and metastasis is closely related to its motility. For example, when the tumor cells pass through blood vessel walls or invade surrounding normal tissues, a certain movement or migration is required. However, compared to the molecular mechanism of transcriptional activity of BMPs, little is known about the molecular mechanism by which BMP-2 regulates cell migration. Sotobori *et al*. (2006) suggested that BMP-2 promotes the migration of osteosarcoma cells through fibronectin-integrin β1

signaling, and Lai *et al*. (2008) reported that BMP-2 promotes the migration of prostate cancer cells by activating integrins β1 and β3. Recently, Gamell *et al*. (2008) proposed that BMP-2 influences the reorganization of the cytoskeleton by activating PI3K and Cdc42 of the Rho GTPase superfamily and its downstream molecules (PAK1, LIMK1, etc.), so as to regulate cell migration further. This is consistent with the recent findings of Hocking *et al*. (2009) in the nervous system. Interestingly, the BMP Type II receptor (BMPRII) cytoplasmic tail has been shown to interact directly with and regulate the activity of LIMK1 in vitro (Foletta *et al*., 2003). Based on these new findings, in this study, a change of cell migration in COS-7 cells caused by high-level expression of *BMP-2* gene was observed, and the effects of BMP-2 on the activities of LIMK1 and cofilin were explored. It was found that the high-level expression and secretion of BMP-2 by transfection of a recombinant plasmid significantly promoted cell migration and increased phosphorylation levels of LIMK1, which in turn inactivated cofilin by phosphorylation. Cofilin is capable of accelerating the rate of F-actin polymerization and depolymerization, resulting in cytoskeleton remodeling and cell migration (Matsui *et al*., 2002; Vardouli *et al*., 2005).

In summary, the secretory eukaryotic expression vector pEGFP-C1-BMP-2 was generated and employed to intervene in the expression and secretion of BMP-2 in COS-7 cells, and an enhancement of cell migration was observed. For the first time, the present study reported that expression level of BMP-2 in COS-7 cells genetically modified by plasmid transfection modulated the phosphorylation of LIMK1 and cofilin, which are thought to be involved in cytoskeletal remodeling. These results suggest that BMP-2 is likely to promote cell migration via reorganization of the cytoskeleton by regulating the downstream molecules of the Rho GTPase signaling pathway.

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