·Basic Research·

Distribution of bone morphogenetic protein receptors in human scleral fibroblasts cultured *in vitro* and human sclera

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Abstract

• AIM: To investigate the distribution of bone morphogenetic protein receptors (BMPRs) in human scleral fibroblsasts (HSFs) and in human sclera.

• METHODS: Primary HSFs were cultured *in vitro*. The mRNA levels of BMP-2 and BMPRs in HSFs were assayed by reverse transcription-polymerase chain reaction (RT-PCR). The protein distributions of BMP-2 and BMPRs in HSFs were further detected by immunocytofluorescence and western blot. Their protein expression was also detected in frozen human posterior scleral sections by immunohistofluorescence.

• RESULTS: BMP-2 and BMPRs were expressed in both HSFs and human sclera not only at mRNA level but also at protein level. The expressions of BMPRIA and BMPRII were higher than that of BMPRIB in the cytoplasm and cell membrane of HSFs *in vitro* Western blot further verified the results of immunocytofluorescence. In human sclera, BMP2, BMPR IB and BMPR II were found to be expressed in the cytomatrix of HSF, and weak signal was detected about BMPRIA.

• CONCLUSION: BMP-2 and all three subtypes of BMPRs were found in HSFs and may play a role in scleral remodeling.

• KEYWORDS: human scleral fibroblasts; BMP-2; BMPRs

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INTRODUCTION

Myopia is a common visual disorder, and the prevalence of myopia and the degree of severity are rapidly increasing, especially in Asian country [1-5]. It is well known that the scleral fibroblasts are involved in scleral remodeling during axial elongation in myopia, during which the posterior sclera becomes thinner and the diameter of collagen fibrils smaller compared to sclera from normal eyes [6-8]. In recent years bone morphogenetic proteins (BMPs) has been found to be expressed in several ocular tissues, such as conjunctiva [9], cornea [10], trabecular meshwork [11], lens [12], sclera [13, 14], optic nerve[11]and retina[15]. BMPs are believed to play an important role in eye development and differentiation. Other cellular functions include morphogenesis, cell proliferation, apoptosis and extracellular matrix synthesis [16].

BMPs are multi-functional growth factors belonging to the superfamily of the transforming growth factor-β (TGF-β), which interact with bone morphogenetic protein receptors (BMPRs). BMPRs include three distinct subtypes, BMPR IA, BMPR IB and BMPR II. Bone morphogenetic protein 2 (BMP-2) is a member of the BMPs and displays the strongest activity of the BMPs [17]. Previous studies have shown that BMP-2 and BMPRs are expressed in the sclera of both human and guinea pigs at the mRNA level, but the expression at protein level and the distribution of BMPRs does not seem to be fully documented [13,14]. In present study, the aim was to clarify the distribution of BMP2 and BMPRs in normal human sclera and in HSFs *in vitro*.

SUBJECTS AND METHODS

Subjects This study was approved by the Ethics Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University (Guangzhou, China) and complied with the tenets of the

Table 1 Primer sequences used in polymerase chain reaction

Name	Genbank accession number	Upstream primer	Downstream primer	Size(bp)
BMP-2	NM_001200.2	AAACGTCAAGCCAAACAC	GAGCCACAATCCAGTCAT	109
BMPR IA	NM_004329.2	TATGGCACCCAAGGAAAG	CCTGTGATACAACGACGAG	293
BMPR IB	NM_001203.2	ACCCTACACTGCCTCCATTG	AACCCAATGCTGTATCGAGG	169
BMPR II	NM_001204.6	ACCCTACACTGCCTCCATTG	AACCCAATGCTGTATCGAGG	224
GAPDH	NM_002046.3	GCTCAGACACCATGGGGAAGGT	GTGGTGCAGGAGGCATTGCTGA	474

Declaration of Helsinki for biomedical research involving human subjects. Normal human eyes from donors ranging from 18 to 25 years of age were obtained from the Guangdong Province Eye Bank (Sun Yat-sen University).

Methods

Frozen section of human sclera preparation From donor eyeball number 1 and 2, all eye contents were removed and both of the sclera surfaces were cleaned. The posterior sclera was cut into 5mm×5mm pieces, embedded with optimum cutting temperature compound (OCT), then cut into 5μm sections at -20℃. They were tiled onto carrier slices, and fixed with paraformaldehyde for 15 minutes at 37℃, air-dried, and kept frozen at -20℃ until use.

Human scleral fibroblast isolation and culture Two eyes were washed immediately in phosphate buffered solution(PBS),containing 1×antibiotic/antimycotic (penicillin-streptomycin solution) (Invitrogen Corp. Carlsbad, CA). The retina and choroid were removed from the sclera and both surfaces of sclera were cleaned. The sclera was trimmed into pieces approximately 1mm×1mm, placed in 25mm^2 plastic culture bottles (Corning Ltd) in DMEM/F12 (Gibco) with 1× antibiotic/antimycotic and 15% fetal bovine serum (FBS, Gibco), and then incubated at 37℃ in a humidified incubator containing 5% CO_2. The growth medium was changed every 3 or 4 days. When a heavy primary monolayer was achieved, the cells were trypsinized for 3 minutes at 37℃ in 0.25% trypsin/EDTA solution in PBS and subcultured at a split ratio of 1:2 or 1:3 in a 25mm^2 plastic bottle. The fourth to sixth fibroblast passage was used for this experiment. The purity of fibroblast cultures was confirmed by staining for vimentin and stain resistance for cytokeratin, desmin, and S-100, using the indirect immunofluorescence procedure, as previously described [18]. The HSFs were grown in 25mm^2 plastic culture bottles for extraction of RNA and were grown on coverslips in six well plates (Corning Ltd) to 50% confluence. The cells were washed with PBS three times, fixed with cold methyl alcohol for 20 minutes at -20℃, air-dried, and kept frozen at -20℃ until use. The HSFs morphology was observed with light microscopy.

RNA isolation and reverse transcription polymerase chain reaction Total RNA was extracted from HSFs or human sclera using the PureLink RNA mini kit (Invitrogen Corp. Carlsbad CA USA) according to the manufacturer's instructions. Complementary DNAs (cDNAs) were synthesized using primescriptTM 1st strand DNA synthesis kit (TaKaRa Biotechnology, Japan) with 1μL of total RNA, 5×Prime Script buffer 2μL and RNase free ddH_2O 7μL at 37℃ for 15 minutes, immediately at 85℃ for 5 seconds, then keeping at 4℃. The primer sequences used in polymerase chain reaction were shown in Table 1. Each PCR was performed in a 25μL solution containing 1μL (10μmol/L) of each forward and reverse primer, 12.5μL of Premix Ex Taq DNA polymerase (TaKaRa Biotechnology, Japan), and 1μL reverse transcription reaction products. Amplification was conducted for 30 cycles in a professional thermo cycler (Biometra, Germany). Each cycle consisted of 45 seconds at 94℃ for denaturation, 30 seconds at 55℃ for annealing, and 60 seconds at 72℃ for extension. PCR amplification of GAPDH was performed in parallel as an internal control. The amplified products were analyzed with 1% agarose gel electrophoresis (Invitrogen, Life Technologies, Grand Island, NY) containing ethidium bromide and then photographed under ultraviolet light illumination. A standard DNA ladder was used as a size marker. All experiments were done in triplicate. All RT-PCR products were compared to GAPDH cDNA products from corresponding samples, and all band intensities were evaluated by densitometry.

Indirect immunofluorescence The slides were washed three times with PBS, and incubated with 10% normal goat serum (Boster Biological Technology, Wuhan, China) diluted in PBS for 30 minutes at 37℃. Then, the slides were incubated at 4℃ over night with primary antibody (BMP-2, BMPR IA, BMPR IB, BMPR II, Santa Cruz Biotechnology, Inc. CA, USA), diluted at 1:50 in PBS. Cells were incubated in PBS without primary antibodies as a negative control. The slides were washed with PBS and exposed to fluorescein isothiocyanate-conjugated (FITC) anti-rabbit/goat IgG fragment secondary antibody (Santa Cruz Biotechnology, Inc.CA, USA), diluted at 1:100 in PBS at 37℃ for one hour. The slides were washed in PBS three times, and then stained with propidiumiodide (PI) (Sigma Aldrich, USA) to stain the cell nuclei red for 10 minutes. Immunofluorescent images were taken with a confocal microscope (LSM 510 META, Carl Zeiss, Jena, Germany).

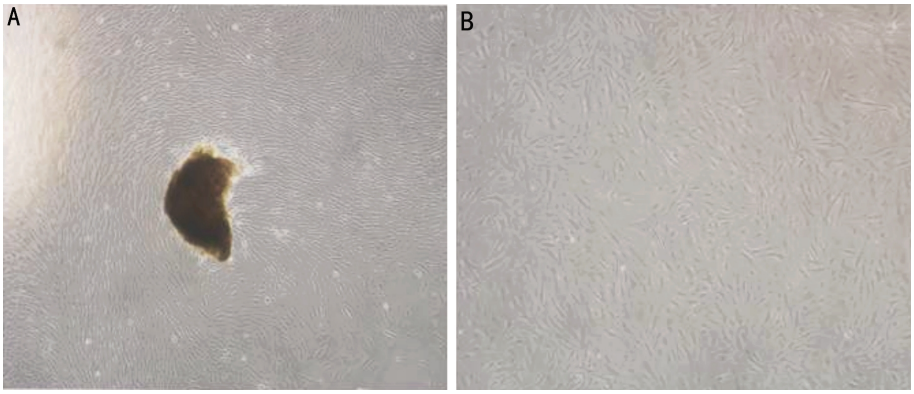

Figure 1 Primary culture of human scleral fibroblasts (HSFs) HSFs migrated from the piece of sclera tissue, populated the surroundings and exhibited a uniform, fusiform shape. The growth was compact and arranged in a radiating pattern. A: ×50; B: ×100.

Western blot analysis HSF were cultured in DMEM/F12 with 10% FBS 3 or 4 days, then cells were washed with PBS three times and lysed in ice-cold lysis buffer (Shanghai XinghanSci & Tech Co. Ltd, Shanghai, China). After cell debris was centrifuged at 15 000g at 4℃ for 30 minutes, the protein was detected by BAC kits. Protein (40μg) was loaded in each lane of 10% SDS polyacrylamide gels, transferred onto polyvinylidenedifluoride membranes for electrophoresis, blocked in TBST (5% fat-free dry milk, 0.1% Tween 20, 150mmol/L NaCl, and 50mmol/L Tris at pH 7.5) for 1 hour. The membranes were exposed to 1μg/mL of anti-BMP2, anti-BMPR IA, anti-BMPR IB, and anti-BMPR II polyclonal antibody and incubated overnight at 4℃. The same blots were then stripped and reanalyzed using anti-GAPDH antibodies (Proteintech Group, Inc., USA) as an internal control. The membranes were then incubated with a secondary horseradish peroxidase-labeled antibody for 1 hour. Protein bands were visualized with the use of a chemiluminescence Phototope (R)-HRP Western Blot detection system (Cell Signaling Technology, Inc., Danvers, MA) and exposed onto a negative film, developed, and fixed. The film was scanned and then analyzed with Bio-Rad Quautity Imaging software (Bio-Rad Laboratories, Inc. USA).

RESULTS

Primary Culture of HSFs After two weeks cultured, the HSFs migrated from the pieces of sclera tissue and populated the surroundings. HSF morphology was observed under light microscopy. The HSFs exhibited a uniform, fusiform shape and the growth was compact and arranged in a radiating pattern, as showed in Figure 1.

BMP -2 and BMPRs expressions in human scleral fibroblasts Because BMP-2 plays a role in HSFs through the BMPRs, the mRNA expressions of BMP-2 and all the BMPRs at mRNA levels in HSFs were detected by RT-PCR, as shown in Figure 2. BMP-2 and the BMPRs were both expressed in HSFs. Compared to BMPR IA and BMPR II, the expression of BMPR IB in HSF cultured *in vitro* is weak.

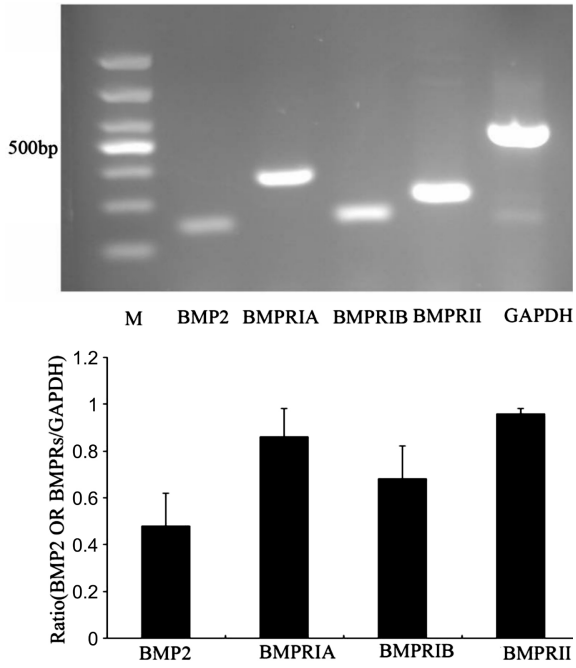

Figure 2 Bone morphogenetic protein 2 (BMP-2) and the bone morphogenetic protein receptors (BMPRs) mRNA levels detection in human scleral fibroblasts (HSFs) Semi-quantitative RT-PCR analysis of total RNA from normal human sclera and HSFs using specific primers for BMP-2, BMPR I A, BMPR I B, BMPR II normalized to GAPDH at 30 cycles (M: molecular size marker). It shows that BMP-2 and the BMPRs are both expressed in HSFs, BMPR IA and BMPR II are strongly expressed, the expressions of BMPRI A and BMPRII are higher than that of BMPRI B.

To further confirm the mRNA result, we detected BMP-2 and the BMPRs in protein levels in cultured HSFs by immunocytofluorescence and western blot methods. As show in Figure 3 and 4, BMP-2 and BMPRs were expressed in the primary cultured HSFs. The protein result was similar to the mRNA result, as the expressions of BMPR IA and BMPR II were stronger than BMPR IB.

BMP-2 and the BMPRs in protein levels in normal human sclera As shown in Figure 5, immunohistofluorescence showed expressions of the protein of BMP-2 and BMPRs in

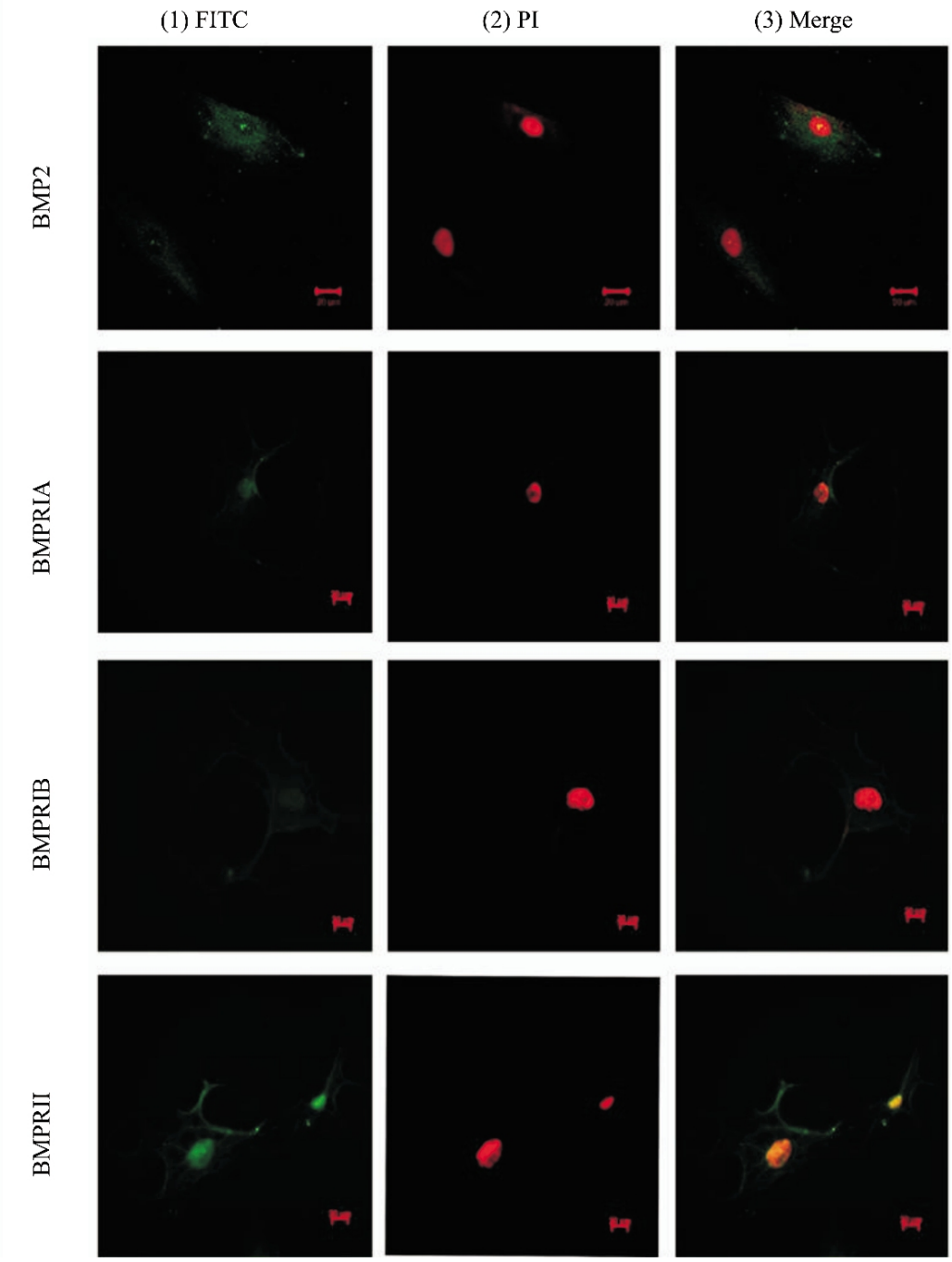

Figure 3 Distributions of bone morphogenetic protein 2 (BMP−2) and bone morphogenetic protein receptors (BMPRs) in human scleral fibroblasts (HSFs) by indirect immunocytofluorescence FITC marked the secondary antibody (green, 1) and PI dyed the nucleus (red, 2). (1) and (2) are combined into (3). The original magnification was ×400 and the scale bar was 20μm.

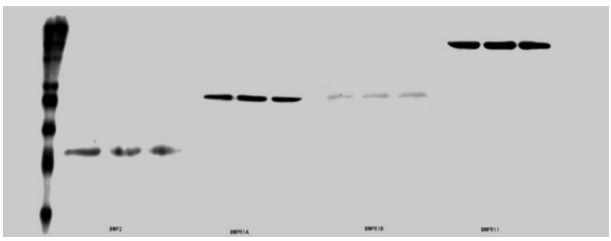

Figure 4 Expression of bone morphogenetic protein 2 (BMP−2) and bone morphogenetic protein receptors (BMPRs) in human scleral fibroblasts (HSFs) by western blot method The result shows that BMP-2 and the BMPRs are both expressed in HSFs, the expressions of BMPRI A and BMPRII are strong, but only weak signal is detected about BMPRI B.

the human sclera. Thus, BMP2, BMPR IB and BMPR II were found to be expressed in cytomatrix of HSF. In contrast, only weak signal was detected about BMPRI A.

DISCUSSION

In the current study, the expressions of BMP-2 and BMPRs in mRNA and protein levels in human sclera as well as in HSFs cultured *in vitro* were investigated. Although previous studies have shown that BMP-2 and BMPRs are expressed in both human and guinea pig's scleras at the mRNA level, the expression at protein level and the distribution of BMPRs remains uninvestigated [13,14]. Our findings of only a weak expression of BMP-2 in normal human sclera and

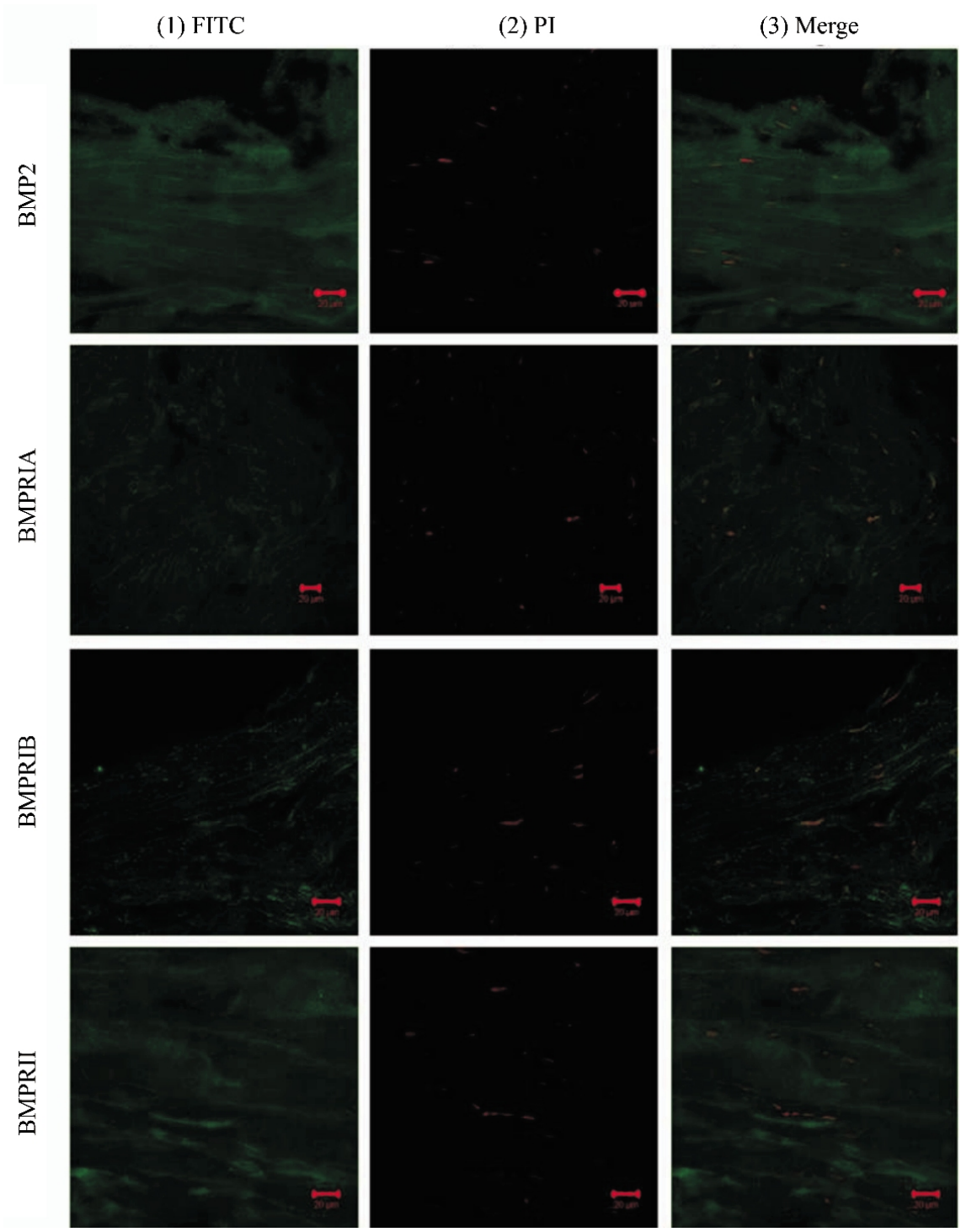

Figure 5 Distribution of bone morphogenetic protein 2 (BMP-2) and bone morphogenetic protein receptors (BMPRs) in normal human sclera by indirect immunohistofluorescence FITC marked the secondary antibody (green,1) and PI dyed the nucleus (red, 2). (1) and (2) are combined into (3). The original magnification was × 400 and the scale bar was 20μm.

HSFs are in accordance with earlier findings. BMP-2 appears to be localized in the nucleus and the cytoplasm of HSFs. The differential localization of the BMPs, with BMPR IA and BMPR II localized in both cytoplasm and cytomembrane, and BMPR IB weakly expressed in cytoplasm and cytomembrane, can be ascribed to different cellular functions of these receptor subtypes.

BMP-2 is a member of the BMP subgroup of the TGFβ superfamily. Human BMP-2 is synthesized as a 396 amino acid (AA) preproprotein that contains a 23 AA signal sequence, a 259 AA prosegment, and a 114 AA mature region. BMP-2 may play a major role in regulating function of ocular tissues. Expression of BMP-2 and its receptors in conjunctiva has been reported, suggesting that modulation of BMP/activin activities may help controlling postoperative conjunctival scarring responses in glaucoma patients [9]. BMP-2 and BMP-4 are expressed in cultured human corneal cells, where BMP-2 as a heparin-binding cytokine may

modulate corneal fibroblast apoptosis [10,19]. BMP-2 /BMP-4 may act as negative growth regulators in adult retinal pigmented epithelium (RPE) [15]. BMP-2 is expressed in the human sclera, where it is believed to promote cell proliferation and induce changes in extracellular matrix (ECM) components such as Type I collagens, aggrecan, MMP-2,TIMP-2 [13,14,20]. The scleral remodeling of myopic eyes, which leads to thinning and ectasia of the posterior sclera, is accompanied by changes in the composition of the ECM [21].

BMPRs include three distinct subtypes, BMP IA, BMP IB and BMPR II, which are expressed at the cell surface as homeric and heteromeric complexes. Theserine/threonine kinase domains of the type II receptor are constitutively active and, upon BMP binding, phosphorylate Gly-Ser domains in the type I receptor [16]. BMPs exert their effects through distinct combinations of BMP I and BMP II serine/threonine kinase receptors [22,23]. The oligomerization pattern of the BMPRs appears to be different from that of receptors used by other members of the TGF-b superfamily [24]. In present study the amount of the expressions of BMPIA and BMPIB were significantly different, which could explain the pleiotropic effects of BMP-2 action in human sclera.

In conclusion, we have shown that BMP-2 and BMPRs are expressed in normal human sclera and HSFs cultured *in vitro*. There are indications that BMP-2 plays an important role in remodeling of the sclera, but the exact mechanisms remain unclear. Further research is needed to determine which of the BMPR subtypes are activated by BMP in the sclera.

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