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Over-expression of BMP4 inhibits experimental choroidal neovascularization by modulating VEGF and MMP-9

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Abstract

Bone morphogenetic protein (BMP)-4 has been shown to play a pivotal role in eye development; however, its role in mature retina or ocular angiogenic diseases is unclear. Activating downstream Smad signaling, BMP4 can be either pro-angiogenic or anti-angiogenic, depending on the context of cell types and associated microenvironment. In this study, we generated transgenic mice over-expressing BMP4 in retinal pigment epithelial (RPE) cells (*Bmp4-Vmd2* Tg mice), and used the laser-induced choroidal neovascularization (CNV) model to study the angiogenic properties of BMP4 in adult eyes. *Bmp4-Vmd2* Tg mice displayed normal retinal histology at 10 weeks of age when compared with age-matched wildtype mice. Over-expression of BMP4 in RPE in the transgenic mice was confirmed by real-time PCR and immunostaining. Elevated levels of Smad1,5 phosphorylation were found in BMP4 transgenic mice compared to wildtype mice. Over-expression of BMP4 was associated with less severe CNV as characterized by fluorescein angiography, CNV volume measurement and histology. While control mice showed increased levels of vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP)-9 after laser injury, *Bmp4-Vmd2* Tg showed no increase in either VEGF or MMP-9. Further, we found that TNF-induced MMP-9 secretion *in vitro* was reduced by pretreatment of RPE cells with BMP4. The inhibition of MMP-9 was Smad-dependent because BMP4 failed to repress TNF-induced MMP-9 expression when Smad1,5 was silenced by siRNA. In summary, our studies identified an anti-angiogenic role for BMP4 in laser-induced CNV, mediated by direct inhibition of MMP-9 and indirect inhibition of VEGF.

Keywords

BMP4; MMP-9; VEGF; Choroidal neovascularization (CNV); Retinal pigment epithelium (RPE)

Introduction

Bone morphogenetic proteins (BMPs) are a group of growth factors that participate in a wide variety of cellular processes [1]. Among BMPs, BMP4 plays a key role in eye morphogenesis in the mouse and specification of the retinal pigment epithelium (RPE) in the chick retina [2, 3]. While BMP4 and associated Smad signaling are well studied developmentally in the eye [4], little is known about the physiological and pathological roles

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of BMP4 in the mature retina or in retinal disease. Age-related macular degeneration (AMD) is the most common cause of irreversible blindness in the elderly and can progress to two distinct late forms of the disease. Atrophic AMD is characterized by degeneration and death of the RPE in the macular region with associated loss of photoreceptors [5]. In contrast, neovascular AMD is an angiogenic complication of the disease in which choroidal endothelial cells proliferate and migrate through the adjacent Bruch's membrane to the sub-RPE or sub-retinal space where they can leak and hemorrhage; a process known as choroidal neovascularization (CNV) [6]. CNV accounts for the majority of cases of severe visual loss in AMD [6]. In CNV lesions, RPE are activated, and are a source of vascular endothelial growth factor (VEGF) [7]. We recently showed that BMP4 is highly expressed in the RPE of patients with atrophic AMD and that BMP4 induces senescence in RPE cells in culture [5]. In contrast, our evaluation of surgically excised CNV lesions from patients with AMD showed that BMP4 was immunohistochemically absent from RPE in the neovascular lesions suggesting that BMP4 might be a molecular switch that determines which late form of AMD a patient will develop [8].

Laser-induced rupture of Bruch's membrane in rodents or primates results in a reproducible wound healing response under the retina that is widely used as a model of CNV, as well as a general model of pathologic angiogenesis [9, 10]. VEGF has been identified as a key player in the pathogenesis of CNV in both animal studies and patients with AMD [11]. However, transgenic mice over-expressing VEGF either in photoreceptor cells or RPE fail to develop CNV spontaneously [12, 13], indicating VEGF alone is not sufficient for CNV formation. Instead, damage to Bruch's membrane, induced with either physical damage (eg. laser photocoagulation) or chronic inflammation (eg. subretinal matrigel implantation), is required for angiogenesis to occur [14]. In addition, integrity of RPE is required for CNV development because CNV does not occur when sodium iodate is used to eliminate RPE prior to laser photocoagulation [15].

RPE cells, sitting on Bruch's membrane, form the outer blood-retinal barrier between the vascular choriocapillaris and the neural retina, and support the bidirectional exchange of metabolic waste and nutrients [16]. RPE contribute to CNV development in several ways. RPE secrete pro-angiogenic factors, such as VEGF and angiopoietin-2, whose expression is up-regulated by macrophage-derived tumor necrosis factor (TNF) [17], itself a crucial mediator in experimental CNV [18]. In addition to proangiogenic factors, the migration and proliferation of new vessels in CNV also involves degradation of the extracellular matrix by matrix metalloproteinases (MMPs) [19]. Among MMPs, MMP-9 has been shown to be up-regulated by TNF in RPE cells [20], and MMP-9 knockout mice display reduced CNV phenotypes after laser photocoagulation [21]. Recently, we identified TNF as a negative regulator of BMP4 in RPE cells, and showed an inverse relationship between TNF and BMP4 expression in the laser-induced CNV mouse model [22]. However, it is unknown whether BMP4 plays a role in CNV pathogenesis. BMP4 has been reported to be either pro-angiogenic or anti-angiogenic in different conditions. For example, BMP4 was found to be strongly expressed in malignant melanoma cells where it had a paracrine pro-angiogenic effect on the tumor vasculature [23]. It has also been shown to induce capillary sprouting, migration and tube formation of human umbilical vein endothelial cells (HUVEC) [24, 25]. On the other hand, BMP4 was shown to induce apoptosis of capillary and venous endothelial cells but not arterial endothelial cells [26, 27]. Since BMP4 expression was increased in RPE in atrophic AMD and was not expressed in RPE in human CNV specimens, we evaluated the potential regulatory role of BMP4 in experimental CNV.

In this study, we evaluated the pathogenesis of laser-induced CNV in transgenic (Tg) mice over-expressing BMP4 under the control of the RPE-specific human vitelliform macular dystrophy type (VMD) 2/*BEST1* promoter [28, 29]. Best1 protein is first expressed in mice

at postnatal d10, so use of this promoter avoids the complication of modulating BMP4 levels during eye development [30]. Tg mice 6–10 weeks were used since independent age-effects have been identified in laser-induced CNV [31, 32]. The functional impact of BMP4 on CNV development was established by evaluating CNV pathogenesis in BMP4 Tg mice compared to age-matched wildtype (Wt) mice, with particular emphasis on VEGF and MMP-9 gene regulation by BMP4 *in vivo* and *in vitro*.

Materials and Methods

Generation of transgenic mice over-expressing BMP4 in RPE (*Bmp4-Vmd2* Tg mice)

A 661bp DNA fragment was PCR-amplified from the promoter region (–609 to +52bp) of the human *VMD2/BEST1* gene using human genomic DNA as a template [33]. Mouse BMP4 cDNA, digested from PMT-BMP4 vector, were subcloned directionally after the human *VMD2/BEST1* promoter into a PSKII vector (Stratagene, La Jolla, USA). The sequence of the DNA construct was confirmed by DNA sequencing. The DNA construct was micro-injected into fertilized mouse oocytes to generate transgenic mice. All the transgenic founder lines were genotyped with southern blot and PCR. The transgenic mice were backcrossed for 5 generations and maintained in the C57BL/6 background. Age-matched C57BL/6 mice were purchased from the National Cancer Institute (Frederick, USA) and used as controls. All experiments were performed in accordance with protocols approved by USC's Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Laser-induced CNV

The Wt and Tg mice were anesthetized for all procedures as previously described [34]. Four photocoagulation lesions were delivered with a diode laser (150 mW, 0.05 s, 75 μ m) between the retinal vessels in a peripapillary distribution in each fundus. Production of a subretinal bubble at the time of laser treatment confirmed the disruption of Bruch's membrane.

Fluorescein angiogram (FA)

The development of CNV in Wt and Tg mice was evaluated by semi-quantitative assessment of late-phase fluorescein angiograms, captured 3 minutes after intraperitoneal injection of 0.1 mL of 2.5% fluorescein sodium (Akorn, Decatur, USA) as previously described [34]. Leakage was defined as the presence of a hyperfluorescent lesion that increased in size with time in the late-phase angiogram. Angiography was graded in a masked fashion using reference angiograms. Angiograms were graded as follows: 0, no leakage; 1, slight leakage; 2, moderate leakage; and 3, prominent leakage.

Whole mount staining

After eyes from Wt and Tg mice were enucleated, the cornea, lens and retina were removed. Dissected eye-cups were fixed with 4% paraformaldehyde overnight and washed with PBS buffer containing 0.5% Triton-X (3 times). After blocking with 1% BSA in PBS/Triton-X for 2 hrs, rabbit anti-isolectin B4 (specific endothelial cell marker, 1:100; Vector Laboratories, Burlingame, USA) or rabbit anti ZO-1 (1:100, Invitrogen, Carlsbad, USA) antibodies was added and incubated at 4°C overnight. Eye-cups were washed with PBS/Triton-X (3 times) and incubated with fluorescein-conjugated anti-rabbit for 30 min, followed by three washing steps. Eye-cups were placed on the slide, and incisions were made to flatten the eye-cup, and additional muscle and fat tissue removed. Samples were then mounted with Vectashield mounting media (Vector Laboratories). Images were

obtained using a laser scanning confocal microscope (LSM; model 510; Carl Zeiss, Thornwood, USA) confocal microscope.

CNV volume quantification

After being stained with isolectin B4, retinal flatmounts were visualized with a laser scanning confocal microscope (LSM510; Carl Zeiss) using a 25× objective lens. Z stack images of CNV lesion were taken. The image stacks were rendered in 3D using Volocity imaging software (Improvision Inc., Waltham, USA) and processed to digitally extract the fluorescent lesion volume. CNV volume was measured in micrometers cubed.

Retinal section histology or immunohistochemistry

Cryostat sections (8 μm) of snap-frozen posterior eye-cups were obtained from Wt and Tg mice at Day 7 after laser treatment. Sections were obtained from the central region of each lesion. After fixation with 4% paraformaldehyde for 30 minutes, the sections were used for hematoxylin and eosin (H&E) staining or immunohistochemistry. For immunohistochemistry, the fixed sections were rinsed in PBS twice and incubated with a blocking agent of 10% normal goat serum for 30 minutes. Sections were then incubated with either rabbit anti-VEGF antibody (1: 100 dilution, Santa Cruz Biotechnology, Santa Cruz, USA) or goat anti-MMP-9 antibody (1:100 dilution, R&D Systems, Minneapolis, USA) overnight, followed by Cy5-conjugated anti-rabbit or anti-goat IgG secondary antibody for 30 minutes. Images were obtained using a Leica microscope.

Gross measurement of RPE, outer nuclear layer (ONL) and inner nuclear layer (INL) thickness

After H&E staining, slides were scanned at 40× using an automated ScanScope digital slide scanner (Aperio, Vista, USA). The thickness of RPE, ONL and INL was measured using the ruler tool of Aperio ImageScope software (v10.2.2.2319). Data was the average of total 18 measurements (six randomly-chosen measurements per animal; n=3 per group).

Real-time RT-PCR

Total RNA was isolated (TRIzol extraction protocol; Invitrogen), and reverse transcription was performed with 1 μg total RNA according to the manufacturer's protocol (Promega, Madison, USA). The PCR was performed on a thermocycler (model LC 480 light cycler; Roche Diagnostics). Quantification of mRNA was normalized with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. Reaction conditions were as follows: 5 min at 95°C followed by 45 cycles of 10 sec at 95°C, 20 sec at 55°C, and 20 sec at 72°C. The sequences of primers used for mouse VEGF-A were forward: 5'- ACT GGA CCC TGG CTT TAC TG-3', reverse: 5'- TCT GCTCTC CTT CTG TCG TG-3'; mouse GAPDH forward: 5'-ATGGTGAAGGTCGGTGTGA -3', reverse: 5'-AATCTCCACTTTGCCACTGC -3'. Relative changes in mRNA expression (the target gene / GAPDH) were determined by calculation of $-2^{\Delta\Delta CT}$.

Cell culture and treatment

Human RPE cells were isolated from fetal eyes of 18–20 weeks' gestation obtained from Advanced Bioscience Resources, Inc. as previously described [35], and were cultured in Dulbecco's modified Eagle's medium (DMEM). Second- to fourth-passage cells grown to confluence for 48 to 72 h were used for these experiments. Confluent primary human RPE cells were adapted to serum-free DMEM overnight. The cells were then treated with or without recombinant human BMP4 (50 ng/ml, R&D Systems) in serum free medium for 24 h. The next day, the cells were incubated in fresh serum-free medium with TNF (10 ng/ml, R&D Systems) with or without BMP4 for another 18 h.

Enzyme-linked immunosorbent assay (ELISA)

Enucleated posterior eye-cups of Wt and Tg mice at Day 4, 6, 8, 11 and 14 after laser were homogenized and stored at -80°C until further analysis. VEGF concentrations in these samples were measured by ELISA according to the manufacturers' protocols (Quantikine; R&D Systems). Total protein concentration was measured using Bio-Rad protein assay (Bio-Rad, Hercules, USA). Data derived from standard curves were expressed as picograms per milliliter per mg of total cellular protein.

Western Blot analysis

Proteins were extracted from cultured RPE cells using T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, USA) or from mouse posterior eye-cups using RIPA buffer (Cell Signaling, Danvers, USA) according to the manufacturer's protocol. The protein concentration was measured using Bio-Rad protein assay (Bio-Rad). Equal amounts of protein lysate (20–60 μg) were resolved on 4–10% Tris-HCl polyacrylamide gels (Ready Gel; Bio-Rad) and transferred to a PVDF blotting membrane (Millipore, Billerica, USA). Next, the membrane was blocked with 5% nonfat milk and incubated with antibodies specific for MMP-9 (R&D) or phospho-Smad1,5 (Cell Signaling). After incubation with a horseradish peroxidase-conjugated secondary antibody (Vector Laboratories), protein bands were detected by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, USA). The membrane was re-probed with anti-GAPDH (Millipore) for equal protein loading. Band intensities were quantitated from three independent experiments using image processing and analysis software (NIH Image). JNK pathway inhibition was achieved using 5 μM SP600125 (EMD 4 BioSciences; Rockland, MA).

Zymography

The conditioned medium collected from RPE cells was concentrated sixty-fold using Nanosep MF Centrifugal Devices with a 10K MWCO (Pall Life Sciences, Covina, USA). The concentrated medium was mixed 1:1 with the loading buffer and loading onto a pre-cast gel with 1% gelatin (Bio-Rad). After electrophoresis, the gel was incubated for 30 minutes at room temperature in the renaturing buffer containing 0.25% Triton X-100 (Bio-Rad) to remove sodium dodecyl sulfate. The gel was then incubated overnight at 37°C with the developing buffer (Bio-Rad) containing 5 mM Tris-HCl (pH 7.5), 20 mM NaCl and 0.5 mM CaCl_2 . MMP-2 and MMP-9 activities were visualized as negative bands on the gel by staining the gel with Coomassie blue (Bio-Rad). Band intensities were quantitated from three independent experiments using image processing and analysis software (NIH Image).

siRNA transfection

Human fetal RPE cells (2×10^5 cells/well) were seeded in 6-well plates 24 hours before transfection with small interfering RNA (siRNA) targeting Smad1 (25 nM), Smad5 (25 nM) or scrambled control siRNA (50 nM, Qiagen, Valencia, USA) using HiPerFect (Qiagen) according to the manufacturer's protocol. Cells were used 48 hours after transfection.

Statistical analysis

Statistical software package SAS, Version 9.2 (SAS Institute Inc., Cary, USA) was applied to VEGF mRNA and protein analyses in this study. Multivariable linear regression models were used to examine the differences of VEGF expression between Tg and Wt mice. Tukey-Kramer method was used to adjust for multiple comparisons. Other statistical analyses were performed with Student's t-test. Values in the figures were expressed as the mean $\pm$ SEM. All p-values reported were 2-sided, and $P < 0.05$ was considered statistically significant.

Results

Transgenic mice over-expressing BMP4 in RPE layer

We created RPE-specific BMP4 over-expressing mice, whose BMP expression was driven by human *VMD2* gene promoter. Seven transgenic founder lines were generated and confirmed by Southern blot and PCR (data not shown). These transgenic mice did not show any gross abnormalities at birth and had normal fertility and life spans. Among seven founder lines, 3 lines of transgenic retinas expressed significantly higher level of BMP4 using real time PCR, which were about 2.3 times, 1.8 times and 1.6 times higher than that of wild-type littermates, respectively (Fig. 1A, $P<0.01$, $n=6$ /group). No increase in BMP4 was found in neural retina or brain of Tg mice (results not shown). Immunofluorescence staining of retinal sections from 4 week old animals showed increased BMP4 protein expression in RPE and Bruch's membrane in Tg mice than in Wt mice (Fig. 1B–J). Higher expression of phosphorylated Smad1,5, the downstream mediators of BMPs, was detected in Tg mice as compared to age-matched control Wt mice. The phosphorylated Smad1,5 was identified in RPE, and cells within the choroid (Fig. 1K). Our experimental data indicated that BMP4 was over-expressed in the RPE of Tg mice with downstream signaling effects in both RPE and choroid.

Comparing Tg and Wt mice (4–10 weeks old), we found no difference in retinal morphology by fundus images (data not shown) or H&E staining of retinal sections (Fig. 2A& B). The thickness of RPE, ONL and INL layers were measured and no significant differences were found between Tg and Wt mice (Fig. 2C). In addition, ZO-1 staining of retinal flat mounts showed hexagonal shape of normal RPE cells in both Tg and Wt mice (Fig. 2D).

Over-expressed BMP4 inhibited CNV formation

At Day 7 after laser, CNV lesion size and leakage, as evaluated by FA score, was significantly lower in Tg mice than Wt (Fig. 3A, $*P<0.01$, $n=8$ mice/group). Although the trend was the same, the FA scores between Tg and Wt mice were not significantly different at Day 14 after laser (Fig. 3A, $n=6$ mice/group). A representative FA image at Day 7 after laser is shown in Fig. 3B.

The volume of the CNV lesions on Day 7 after laser photocoagulation was evaluated by labeling choroidal vessels with FITC-conjugated isolectin B4 on flatmounts (Fig. 3C). Quantitative analysis of the CNV volume showed there was a 54% reduction in choroidal vascular lesion volume in Tg mice compared with that in Wt mice ($P<0.05$, Fig 3D, WT $n=8$ mice, 20 lesions; Tg $n=10$ mice, 29 lesions) on Day 7 after laser injury, which is consistent with our FA analysis.

Since there was a significant difference in CNV lesion between Tg and Wt mice on Day 7 after laser treatment, the histology of CNV lesions at this time point was further characterized. A reduction in lesion diameter and thickness was observed in Tg mice, compared to that of the Wt mice (Fig. 3E, $n=4$ mice/group). In addition, Tg mice contained decreased subretinal fluid, indicated by smaller subretinal space in the CNV lesion; there was also less accumulation of mononuclear cells within the space. Instead, pigmented cells show increased coverage of the CNV lesion in Tg mice (arrows in Fig. 3E).

Over-expressed BMP4 down-regulates VEGF and MMP-9 expression *in vivo*

VEGF is known as one of the most potent pro-angiogenic factors in pathologic neovascularization [36, 37]. We examined VEGF mRNA expression in posterior eye-cups of Tg and Wt mice using real-time PCR. VEGF mRNA levels between Tg and Wt mice did not differ at baseline; however, VEGF mRNA levels were significantly higher in Wt compared

to Tg at d4, 6, 8, and 11 after laser (Fig. 4A, # $P<0.05$; $n=3-6$ /group), while VEGF mRNA levels did not increase at any time point in the Tg mice. At d14 both Tg and Wt VEGF mRNA levels were not significantly different from baseline.

The VEGF protein levels (VEGF₁₂₀ and VEGF₁₆₄) were measured in posterior eye-cups of Tg and Wt mice using ELISA. VEGF protein levels between Tg and Wt mice did not differ at baseline; however, VEGF protein levels were significantly higher in Wt compared to Tg at d4, 6, 8, 11 and 14 after laser (Fig. 4B, # $P<0.05$; $n=3-6$ /group), while VEGF protein levels did not increase at any time point in the Tg mice (Fig. 6–4B).

We also compared VEGF protein expression in the Wt and Tg mice by immunofluorescence staining. No difference in VEGF expression was found in non-lasered Tg and Wt retinas (Fig. 4C). In Wt mice on day 7 after laser injury, there was increased VEGF expression in radial cells with the appearance of Muller cells in the retina and in cells within the lesion compared to the Tg (Fig. 4D).

The importance of MMP-9 in CNV formation has also been demonstrated in several studies [19,21]. It has been suggested that MMP-9 could act both upstream and downstream of VEGF in angiogenesis [38]. We evaluated MMP-9 expression in mouse retinal sections by immunofluorescence staining. One week after laser induction, in the CNV lesion areas, MMP-9 expression levels in Tg mice were much weaker compared with Wt mice (Fig. 5A, $n=4$ /group). To confirm reduced MMP-9 expression in the Tg mice after laser photocoagulation, we also utilized Western Blots to assess MMP-9 level using total protein extracts from mouse posterior eye-cups. One week after laser treatment, MMP-9 expression in Tg mice was much less than that in WT mice (Fig. 5B, $n=3$ /group). However, baseline MMP-9 expressions in non-treated Wt and Tg mice were similar with very low expression (data not shown).

BMP4 repressed MMP-9 induced by TNF in RPE cells

One source of the MMP-9 present in the CNV lesions is the RPE [20]. We used early passage primary cultured human RPE cells to determine whether BMP4 exposure could affect MMP-9 expression. The culture media were concentrated and used in zymography to detect expression and activity of MMPs. Consistent with our previous studies¹⁷, resting RPE cells did not secrete MMP-9 but rather MMP-2. BMP4 alone did not change the MMP-9 or MMP-2 expression (Fig. 6A). Since the secretion of MMP-9 was so low in resting RPE, we could not identify a suppressing effect of BMP4 on MMP-9 expression. Therefore, we induced MMP-9 expression by TNF stimulation. As expected, TNF induced MMP-9 secretion prominently ($p<0.05$) but did not affect MMP-2 secretion (Fig. 6A). We then examined whether BMP4 had any effects on MMP-9 induction by TNF. RPE cells were incubated with or without BMP4 overnight, followed by incubation with TNF in fresh medium with or without BMP4. MMP-9 secretion and activity in the conditioned medium were evaluated using zymography. We found that there was a significant reduction of MMP-9 secretion if RPE cells were pretreated with BMP4 and continuously exposed to BMP4 + TNF as compared to TNF alone (Fig. 6B; $n=3$; $p<0.05$).

Our previous studies showed that in RPE cells down-regulation of BMP4 by TNF required JNK signaling but not NF- κ B¹⁹. RPE cells were pre-treated with either BMP4 or chemical inhibitors for JNK or NF- κ B, followed by TNF stimulation for 24 h. Our zymography study of supernatants from treated cells showed that inhibition of NF- κ B (data not shown) and inhibition of JNK activation significantly reduced the ability of TNF to induce MMP-9 expression (Fig. 6C; $n=3$; $p<0.05$). The treatment of RPE cells with NF- κ B or JNK inhibitors or TNF at the same concentrations and time points did not cause increased RPE cell death (data not shown).

Smad1,5 activation was required for the down-regulation of TNF-induced MMP-9 by BMP4

Next, we sought to determine whether BMP4's inhibition of MMP-9 was Smad-dependent. RPE cells were treated with BMP4 for 0, 5, 15, 30, 60 and 90 minutes. Western Blot analysis showed that phosphorylation of Smad1,5 was increased at 30 min after BMP4 stimulation, the signal reached its apparent maximum at 60 min and was still elevated at 90 minutes (Fig. 7A; $n=3$; $p<0.05$). The necessity of Smad1,5 activation in this regulation was tested by siRNA. Our results (Fig. 7B) showed that the combination of siRNAs for Smad1 and Smad 5 efficiently blocked BMP4-mediated Smad phosphorylation, compared to untreated cells or cells transfected with scrambled siRNAs. We then examined whether BMP4 could inhibit TNF-induced MMP-9 expression when Smad1,5 was knocked down by siRNAs. The zymography studies showed, in cells transfected with siRNA targeting Smad1,5, BMP4 failed to repress TNF-induced MMP-9 secretion, while BMP4 repressed TNF-induced MMP-9 secretion in control cells ($n=3$; $p<0.05$) or cells transfected with scrambled siRNAs ($n=3$; $p<0.05$). These results indicate that Smad1,5 activation is required for BMP4's inhibitory effects (Fig. 7C).

Discussion

In this study, we identified BMP4 as an anti-angiogenic factor in CNV development and found that BMP4 suppressed VEGF and MMP9 up-regulation at the site of angiogenesis using a transgenic mouse model.

We generated transgenic mice that specifically over-expressed BMP4 in RPE, using the promoter of the human VMD2 gene. This is consistent with previously published data that expression of the LacZ reporter gene under the VMD2 promoter is specifically and sufficiently expressed in mouse RPE cells [33]. Postnatal activity of the VMD2 promoter is well suited for our study since we want to over-express BMP4 in adult mice without disturbing the development of the retina and RPE during early embryonic stages [26, 39]. We confirmed BMP4 over-expression in adult Tg mice. However, the level of BMP4 expression measured by real-time PCR was approximately 2 times higher in Tg mice than that in Wt control mice. At the same time, immunofluorescent staining showed a prominent increase in BMP4 in RPE. A possible reason for the relatively low increase in mRNA, is that total RNA for measuring BMP4 expression was isolated from whole posterior eye-cups, and the expression level of the transgene was diluted by the endogenous BMP4 expressed by mouse retina.

RPE-derived VEGF has been shown to be necessary for the maintenance of choroidal capillaries [40] and increased VEGF has been associated with development of CNV when associated with damage to Bruch's membrane [7, 12–14]. Previous studies evaluating how VEGF is regulated by BMP4 in RPE are controversial. In non-polarized cells, BMP4 has been shown to either have no effect on VEGF expression in primary RPE [41], or to increase VEGF secretion in ARPE-19 cells, a human RPE cell line [42]. Our recent work also showed that BMP4 had no effect on VEGF secretion in non-polarized RPE cultures; however, basolateral VEGF secretion increased with BMP4 treatment in polarized RPE cultures [43]. In the CNV lesion, RPE lose their polarization and become transdifferentiated; therefore based on results above, it is likely that BMP4 results in decreased VEGF expression indirectly through effects on other cell types or mediators [7]. Here, we extend this work to in vivo studies and show that basal levels of VEGF in Wt and BMP4 Tg posterior eye cups were not significantly different using real-time PCR and ELISA. Since we measured VEGF expression using posterior eye-cups, not only RPE cells but Muller cells and other cell types in the sample could contribute to total VEGF expression, as shown in our VEGF immunofluorescent staining. VEGF can stimulate endothelial cell proliferation and migration as well as decrease vascular permeability. Increased VEGF levels in Wt mice

after laser treatment were not observed in Tg mice, which may explain the reduced laser-induced CNV phenotypes (lesion size and leakage, CNV volume and histology) in Tg mice over-expressing BMP4.

In addition to VEGF, we showed the absence of up-regulation of MMP-9 after laser treatment in BMP4 Tg mice compared to Wt mice. We further confirmed a direct inhibition of TNF-induced MMP-9 expression in human RPE cells by BMP4. However, BMP4 failed to inhibit VEGF expression with TNF treatment in human RPE cells (data not shown). Since MMP-9 is able to act upstream of VEGF in angiogenesis and a positive feedback exists between these mediators [38], it is possible that reduction of MMP-9 is one of the factors that is responsible for absence of elevated VEGF expression in BMP4 Tg mice after laser injury. Similarly, suppression of MMP-9 activity by exogenous BMP4 has been reported using MDA-MB-231 and PMA-treated MCF-7 cells [44], which helped to explain the inhibitory effects of BMP4 on metastatic potential of breast cancer cells. BMP4 was also reported to block MMP-3 and MMP-13 expression in C3H10T1/2 stem cells [45]. Although the regulation of MMPs by BMP4 in RPE cells has not been studied, some studies have suggested that BMP4 could modulate the production and/or organization of the ECM in the eye. One study showed that BMP4 haploinsufficient mice developed developmental ocular defects including ECM abnormalities [39]. Another group found that in optic nerve head cells BMP4 inhibited extracellular matrix proteins induced by TGF- β 2 [46]. However, our study is the first study to demonstrate the ability of BMP4 to modulate MMP-9 in angiogenesis regulation both *in vitro* and *in vivo*.

Signaling pathways underlying induction of MMP-9 by TNF have been widely investigated [47, 20]. In addition to NF- κ B activation, we also showed that TNF-induced MMP-9 expression required JNK pathway activation, complementary with our previous work showing the role of the JNK pathway in the down-regulation of BMP4 by TNF in RPE cells [22]. Therefore, our studies indicated a negative feedback between BMP4 and MMP-9 through JNK in CNV formation. When JNK is activated by increased pro-inflammatory factors such as TNF in CNV, BMP4 levels goes down, and its inhibitory effects on TNF-induced MMP-9 expression is impaired. TNF itself further up-regulates MMP-9 and other pro-angiogenic factors, promoting an environment in favor of new vessel growth.

We further showed that Smad activation was required for BMP4's repression of TNF-induced MMP-9 expression. BMP4 can also activate Smad-independent pathways, and our study did not rule out the possibility of down-regulating MMP-9 by BMP4 through non-Smad pathways. The detailed downstream signaling of BMP4 in the down-regulation of MMP-9 is beyond the focus of this study but we proposed an interesting hypothesis regarding to the emerging role of Smad signaling in miRNA maturation. In primary pulmonary smooth muscle cells, Smad proteins, the transducer of BMP4 signaling, have been shown to induce miRNAs including miR-212 [48]. The miR-212/132 family has been found to target MMP-9 *in vitro* and *in vivo* [49]. Therefore, it would be possible that repression of MMP-9 expression is mediated by an induction of miR-212 with BMP-4 in RPE cells. Detailed studies on how distinct signaling activation and interactions lead to transcriptional regulation of MMP-9 expression by BMP4 are needed in the future.

In summary, this study shows that transgenic over-expression of BMP4 in RPE cells plays an anti-angiogenic role and it accounts for the less severe laser-induced CNV phenotypes in BMP4 Tg mice compared to Wt mice. In Tg mice with laser-induced CNV, BMP4 over-expression in RPE cells disrupts TNF-mediated up-regulation of MMP-9 and VEGF expression. The direct down-regulation of TNF-induced MMP-9 by BMP4 was verified using primary RPE cells, which was Smad-dependent. The finding that BMP is anti-angiogenic and enable to regulate extracellular matrix proteins such as MMP-9, could also

apply to other pathological conditions like cancer, where up-regulated BMP4 has been shown to be anti-angiogenic by modulating the ECM.

This work provides further support for our hypothesis that BMP4 is a mediator in the molecular switch that determines whether an individual AMD patient would develop late atrophic or neovascular forms of the disease [8]. Chronic oxidative stress increases BMP4 expression in RPE cells [5] and up-regulated BMP4 could inhibit MMP-9 expression directly, and VEGF indirectly, creating an anti-angiogenic environment. However, in the presence of inflammatory mediators such as TNF, BMP4 would be down-regulated [22], and the inhibitory effects on MMP-9 and VEGF expression would be compromised, resulting in a pro-angiogenic environment that favors development of CNV.

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Fig. 1. Over-expression of BMP4 in RPE in *Bmp4-Vmd2* Tg mice

A. BMP4 mRNA expression by real time PCR in posterior RPE/choroid eye-cups from Wt and three lines of Tg mice at 10 weeks of age. BMP4 mRNA levels were 1.8, 1.6 and 2.3 times higher in Tg mice than that in Wt mice (* $P < 0.01$; $n = 6$ mice/group). B–J. BMP4 expression by immunofluorescent staining in retinal sections from 4 weeks old Tg and Wt mice. Tg mice showed stronger fluorescence of BMP4 in the RPE than Wt mice. Representative images were shown from two lines of Tg mice (B and E) and Wt mice (H). Pigmented RPE cells in the same section were also visualized under light microscopy (C, F,

and I) and shown as BMP-4/ light microscopy merged images (D, G, J). K. Evaluation of Smad1,5 phosphorylation using immunofluorescent staining. Smad activation was seen in the nuclei of cells of both Tg and Wt RPE. However, many more Smad phosphorylation-positive cells were observed in RPE (solid arrows) and choroid (dashed arrows) in Tg mice (Phospho-Smad1,5: red; DAPI: blue; n=3/group). Scale bars equal 10 μ m in J and 100 μ m in K.

Fig. 2. *Bmp4-Vmd2* Tg mice (4–10 weeks old) showed no retinal abnormality
A–B. Wt and Tg mice retinal morphology. There was no morphologic differences between Tg and Wt mice in ganglion cell layer, inner nuclear layer (INL), outer nuclear layer (ONL) and RPE layer. Representative images were shown. C. Quantification of INL, ONL and RPE thickness in retinal sections. Average RPE, ONL and INL thickness was not significantly different between Wt and Tg mice (n=3/group). D. ZO-1 staining of retinal flat mounts (n=3/group). RPE cells in both Wt and Tg mice showed normal hexagonal morphology. Representative images were shown. Scale bars equal 10 μm in A and D.

Fig. 3. Attenuation of laser-induced CNV in *Bmp4-Vmd2* Tg mice

A. Fluorescein angiography (FA) scores: CNV was induced by laser photocoagulation in Tg and Wt mice, and FA was quantified in Tg and Wt mice at 7 or 14 days after laser. FA scores after 7 days after laser treatment were significantly lower in Tg mice than Wt (* $P<0.01$; $n=8$ /group and 6/group at 1 and 2 weeks, respectively). B. Representative FA images from Wt and Tg mice at 7 days after laser treatment were shown. Leakage of fluorescein was reduced in the Tg mice compared to Wt mice. C. CNV volume was compared between Wt and Tg mice at 7 days after laser treatment with isolectin-B4 labeling of endothelial cells. Representative images are shown. D. Quantification of CNV volume. There was a 54% reduction of CNV volume in Tg mice compared to Wt mice (* $P<0.05$, WT $n=8$ mice, 20 lesions; Tg $n=10$ mice, 29 lesions). E. H&E staining of retinal sections from Tg and Wt mice at 7 days after laser treatment. CNV size was smaller in Tg mice than the control ($n=4$ mice/group). In the Tg retinal section, pigmented cells cover the CNV lesion (arrows). Scale bar=50 μm .

D

Fig. 4. Lower VEGF expression in *Bmp4-Vmd2* Tg mice mice after laser treatment

A. VEGF expression detected by real-time PCR. Total RNA extracted from mouse posterior eye-cups was used to measure VEGF mRNA expression (n=3/group at each time point). VEGF mRNA was significantly decreased in Tg compared to Wt mice at all time points post laser injury except day 14 (* $P < 0.05$ Tg compared to Wt controls; # $P < 0.05$ compared between Wt and Tg mice on the same day of laser treatment). B. ELISA was used to measure VEGF concentrations in posterior eye cups from Wt and Tg mice after laser injury (n=3/group at each time point). Compared to Wt mice on the same day after CNV induction, VEGF levels were significantly lower in Tg mice at every time point post laser photocoagulation. (* $P < 0.05$ compared to Wt controls; # $P < 0.05$ compared between Wt and Tg mice on the same day of laser treatment). C. VEGF expression was evaluated in retinal sections from non-lasered control Wt and Tg; no differences were seen.. D. VEGF expression was evaluated in retinal sections from Wt and Tg mice one week after laser photocoagulation using immunofluorescence staining (Red: VEGF staining; Blue: nuclear staining; Dashed circle: CNV lesion area; n=3/group). VEGF expression was much weaker in Tg mice compared to that in Wt mice after laser injury. Arrows show strong fluorescence in Muller cells. Scale bar=50 μ m.

Fig. 5. Less MMP-9 expression in *Bmp4-Vmd2* Tg mice after laser treatment

A. MMP-9 expression in CNV lesions from Wt and Tg mice using immunostaining (Red: MMP-9 positive staining, Blue: nuclear staining; n=4/group). Compared to Wt mice, MMP-9 expression in CNV lesions were reduced in Tg mice at one week after laser treatment. Representative images were shown. CNV lesion area was circled by dashed lines. Scale bar=50 μ m. B. MMP-9 expressions in Wt and Tg mice detected using Western Blots. Total proteins extracted from mouse posterior eye-cups after laser treatment. MMP-9 expression, at one week after laser treatment, was much weaker in Tg mice than that in Wt mice. The same membrane was reprobed for GAPDH expression to confirm equal loading of total proteins. Representative results were shown from three independent experiments.

Fig. 6. BMP4 inhibits TNF induced MMP-9 expression in human fetal RPE cells

A. RPE cells were treated with TNF or BMP4 alone, the cell culture media were collected, concentrated and resolved by zymography (representative gel presented). Quantitation of relative band intensity for 3 independent experiments is shown below. BMP4 alone did not induce MMP-9 secretion or affect MMP-2 secretion in RPE cells while TNF alone robustly increased MMP9 secretion ($*p<0.05$). B. RPE cells were pre-treated with BMP4 followed by TNF stimulation, the cell culture medium were collected, concentrated and resolved by zymography (representative gel presented). Quantitation of relative band intensity for 3 independent experiments is shown below. Pretreatment of RPE cells with BMP4 inhibited MMP-9 secretion induced by TNF compared to treatment with TNF alone ($*p<0.05$). However, MMP-2 expression remained largely unaltered throughout. C. Signaling pathways for MMP-9 induction by TNF. RPE cells were pretreated with BMP4 or specific JNK pathway inhibitor (5 μ M SP600125) before TNF stimulation, and the supernatants were collected and analyzed by zymography to visualize MMP-2 and MMP-9 secretion (representative gel presented). Quantitation of relative band intensity for 3 independent experiments is shown below. The results showed that BMP4 inhibits TNF-induced MMP9 induction ($*p<0.05$) and that inhibition of JNK activation also suppresses MMP-9 induction by TNF ($*p<0.05$).

Fig. 7. BMP4 inhibits MMP-9 expression through Smad1,5 pathway

A. Smad1,5 phosphorylation using Western Blots. BMP4 activated Smad1,5 phosphorylation in human RPE cells in a time-dependent manner at 30, 60 and 90 minutes (* $p < 0.05$). GAPDH was used as the loading control. (representative gel presented).

Quantitation of relative band intensity for 3 independent experiments is shown below. B. Smad1,5 expression and phosphorylation inhibited by siRNA. Forty-eight hours after siRNA transfection, human fetal RPE cells were treated with BMP4 for 1 hour. Whole cell lysates were used in Western Blots to test Smad pathway activation. BMP4 alone and BMP4 treatment of cells transfected with scrambled siRNA induced Smad1,5 phosphorylation (* $p < 0.05$). However, there was no induction of Smad1,5 phosphorylation when cells were transfected with siRNA targeting Smad1,5. C. MMP9 expression and activity restored by Smad1,5 specific siRNA. Forty-eight hours after siRNA transfection, human RPE cells were incubated with fresh medium and pre-treated with BMP4 overnight followed by TNF stimulation. Zymography was used to evaluate secreted MMP-9 and MMP-2 in the supernatant. When Smad1,5 was knocked down, BMP4 failed to inhibit MMP-9 expression induced by TNF, while treatment with BMP4 alone or BMP4+scrambled siRNA effectively inhibited MMP-9 secretion as compared to TNF alone (* $p < 0.05$).