Bone Morphogenetic Protein 7 Increases Chick Photoreceptor Outer Segment Initiation

Rachna Sehgal,1 Dirk J. Andres,1 Ruben Adler,2 and Teri L. Belecky-Adams1

PURPOSE. The purpose of this study was to investigate the regulation of photoreceptor differentiation and outer segment elongation by the growth factor BMP7.

METHODS. Dissociated low-density embryonic day 6 (E6) chick retinal cultures were grown for 6 days in the presence of BMP7, other members of the TGF-β family of growth factors, or control vehicle. Cultured cells were characterized using microscopy, immunocytochemistry, and RT-PCR. Antibodies against visinin and GABA were used to distinguish photoreceptors from nonphotoreceptor cells, and monoclonal antibodies rhodopsin (rho) 4D2, OS-2, and COS-1 were used to distinguish subpopulations of cones and rods. RT-PCR was used to investigate mRNAs encoding visual pigments.

RESULTS. Photoreceptors treated with BMP7 initiated outer segment elongation more frequently than photoreceptors in control cultures. The effect on outer segment initiation was confined to rods and to green opsin-expressing cones and appeared not to involve an increase in outer segment length. BMP7 did not appear to affect the survival, proliferation, or differentiation of progenitors or the fate of photoreceptors or amacrine cells in vitro. BMP5 and GDF5 showed weaker stimulatory effects than BMP7 on outer segment formation, whereas activin, BMP2, and BMP4 inhibited visual pigment expression and outer segment formation, and BMP6 had no detectable effects.

CONCLUSIONS. BMP7 must be added to the list of candidate molecules capable of stimulating outer segment formation. (Invest Ophthalmol Vis Sci. 2006;47:3625–3634) DOI:10.1167/iovs.06-0079

Rod and cone photoreceptor cells are essential components of the retina that initiate vision by transducing electromagnetic energy into electrochemical signals. Phototransduction takes place in the photoreceptor outer segment, a highly specialized structure containing stacks of parallel disks that increase photon capture efficiency. At the molecular level, outer segments are characterized by the presence of a complex and well-characterized phototransduction machinery, including visual pigment, transducin, cGMP, arrestin, ion channels, transporters, and a variety of enzymes.1,2 Structural proteins, such as tubulin, peripherin, and ROM1, are necessary for outer segment formation and maintenance.3,4 In addition to its role in phototransduction, the outer segment appears to be critical for photoreceptor survival; outer segment abnormalities frequently are the initial signs of photoreceptor degeneration caused by genetic mutations,5–7 nutritional deficiencies,8–10 or toxic agents.11 The onset of outer segment formation is an important landmark in photoreceptor development. The differentiation of rod and cone photoreceptors appears to occur in at least two distinct stages in the vertebrate species thus far studied. Some photoreceptor-specific genes are expressed at, or shortly after, the time of photoreceptor birth. Examples of these early genes, analyzed by in situ hybridization, immunocytochemistry, or both, include those encoding interphotoreceptor binding protein (IRBP) and visinin in the chick,12–15 IRPB in the goldfish,14 rhodopsin in Xenopus,15 and arrestin and recoverin in the ferret.16 Photoreceptors appear to remain quiescent for a significant period thereafter, until outer segments begin to develop many days or even several weeks after photoreceptor birth (see, for example, Cepko17 and Bumsted et al.18). Outer segment formation is accompanied by expression of a “late” group of genes, such as β- and γ-transducin, cGMP phosphodiesterase, phosducin, rhodopsin kinase, rod cGMP-gated cation channel, peripherin, and short- and medium-wavelength cone opsins in the ferret,19 and visual pigments, arrestin, transducin-γ, and peripherin in the chick.12,13 In the chick embryo, the subject of the present study, fundal photoreceptors are generated on or before embryonic day (E) 6, whereas outer segment formation and the late phase of gene expression start at approximately E14 to E15.12,19

The signal(s) that control the onset and progression of visual pigment expression and outer segment formation during the terminal differentiation of photoreceptor cells remain unknown. The release of photoreceptors from the effects of hypothetical inhibitory signals could play a role in this process. Activin, for example, has been shown to downregulate the expression of the red cone pigment and the morphologic maturation of chick embryo photoreceptors in vitro.20 Moreover, the expression of activin subunits appears to be markedly downregulated in the embryonic retina at approximately E15, the time of visual pigment expression onset and outer segment formation (Belecky-Adams TL and Adler R, unpublished observations, 2003). Another inhibitory factor is ciliary neurotrophic factor (CNTF), which inhibits rhodopsin expression in rat photoreceptors.21–23 Inductive/stimulatory molecules are also likely to exist, but the list of identified candidates is limited. The retinal pigment epithelium (RPE) appears to be necessary for the formation and maintenance of photoreceptor outer segments.24–25 It can be replaced by lactose in amphibians30 or by brain-derived neurotrophic factor (BDNF) in mammalian models of retinal detachment.31 It has also been proposed that Müller glial cells and the interphotoreceptor matrix are necessary for outer segment development, maintenance, or both.35,38,39–42

From the 1Department of Biology, Center for Regenerative Biology and Medicine, Indiana University–Purdue University, Indianapolis, Indiana; and the 2Wilmer Eye Institute, Johns Hopkins School of Medicine, Baltimore, Maryland.

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Corresponding author: Teri L. Belecky-Adams, Department of Biology, Center for Regenerative Biology and Medicine, Indiana University–Purdue University, 723 West Michigan Street, Indianapolis, IN 46202; thadams@iupui.edu.

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Photoreceptors present in dissociated retinal cultures form outer segments detectable by visual pigment immunocytochemistry and transmission electron microscopy. With this bioassay, we have now shown that BMP7, a member of the bone morphogenetic factor family, promotes the formation of outer segments in rods and green opsin–expressing cones. These effects were not mimicked by other BMPs and were not accompanied by changes in the frequency of photoreceptors in the cultures or by overall changes in cell proliferation or cell death. BMP7, therefore, must be added to the list of putative regulators of outer segment formation by photoreceptor cells.

**Materials and Methods**

Reagents used were as follows: Trizol, phenol-chloroform, chloroform, Superscript II, DNase I, oligo d(T), and random hexamers (Invitrogen, Carlsbad, CA); RNase H; isopropanol, sucrose, EDTA, Tris, NaCl (Fisher Scientific, Hanover Park, IL); GABA antibody, staurosporine, paraformaldehyde, Tris, 199 culture medium with HEPEP, high glucose DMEM, bovine serum albumin, 10× HBSS, 10× calcium and magnesium-free HBSS, bromodeoxyuridine, Triton X-100, linoleic acid, ethidium bromide (Sigma, St. Louis, MO); RQ1 DNase I (Promega, Madison, WI); iScript Reverse Transcription Kit (Bio-Rad, Hercules, CA); culture dishes and other plastics (Dot Scientific, Burton, MI); CNTF, BMP2, BMP4, BMP5, BMP6, Activin A (R&D Systems, Minneapolis, MN); fetal bovine serum, 0.25% Trypsin (1:250) without Mg2+, Ca2+, or sodium bicarbonate (Irvine Scientific, Santa Ana, CA); BrDU antibody was developed by Stephen J. Kaufman and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences, University of Iowa (Iowa City, IA).

**Dissociated Cultures**

White Leghorn chick embryos, obtained from the Purdue Poultry Farm (West Lafayette, IN) or Ohio State University (Columbus, OH), were incubated at 37°C in a humidified incubator (Kuhl, Flemington, NJ). Low-density cultures of neural retina were prepared as described. Briefly, neural retina was dissected from surrounding tissues and vitreous, cut into small fragments with tungsten needles, and digested with 0.25% trypsin (1:250) for 20 minutes at 37°C. After three rinses in Dulbecco minimal Eagle medium (DMEM) containing 1% bovine serum albumin (fraction V), tissue fragments were triturated with siliconized glass pipettes, and the resultant cell suspension was diluted to 4 × 10^5 cells/mL in culture medium containing HEPES-buffered 199 supplemented with 5% fetal calf serum, penicillin, and glutamine and was seeded in 35-mm culture dishes at 8 × 10^5 cells/dish. Cultures were maintained at 37°C with 5% CO2 in air for 4 to 6 days, fixed with 4% paraformaldehyde, rinsed twice with phosphate-buffered saline (PBS), and stored at 4°C until use. Quantification of photoreceptor number and outer segment length was performed as described in Adler and Belecky-Adams. We did not observe obvious differences in cell density, although BMP7 cultures appeared to have less debris. An effect of BMP7 on outer segment development was discovered during statistical analysis of E6 low-density retinal cell cultures by phase-contrast or Nomarski microscopy suggested that neurite development was more extensive in cultures grown in the presence of 50 ng/mL recombinant BMP7 than in control cultures (Figs. 1A, 1B). We did not observe obvious differences in cell density, although BMP7 cultures appeared to have less debris. An effect of BMP7 on outer segment development was discovered during statistical analysis of E6 low-density retinal cell cultures by phase-contrast or Nomarski microscopy suggested that neurite development was more extensive in cultures grown in the presence of 50 ng/mL recombinant BMP7 than in control cultures (Figs. 1A, 1B). We did not observe obvious differences in cell density, although BMP7 cultures appeared to have less debris. An effect of BMP7 on outer segment development was discovered during immunocytochemical study initially aimed at ascertaining possible changes in the relative frequency of amacrine and photoreceptor cells, the most abundant cell types in these cultures. Qualitative analysis of cultures immunoreacted with the rho 4D2 monoclonal antibody, which labels rhodopsin, and the green cone opsin showed three types of positive photoreceptors in all cultures: (1) those that were immunoreactive throughout the cell body and neuritic processes appeared to be higher in BMP7-treated cultures than in controls.

**Reverse Transcription—Polymerase Chain Reaction**

RNA was isolated from 100-mm culture dishes using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The RNA pellet was washed with RNa-free 75% ethanol, spun at 10,000 rpm for 10 minutes, air dried for 10 minutes at room temperature, and rehydrated in RNa-free water at 37°C for 20 minutes with periodic trituration using a 200-µL pipetman. Samples were stored at –80°C until use. Reverse transcription was performed with iScript RT (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions, and 2 µL RT reaction was then used for PCR. Primers to detect visual pigments and other photoreceptor-specific genes, and the conditions used for each primer pair, were reported in Adler et al. and Bradford et al. For semiquantitative PCR, samples of reaction product were collected every 2 cycles after the 17th round of amplification.

**Quantitation of Photoreceptor Number and Outer Segment Length**

Outer segments were identified as described by Saga et al. as an apical process connected to the photoreceptor inner segment and strongly immunoreactive with a visual pigment antibody (rho 4D2, OS-1, or COS-2). Outer segment measurements were made on digital images obtained with an inverted microscope (Eclipse TE-2000; Nikon, Melville, NY) with an attached digital camera (DXM200; Nikon); outer segment length, from base to tip, was determined with Axiovision software (Carl Zeiss, Oberkochen, Germany).

**Statistical Analysis**

Student paired t test (Instat; GraphPad Software, Inc., San Diego, CA) was used to analyze data.

**Results**

**Qualitative Evaluation of the Effects of BMP7 on Chick Retinal Cells in Low-Density Cultures**

Analysis of E6 low-density retinal cell cultures by phase-contrast or Nomarski microscopy suggested that neurite development was more extensive in cultures grown in the presence of 50 ng/mL recombinant BMP7 than in control cultures (Figs. 1A, 1B). We did not observe obvious differences in cell density, although BMP7 cultures appeared to have less debris. An effect of BMP7 on outer segment development was discovered during an immunocytochemical study initially aimed at ascertaining possible changes in the relative frequency of amacrine and photoreceptor cells, the most abundant cell types in these cultures. Qualitative analysis of cultures immunoreacted with the rho 4D2 monoclonal antibody, which labels rhodopsin, and the green cone opsin showed three types of positive photoreceptors in all cultures: (1) those that were immunoreactive throughout the cell body and neuritic process but that had no visible outer segment–like process (Fig. 1C); (2) similarly labeled cells that, in addition, had a heavily labeled outer segment–like process (Fig. 1D); and (3) cells showing a heavily labeled outer segment–like process in the absence of immunostaining in the rest of the cell (Fig. 1E). Interestingly, the frequency of rho 4D2–positive photoreceptors with outer segment processes appeared to be higher in BMP7-treated cultures than in controls.
FIGURE 1. Microscopic and immunocytochemical analysis of dissociated retinal cells cultured in the presence of vehicle or 50 ng/mL BMP7 for 6 days (A, B). Analysis by phase contrast showed a generally similar appearance in cultures treated with vehicle (A) or BMP7 (B), though neurite development appeared more extensive in the latter. (C-H) Immunocytochemical analysis with rho 4D2, which labels rhodopsin and green opsin–expressing photoreceptors. Three distinct patterns of immunoreactivity distribution could be observed: in the cell body alone (C), in both the cell body and the outer segment process (D), and in the outer segment alone (E). (F–H) Double labeling with visinin (green) and rho 4D2 (red) examples of cells with conspicuous outer segments in cultures treated with vehicle (F) or BMP7 (G–H).

BMP7 Increases the Number of Outer Segments Formed by Photoreceptors Immunoreactive with the Rho 4D2 Monoclonal Antibody

Quantitative analysis supported subjective impressions (Fig. 2A). Photoreceptors with rho 4D2–positive outer segments and cell bodies were nearly 500% more numerous in BMP7 cultures (20,256 ± 1954) than in vehicle-treated dishes (6752 ± 2954) (Fig. 2A). This increase in photoreceptors with outer segments was accompanied by a decrease in the frequency of photoreceptors with rho 4D2 immunoreactivity restricted to their cell bodies (Fig. 2A). The effects appeared time dependent (Fig. 2B). Thus, no significant differences in outer segment frequency were observed after 4 days in vitro, when the frequencies of rho 4D2–positive outer segments were 7061 ± 1529 and 7479 ± 877 in control and BMP7-treated cultures, respectively (Fig. 2B). By 6 days in vitro, the frequency of rho 4D2–positive segments remained relatively stable in control cultures (6870 ± 1231) but increased markedly in BMP-treated dishes, to 14,357 ± 1227.

To determine whether BMP7 actions were limited to rho 4D2–positive cells, we analyzed cultures immunoreacted with the COS-1 antibody (which recognizes chick red cones) or with the OS-2 antibody, which has much broader visual pigment specificity.38 No differences between BMP-7 and control cultures were detected in the distribution patterns of immunoreactivity in the labeled cells or in the frequency of photoreceptors with outer segments (Figs. 2C, 2D). Semiquantitative PCR using visinin (photoreceptors) as a control confirmed there were no changes in the expression of red opsin (Fig. 2F). Green opsin expression appeared to decrease slightly in BMP-treated cultures in comparison with control (Fig. 2F). Both isoforms of rhodopsin mRNA, 1.6 kb and 2.5 kb, were noted in the RT-PCR of control and vehicle-treated dishes. There appeared to be a small increase in the higher molecular weight isoform of rhodopsin after BMP treatment in comparison with vehicle treatment (Fig. 2F). As has been noted in previous studies and confirmed here, neither blue opsin nor violet opsin was expressed in control vehicle–treated culture, and each remained undetectable by RT-PCR in BMP-treated culture (data not shown).

Effects of BMP7 Concentration Dependent

E6 cultures were treated with vehicle or with different concentrations of BMP7, between 1 ng/mL and 100 ng/mL; after 6 days in vitro, the cells were fixed and immunolabeled with rho 4D2. Quantitative analysis showed concentration-dependent increases in the number of cells with immunoreactive outer segments, regardless of whether they were accompanied by immunoreactivity in the cell body (Fig. 2E). These increases reached a plateau at 50 ng/mL, with calculated half-maximal values at 1.2 ng/mL (Fig. 2E). BMP concentrations higher than 50 ng/mL appeared to be inhibitory, toxic, or both; cultures treated with 100 ng/mL BMP7 showed a marked decrease in the number of rho 4D2–positive cells that appeared to be less elongated and differentiated than the rho 4D2–positive cells in control cultures or in cultures treated at lower concentrations of BMP7 (data not shown).

Rod and Green Cones Response to BMP7 Treatment

Rhodopsin and green cone opsin are highly homologous39 and are similarly recognized by the rho 4D2 antibody.40 To evaluate whether rods and green cones were responsive to BMP7, we used treatments that specifically upregulated or downregulated the expression of these visual pigments. CNTF has been shown to increase substantially the number of photoreceptors that express the green cone pigment in cultures of chick retinal cells, without affecting rhodopsin expression.13,40 Therefore, CNTF-induced increases in the frequency of rho 4D2–positive cells that form outer segments in response to BMP7 treatment would identify those cells as green cones. To investigate possible synergistic effects of CNTF and BMP7, E6 retinal cells were cultured for 6 days in the presence of vehicle, 10 ng/mL CNTF, 25 ng/mL BMP7, or a combination of CNTF and BMP7. As previously shown,13,40 CNTF did not change the total number of photoreceptors in the cultures (Fig. 3B) but did significantly increase the number of rho 4D2–positive photoreceptors from 44,522 in the control to 65,200 in the CNTF-treated culture (Fig. 3A). The figure also shows that the number of photoreceptors with outer segments in CNTF-treated cultures
BMP7 treatment of retinal cultures significantly stimulated the formation of outer segments by a subpopulation of photoreceptors immunoreactive with the rho 4D2 antibody. (A, B) Cultures labeled with rod- and green-cone-specific antibody rho 4D2. (A) Cultures treated with BMP7 showed an increase in the total number of rho 4D2-positive outer segments (OS), reflecting increases in cells with immunoreactivity restricted to both the OS and the cell body. These increases were accompanied by a concomitant decrease in the number of photoreceptors with rho 4D2 immunoreactivity restricted to the cell body. (B) The effects of BMP7 appeared time dependent because they were clearly observed after 6 days in vitro but were not detectable 2 days earlier. (C, D) Control and BMP7 treated cultures immunoreacted with the COS-1, which labels red cones in chickens, and OS-2, which labels red cones and a small subpopulation of other cones (approximately 4%). BMP7 had no detectable effects on the total number of photoreceptors immunoreactive with these antibodies or in the frequency at which they formed outer segment processes. (C) Although COS-1 and OS-2 are cone specific and labeled approximately 56% of the photoreceptors in culture, a large subpopulation of photoreceptors remained unaccounted for in our analysis. (E) Dose dependence of BMP7 effects. Cultures treated with concentrations of BMP7 varying from 0 to 100 ng/mL showed increasing numbers of rho 4D2-positive outer segments up to and including 50 ng/mL. Cultures treated with concentrations of BMP7 greater than 50 ng/mL showed a decrease in the number of rho 4D2-positive cells with outer segments. RT-PCR using primers specific for opsins was performed to investigate the differentiation state of E6 cultures treated with vehicle. Alternatively, BMP7 samples were removed at odd-numbered cycles from 19 to 31 for gel analysis. Although analysis of visinin and green opsin revealed no differences between cultures treated with vehicle or BMP, there was a small increase in the large rhodopsin transcript in cultures to which BMP was added compared with vehicle-treated dishes (F). *P ≤ 0.05; **P ≤ 0.01.

(22,367 ± 3063) was higher than in controls (7807 ± 2680) but lower than in BMP7-treated cultures (29,224 ± 5636). On the other hand, combined treatment with CNTF and BMP7 caused a statistically significant increase in the number of rho 4D2-positive with outer segments, to 38,402 ± 3063 (Fig. 3A).

The responsiveness of rods to BMP7 treatment was investigated using staurosporine (a general inhibitor of protein kinases), which induces the expression of rhodopsin while suppressing the expression of green and red cone pigments. In agreement with these reports, the total number of photoreceptors was not changed by staurosporine treatment (Fig. 3D), but cultures grown in the presence of 25 mM or 50 mM staurosporine showed increases in rho 4D2-positive cells, from 40,512 ± 5168 in control cultures to 56,654 ± 6041 and 69,103 ± 9995, respectively (Fig. 3C). Many of these rhodopsin-positive cells in staurosporine-treated cultures had outer segments (Fig. 3C), but their frequency was significantly increased in cultures treated with staurosporine and BMP7 (Fig. 3C). These experiments, therefore, demonstrate that rods can also respond to BMP7 with an increase in outer segment formation.

**No Increase in Outer Segment Length with BMP7 Treatment**

To determine whether increases in outer segment initiation were accompanied by changes in outer segment elongation, E6 cultures were grown in the presence of vehicle or 25 ng/mL BMP7 for 6 days, fixed, and immunolabeled with rho 4D2 antibodies for computer-assisted measurement of outer segment length, as described in “Materials and Methods.” The average length of rho 4D2-labeled outer segments in control dishes was 4.48 μm (±1.84 μm), which was not significantly different from values in BMP7-treated cultures (4.93 μm ± 2.92 μm). No differences between BMP7-treated and control cultures were observed in plots of the length of individual outer segments, arranged from longest to shortest (Fig. 4A), or in
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**Figure 3.** BMP7 increases outer segment formation in rods and green cones. Given that the rho 4D2 antibody recognizes rhodopsin and the green cone opsin, the number of rho 4D2-positive outer segments was evaluated under conditions that specifically induced the expression of the green cone pigment (CNTF treatment; A, B) or that induced rhodopsin while suppressing other visual pigments (staurosporine treatment; C, D). (A) E6 retinal cells treated with CNTF alone exhibited an increase in the number of green cones, as shown in previous studies. Cultures cotreated with BMP7 and CNTF exhibited a statistically significant increase in the number of cells that formed outer segment processes in comparison with cells treated with either factor alone or with vehicle. (B) The total number of visinin-positive photoreceptors was similar in cultures treated with vehicle, BMP7, and CNTF, or any combination of them. (C) Cultures treated with increasing concentrations of staurosporine; a general inhibitor of protein kinases, showed increasing numbers of rods compared with vehicle-treated cultures; cultures treated simultaneously with BMP7 and 25 mM or 50 mM staurosporine showed increasing numbers of Rho 4D2-positive outer segment processes. (D) The total number of visinin-positive photoreceptors was not changed by staurosporine either by itself or in combination with BMP7. *P ≤ 0.05; **P ≤ 0.01.

**Cell Survival, Cell Proliferation, or Frequency of Cell Types Not Affected by BMP7**

To determine whether BMP7 had effects other than the stimulation of outer segment development, we investigated cell survival, proliferation, and differentiation in BMP7-treated and control cultures. Cell survival was evaluated 3 and 6 days after culture onset by immunocytochemistry for phospho-histone-3, an antigen present during the M-phase of the cell cycle. There were 5627 (± 739) and 6541 (± 693) in vehicle- and BMP7-treated dishes, respectively, representing 1% of the total cell population in either group.

**BMP7 Effects Different from Those of Other TGF-β Family Members**

Possible BMP7 effects on the relative frequency of different cell types were investigated in cultures immunolabeled with the cell type-specific markers visinin (for photoreceptors) and GABA (for amacrine cells). In vehicle-treated dishes, there were 110,144 ± 4509 visinin-positive, 73,597 ± 14,179 GABA-positive, and 50,125 ± 3249 unlabeled cells per dish. Similar numbers of visinin-positive, GABA-positive, and unlabeled cells were found in BMP-treated dishes (118,498 ± 4176, 66,634 ± 3925, and 42,187 ± 3655 respectively; Fig. 5C).

To determine whether BMP7 was the only member of the TGF-β family of growth factors capable of stimulating the development of outer segments in rho 4D2-positive photoreceptors, we compared its effects with those of other family members. Activin A, BMP2, and BMP4 caused a decrease in rho 4D2-positive outer segments in culture (Fig. 6A). All three factors also caused a substantial decrease in the number of rho 4D2-positive photoreceptors (Fig. 6B) without affecting the total number of photoreceptors in the cultures (Fig. 6C); this suggested that the decrease in outer segments probably reflected an inhibitory effect on visual pigment expression. BMP5 and GDF5, on the other hand, showed stimulatory effects on outer segment formation with respect to controls, which, however, did not reach the magnitude of the increases observed with BMP7 (Fig. 6A).
DISCUSSION

The experiments reported here describe the effects of BMP-7 on cultured photoreceptor cells. These results can be summarized as follows. First, BMP-7 significantly increased the number of outer segment processes formed by cultured photoreceptors, without affecting outer segment length. Second, visual...
receptors, identified by visual pigment immunoreactivity with the rho 4D2 antibody and by simultaneous treatment with BMP7 and CNTF or staurosporine, which regulate the expression of rhodopsin and the green cone pigment. Fourth, BMP-7 effects on outer segment formation were concentration and time dependent and were not accompanied by changes in photoreceptor survival, proliferation, or differentiation. Fifth, among other members of the TGF-β family of growth factors that were tested, BMP-5 and GDF-5 showed weaker stimulatory effects than BMP-7 on outer segment formation, whereas activin, BMP-2, and BMP-4 inhibited visual pigment expression and outer segment formation, and BMP-6 had no detectable effects. Sixth, overall cell survival and proliferation and the relative frequency of different cell types were similar in BMP-7-treated and control cultures.

The finding that rods and green cones were the only photoreceptor subtypes that responded to BMP-7 with an increase in outer segment formation was unexpected because we were unaware of any evidence suggesting that outer segment formation could be regulated by different molecular signals in different types of photoreceptors. Green cones and rods, however, are unique in other respects, particularly in birds and fish. In contrast to human and bovine green cone pigments, which are highly homologous to the red cone pigment, the chicken green cone pigment has much higher homology to rhodopsin than to other cone opsins. Similar findings were subsequently reported in other species, such as goldfish and zebrafish. Other differences between green cones and other cone types to the regulatory effects of growth factors because the green cone pigment is the only cone pigment that is upregulated by CNTF. Together with these earlier findings, our results with BMP-7 add support to the notion that, at least in some species, green cones differ in many respects from other cone subtypes and have similarities to rods.

Several of our findings suggest that the effects of BMP-7 on outer segment formation are fairly, though not absolutely, specific. Pharmacologically, BMP-7 effects were different from those of other members of the TGF-β superfamily of growth factors tested, were time and concentration dependent, and showed maximal activity at a 2-nm concentration. BMP-7 did not appear to have generalized effects on the health or behavior of the cultures because BMP-7-treated and control cultures were similar in total cell number, the relative proportion of photoreceptor and nonphotoreceptor cells, and cell proliferation. We did observe, however, some indications of increased neurite formation by amacrine cells and an increase in the number of Hu C/D–expressing cells (Belecky-Adams T, Sehgal R, unpublished data, 2005). Those effects of BMP-7 will be reported in detail elsewhere.

Although no changes were observed in BMP-7-treated cultures in the total number of photoreceptor cells, the number of cells expressing Rho 4D2-immunoreactive materials, or the expression levels of the green cone pigment and rhodopsin genes, increased outer segment formation by rods and green cones in response to BMP-7 was accompanied by a conspicuous change in the distribution of visual pigment immunoreactive materials within the photoreceptors. The disappearance of visual pigment immunoreactive materials from the photoreceptor cell body, accompanied by their increased restriction to the outer segment, resembled the transition that occurs during photoreceptor differentiation in vivo after the onset of outer segment formation. The mechanisms that control polarized opsin transport remain poorly understood, but considerable evidence suggests the involvement of cytoskeleton and cyto-
toskeletal phosphorylation mediators such as GTPases.\textsuperscript{48–51} This could provide a target for BMP7 regulation because this factor has been reported to regulate neurite formation and growth through mechanisms that may involve changes in actin dynamics.\textsuperscript{52}

An extensive body of literature describes factors that can interfere with the formation and maintenance of photoreceptor outer segments, including experimental or pathologic detachment of the retina from the pigment epithelium,\textsuperscript{53–55} mutational and metabolic deficits in vitamin A, carbohydrate metabolism,\textsuperscript{56–57} changes in choline uptake, and mutations in proteins such as peripherin and rhodopsin.\textsuperscript{58–60} On the other hand, the list of molecules that act as specific regulators of photoreceptor development and maintenance is more limited and includes the protective effects of BDNF in a retinal detachment model and the stimulation of outer segment formation by lactose in the amphibian retina.\textsuperscript{5,30,67,68} BMP-7 must now be added to this list of outer segment–promoting agents, though it is uncertain whether this effect is physiologic or only pharmacologic. A possible physiologic role is suggested by the demonstration that BMP-7 is expressed in the retina and in the retinal pigment epithelium,\textsuperscript{69–74} but it is uncertain whether photoreceptor cells in general and rods and green cones in particular express the appropriate BMP-7 receptors. Although most studies have focused on the role of BMPs in patterning of the optic cup or differentiation of tissues other than the neural retina, a few studies have shown the presence of BMPs in the retina or RPE at the developmental stages when photoreceptors are undergoing differentiation and outer segment elongation.\textsuperscript{3,57,75} Similarly, at least some of the cогnate receptors are also present in the photoreceptors, including BMP receptors IA and II and activin type II receptors.\textsuperscript{76} In addition, BMP signaling molecules have been localized predominantly in the rod photoreceptors of mature mice.\textsuperscript{77}

Our attempts to carry out loss-of-function experiments to investigate the role of BMP-7 in ovo were complicated by the time lag between the stages when the chick embryo eye is accessible to gene delivery methods such as electroporation, on E3 to E4, and the time of onset of outer segment formation (E15). Thus, although the physiologic role of BMP-7 in outer segment regulation awaits experimental verification, its potential as a pharmacologic agent is nevertheless significant because it could help alleviate vision loss caused by outer segment degeneration.

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