Cross-Talk between Fibroblast Growth Factor and Bone Morphogenetic Proteins Regulates Gap Junction-mediated Intercellular Communication in Lens Cells

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Homeostasis in the lens is dependent on an extensive network of cell-to-cell gap junctional channels. Gap junction-mediated intercellular coupling (GJIC) is higher in the equatorial region of the lens than at either pole, an asymmetry believed essential for lens transparency. Primary cultures of embryonic chick lens epithelial cells up-regulate GJIC in response to purified fibroblast growth factor (FGF)1/2 or to medium conditioned by vitreous bodies, the major reservoir of factors (including FGF) for the lens equator. We show that purified bone morphogenetic protein (BMP)2, -4, and -7 also up-regulate GJIC in these cultures. BMP2, -4, or both are present in vitreous body conditioned medium, and BMP4 and -7 are endogenously expressed by lens cells. Remarkably, lens-derived BMP signaling is required for up-regulation of GJIC by purified FGF, and sufficient for up-regulation by vitreous humor. This is the first demonstration of an obligatory interaction between FGF and BMPs in postplacode lens cells, and of a role for FGF/BMP cross-talk in regulating GJIC in any cell type. Our results support a model in which the angular gradient in GJIC in the lens, and thus proper lens function, is dependent on signaling between the FGF and BMP pathways.

INTRODUCTION

The development and function of the lens is profoundly affected by growth factors in the ocular environment. Differentiation of lens epithelial cells into secondary fibers is initiated at the lens equator, the region where epithelial cells are first exposed to the high levels of fiber-promoting growth factors, in particular fibroblast growth factor (FGF)1 and -2, that diffuse out of the vitreous body (Schulz *et al.*, 1993; for reviews, see Lovicu and McAvoy, 2005 and Robinson, 2006). The equator is also the area of the lens that has the highest levels of gap junction-mediated intercellular coupling (GJIC) in all species examined (Baldo and Mathias, 1992; Mathias *et al.*, 1997, 2007). Gap junctions are clusters of reversibly gated intercellular channels that allow the cell-to-cell transfer of a variety of ions and low molecular mass second messengers and nutritional metabolites. They are especially important and abundant in the avascular lens, in which they serve as a means to move the aforementioned substances between the functionally and structurally distinct subdomains of the organ (for review, see Goodenough, 1992). Defective expression of the proteins that comprise lens fiber cell gap junctions (connexins 50 and 46) causes cataracts and/or growth defects in humans and genetically engineered mice (Gong *et al.*, 1997; White *et al.*, 1998; for

review, see Gong *et al.*, 2007 and Gerido and White, 2004). It has been proposed and experimentally supported (Mathias and Rae, 1985; Mathias *et al.*, 1997; Donaldson *et al.*, 2001) that the increased level of GJIC at the equator of the lens relative to either the anterior or posterior poles serves to direct the overall pattern of current and solute flow in the organ and is thus essential for the nonvascular microcirculatory system believed to keep the lens transparent. Genetic manipulation of gap junctional coupling in the lens leads to the regional changes in calcium concentration predicted by this model (Gao *et al.*, 2004).

Despite its apparent importance for lens clarity, it is not known how the asymmetry in gap junctional coupling in the lens is established and maintained. We have addressed this question using dissociated cell-derived monolayer cultures (DCDMLs) prepared from E10 chick lens and maintained in the absence of serum. For as yet unknown reasons, chick cultures more closely recapitulate the *in vivo* processes of epithelial-to-fiber differentiation and fiber-type gap junction formation than any existing mammalian system. Unlike central epithelial explants, DCDMLs contain cells from the equatorial and pre-equatorial region of the lens (Menko *et al.*, 1984, 1987; Le and Musil, 1998). They express all three lens connexins, Cx43, Cx45.6, and Cx56 (the latter two the chick orthologues of mammalian Cx50 and Cx46). We have reported that FGF-1 and FGF-2, either as purified recombinant proteins or as components of vitreous body conditioned medium, increase gap junction-mediated intercellular dye coupling as well as expression of fiber differentiation markers in these cells. Moreover, removing FGF from vitreous humor by absorption onto heparin beads under high salt conditions blocked its ability to up-regulate GJIC. Neither vitreous humor nor purified FGF significantly increased the number of immunocytochemically detectable gap junctions in DCDMLs, suggesting that up-reg-

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Abbreviations used: DCDML, dissociated cell-derived monolayer culture; ERK, extracellular signal-regulated kinase; GJIC, gap junction-mediated intercellular coupling; VBCM, vitreous body conditioned medium.

ulation of gap junction function occurred at the level of gap junction gating (Le and Musil, 2001a,b). Given that the fraction of active gap junction channels can be ≤ 0.2 (Lin and Faber, 1988) (Bukauskas *et al.*, 2000), a large increase in intercellular coupling could be achieved by opening preexisting, previously functionally silent, gap junction channels. In vivo, this mechanism would account for the >14 -fold higher levels of gap junction coupling conductance measured in the equatorial region in the intact lens relative to either pole despite the absence of a proportional increase in gap junction number (Baldo and Mathias, 1992; Rae *et al.*, 1996; Mathias *et al.*, 2007).

An important question in lens biology is whether any of the many other growth factors known or suspected to be present in the eye also affect lens gap junctional coupling. Although originally shown to play a role in bone development, bone morphogenetic proteins (BMPs) are very widely expressed and are major determinants of cell fate in embryonic and postnatal tissues throughout the body. Mice null for either BMP4 or BMP7 have severe defects in lens induction at the placode stage, a process that may require cooperation between BMP and FGF signaling (Furuta and Hogan, 1998; Wawersik *et al.*, 1999; Faber *et al.*, 2001). Other studies have indicated that BMP signaling is also required for subsequent normal formation of primary lens fiber cells (Belecky-Adams *et al.*, 2002; Faber *et al.*, 2002). At later stages of lens development, expression of BMP receptors has been reported to be high in the equatorial region, as is BMP-mediated signaling as indicated by nuclear staining of activated Smad1 (a core intracellular mediator in the canonical BMP signaling pathway) (Belecky-Adams *et al.*, 2002; de Iongh *et al.*, 2004). Although the latter findings suggest that BMPs could also play an important role at the lens equator, this possibility cannot be addressed by using the BMP knockout mice mentioned above because the early stages of lens development are severely perturbed. We report here that purified, recombinant BMP2, -4, and -7 up-regulate GJIC in DCDMLs to an extent comparable with that obtained with FGF. Moreover, highly specific blockers of BMP2, -4, and -7 signaling prevent both vitreous humor and purified recombinant FGF from enhancing gap junctional coupling, an effect that can be attributed to inhibition of lens-derived BMP4 and -7. Together, our results support a model in which the angular gradient in gap junctional coupling believed to be required for lens transparency is dependent on a novel type of cross-talk between the BMP and FGF signal transduction cascades within the organ.

MATERIALS AND METHODS

Materials

Recombinant bovine FGF-2, human BMP4, mouse noggin/Fc chimera, and mouse chordin were purchased from R & D Systems (Minneapolis, MN), as was the monoclonal anti-BMP2/4 antibody (MAB3552). FGF-1 and low-molecular-weight heparin (sodium salt, from porcine intestinal mucosa) were from Sigma-Aldrich (St. Louis, MO), and fetal calf serum was obtained from HyClone Laboratories (Logan, UT). R³IGF-1, an analogue of human insulin-like growth factor (IGF)-1 with reduced affinity for IGF binding proteins, was from GroPep (Adelaide, Australia). Recombinant human BMP2 was from Genetics Institute (Cambridge, MA). Initial experiments were conducted with recombinant bovine FGF-2 purified as described in Le and Musil (2001a). These preparations up-regulated lens epithelial cell proliferation at 1 ng/ml, and fiber marker expression at ≥ 10 ng/ml, similar to the values reported in the literature (McAvoy and Chamberlain, 1989) and those obtained with FGF-2 purchased from Sigma-Aldrich. FGF-2 enhanced GJIC only at the higher concentration (Le and Musil, 2001b). Our later studies have used recombinant FGF-2 purchased from R & D Systems (2099-FB-025), which up-regulates fiber marker expression and GJIC at a concentration given by the manufacturer as 5 ng/ml. To facilitate comparison with previous studies, all FGF concentrations are given as if the specific bioactivity of the R & D preparation was

equal to that of the others. UO126, PD173074, and SU5402 were from Calbiochem (San Diego, CA). BMP7 and anti-BMP7 antibodies (rabbit polyclonal 5086 and mouse monoclonal 1B12) were from Creative Biomolecules (now Curis, Cambridge, MA) (Vukicevic *et al.*, 1994; Lein *et al.*, 2002).

Cell Culture and Treatments

Cultures were prepared from E10 chick lenses and plated at 1.6×10^5 cells/well onto laminin-coated 96-well tissue culture plates as described previously in Le and Musil (1998). Cells were cultured for up to 7 d in M199 medium plus BOTS (2.5 mg/ml bovine serum albumin, 25 mg/ml ovotransferrin, and 30 nM selenium), penicillin G, and streptomycin (M199/BOTS), with or without additives at 37°C in a 5% CO₂ incubator. Cells were fed every 2 d with fresh medium. We refer to these cultures as dissociated cell-derived monolayers (DCDMLs) to distinguish them from related, but functionally distinct, systems such as central epithelial explants and immortalized lens-derived cell lines. Anti-BMP antibodies, rabbit immunoglobulin G (IgG), and chordin were used at a concentration of 40 μ g/ml; noggin was used at 0.5 μ g/ml. DCDMLs were incubated with kinase inhibitors (15 μ M UO126, 100 nM PD173074, and 20 μ M SU5402) for 45 min at 37°C before addition of growth factors. FGF reversal experiments (Figure 10B) were conducted as described previously (Le and Musil, 2001b).

Scrape-Loading/Dye Transfer Assay for Gap Junctional Intercellular Communication

DCDML cultures grown on laminin-coated coverslips were assessed for gap junction-mediated intercellular coupling as described previously (Le and Musil, 1998). In brief, the culture medium from a confluent monolayer of lens cells was removed and saved. The cells were rinsed three times with Hank's balanced salt solution containing 1% bovine serum albumin (HBC), after which a 27-gauge needle was used to create two longitudinal scratches through the cell monolayer in the presence of a solution of Dulbecco's phosphate-buffered saline containing 0.75% rhodamine dextran ($M_r = 10$ kDa; Invitrogen, Carlsbad, CA) and 1% Lucifer yellow (Sigma-Aldrich). After exactly 1 min, the culture was quickly rinsed three times with HBC and then incubated for an additional 8 min in the saved culture medium to allow the loaded dye to transfer to adjoining cells. The culture was then rinsed three times with phosphate-buffered saline and fixed. Rhodamine dextran and Lucifer yellow were visualized by fluorescence microscopy. Unless otherwise indicated, cells were assayed on day 3 after plating. For all figures except Figure 1A, only the Lucifer yellow images are shown.

Scrape-loading/dye transfer assays were quantitated using the averaged fluorescence plot profile method as described previously; similar results were obtained if the size of the total fluorescent surface area was measured (Le and Musil, 2001b). In brief, a DM LD photomicrography system (Leica Microsystems, Deerfield, IL) and Scion Image 1.60 software (Scion, Frederick, MD) were used to generate digital images of the scrape-loaded and fixed monolayers. The distance from the proximal edge of intercellular Lucifer yellow dye transfer (defined as the point nearest to the scrape edge with a mean pixel value >10 U above background) to the distal edge of transfer (defined as the point at which the mean pixel value returned to this level) was measured along lines perpendicular to the scrape, taking care to avoid regions where the cell monolayer was incomplete or contained enlarged cells undergoing epithelial-to-fiber differentiation. At least three lines were measured per image. Because none of the compounds tested significantly altered the size of epithelial cells within the monolayer (data not shown), the distance of intercellular Lucifer yellow transfer was directly proportional to the number of coupled cells. The scrape-loading dye transfer assay has the advantage over microinjection techniques of allowing simultaneous monitoring of dye coupling in a large population of cells; its utility in the assessment of gap junction-mediated intercellular communication has been well documented (el-Fouly *et al.*, 1987; Venane *et al.*, 1995). Opsahl and Rivedal (2000) have reported that scrape-loading of Lucifer yellow followed by image analysis (by either the averaged fluorescence plot profile or the total fluorescent surface area method) leads to the same conclusions as dye microinjection, with less data variation.

Immunoblot Analysis of DCDML Cultures

DCDML cultures were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer (7.1% glycerol, 1.92 mM Tris, pH 6.8, 2.5% SDS, and 2% 2-mercaptoethanol), boiled for 3 min, and the entire cell lysate from each well of a 96-well culture plate analyzed per lane of a 10% (extracellular signal-regulated kinase [ERK]) or 7.5% (pSmad1) SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene fluoride membranes (Immobilon; Millipore, Billerica, MA). Blots were probed with the following primary antibodies: anti-p44/42 mitogen-activated protein (MAP) kinase polyclonal rabbit antibody (recognizes both activated and inactive forms of ERK), anti-phospho-p44/42 MAP kinase E10 monoclonal mouse antibody (specific for activated ERK) (both purchased from Cell Signaling Technology, Danvers, MA), or anti-phospho-Smad1 (Ser463/465) (06-702; Millipore). Immunoreactive protein bands were detected using secondary antibodies conjugated to either IRDye800 (Rockland Immunochemicals, Gilbertsville, PA) or Alexa Fluor 680 (Invitrogen) and directly quantified using the Odyssey infrared imaging

Figure 1. Up-regulation of gap junctional intercellular communication in chick lens DCDMLs by BMPs. The indicated factors were added to DCDMLs on day 1 of culture. On day 3, the cultures were assessed for their ability to mediate the intercellular transfer of the gap junction tracer Lucifer yellow (LY) by using the scrape-loading/dye transfer assay. The $M_r = 10$ kDa rhodamine dextran (RD) remained confined to the cells at the wound edge into which dye had been directly introduced during the scrape-loading process. In contrast, LY was transferred to adjacent cells via open gap junctional channels. Each panel depicts a portion of the right half of the scrape/load wound. (A) DCDMLs were treated for 48 h with either 10 ng/ml FGF-2; 15 ng/ml IGF-1; or 10 ng/ml BMP2, -4, or -7. The loading pattern of each dye is shown separately (LY and RD), as well as superimposed (LY/RD). Relative to untreated control cells within the same experiment, the fold increase in the distance of gap junction-mediated transfer of Lucifer yellow into the cell monolayer was for FGF-2, 2.42 ± 0.25 ($n = 16$); for IGF-1, 1.07 ± 0.098 ($n = 3$); for BMP2, 2.88 ± 0.21 ($n = 6$); for BMP4, 3.11 ± 0.59 ($n = 5$); and for BMP7, 3.03 ± 0.52 ($n = 6$). (B) DCDMLs were treated for 48 h with BMP2, -4, or -7. The indicated cultures were coincubated with a function-blocking antibody directed against either BMP2 and 4 (α BMP2,4) or BMP7 (α BMP7; 1B12), or with nonimmune IgG purified from normal rabbit serum (ctl IgG). Other cultures were treated on day 3 for 1 h with the gap junction blocker 18 β -glycyrrhetic acid (β GA) (Le and Musil, 1998, 2001) before measuring dye transfer. Only the Lucifer yellow images are shown; representative of two to five independent experiments.

system (LI-COR Biosciences, Lincoln, NE) and associated software. Unless indicated otherwise, all experiments were repeated a minimum of three times and representative experiments are shown. As assessed by immunoblotting of total ERK and inspection of total protein after Western transfer, all samples within the same experiment contained equal amounts of cellular protein.

Preparation of Vitreous Humor and Vitreous Body Conditioned Medium (VBCM)

Vitreous bodies were dissected from the eyes of either E10 chicks or adult pigs. Vitreous humor was prepared by subjecting vitreous bodies to centrifugation for 10 min at 4°C at $18,000 \times g$ to remove cells and fibrous elements. For vitreous body conditioned medium, intact E10 chick vitreous bodies were transferred to the top chamber of Transwell filter unit containing M199 medium in the top and bottom compartments. After an overnight incubation at 37°C in a 5% CO₂ incubator, the bottom compartment medium was collected.

Depletion with Noggin Beads

VBCM, 30% vitreous humor, and 50 ng/ml BMP2 (the latter two diluted in M199 medium) were depleted of noggin-binding BMPs by taking advantage of the ability of the recombinant noggin-human Fc chimera to bind to protein A and G without compromising its affinity or specificity for BMP2, -4, and -7 (Zimmerman *et al.*, 1996). Protein A agarose and an equal amount of protein G-Sepharose (Invitrogen) beads were mixed, washed three times with PBS,

resuspended in M199, and incubated with noggin-Fc (per 100 μ l of packed beads resuspended in 500 μ l of M199, 33 μ l of 50 μ g/ml noggin/Fc) for 1 h at room temperature while rotating. The beads were then washed three times with M199 to remove any unbound noggin. For each 100 μ l of VBCM, 30% vitreous humor, or BMP2 solution to be depleted, 7.5 μ l of packed noggin-bound beads was added, and the sample was incubated for 1 h at room temperature. The beads (and any associated noggin-bound material) were removed from the sample by centrifugation; the supernatant was then sedimented again to ensure that no beads were carried over. The supernatant of the second spin was then incubated with new noggin-bound beads (7.5 μ l of packed beads/100 μ l of original volume of VBCM, 30% vitreous humor, or BMP2), and the depletion was repeated. The final supernatant (=noggin-depleted VBCM, 30% vitreous humor, or BMP2) was then supplemented to $1\times$ with BOTS before incubation with DCDMLs as indicated in the text. All procedures were conducted under sterile conditions; all centrifugation steps were for 1 min at $12,000 \times g$. Control experiments confirmed that depletion of Smad1-activating activity from vitreous is dependent on the presence of noggin on the protein A/G beads.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from DCDMLs or freshly isolated E10 chick lens by using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's

protocol. RNA samples (1 μ g) were reverse transcribed using random primers and the SuperScript III first-strand synthesis system (Invitrogen). The resulting cDNA was amplified using Taq DNA polymerase and previously published primer sets for chicken BMP2, BMP4, BMP7, and (as a positive control) chicken glyceraldehyde-3-phosphate dehydrogenase (Li *et al.*, 2003). The PCR protocol included a 95°C denaturation step for 5 min, followed by 35 cycles of 95°C denaturation (20 s), 47°C annealing (20 s), and 72°C extension (30 s), with a final 7-min extension step at 72°C.

RESULTS

BMPs Increase Gap Junctional Intercellular Communication in Chick Lens DCDMLs

The effect of purified recombinant BMPs on GJIC in DCDML lens cell cultures was assessed using the scrape-load dye transfer assay. In brief, a 27-gauge needle was used to make a vertical scratch through the center of a confluent monolayer of cells in the presence of a mixture of the gap junction tracer Lucifer yellow (M_r = 457 Da) and rhodamine dextran (M_r = 10 kDa). The latter is too large to pass through gap junctions and remains confined to the wounded cells bordering the scratch, whereas Lucifer yellow can be transferred to adjacent, unperturbed cells via open gap junctional channels. The ability of this technique to reliably and reproducibly assess GJIC in DCDMLs has been established previously (el-Fouly *et al.*, 1987; Venance *et al.*, 1995; Opsahl and Rivedal, 2000). We found that 10 to 50 ng/ml BMP2 increased the spread of Lucifer yellow into the cell monolayer to an extent similar to that obtained with maximally effective concentrations of FGF (~10 ng/ml) (Figure 1A) or vitreous body conditioned medium (VBCM; Figure 3). As was true for FGF and VBCM (Le and Musil, 2001b), a detectable increase in Lucifer yellow transfer in response to BMP2 required more than 6 h of continuous exposure to the growth factor at concentrations greater than 1 ng/ml. BMP2-treated cells retained their epithelial morphology and continued to express lens-specific proteins such as MP26, δ -crystallin, and CP49 at levels comparable with cells cultured with FGF or IGF-1 (data not shown). The increase in dye transfer induced by BMP was due to a bona fide up-regulation of GJIC in that it was abolished by the gap junction blocker 18 β -glycyrrhetic acid (Figure 1B), and it was also observed with a gap junction-permeable compound with physical properties distinct from Lucifer yellow (biocytin; data not shown). Similar results were obtained with recombinant human BMP4 and BMP7, the amino acid sequences of which are, respectively, ~90 and 60% identical to that of BMP2 (Figure 1). Coculture with the corresponding function-blocking anti-BMP antibodies prevented up-regulation of dye transfer by these BMP preparations, ruling out any possibility that their activity was due to a non-BMP contaminant (Figure 1B). In contrast, neither IGF nor insulin increased GJIC despite the ability of DCDMLs to respond to these factors by enhanced expression of fiber differentiation markers (Figure 1A) (Le and Musil, 2001a,b). Relative to measurements of electrical coupling, scrape-load dye transfer is a less sensitive means to assess GJIC. The 2- to 3-fold increases we detect with this assay after addition of BMPs or FGF are therefore likely to be an underestimate of the true extent to which junctional coupling is up-regulated. To our knowledge, this is the first demonstration that BMP2, -4, and -7 increase GJIC in any nonneuronal system.

Exogenous BMPs and FGF Up-Regulate GJIC by Distinct, but Cooperative, Signaling Pathways

We have shown that at concentrations that increase GJIC, FGF induces sustained (>8-h) activation of the ERK cascade in chick and rodent lens cells, an event that can be assayed

using anti-phospho-ERK-specific antibodies and quantitative Western blotting (Le and Musil, 2001b). Up-regulation of GJIC by FGF-2 and FGF-1 is completely dependent on this sustained activation of ERK and can be blocked with 15 μ M UO126, a highly specific inhibitor of the kinases (mitogen-activated protein kinase kinase [MEK]1/2) immediately upstream of ERK in the MAPK cascade (Davies *et al.*, 2000; Le and Musil, 2001b). The canonical BMP signaling pathway is via Smad1/5/8 phosphorylation, and does not include ERK (Massague, 1998). Neither BMP2, -4, or -7 increased activation of ERK in DCDMLs after a 15-min up to 48-h treatment (Figure 2A), nor did UO126 affect their ability to enhance intercellular dye transfer in nine of nine trials (Figure 2B; data for BMP2 graphed in Figure 3). Thus, the effects of BMP and FGF on GJIC, although similar in some respects, are mediated by nonidentical signaling pathways.

In nonlenticular systems, FGF and BMP can have antagonistic, synergistic, or unrelated actions on downstream events. We examined the interactions between these two types of growth factors on GJIC in a series of scrape-load dye transfer experiments summarized in Figure 3. Although ineffective when added separately, combining 1 ng/ml BMP with 5 ng/ml FGF enhanced GJIC in DCDMLs to near the level obtained with maximally effective concentrations of either growth factor alone (15 ng/ml FGF or BMP). Notably, the ability of this mix to up-regulate GJIC was not blocked by inhibition of the ERK pathway by 15 μ M

Figure 2. Up-regulation of GJIC by BMP, unlike by FGF, is independent of ERK activation. (A) As assessed by anti-phospho-ERK immunoblotting, a 45-min treatment with 10 ng/ml FGF induces activation of the ERK pathway in DCDMLs that is completely blocked by the MEK inhibitor UO126 at 15 μ M (note that chick lens cells express only the ERK2 isoform). In contrast, 1–50 ng/ml BMP2, -4, or -7 did not induce ERK phosphorylation. Levels of total ERK protein were not affected (data not shown). (B) DCDMLs were cultured for 48 h with 10 ng/ml FGF-2, BMP4, or BMP7 in either the absence or continuous presence of 15 μ M UO126. Cells were assessed for GJIC as in Figure 1; only the Lucifer yellow images are shown.

Figure 3. Exogenously added BMP and FGF act cooperatively to up-regulate GJIC in an ERK-independent manner that mimics up-regulation by vitreous humor. DCDMLs were cultured for 48 h with FGF-2 and/or BMP2 at the concentrations indicated (in nanograms per milliliter), VBCM, or with 30% chick vitreous humor (VH) in either the absence or continuous presence of 15 μ M UO126. Cells were assessed for gap junction-mediated intercellular transfer of Lucifer yellow as in Figure 1; results are expressed relative to dye transfer in untreated control cells within the same experiment. For all conditions, $n \geq 4$. The asterisks denote values significantly different from control ($p < 0.015$) as assessed by the two-tailed paired Student's t test. For the remaining data sets, $p > 0.5$.

UO126. Moreover, the GJIC-enhancing effect of 15 ng/ml FGF, which is completely dependent on sustained activation of ERK when added to otherwise unsupplemented cells (Figure 2B) (Le and Musil, 2001b), was rendered UO126-insensitive by addition of 1 ng/ml BMP ($n = 5$). Similar results were obtained with BMP2, -4, and -7; control experiments confirmed that the ability of UO126 to block FGF-induced activation of ERK was unaffected by the presence of added BMP (data not shown). These results indicate that exogenously added BMPs and FGFs can act cooperatively to up-regulate GJIC via a novel, ERK-independent pathway.

In vivo, only factors that can diffuse out of the intact vitreous body have access to the lens equator. We have reported increased gap junction-mediated dye coupling in DCDMLs after a 2-d incubation with VBCM, produced by loading intact, isolated vitreous bodies into the top chamber of a Transwell unit in M199 medium and collecting the medium on the opposite side of the 0.45 μ m filter 12–18 h later (Le and Musil, 2001b). If FGF were the sole growth factor in VBCM responsible for up-regulation of GJIC, then the ability of VBCM to up-regulate GJIC would be blocked by UO126. Instead, VBCM increased dye coupling in an ERK-independent manner and thus behaved like a mixture of BMP and FGF (Figure 3). Similar results were obtained with 30% vitreous humor, made by diluting the soluble fraction of crushed vitreous bodies 1:2.3 with M199/BOTS medium.

Expression of BMPs in Vitreous Humor and Their Role in Vitreous-Induced Up-Regulation of GJIC

The results shown in Figure 3 raised the question as to whether vitreous humor contained BMP bioactivity in a form accessible to the lens. This was addressed by Western blotting by using an antibody specific to the activated (phosphorylated on serine residues 463 and 465) form of the transcriptional regulator Smad1 to assess stimulation of the canonical BMP signaling cascade (Massague, 1998). As expected, a 45-min treatment of DCDMLs with >1 ng/ml BMP2, -4, or -7 consistently increased anti-phospho-Smad1 immunoreactivity in whole cell lysates that was specifically abolished if the cells had been coincubated with the appropriate function-blocking anti-BMP antibody (Figure 4A). Vitreous humor (diluted to 30% with M199 medium) also activated Smad1 in DCDMLs, as did VBCM. Similar results were obtained with 30% vitreous humor prepared from adult pig (Figure 4B). Moreover, vitreous induced expression of alkaline phosphatase in C3H10t1/2 cells, a standard bioassay for BMP activity (Ruppert *et al.*, 1996; Katagiri *et al.*, 1990) (data not shown).

Association of BMP2, -4, and -7 with BMP receptors is selectively and potently blocked by noggin, a naturally produced, secreted protein that binds to these BMP species with very high affinity and specificity (Zimmerman *et al.*, 1996) (Groppe *et al.*, 2002). A 45-min preincubation with recombinant noggin abolished the ability of all three BMPs to acti-

Figure 4. Vitreous humor contains diffusible BMP activity sensitive to noggin and inhibitors of BMP2 and -4, but not of BMP7. DCDMLs were treated for 45 min with either BMP2, -4, -7 at 15 ng/ml, VBCM, or 30% VH from chick or pig. Before addition to the cells, the factors had been preincubated for 1 h at room temperature with one or more of the following as indicated: noggin (nog), chordin (chor) (both of which block BMP2, -4, and -7), antibodies specific to either BMP2,4 ($\alpha 2,4$) or BMP7 ($\alpha 7$; 1B12), or IgG purified from non-immune rabbit serum (ctl IgG). Control cultures were untreated (control), or incubated with noggin only (noggin). Activation of Smad1 was assessed by Western blot of whole cell lysates with a phospho-Ser463,465-specific Smad1 antibody. Representative of at least three experiments.

vate Smad1 in DCDMLs (Figure 4A). Notably, activation of Smad1 by VBCM was also blocked by noggin or (to a slightly lesser extent) with chordin, a protein structurally distinct from noggin that has similar functional properties (Figure 4C) (Piccolo *et al.*, 1996; Balemans and Van Hul, 2002). Smad1 was not activated in response to medium conditioned by ribbons of ciliary epithelium dissected from E10 chick eyes, ruling out the possibility that the source of BMP in our VBCM preparations was contaminating fragments of ciliary processes that might have remained attached to the lens capsule (data not shown). Antibodies active against both BMP2 and -4 (Figure 4A) reduced VBCM-induced Smad1 activation by 90.5% (± 5 ; $n = 4$), whereas a reagent that specifically blocks BMP7 activity (the 1B12 monoclonal antibody [mAb]; Figure 4A) had no significant effect either alone or in combination with anti-BMP2,4 ($n = 7$; Figure 4C). Together, these results indicate that the major, and possibly only, diffusible BMPs present in vitreous humor are BMP2 and/or -4, with little or no BMP7.

To assess the role of BMP signaling in up-regulation of GJIC by vitreous, DCDMLs were incubated with VBCM in the presence or absence of noggin for 48 h. Control experi-

ments verified that noggin potently blocked the ability of recombinant BMP2, -4, or -7 to enhance intercellular dye transfer under these conditions (Figure 5B). Noggin also strongly inhibited up-regulation induced by VBCM, as did chordin (Figure 5A; data graphed in Figure 5B). Incubation with noggin (either with or without VBCM) did not detectably alter the morphology, viability, or confluence of the cells, nor did it induce epithelial-mesenchymal transition as assessed by expression of α -smooth muscle actin-containing stress fibers (data not shown). Noggin also inhibited up-regulation induced by 30% vitreous humor generated from either E10 chick or adult pig (Figure 5B). Noggin did not, however, diminish the ability of fetal calf serum to increase GJIC, further indicating the specificity of its effect against vitreous (Figure 5B).

The 1B12 anti-BMP7 mAb (Vukicevic *et al.*, 1994) abolished Smad1 activation (Figure 4A) and GJIC (Figure 1B) induced by recombinant BMP7, but not by BMP2 or -4. This antibody partially inhibited the ability of VBCM to up-regulate GJIC, and when combined with the anti-BMP 2,4 antibody further reduced dye coupling to control levels (Figure 5A; quantitation given in Figure 5B). These findings

Figure 5. Up-regulation of GJIC by vitreous humor is sensitive to noggin and inhibitors of BMP2, -4, and -7. DCDMLs were cultured for 48 h with VBCM, BMP2, -4, or 7 (at 15 ng/ml), 15% bovine fetal calf serum (FCS), or 30% VH from chick or pig. Where indicated, the incubations included noggin (nog), chordin, function-blocking anti-BMP2,4 antibodies (α BMP2,4), anti-BMP7 antibodies (α BMP7; 1B12), or IgG purified from nonimmune rabbit serum (ctl IgG). Control cultures were untreated (control), or incubated with noggin only (noggin). Cells were assessed for gap junction-mediated intercellular transfer of Lucifer yellow. In B, results are expressed relative to dye transfer in untreated control cells within the same experiment. For all conditions, $n \geq 3$. The asterisks denote values significantly different from control ($p < 0.002$) as assessed by the two-tailed paired Student's *t* test. For the remaining data sets, $p > 0.3$.

Figure 6. Depleting vitreous of noggin-binding BMPs does not block its ability to up-regulate GJIC. DCDMLs were incubated for either 48 h or (insets) 45 min with no additions (control), BMP2 (50 ng/ml) (A), 30% VH (B), or VBCM (C) in either the absence or presence of noggin. Where indicated, the BMP, VH, or VBCM had been preabsorbed with noggin-bound beads to create noggin-depleted BMP2 (A), vitreous humor (B), or VBCM (C). One set of cultures was lysed 45 min later and assessed for activation of Smad1 by Western blotting as in Figure 4 (inset); the other set was evaluated for gap junction-mediated intercellular transfer of Lucifer yellow 48 h later. Typical of results from six independent experiments.

suggest that the ability of VBCM to maximally increase GJIC requires not only BMP2 and/or BMP4 but also the activity of a BMP sensitive to Mab1B12.

The source of the BMP required for DCDMLs to increase GJIC in response to VBCM could either be vitreous humor or the lens cells themselves. To address the role of vitreous-derived BMP, we incubated 30% vitreous humor with beads to which noggin had been immobilized and then removed the noggin-bound BMPs by low speed centrifugation as described in *Materials and Methods*. Control experiments demonstrated that this procedure efficiently removed Smad1- and GJIC-activating activity from a 50 ng/ml solution of recombinant BMP2 (Figure 6A). Depletion of vitreous with noggin beads specifically blocked its ability to increase phosphoSmad1 levels, but it did not significantly diminish its enhancement of GJIC. Up-regulation of dye coupling was, however, blocked if DCDMLs were incubated with noggin-depleted 30% vitreous humor in the presence of (soluble) noggin, which has access to lens cell-derived BMPs (Figure 6B). Similar results were obtained with VBCM (Figure 6C). Together, these findings indicate that the BMP required for up-regulation of GJIC in response to vitreous originates from the lens cells themselves instead of from vitreous. This explains why anti-BMP7 antibodies block up-regulation of GJIC by VBCM, even though VBCM does not contain BMP7 bioactivity (Figure 8).

Up-Regulation of GJIC by FGF Requires Lens-derived BMP4 and -7

When purified from VBCM or vitreous humor by using heparin beads, FGF is capable of increasing dye coupling to a level comparable with that obtained with unfractionated vitreous (Le and Musil, 2001b). How can this be reconciled with the current finding that up-regulation of GJIC by vitreous also depends on lens cell-derived BMP? The simplest explanation would be that enhanced junctional coupling requires both FGF from vitreous humor and BMP produced by DCDMLs. If true, then it would be expected that up-regulation of GJIC in response to purified, recombinant FGF would also require endogenous BMP activity, even though the canonical FGF signaling pathway does not involve BMPs or BMP-regulated Smads. In keeping with this hypothesis, coculturing DCDMLs with noggin and FGF-2 (or FGF-1; data not shown) abolished the ability of FGF to up-regulate Lucifer yellow dye coupling ($n = 14$; Figure 7A). Cells treated with FGF plus noggin were viable, of normal confluence and morphology, and did not up-regulate expression of the epithelial-mesenchymal transition marker α -smooth muscle actin (data not shown). Noggin had no

effect on the ability of FGF to induce either transient (1-h) or sustained (8-h) activation of ERK in DCDMLs even after an extended (3- to 9-h) preincubation of either the growth factor (Figure 7C) or cells (Figure 7D) with noggin, ruling out the possibility that noggin artifactually interfered with the capacity of FGF to productively interact with its receptor. As was the case for VBCM, up-regulation of GJIC by FGF was partially inhibited by the BMP7-specific antibody 1B12 (by $57 \pm 15.6\%$), and more completely (by $98 \pm 10\%$; $n = 4$) when it was combined with the anti-BMP2,4 reagent (Figure 7A). The ability of FGF to enhance dye coupling was also partially inhibited (by $66 \pm 12\%$; $n = 4$) by a polyclonal anti-BMP7 antibody (5086) that we confirmed blocked the ability of BMP7, but not of BMP4, to activate Smad1 (Figure 7B) and up-regulate GJIC (data not shown).

Based on the results shown in Figure 7A, it would be predicted that lens cells express BMP2 and/or 4 as well as BMP7. This was supported by RT-PCR using published primer sets and conditions. Bands of the size expected for BMP4 and BMP7 (~300 base pairs; Li *et al.*, 2003) were amplified by their respective primer pairs from total RNA prepared from DCDMLs and from whole chick lens (Figure 8). The BMP2 primer set yielded no product, although an appropriately sized band was readily recovered when genomic DNA from foot or beak was used as the source of template (Figure 8). Together, our findings indicate that lens-derived BMP4 as well as BMP7 play a role in up-regulating GJIC in response to FGF. This would explain why the ability of VBCM to enhance GJIC is reduced by BMP7-specific antibodies (Figure 5), even though VBCM does not contain Smad1-activating levels of BMP7 (Figure 4C). Both BMP4 and -7, but not BMP2, have also been reported to be expressed by embryonic and postnatal rodent lens (Furuta and Hogan, 1998) (Wawersik *et al.*, 1999) (Hung *et al.*, 2002) (Thut *et al.*, 2001), and they have been detected in gene microarrays of human lens epithelial cells (Dawes *et al.*, 2007). Attempts to use Western blotting to detect BMPs from DCDMLs were unsuccessful, most likely because of the small quantities of protein produced (see *Discussion*).

Cross-Talk between the BMP and FGF Pathways Is Nonreciprocal with Respect to Regulation of GJIC

Lens cells from many species express FGF-2 or -1 mRNA and/or protein in vivo and in tissue culture, and message for both FGFs has been reported to be highest at the lens equator (Schulz *et al.*, 1993; Lovicu *et al.*, 1997; Schweigerer *et al.*, 1988). If endogenous, lens-derived BMP is required for cells to up-regulate GJIC in response to FGF, is endogenous, lens-derived FGF necessary for exogenous BMP to enhance

Figure 7. Up-regulation of GJIC by FGF is sensitive to noggin and inhibitors of BMP2, -4, and -7. (A) DCDMLs were cultured for 48 h with no additions (control) or with 10 ng/ml recombinant FGF-2. As indicated, the incubations included one or more of the following: noggin, function-blocking anti-BMP2,4 antibodies (α BMP2,4), anti-BMP7 antibodies (α BMP7; 1B12 or 5086), or IgG purified from nonimmune rabbit serum (ctl IgG). Cells were assessed for gap junction-mediated intercellular transfer of Lucifer yellow. (B) DCDMLs were treated for 45 min with either 15 ng/ml BMP4 or -7, with or without preincubation of the BMPs with 5086 anti-BMP7 antibodies. Activation of Smad1 was assessed by Western blot. (C) FGF (10 ng/ml) was incubated for 3 h at room temperature with noggin before addition to DCDMLs. One or 8 h later, cells were lysed and evaluated for activation of ERK by anti-pERK Western blot. (D) DCDMLs were cultured for 9 h with noggin, after which the medium was supplemented with 10 ng/ml FGF. One or 8 h later, cells were lysed and evaluated for activation of ERK by anti-pERK Western blot. Even after the longest treatment period, the levels of total ERK in each sample were nearly identical as quantitated by Western blotting (in Figure 7D, lane 1 = 0.82, lane 2 = 0.85, lane 3 = 0.8, lane 4 = 0.80, and lane 5 = 0.85; arbitrary units).

Figure 8. Chick lens epithelial cells express BMP4 and -7, but not BMP2. Total RNA prepared from either DCDMLs on day 6 of culture or freshly isolated E10 chick lenses was assayed by RT-PCR for mRNA encoding BMP2, -4, and -7, and, as a positive control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PD). The BMP primer pairs were also used in PCR reactions in which genomic DNA from E10 chick foot was used as the source of template. PCR products were subjected to electrophoresis on 1% agarose gels in Tris-acetate-EDTA. No bands were detected from negative controls in which the reverse transcription reactions were conducted in the absence of either RNA template or RT enzyme (data not shown).

the same process? We addressed this question using two compounds, SU5402 (Mohammadi *et al.*, 1997) and PD173074 (Mohammadi *et al.*, 1998) (the latter is also known as SB402451). Both are commonly used as inhibitors of FGF receptor 1-4 kinase activity in a variety of culture and whole animal systems, with PD173074 considered to be the more potent and specific of the two (e.g., Bansal *et al.*, 2003). Control experiments verified that an hour preincubation with either 20 μ M SU5402 or 100 nM PD173074 effectively blocked activation of ERK in response to a 45 min treatment with FGF (Figure 9A; $n = 11$). Under these conditions, neither compound had any significant effect on up-regulation of ERK induced by IGF (Figure 9A; $n = 7$) or by phorbol ester (data not shown; $n = 3$), which stimulate ERK by processes independent of FGF receptors, or on activation of Smad1 in response to BMPs (Figure 9B; $n = 5$). Similar results were obtained when activation of p38 kinase was

used to assess FGF and IGF signaling (data not shown). As expected, up-regulation of intercellular dye coupling induced by FGF was completely blocked by either SU5402 or PD173074 (Figure 9C; $n = 8$), demonstrating that it requires FGF receptor activation. Neither compound diminished the extent to which GJIC was enhanced by BMP2, -4, or -7 (data for BMP4 shown in Figure 9C; $n = 7$).

Role of Lens Cell-Derived BMP in the Establishment and Maintenance of Increased GJIC-induced FGF or VBCM

The effect of FGF on GJIC can be divided into two phases: 1) initiation, in which a marked increase in intercellular transfer of Lucifer yellow first becomes detectable (under our standard assay conditions, ~ 12 h after addition of FGF); and 2) maintenance, in which the high levels of GJIC that are attained by ~ 24 h are sustained for longer periods. Both phases require the continuous presence of FGF in the culture medium (Le and Musil, 2001b). Lens-derived BMP is essential for the first phase, as evidenced by the ability of noggin to completely block up-regulation of GJIC by FGF after a 12- to 24-h coincubation (Figure 10A). To address the role of endogenous BMP in the second phase, cells were incubated with FGF for 48 h to maximally increase dye coupling. FGF was then stripped from the cell surface using heparin, and the cells cultured for two additional days under various conditions. If FGF was not readded (or added in the presence of the FGFR inhibitor SU5402; data not shown), GJIC returned to near baseline levels within the second 48 h (Figure 10B). Remarkably, cells refed with FGF under conditions in which the ERK pathway was blocked (+ UO126) had high levels of GJIC (Figure 10B), a result very different from that obtained when cells are exposed to FGF in the presence of UO126 on day 1 (Figure 2; Le and Musil, 2001b). We have previously shown that addition of UO126 reduces FGF-induced activation of ERK in DCDMLs to baseline levels within 90 min (Le and Musil 2001b). In contrast, cells were unable to maintain high-level GJIC if refed with FGF

Figure 9. Up-regulation of GJIC by exogenous BMP does not require lens-derived FGF signaling. DCDMLs were incubated for either 1 h (A and B) or 48 h (C) with no additions (control), 15 ng/ml FGF-2, 10 ng/ml BMP4, or 15 ng/ml IGF-1 in either the absence or presence of the FGF receptor kinase inhibitors SU5402 (SU) or PD173074 (PD) (inhibitors added 1 h before addition of growth factors.) Some cultures were lysed 1 h later and assessed for activation of ERK (A) or Smad1 (B) by Western blotting; the others were evaluated for gap junction-mediated intercellular transfer of Lucifer yellow 48 h later (C).

Figure 10. BMP is required for both the establishment (A) and maintenance (B) of FGF- or VBCM-enhanced GJIC. (A) On day 2 of culture, DCDMLs were incubated with 10 ng/ml FGF-2 or VBCM in the absence or presence of noggin for 18 more hours before scrape-load dye transfer analysis of GJIC with Lucifer yellow. (B) One-day-old DCDMLs were cultured for 48 h with FGF-2 (10 ng/ml) or VBCM. On day 3, the cells were washed three times with heparin to remove extracellular FGF and incubated for an additional 48 h in culture medium with no additions (0), or one or more of the following: 10 ng/ml FGF-2, 15 μ M UO126 (UO), noggin (nog), or VBCM. Cells were assessed for GJIC as described in A.

plus noggin (Figure 10B). Thus, initiation of increased dye coupling by FGF is dependent on both ERK and BMP signaling, but its maintenance requires only the latter.

Using a similar approach, we found that initiation of enhanced GJIC in response to VBCM also required BMP signaling (Figure 10A). In contrast to the results obtained with FGF, removing VBCM from the culture medium did not cause GJIC to return to untreated control levels within 48 h (Figure 10B). The effects of VBCM were, however, completely reversed if cells were incubated in the presence of noggin (Figure 10B). We conclude that maintenance as well as initiation of increased GJIC induced by either FGF or VBCM requires ongoing signaling from noggin-sensitive BMPs. The potential physiological role of this coregulation by FGF and BMPs is discussed.

DISCUSSION

Previous studies have supported the hypothesis that vitreous humor-derived FGF plays a major role in increasing the levels of GJIC at the lens equator, an up-regulation essential to the nonvascular microcirculatory system believed to be important for lens homeostasis (Le and Musil, 2001b; Mathias *et al.*, 2007). We report here that although FGF may be necessary for this process, it is not sufficient. Instead, enhanced GJIC in response to either vitreous humor-derived or purified recombinant FGF also requires signaling by BMPs endogenous to the lens cells. Our findings demonstrate that cooperation between FGF and BMPs extends past the earliest stage of lens development (Faber *et al.*, 2001) and regulates one of the organ's essential postnatal processes, namely gap junctional intercellular coupling.

The effect of BMPs on gap junctional coupling is highly dependent on their concentration, as has been described for certain other functions of BMPs in other primary culture systems (Reddi, 1997; Wilson *et al.*, 1997; Shou *et al.*, 2000). BMPs have been reported to have biological activities in the subpicomolar range (Reddi, 1997). Although we have not directly measured the amount of BMP4 and -7 produced by lens cells, it would seem to be well below the minimum concentration of exogenous BMP that must be added to detectably up-regulate dye intercellular transfer (~ 2 ng/ml). Medium conditioned by either DCDMLs or intact lenses does not detectably activate Smad1 when added to C3H10t1/2 cells as assessed by Western blot, indicating that the levels of diffusible BMP produced by lens cells are below the threshold of sensitivity of this assay (~ 1 ng/ml BMP4; data not shown). Endogenous levels of BMP, do, however, confer upon FGF the ability to up-regulate GJIC in an ERK-dependent manner. At relatively high concentrations (>2.5 ng/ml), exogenously added BMP2, -4, and -7 can stimulate GJIC in lens cells without a requirement for FGF (Figure 9) or ERK (Figure 2) signaling. At intermediate levels, BMPs can promote ERK-independent GJIC, but only in the presence of FGF (Figure 3). One possibility is that exogenous FGF utilizes ERK-dependent processes to enhance signaling by noggin-sensitive BMPs in lens cells by either up-regulating a BMP pathway activator or down-regulating a BMP antagonist. This enhancement is not necessary when cells are concomitantly exposed to exogenous BMP from vitreous humor or another source. Alternatively or in addition, endogenous BMP signaling may be required to keep lens cells in a maximally FGF-responsive state, perhaps by up-regulating the functional levels of a component in the FGF sig-

naling pathway or by reducing the expression of an ERK pathway inhibitor. The converse possibility—that endogenous FGF signaling is necessary for cells to respond to the GJIC-enhancing effects of exogenous BMP—is ruled out by the finding that blocking FGF signaling with FGF receptor antagonists does not abrogate the ability of cells to increase dye transfer when stimulated with >2.5 ng/ml BMP (Figure 9). It is also conceivable that BMPs and FGFs activate independent signaling pathways that converge only at the level of transcription of one or more genes involved in up-regulation of GJIC. High-level BMP signaling can compensate for a lack of FGF-mediated input, but not *visa versa*. We are unaware of a precedent for this type of nonreciprocal cross-talk between FGF and BMP in the eye, or in the regulation of gap junction function in any organ. The amount of noggin that must be added to block a BMP-mediated effect is proportional to the concentration of BMP present (Zimmerman *et al.*, 1996; Groppe *et al.*, 2002). Because less noggin is required to inhibit FGF from up-regulating GJIC in DCDMLs than is needed to prevent up-regulation by the minimally effective concentration of exogenously added BMP4 (data not shown), it is unlikely that FGF enhances coupling by stimulating lens cells to increase their synthesis of BMPs to above this threshold.

What purpose would be served by having gap junctional coupling at the lens equator regulated by both BMP and FGF instead of by FGF alone? Up-regulation of GJIC in lens cells by FGF (either recombinant, or purified from vitreous humor) requires activation of ERK (Figure 2; Le and Musil, 2001b). If this were the sole mechanism that controlled gap junctional coupling *in vivo*, then GJIC would be elevated only in cells newly exposed to FGF from the vitreous humor for ≤ 24 h, the period for which ERK is strongly activated by this level of FGF (Le and Musil, 2001b; Iyengar *et al.*, 2007). This is inconsistent with the high level of coupling measured throughout the equatorial region of the lens cortex *in vivo* (Mathias *et al.*, 1997), and after incubation of DCDMLs with FGF for >24 h. Our data indicate that BMPs play an essential role in maintaining the high levels of GJIC in the entire equatorial cell population that are required for the proposed gap junction-dependent microcirculation of the lens. Two mechanisms are operative. In the first, BMP at the levels at which it diffuses out of vitreous bodies confers upon FGF the ability to up-regulate GJIC in DCDMLs even in the absence of ERK activation (Figure 3). In the second, high-level coupling initiated by FGF or VBCM is maintained for >24 h due to the activity of lens-derived BMP (Figure 10B). The latter mechanism would ensure that GJIC remains high at the equatorial region even if BMP is no longer available from the vitreous body, as might result from postnatal down-regulation of BMP production (or increased expression of secreted BMP antagonists) in the retina or ciliary body. *In vivo*, BMPs often function in a paracrine or autocrine manner due to their tendency to bind tightly to extracellular matrix components (Reddi, 1997) (Christian, 2000). Whether enough functional BMP can diffuse out of the vitreous body, cross the matrix-rich lens capsule, and diffuse through the intercellular space to access BMP receptors on all of the equatorial cells that require BMP signaling to facilitate FGF-dependent up-regulation of GJIC is unclear. This would be unnecessary if (as our data suggest; Figure 6) equatorial cells can rely on lens-derived BMP instead of from extralenticular sources. In older (e.g., pole-to-pole) fiber cells, BMP signaling seems to decline, perhaps as a consequence of decreased expression of BMP receptors (de Jongh *et al.*, 2004). This could contribute to the observed reduction in GJIC at the

posterior pole of the lens despite the continued exposure to vitreous-derived FGF.

Based on our findings, we would predict that inhibition of lenticular BMP signaling after formation of the lens would perturb gap junctional coupling in the organ and could lead to defects similar to those caused by direct disruption of connexin expression (e.g., postnatal cataracts and/or reduced lens growth). Consistent with this possibility, Zhao *et al.* (2002) have reported that lens-specific expression of a noggin transgene that had no obvious prenatal effects caused cataracts and microphthalmia within a few weeks after birth. Unfortunately, the rapid onset and severity of these abnormalities, combined with the extralenticular effects of (secreted) noggin, would make it difficult to determine whether these defects were a direct downstream consequence of any changes in GJIC observed. Future studies are directed toward developing models to better address the role of lens-derived BMPs in the regulation of lens gap junctions.

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