

Synergistic interaction between the fibroblast growth factor and bone morphogenetic protein signaling pathways in lens cells

Bruce A. Boswell and Linda S. Musil

Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, OR 97239

ABSTRACT Fibroblast growth factors (FGFs) play a central role in two processes essential for lens transparency—fiber cell differentiation and gap junction–mediated intercellular communication (GJIC). Using serum-free primary cultures of chick lens epithelial cells (DCDMLs), we investigated how the FGF and bone morphogenetic protein (BMP) signaling pathways positively cooperate to regulate lens development and function. We found that culturing DCDMLs for 6 d with the BMP blocker noggin inhibits the canonical FGF-to-ERK pathway upstream of FRS2 activation and also prevents FGF from stimulating FRS2- and ERK-independent gene expression, indicating that BMP signaling is required at the level of FGF receptors. Other experiments revealed a second type of BMP/FGF interaction by which FGF promotes expression of BMP target genes as well as of BMP4. Together these studies reveal a novel mode of cooperation between the FGF and BMP pathways in which BMP keeps lens cells in an optimally FGF-responsive state and, reciprocally, FGF enhances BMP-mediated gene expression. This interaction provides a mechanistic explanation for why disruption of either FGF or BMP signaling in the lens leads to defects in lens development and function.

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INTRODUCTION

The vertebrate lens consists of a monolayer of epithelial cells and the highly elongated, crystallin-rich lens fiber cells that differentiate from them. Epithelial-to-fiber differentiation continues throughout life and takes place at the border of the anterior and posterior faces of the organ in a region referred to as the lens equator (Piatigorsky, 1981; Mochizuki and Masai, 2014). Environmental or genetic factors that perturb fiber formation cause vision-disrupting cataracts and/or microphthalmia (Reneker and Overbeek, 1996; Lovicu and Overbeek, 1998; Nishiguchi *et al.*, 1998; Zhao *et al.*, 2008).

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Address correspondence to: Linda S. Musil (musill@OHSU.edu).

Abbreviations used: BMP, bone morphogenetic protein; BMPR, BMP receptor; BRE, BMP response element; DCDML, dissociated cell–derived monolayer culture; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; ERK, extracellular signal–regulated kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; GJIC, gap junction–mediated intercellular communication; TGF β , transforming growth factor β ; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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As reported in >30 primary publications spanning >20 years, multiple lines of evidence indicate that one or more members of the fibroblast growth factor (FGF) family play a central role in the early stages of epithelial-to-fiber differentiation (McAvoy *et al.*, 1999; Robinson, 2006; reviewed by Lovicu and McAvoy, 2005; Lovicu *et al.*, 2011). It is believed that fiber formation is initiated at the lens equator because this is where epithelial cells are first exposed to the high concentrations of FGFs that diffuse out of the vitreous body. All four members of the FGF receptor (FGFR) family are expressed in the mammalian lens and share the same signaling mechanisms (Reuss and von Bohlen und Halbach, 2003). Studies in which one or more FGFRs were conditionally deleted in the mouse lens elegantly demonstrated that FGFRs are required for fiber cell differentiation and that concurrent knockout of FGFRs 1–3 is necessary to block fiber formation (Zhao *et al.*, 2008). FGF appears to be the main activator of the extracellular signal–regulated kinase (ERK) mitogen-activated protein kinase (MAPK) in the lens in vivo (Govindarajan and Overbeek, 2001; Zhao *et al.*, 2008).

We use as our standard experimental system dissociated cell–derived monolayer cultures of primary embryonic chick lens epithelial cells (DCDMLs; Le and Musil, 1998). Unlike central epithelial explants, the cells in DCDMLs include those from the peripheral (= pre-equatorial and equatorial) epithelium (Menko *et al.*, 1984; Le and Musil, 2001a). We have shown that DCDMLs are an appropriate

model system for the study of processes localized to the peripheral region of the mammalian lens *in vivo*, including epithelial-to-fiber cell differentiation and fiber cell-type gap junction formation, regulation, and function (Musil, 2012). As reported by Menko *et al.* (1984), dissociated cell-derived cultures of embryonic chick cells so closely recapitulate the *in vivo* process of epithelial-to-fiber differentiation that the most differentiated cells in these cultures are ultrastructurally indistinguishable from cortical fibers in the intact lens. When added to serum-free DCDMLs on day 1 of culture, purified recombinant FGF2 (or FGF1 with its cofactor heparin) at “high” concentrations (defined hereafter as ≥ 10 ng/ml FGF) maximally increases the expression of markers indicative of fiber cell differentiation (e.g., δ -crystallin; the beaded intermediate filament proteins CP49 and CP115) when assayed on day 6 (Le and Musil, 2001a). These levels of FGF1 and 2 also increase expression of both crystallin and noncrystallin genes in central epithelial explants prepared from either chick (Le and Musil, 2001a) or rat (McAvoy and Chamberlain, 1989) lens.

Members of the bone morphogenetic protein (BMP) family of growth factors are key regulators of early embryogenesis and the morphogenesis of a wide variety of tissues and organs (Whitman, 1998). BMP receptor 1A (ALK3) and BMP receptor II have been consistently detected in lens epithelium (Obata *et al.*, 1999; Hung *et al.*, 2002; de longh *et al.*, 2004). We have shown that the ability of FGF to up-regulate fiber marker expression in DCDMLs is blocked when cells are cultured under conditions that prevent BMPs from binding to BMP receptors (e.g., with anti-BMP2/4/7 antibodies or with noggin; Boswell *et al.*, 2008b; reviewed in Lovicu *et al.*, 2011). This effect is attributable to inhibition of BMPs produced by the lens cells themselves, which act as bona fide differentiation-promoting factors. Expression of noggin in the lenses of transgenic mice (Zhao *et al.*, 2002) resulted in a postnatal block of epithelial-to-fiber cell differentiation, consistent with (although not proof of) a role for BMP in FGF-induced fiber cell differentiation *in vivo* (Boswell *et al.*, 2008b). In other studies, we showed that treating DCDMLs with purified BMP2, 4, or 7 at “high” concentrations (defined hereafter as ≥ 4 ng/ml BMP) induces the expression of markers of epithelial-to-fiber differentiation in a process that does not require signaling from endogenously produced FGF. This is unlike up-regulation of fiber cell differentiation in response to exogenously added FGF, which is obligatorily linked to lens-derived BMP signaling (Boswell *et al.*, 2008b). We are not aware of a precedent for this type of nonreciprocal interaction between FGF and BMP in any system. This includes early retina/lens explants, in which exogenously added BMP cannot induce either cell cycle exit or normal equarion expression in the absence of FGF (Jarrin *et al.*, 2012).

Growth factor signaling also regulates another process essential for lens function, gap junction-mediated intercellular communication (GJIC). Gap junctional coupling is much higher at the lens equator than at either pole (Baldo and Mathias, 1992; Mathias *et al.*, 1997), a distribution believed to be required for lens homeostasis (Mathias *et al.*, 1997; Donaldson *et al.*, 2001; Sweeney *et al.*, 2003). We reported that FGF enhances GJIC in DCDMLs in a concentration-dependent manner and that the pole-to-equator gradient in FGF signaling in the lens *in vivo* may be responsible for the observed asymmetry in GJIC (Le and Musil, 2001b). Up-regulation of GJIC by FGF in DCDMLs occurs before, and is not a prerequisite for, fiber differentiation, demonstrating that they are independently regulated processes. Nonetheless, the ability of FGF to up-regulate GJIC, like its effect on fiber formation, depends on signaling from lens-derived BMPs. Similar to fiber differentiation, GJIC can be enhanced in the absence of FGF signaling by culturing DCDMLs with “high” levels of BMP2, 4, or 7 (Boswell *et al.*, 2008a).

In many nonlens systems, FGF and BMP signals act in an opposing manner to regulate a myriad of processes, including limb growth (Niswander and Martin, 1993), neural induction (Wilson and Edlund, 2001), placode formation (Sjödäl *et al.*, 2007), cranial suture closure (Warren *et al.* 2003), patterning of the olfactory epithelium (Maier *et al.*, 2010), chondrogenesis (Yoon *et al.*, 2006), and lung bud morphogenesis (Weaver *et al.*, 2000). Although it has long been recognized that this antagonistic relationship cannot hold true in all situations (Whitman, 1998; Sapkota *et al.*, 2007), knowledge of how FGFs and BMPs can positively cooperate is limited. In this study, we investigate the synergistic interaction between the BMP and FGF pathways that up-regulates secondary fiber differentiation and GJIC in lens cells. We present evidence that two types of mechanisms are operative. In the first, signaling from lens-derived BMPs is required to maintain lens cells in an optimally FGF-responsive state. In the second, FGF potentiates expression of BMP target genes and of BMP4.

RESULTS

BMP signaling is required to maintain lens cells in an optimally FGF-responsive state

Up-regulation of markers of fiber differentiation in chick lens epithelial cell DCDML cultures in response to FGF is blocked if the cells are coincubated during the 6-d culture period with noggin (Boswell *et al.*, 2008b), a highly specific inhibitor of binding of BMP2/4/7 to BMP receptors (Zimmerman *et al.*, 1996; Groppe *et al.*, 2002). One potential mechanism for this effect is that signaling by endogenously expressed, noggin-sensitive BMPs (BMP4 and/or 7; Boswell *et al.*, 2008b) is required for the full activity of one or more key components of the FGF signaling pathway in lens cells. To test this possibility, we examined the consequences of suppressing BMP signaling with noggin on the ability of FGF2 to activate ERK, the best-characterized effector downstream of FGF in lens cells (Figure 1). Levels of FGF sufficient to stimulate fiber differentiation induce sustained (>4 h) activation of ERK (Le and Musil, 2001b; Lovicu and McAvoy, 2001). Normalized to total ERK, dually phosphorylated (activated) ERK (pERK) was increased 5.46 (± 0.98)-fold after an 8-h incubation with 10 ng/ml FGF2 in otherwise untreated cells (Figure 1A, lane 2). This was reduced to 1.93 (± 0.46)-fold in cultures preincubated for 6 d with noggin before addition of FGF ($n = 10$; lane 4). Noggin does not affect lens cell viability, proliferation, or epithelial phenotype (Boswell *et al.*, 2008a,b), nor does it change the expression of total ERK protein (lanes 1–4; bottom). Activation of ERK in response to FGF was also decreased if BMP signaling was suppressed throughout the 6-d culture period by using a function-neutralizing anti-BMP monoclonal antibody (MAB3552) instead of noggin (Figure 1A, lanes 5–8). Control experiments demonstrated that neither noggin nor anti-BMP antibody interferes with the capacity of FGF to productively interact with its receptor (Boswell *et al.*, 2008a). A 6-d preincubation with noggin also markedly reduced activation of ERK in response to a brief (15–60 min) exposure to 5–30 ng/ml FGF (Figure 1B; compare lane 2 to lane 5). Under the same conditions, noggin also down-regulated activation of p38 by FGF, which we show is downstream of ERK in DCDMLs (Supplemental Figure S1). Noggin did not, however, reduce activation of ERK by the FGF-unrelated protein kinase C (PKC) and ERK agonist 50 nM 12-O-tetradecanoylphorbol 13-acetate (TPA; see later discussion of Figure 4A; Chen and Kroog, 2004) (Figure 1B, lane 3 vs. lane 6), showing the specificity of the effect. Time-course studies revealed that >1-d treatment with noggin was required to significantly reduce stimulation of ERK by FGF (Figure 1C).

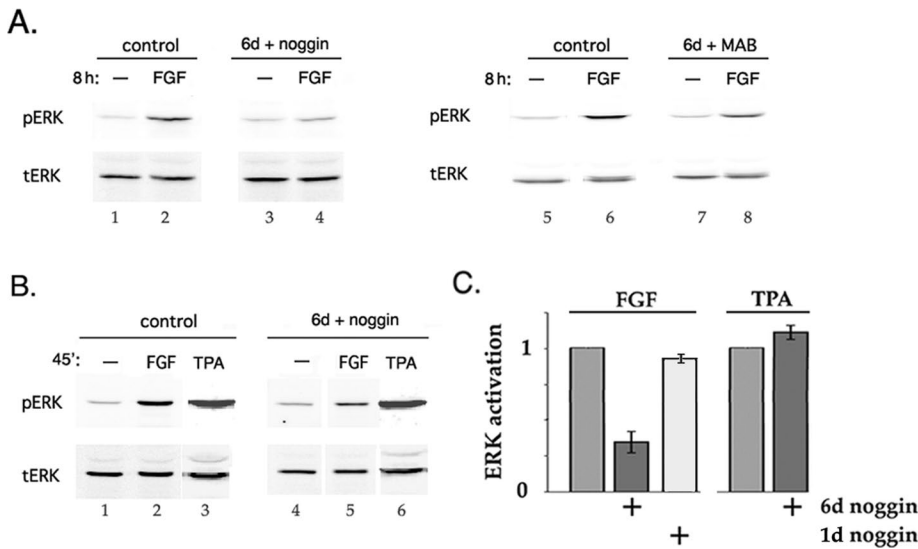


FIGURE 1: Culturing lens cells with a BMP inhibitor decreases their ability to activate ERK in response to FGF. DCDMLs were cultured for 6 d with no additions (control) or with either noggin or the function-blocking anti-BMP antibody MAB3552 (MAB). Cells were then incubated for 8 h (A) or 45 min (B) with no additions (–), 10 ng/ml FGF2, or 50 ng/ml TPA, followed by Western blot analysis of activated (pERK) and total (tERK) ERK kinase. Note that chick cells express only a single ERK isoform (ERK2). (C) Summary of experiments conducted as in B. Results graphed as fold activation of ERK (normalized to total ERK) in response to FGF ($n = 12$) or TPA ($n = 3$) in cells preincubated with noggin for 6 d compared with pERK/tERK in cells without noggin preincubation. Included are results from cells preincubated with noggin for only 1 d ($n = 3$).

Next we inhibited BMP signaling at the level of BMP receptors using dorsomorphin, a selective small-molecule inhibitor of ligand-activated type I BMP receptors (BMPRs; Yu *et al.*, 2008; Kamaid *et al.*, 2010). We confirmed that a 30-min preincubation with 5 μ M dorsomorphin completely blocked BMP4 from activating its downstream effectors Smad1/5 in DCDMLs but did not

prevent activation of Smad3 or ERK in response to transforming growth factor β (TGF β) or FGF, respectively (Figure 2A). To test the effect of dorsomorphin on expression of lens fiber cell markers, we cultured DCDMLs with dorsomorphin for 6 d in the presence of BMP4 or FGF2. Dorsomorphin blocked up-regulation of δ -crystallin and CP49 by either growth factor, without detectable deleterious effects on the cells (Figure 2B). A 6-d incubation with dorsomorphin also down-regulated the ability of subsequently added FGF to activate ERK by 65% (± 7.7 ; $n = 3$; Figure 2C). Thus dorsomorphin has the same inhibitory effect as noggin on FGF-induced lens fiber differentiation and ERK activation, further supporting the role of the canonical BMP signaling pathway in these events.

Experiments using purified FGF in chick lens cells or in the rat lens epithelial explant system have been conducted with either FGF2 or FGF1. Although the identity of the FGF family member(s) essential for lens fiber differentiation *in vivo* is unknown, Robinson (2006) concluded that the most likely candidate in the mammalian and avian lens is FGF9. When assayed as described for FGF2, ≥ 10 ng/ml recombinant, purified FGF9 also up-regulated expression of markers of fiber differentiation (δ -crystallin; CP49; Figure 3A) and induced sustained (>8 h) activation of ERK (Figure 3B). As was the case for FGF2, these FGF9-mediated processes were inhibited by noggin, demonstrating a similar requirement for endogenous BMP signaling. Normalized to total ERK, fold activation of ERK in response to FGF9 dropped from 3.3 \times (± 0.6) in the

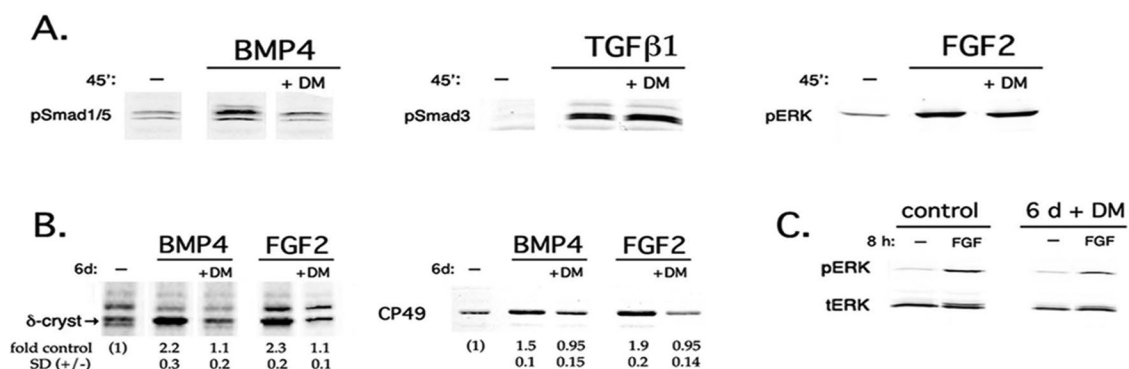


FIGURE 2: BMP-dependent FGF signaling in lens cells requires active BMP receptors. (A) Dorsomorphin is a specific inhibitor of BMP signaling in DCDMLs. Cultures were treated for 45 min without factors (–) or with 5 ng/ml BMP4, 4 ng/ml TGF β 1, or 10 ng/ml FGF2. Where indicated, cells were pretreated with 5 μ M dorsomorphin (DM) before addition of the factor. Whole-cell lysates were probed with antibodies specific for the phosphorylated (activated) forms of Smad1/5, Smad3, or ERK. Representative of three independent experiments; inhibition of either Smad3 or ERK activation never exceeded 20%. (B, C) Dorsomorphin blocks both BMP- and FGF-induced processes in DCDMLs. DCDMLs were incubated without growth factor or with 10 ng/ml BMP4 or FGF2 in either the absence or presence of dorsomorphin as indicated. (B) After 6 d of culture, cells were assayed for synthesis of the fiber differentiation markers δ -crystallin (by [35 S] methionine labeling) and CP49 (by quantitative anti-CP49 Western blotting). Fold increase over control \pm SD given for each condition ($n = 5$). (C) Cultures were assessed for their ability to activate ERK in response to an 8-h incubation with FGF2 as in Figure 1A. Percentage decrease in activation of ERK in response to FGF in dorsomorphin-treated cells relative to cells not cultured with dorsomorphin was 65 \pm 7.7% ($n = 3$).

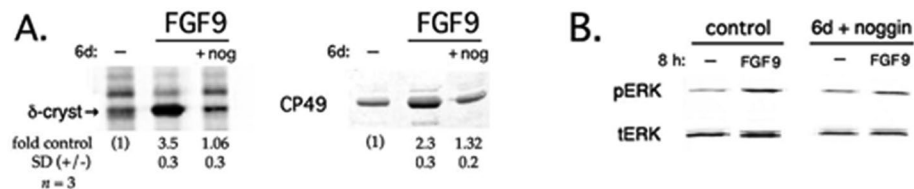


FIGURE 3: Signaling by FGF9 also requires lens-endogenous BMPs. (A) DCDMLs were cultured for 6 d with or without (–) 20 ng/ml FGF9 in either the absence or presence of noggin (nog) as indicated. Up-regulation of markers of fiber differentiation was assessed as in Figure 2B. (B) DCDMLs were cultured for 6 d without (control) or with noggin before being incubated with no additions (–) or 20 ng/ml FGF9 for 8 h. Activation of ERK assessed as in Figure 1.

absence of noggin to $1.2 \times (\pm 0.17)$ after a 6-d preincubation with noggin ($n = 3$), an extent of down-regulation very similar to that obtained with FGF2 (Figure 1).

Having demonstrated that our results with noggin and FGF2 can be reproduced using other BMP and FGF signaling effectors, we next addressed the mechanistic basis for the synergistic interaction between the FGF and BMP pathways. We have shown that FGF stimulates ERK in lens cells via the canonical FGF → Ras → Raf1 → MAPK kinase (MEK) → ERK signaling module (Boswell *et al.*, 2009; Figure 4A). In most cell types, TPA activates PKC, which in turn stimulates Raf1, MEK, and then ERK (Kolch *et al.*, 1993; Ueda *et al.*, 1996). This is also likely to be the case in DCDMLs, as evidenced by the ability of TPA to enhance the phosphorylation (activation) of all three of the last-named kinases (Figure 4B) and the finding that PKC inhibitors block activation of ERK induced by TPA but not by FGF (Figure 4C). The finding that a 6-d incubation with noggin decreases phosphorylation

of ERK in response to FGF but not to TPA (Figure 1) suggests that noggin reduces the activity of a component upstream of Raf1 in the canonical FGF-to-ERK signaling pathway. Consistent with this possibility, a 6-d preincubation with noggin greatly reduced phosphorylation of Raf1 and MEK in response to subsequently added FGF but not the total levels of either kinase (Figure 4D). FRS2 is a docking protein constitutively bound to FGF receptors that mediates most of the downstream effects of FGF-FGFR interaction, including activation of Ras and the

ERK pathway (Eswarakumar *et al.*, 2005; Madakashira *et al.*, 2012; Li *et al.*, 2014). We found that consistently less FRS2 became activated (tyrosine phosphorylated) in response to a 15-min treatment with FGF2 in noggin-preincubated cells than in controls, as assessed with a phosphospecific (Tyr-196) antibody against FRS2 (43.5% inhibition ± 12.1 ; $n = 4$; Figure 4E, compare lane 2 with lane 5). The phospho-FRS2 band was not detected when cells were treated with FGF in the presence of the FGFR blocker PD173074, confirming its identity. The block in FRS2 activation was also detectable using a total anti-phosphotyrosine antibody (Figure 4E, lane 8 vs. lane 11). Taken together, these results show that noggin inhibits FGF-to-ERK signaling upstream of FRS2 activation, supporting a role for endogenous BMP signaling at the level of FGF receptors.

If endogenous BMP signaling were required for FGF receptor function, then noggin pretreatment would be expected to inhibit processes downstream of FGF binding even if they are not

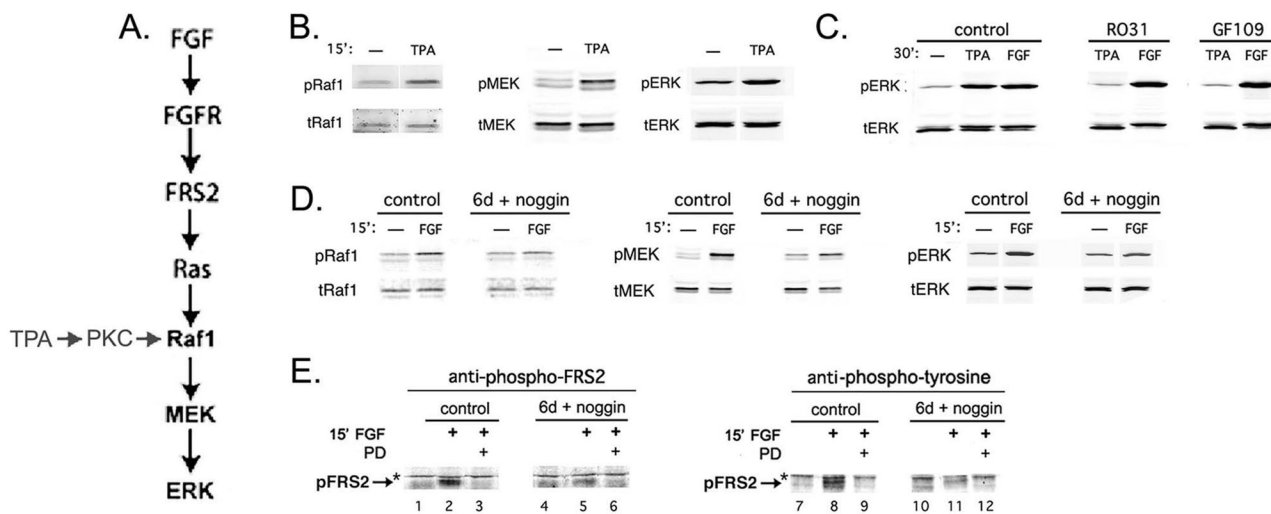


FIGURE 4: Noggin pretreatment inhibits steps upstream of ERK and downstream of FGF receptors in the canonical FGF-to-ERK signaling pathway. (A) Schematic of canonical FGF-to-ERK and TPA-to-ERK signaling pathways. (B) TPA activates Raf1 and MEK, as well as ERK. Lysates from DCDMLs incubated without (–) or with 50 ng/ml TPA for 15 min were analyzed by Western blot using antibodies that recognize either total (t) or activated (phosphorylated; p) forms of the indicated kinases. (C) Activation of ERK by TPA, but not by FGF, is downstream of PKC. Cells were incubated for 30 min without additions (control) or with 5 μ M R031-8220 (R031) or GF109203X (GF109), both inhibitors of PKC. TPA (50 ng/ml) or FGF2 (5 ng/ml) was then added, and the cells were incubated for an additional 30 min before analysis for activated and total ERK. In the gel system used, activation of ERK resulted in a slight decrease in its electrophoretic mobility. (D, E) DCDMLs were cultured for 6 d in the absence (control) or presence of noggin and then treated for 15 min with or without 10 ng/ml FGF2. (D) Effect on activation of kinases. Whole-cell lysates were analyzed by Western blot using antibodies that recognize either activated or total forms of Raf1, MEK, or ERK. FGF-induced activation of Raf1 and of MEK was reduced by $86.6 \pm 23\%$ and by $72.3 \pm 5.1\%$, respectively, in noggin-pretreated cells ($n = 3$). (E) Effect on activation of the FRS2 adaptor. Whole-cell lysates were analyzed by Western blot using antibodies against either phospho-FRS2 (Tyr-196) or phosphotyrosine. Phosphorylated FRS2 was not detected when cells were coincubated with FGF and the FGFR blocker PD173074 (PD). Equal protein loads in each lane. Asterisk, nonspecific band.

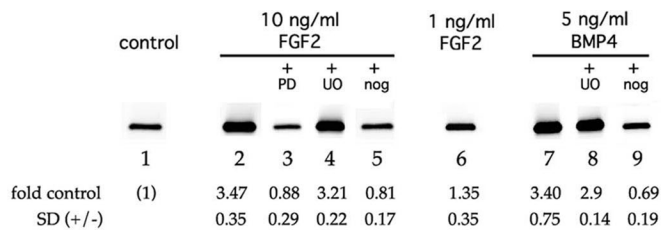


FIGURE 5: Noggin pretreatment inhibits FRS2- and ERK-independent FGF signaling. DCDMLs were transfected with the DCR1- α A-promoter-EGFP reporter construct and cultured for 9 d with no additions (control), 10 ng/ml FGF2, 1 ng/ml FGF2, or 5 ng/ml BMP4. Where indicated, the cells were incubated with growth factor in the presence of PD173074 (PD), UO126 (UO), or noggin (nog). Expression of EGFP was assessed by Western blot analysis (2 μ g total cell lysate/lane). Fold increase over control \pm SD given for each condition ($n \geq 3$).

mediated by FRS2 and ERK. This was confirmed in a series of experiments using a reporter construct driven by upstream elements from the gene encoding mouse α A crystallin, a marker of fiber differentiation whose expression in mouse lens is not dependent on FRS2 or ERK signaling (Li et al., 2014; Figure 5). The DCR1 lens-specific enhancer confers on the DCR1- α A-promoter-enhanced green fluorescent protein (EGFP) construct the ability to be up-regulated by FGF2 in rat lens central epithelial explants (Yang et al., 2006). Addition of FGF at concentrations that induce fiber formation (lane 2), but not below (lane 6), also increased DCR1- α A-promoter-EGFP levels in transiently transfected DCDMLs by 3.47 (± 0.35)-fold ($n = 4$) over a 6- to 9-d period. As expected, this up-regulation was blocked by the FGFR kinase inhibitor PD173074 (lane 3). In contrast, the MEK inhibitor UO126 had no significant effect (lane 4), indicating that expression of the reporter, like that of endogenous crystallin genes in mammalian (Lovicu and McAvoy, 2001) and avian (Le and Musil, 2001a) lens cells, does not require ERK activity. Up-regulation of DCR1- α A-promoter-EGFP by FGF was, however, completely blocked by coculture with noggin (lane 5). Of note, synthesis of DCR1- α A-promoter-EGFP was also stimulated 3.4-fold by 5 ng/ml BMP4 in a UO126-insensitive, noggin-inhibitable manner (lanes 7–9); we did not test the effect of BMP4 + PD173074. Thus the mouse DCR1- α A-promoter-EGFP construct behaved like endogenously expressed δ -crystallin in DCDMLs, in that its expression was increased over a >3-d period by either BMP4 or differentiation-inducing levels of FGF2 in a process that is independent of ERK but sensitive to noggin.

In knockout mice, lens fiber formation is blocked when the expression of FGF receptors 1, 2, and 3 is concurrently reduced by

>50% (Zhao et al., 2008). The simplest explanation for how noggin inhibits FGF from up-regulating fiber differentiation would be that noggin phenocopies these conditions. Repeated attempts to quantitatively detect FGF receptor protein in DCDMLs were unsuccessful, likely due to low levels of endogenous expression. However, no decrease in either *FGFR1* or *FGFR3* was detected by real-time quantitative reverse transcription PCR (qRT-PCR) in DCDMLs treated with noggin for 7 d (Table 1A); *FGFR2* is not expressed in chick lens; Walshe and Mason, 2000). Possible mechanisms by which noggin could reduce FGF receptor function but not expression are examined later (see Discussion).

FGF up-regulates BMP-mediated gene expression in DCDMLs

Having shown that BMP signaling promotes processes downstream of FGF in lens cells, we next addressed whether the converse was also true. As reported in several non-lens cell types (Hollnagel et al., 1999), a 5-h treatment of DCDMLs with 5 ng/ml BMP4 strongly increased the level of mRNA for *Id1*, a well-known direct transcriptional target of canonical, Smad-mediated BMP signaling (Katagiri et al., 2002; Lopez-Rovira et al., 2002; ten Dijke et al., 2003; Table 1A). Remarkably, *Id1* expression was also increased by 10 ng/ml FGF, albeit to a lesser extent ($[4.7 \pm 1.4]$ -fold relative to *GAPDH* measured in the same sample by qRT-PCR in 11 samples from four independent experiments; Table 1B). This is in contrast to several other nonneurological cell types, in which FGF does not affect (or reduces) *Id1* message levels (Rozenblatt-Rosen et al., 2002; Reinhold et al., 2004; Rice et al., 2005; Xu et al., 2005).

In addition to BMP response elements (BREs), the *Id1* promoter contains binding sites for non-Smad transcriptional regulators that could influence the strength and/or kinetics of *Id1* expression (Nehlin et al., 1997; Korchynskiy and ten Dijke, 2002). To demonstrate more definitively that FGF can promote gene expression directly downstream of the canonical BMP signaling pathway in lens cells, we transiently transfected DCDMLs with a luciferase reporter driven exclusively by BREs from mouse *Id1*. Expression of BRE-Luc has been extensively documented to require activation of Smad 1/5 and to be directly proportional to the amount of Smad signaling in multiple cell types (Korchynskiy and ten Dijke, 2002; Monteiro et al., 2004). As detected by anti-luciferase immunostaining, ~70% of the cells in confluent regions of DCDML cultures expressed BRE-Luc after incubation with 5 ng/ml BMP4 for ≥ 5 h (Figure 6C). Given that the efficiency of transient transfection of DCDMLs is also ~70% (Boswell et al., 2009), it appears that all of the transfected cells are transcriptionally responsive to BMP. As expected, expression of BRE-Luc was abolished if cells were cultured with BMP4 in the presence of noggin (Figure 6D).

		<i>Id1</i>	<i>FGFR1</i>	<i>FGFR3</i>	<i>BMP4</i>	<i>BMP7</i>
A	7 d noggin		1.5 \pm 0.06, $n = 6$	1.3 \pm 0.4, $n = 6$		
B	5 h BMP4	42.5 \pm 7.5, $n = 5$				
	5 h FGF2	4.7 \pm 1.4, $n = 11$			4.07 \pm 0.9, $n = 7$	0.7 \pm 0.14, $n = 7$
	5 h CHX	150.5 \pm 3.3, $n = 4$				
	5 h FGF2 + CHX	277.5 \pm 16.1, $n = 4$				
C	5 h DRB + FGF2	4.1 \pm 0.5, $n = 2$			1.2 \pm 0.4, $n = 2$	

Data are expressed as fold change over untreated cells (A, B) and DRB-only cells (C) in the same experiment. DCDMLs were preincubated with 75 μ M DRB for 30 min before addition of FGF2.

TABLE 1: Expression of mRNA for the indicated test gene as measured by qRT-PCR (TaqMan), using *GAPDH* for normalization.

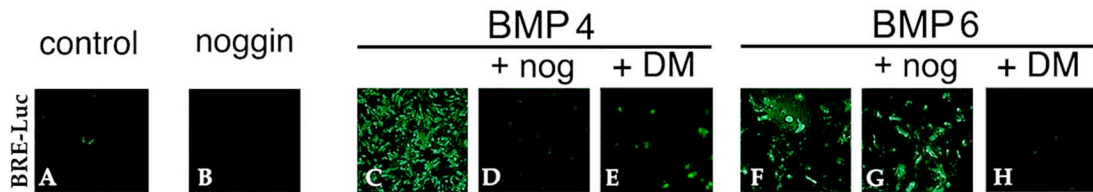


FIGURE 6: Up-regulation of the BMP signaling reporter BRE-Luc by BMPs is specifically blocked by noggin and dorsomorphin. DCDMLs were transfected with the BRE-luciferase reporter construct BRE-Luc and cultured for 2 d with no additions (control), noggin, 5 ng/ml BMP4, 10 ng/ml BMP6, or the indicated BMP plus either noggin or dorsomorphin. Fixed cultures were immunostained with anti-luciferase antibodies, and confluent regions of the monolayer were imaged. See Figure 8C for Western blot quantification of the effect of noggin and dorsomorphin on BRE-luciferase protein levels.

Unlike BMP4, the function of BMP6 is not effectively inhibited by noggin (Song *et al.*, 2010). Noggin had no effect on BRE-Luc induction in response to BMP6 (Figure 6G), demonstrating that noggin is acting by binding to its cognate BMPs instead of by nonspecifically preventing BRE-Luc synthesis, regardless of the stimulus. As expected, up-regulation of BRE-Luc by either BMP species was blocked by the BMP receptor inhibitor dorsomorphin (Figure 6, E and H).

Expression of BRE-Luc has been reported to be insensitive to FGF in several non-lens cell types (Monteiro *et al.*, 2004; Logeart-Avramoglou *et al.*, 2006; Zilberberg *et al.*, 2007), as expected, given that FGF signals via a different set of receptors and effectors than BMP. We reproduced this result in fibroblastic 10 $t_{1/2}$ cells, in which BMP4, but not FGF2, induced expression of BRE-Luc despite robust activation of ERK in response to the same levels of FGF (Supplemental Figure S3). In contrast, FGF markedly increased anti-luciferase

immunostaining in transiently transfected DCDMLs, although less intensely and more slowly than BMP4 (Figure 7, A–C). As quantitated by Western blot, 10 ng/ml FGF2 induced a 10.67 (± 4.09)-fold ($n = 10$) increase in BRE-Luc levels at 22 h, compared with a 123.2 (± 17.9)-fold increase with 5 ng/ml BMP4 (Figure 7B). Up-regulation of BRE-Luc by FGF, but not by BMP, was blocked by the highly selective inhibitor of FGFR kinase activity PD173074. In chick (Le and Musil, 2001a) and mammalian (McAvoy and Chamberlain, 1989) lens cells, FGF up-regulates fiber differentiation only at levels >1 ng/ml. Enhancement of BRE-Luc expression by FGF showed similar concentration dependence. Expression of BRE-Luc was not stimulated by TPA or TGF β 1, the latter a member of the same superfamily of growth factors as BMPs. Conversely, TGF β 1, but neither BMP nor FGF, induced expression of a TGF β - and Smad3- specific reporter, SBE4-Luc (Zawel *et al.*, 1998; Figure 7D).

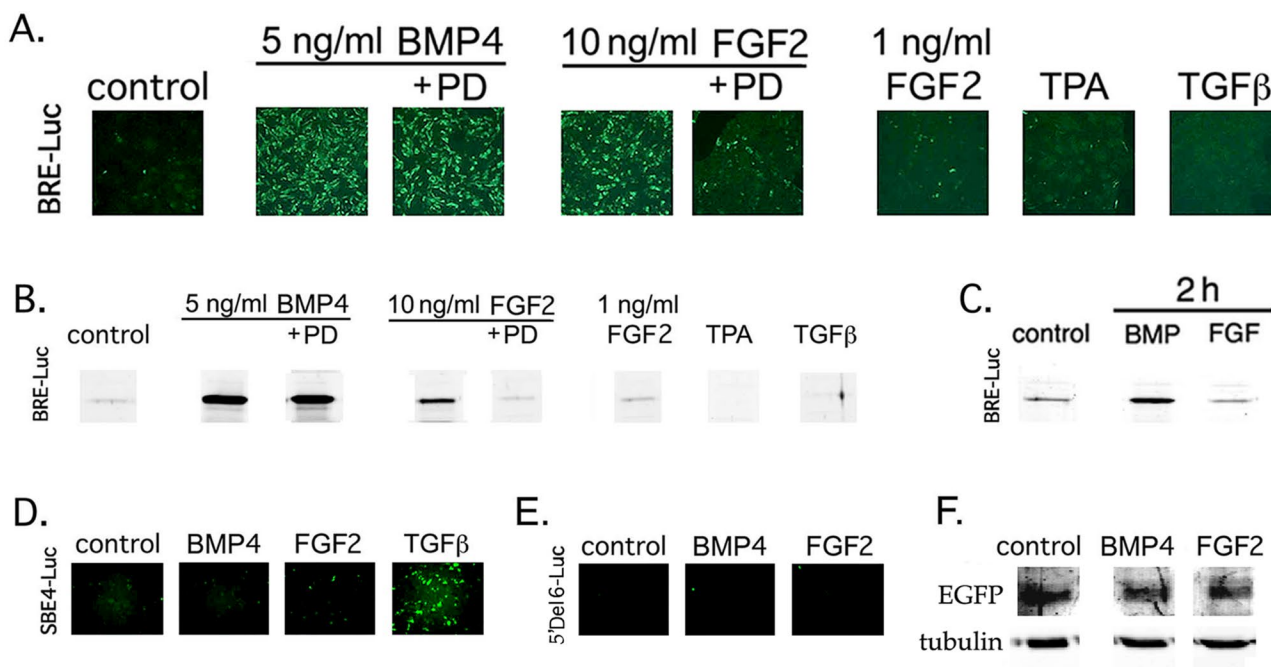


FIGURE 7: FGF specifically increases expression of the BMP signaling reporter BRE-Luc in lens cells. (A–E) DCDMLs were transfected with plasmids encoding BRE-Luc (A–C), SBE4-Luc (D), or 5' Del 6 Id1-Luc (E) and cultured with no additions (control), 5 ng/ml BMP4, 10 ng/ml FGF2, 1 ng/ml FGF2, 4 ng/ml TGF β 1, or 50 nM TPA for 22 h (A, B, D, E), or 2 h (C). Where indicated, the FGFR inhibitor PD173074 (PD) was also present. Cultures were either fixed and immunostained with anti-luciferase antibodies (A, D, E) or lysed and equal amount of total cell protein analyzed by Western blot using the same antibody (B, C). Representative of three or more independent experiments. (F) Experiments in which a plasmid encoding constitutively expressed EGFP was cotransfected with 5' Del 6 Id1-Luc at a ratio of 1:4 demonstrated equal levels of expression of EGFP when normalized to either total protein or tubulin, indicating that the lack of expression of 5' Del 6 Id1-Luc in E was not due to a lack of cell transfection.

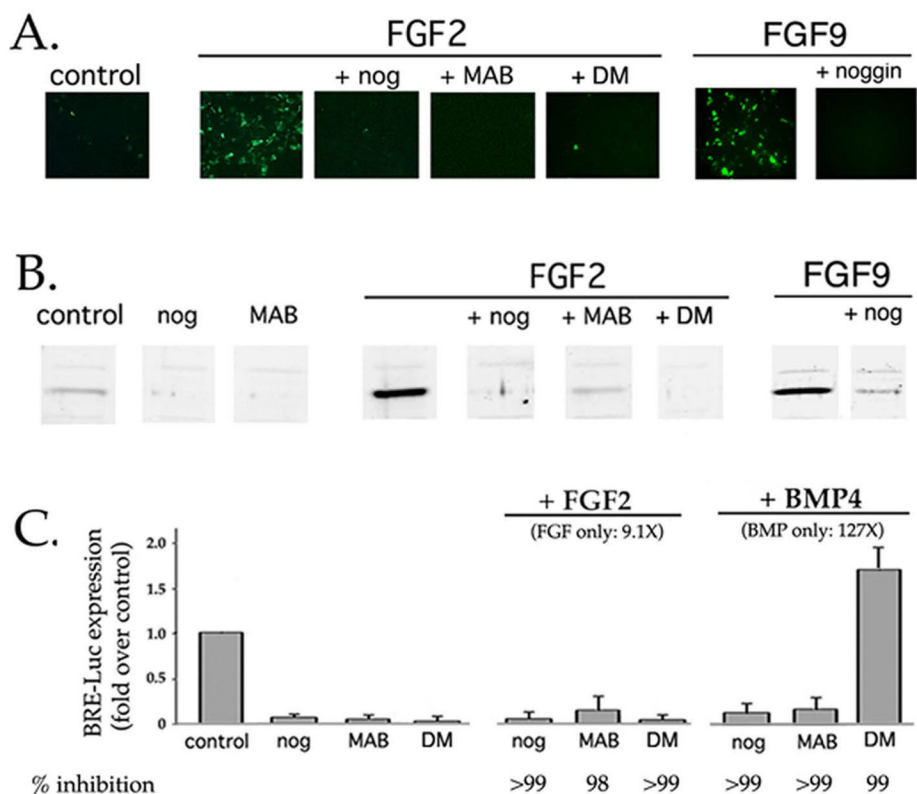


FIGURE 8: Up-regulation of BRE-Luc expression by FGF requires signaling from endogenously expressed, noggin-sensitive BMPs. DCDMLs were transfected with BRE-Luc and cultured with no added growth factor (control), 10 ng/ml FGF2, 20 ng/ml FGF9, or 5 ng/ml BMP4. Incubations were conducted in either the absence or presence of noggin (nog), the anti-BMP antibody MAB3552 (MAB), or dorsomorphin (DM). After 22 h, cultures were either fixed and immunostained with anti-luciferase antibodies (A) or lysed and equal amount of total cell protein analyzed by Western blot using the same antibody (B). (C) Summary of experiments conducted as in B, compared with results obtained in the same experiments from cells cultured for 22 h with 5 ng/ml BMP4. Results graphed as fold increase in luciferase levels relative to untreated control transfectants ($n = 3$). The percentage inhibition relative to growth factor alone is included. The low level of anti-luciferase immunoreactivity in untreated control transfectants is due to endogenous BMP4/7 signaling, as demonstrated by its near absence in cells treated with noggin, MAB3552, or dorsomorphin alone. Immunofluorescence images showing the effect of noggin and dorsomorphin on BMP4-induced BRE-Luc expression are presented in Figure 6.

Neither BMP nor FGF induced the expression of a mouse *Id1* minimal promoter-Luc construct that lacks BREs (5' Del 6; Tournay and Benezra, 1996; Figure 7E). If BREs are both necessary and sufficient for FGF to drive BRE-Luc expression, and if BMP-activated Smad1/5 are the transcriptional regulators that bind to BREs, then how does FGF enhance BRE-Luc expression? The simplest explanation is that FGF acts by promoting canonical Smad1/5 signaling initiated by endogenous BMP4 and/or 7. If so, then the ability of FGF to induce BRE-Luc expression should be inhibited by noggin. This was the result obtained for both FGF2 and FGF9 (Figure 8). Up-regulation of BRE-Luc by FGF was also abolished by the BMPR inhibitor dorsomorphin or the function-neutralizing anti-BMP antibody MAB3552. Similar results were obtained with another BRE-containing transcriptional reporter, pID1SB-Luc (Nehlin *et al.*, 1997; Supplemental Figure S2).

Role of BMP signaling in lens-cell gap junctional communication and proliferation

We reported that exogenously added FGF2 or BMP4 increases the gap junction-mediated intercellular transfer of Lucifer yellow in

DCDMLs in 2 d (Boswell *et al.*, 2008a). Up-regulation of gap junctional coupling by either factor is blocked by noggin or anti-BMP function-neutralizing antibodies (Boswell *et al.*, 2008a); similar results were observed with FGF9 (Figure 9A). Dorsomorphin also blocked BMP- or FGF-induced GJIC within 48 h (Figure 9A).

As in mammalian systems (Lovicu and McAvoy, 2001), low levels of FGF stimulate cell proliferation in DCDMLs (Le and Musil, 2001a). In contrast to gap junctional coupling, however, this process cannot be stimulated by exogenous addition of 0.2–20 ng/ml BMP4 (Figure 9B). Moreover, noggin did not prevent 2 ng/ml FGF2 from enhancing cell proliferation, as assessed using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 9B). Thus BMP signaling is not necessary for stimulation of lens cell proliferation by FGF nor is it sufficient to do so on its own, demonstrating that BMP does not affect all processes downstream of FGF.

Effect of FGF on BMP expression

One mechanism that could explain why noggin blocks the ability of FGF to up-regulate fiber differentiation and GJIC (both of which can also be stimulated by exogenously added BMP) but not cell proliferation (which is BMP insensitive; Figure 9B) would be if FGF enhances the synthesis of BMPs. We used real-time qRT-PCR to examine the effect of FGF on the message levels of BMP7 and BMP4, noggin-sensitive BMPs expressed by both chick (Boswell *et al.*, 2008b) and mammalian (Thut *et al.*, 2001; Hung *et al.*, 2002; Bakrania *et al.*, 2008) lens cells. We found in three of three independent experiments (seven total samples) that 10 ng/ml FGF2 modestly decreased the levels of BMP7 by $\sim 30 \pm 14\%$ within 5 h. In contrast, transcripts for BMP4 were increased 4 (± 0.96)-fold within the same samples (Table 1B).

Increased synthesis of BMP4 may contribute to, but cannot be solely responsible for, up-regulation of BMP target gene expression by FGF in lens cells

As reported in other cell types (Tournay and Benezra, 1996; Katagiri *et al.*, 2002; Lopez-Rovira *et al.*, 2002), treatment of DCDMLs with cycloheximide (CHX; 20 $\mu\text{g/ml}$) alone greatly increased *Id1* transcript levels (Table 1B), possibly by reducing the amount of a short-lived protein required for *Id1* mRNA degradation. *Id1* message levels were higher, however, if cells were treated with FGF and CHX than with CHX alone. This is in contrast to several reports in other systems in which CHX completely abolished FGF-induced transcriptional events (Isaac *et al.*, 2000; Li *et al.*, 2003; Vargas *et al.*, 2005). Further evidence that stimulation of *Id1* by FGF does not require new gene expression was obtained using the transcriptional inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB). In the same samples in which DRB prevented FGF from increasing the level of BMP4 transcripts, *Id1* message levels were still elevated

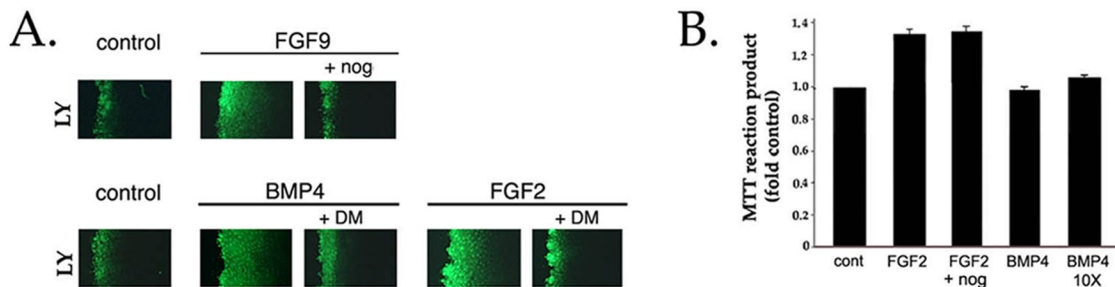


FIGURE 9: Up-regulation of gap junctional communication, but not of cell proliferation, by FGF requires signaling from endogenously expressed BMPs. (A) DCDMLs were incubated without growth factor (control) or with 10 ng/ml BMP4, 10 ng/ml FGF2, or 20 ng/ml FGF9 in either the absence or presence of noggin or dorsomorphin as indicated. After 2 d of culture, cells were assayed for gap junction-mediated intercellular spread of Lucifer yellow (LY) using the scrape-load dye transfer assay. Typical of three independent experiments. (B) DCDMLs were plated at low density (0.7×10^5 cells/well) and incubated with no additions (cont), 2 ng/ml FGF2, 2 ng/ml FGF2 + noggin, 0.2 ng/ml BMP4, or 20 ng/ml BMP4 (10X). After 2 d, the MTT assay was used to colorimetrically assess cell proliferation. Data expressed as fold OD 570 nm experimental/OD 570 nm untreated control. $n = 3$.

approximately fourfold relative to samples from cells treated with DRB alone. Taken together, the findings in Table 1, B and C, demonstrate that augmentation of *Id1* by FGF can occur in the absence of new BMP4 synthesis. We conclude that FGF can up-regulate BMP target genes by at least two mechanisms: 1) by increasing the de novo expression of BMP4, which then stimulates the canonical BMP/Smad signaling pathway, and 2) by more directly affecting BMP target gene transcripts (e.g., *Id1*), possibly by stabilizing them against degradation.

DISCUSSION

Molecular mechanisms by which receptor tyrosine kinase ligands such as FGF inhibit BMP signaling were first elucidated nearly two decades ago (Kretschmar *et al.*, 1997). How RTK ligands can positively cooperate with BMP—a less common, but as physiologically important, occurrence—is poorly understood. We show here that in lens cells, basal signaling from lens-derived, noggin-sensitive BMPs potentiates signaling by FGF. Conversely, FGF enhances the ability of lens-endogenous BMP to stimulate expression of BMP target genes. Our findings explain why levels of endogenous BMP too low to induce fiber differentiation or GJIC in unstimulated lens cells are nonetheless required for up-regulation of these essential processes by FGF. FGF cannot activate BMP receptors, nor can BMP engage FGFRs. In what follows, we discuss possible mechanisms for this synergistic interaction.

Potential of FGF signaling by BMP

We showed that a 6-d, but not a 1-d, exposure of DCDML cultures to the BMP blocker noggin inhibits FGF-induced signaling pathways (Figure 1). This includes the first step downstream of FGF receptor activation in most FGF-stimulated signaling pathways, namely phosphorylation of FRS2 (Figure 3B). The simplest explanation for why such a prolonged pretreatment with noggin is required would be if blocking endogenous BMP signaling reduced the expression of FGFRs at the transcript level. We did not, however, find any evidence for this by real-time RT-PCR (Table 1A). Noggin may instead induce changes in the expression of other genes known to affect FGF signaling. One possibility consistent with our findings is that lens-endogenous BMP signaling is required for the production or function of heparin sulfate proteoglycans that serve to enhance FGF/FGFR affinity and/or prolong receptor half-life (Knights and Cook, 2010). Genetic ablation of the heparin sulfate biosynthetic gene *Ndst1* disrupts binding of multiple FGFS to FGFRs in the early lens, leading to decreased

activation of ERK and defects in lens fiber differentiation (Pan *et al.*, 2006). Another mechanism by which endogenous BMP could promote FGFR signaling at the transcriptional level would be by down-regulating an inhibitor of FGFR function such as c-Cbl or Sef (Wong *et al.*, 2002; Kovalenko *et al.*, 2003; Cho *et al.*, 2004; Xian *et al.*, 2007). It is also conceivable that inhibiting BMP signaling induces the expression of a microRNA (miRNA) that decreases the translation of FGFRs. Noggin-up-regulated miRNAs have been reported in keratinocytes, as has miRNA-mediated reduction of FGFR at the protein, but not transcript, level (Xu *et al.*, 2011). By reducing FGFR activation to below the threshold needed to promote fiber differentiation (McAvoy and Chamberlain, 1989; Le and Musil, 2001a) and gap junctional communication (Le and Musil, 2001b), any of the aforementioned mechanisms would explain how noggin blocks the ability of FGF to enhance GJIC, as well as the expression of a wide range of fiber cell differentiation-associated genes, irrespective of their transcription factor requirements or dependence on ERK activation.

Why does noggin block the ability of FGF to up-regulate lens fiber marker expression and GJIC but not cell proliferation? Even after a 6-d preincubation, noggin only partially reduces FGF signaling, as indicated by the ~65% reduction in FGF-induced activation of ERK (Figure 1). It is possible that this lower level of FGF signaling is sufficient to support normal cell proliferation in response to 2 ng/ml FGF but not fiber differentiation and GJIC, which require higher levels of FGF. Alternatively or in addition, stimulation of cell proliferation by FGF may not require noggin-sensitive changes in gene expression. Because DCDMLs do not tolerate prolonged (>24 h) exposure to transcriptional or translational inhibitors, the role of de novo gene expression in FGF-mediated cell proliferation, differentiation, or GJIC cannot be directly assessed.

Potential of BMP signaling by FGF

It is unlikely that FGF potentiates lens-endogenous BMP signaling by increasing the synthesis of BMP receptors, because addition of high (≥ 5 ng/ml) levels of exogenous BMP4 or 7 increases Smad1 activation in otherwise unstimulated DCDMLs in <15 min, indicating that the number of BMPRs present under basal conditions is more than sufficient to mediate signaling by the lower levels of endogenous BMP. Instead, our data are consistent with two alternative, non-mutually exclusive mechanisms. First, FGF could enhance the signaling capacity of BMP and/or BMPRs by, for example, increasing the release of BMP from the extracellular matrix, increasing the engagement of a BMP coreceptor, or down-regulating a BMPR

phosphatase (e.g., Dullard; Satow *et al.*, 2006) or another inhibitor of BMP signaling (Reinhold *et al.*, 2004). Second, FGF could increase the synthesis of a noggin-sensitive BMP to levels that functionally mimic exogenous addition of BMP2, 4, or 7. Autoactivation of BMP4 expression has been reported in many systems (Schuler-Metz *et al.*, 2000; Wijgerde *et al.*, 2005; Kozmikova *et al.*, 2013; Wang *et al.*, 2013). Although our finding that FGF increases the level of BMP4 mRNA within 5 h is consistent with the second mechanism, it does not rule out the first. Indeed, increasing the expression of BMP4 cannot be the sole mechanism by which FGF promotes BMP target gene expression, given that FGF still increases *Id1* message levels if synthesis of BMP4 is blocked by CHX (Table 1B) or DRB (Table 1C). Because we do not yet have a means to block up-regulation of BMP4 by FGF without also abolishing basal BMP4 expression, we cannot experimentally determine the role of FGF-induced stimulation of BMP4 synthesis.

Comparison to mammalian systems

Wolf *et al.* (2013) conducted an mRNA expression profiling analysis of the effects of FGF on central epithelial explants generated from the lenses of postnatal rats. They reported that FGF2 up-regulated the expression of *Id1* by approximately twofold within 4 h and that of *BMP4* by approximately fivefold within 12 h. *BMP7* transcripts were reduced by ~50% over the same period. These findings mirror our results in chick lens cells and further substantiate the fundamental similarity between bird and mammalian lenses in growth factor signaling. Although the reason for the slower induction of *BMP4* in rat explants compared with DCDMLs is unknown, it could be due to differences in growth factor sensitivity and/or signaling between central and peripheral lens epithelial cells (Richardson *et al.*, 1992, 1993; Boswell *et al.*, 2008b). Wolf *et al.* (2013) also reported that FGF increases the message levels of two other BRE-containing direct BMP target genes, *Id2* and *Id3* (Shin *et al.*, 2013), as well as of *BMP2*. Because the last is not expressed in chick (Boswell *et al.*, 2008b) or human (Dawes *et al.*, 2007) lens cells, the physiological significance of *BMP2* in the lens is unclear.

In mice, germline knockout of *BMP7* or *BMP4* (Furuta and Hogan, 1998; Wawersik *et al.*, 1999) prevents the formation of a lens, as does conditional deletion of both *ALK3* (*Bmpr1a*) and *ALK2* (*Acrv1*) BMP receptors in surface ectoderm at embryonic day 9.5 driven by *LoxP-Cre* (Rajagopal *et al.*, 2009). Conditional deletion of only *ALK3* with *LoxP-Cre* resulted in a smaller lens with defects in fiber cell differentiation (Beebe *et al.*, 2004). Transgenic overexpression of noggin in the lens at a later development stage leads to a block in secondary fiber cell formation at the lens equator (Boswell *et al.*, 2008b). Both lens placodes (Rajagopal *et al.*, 2009) and the equatorial region in older lenses (Beebe *et al.*, 2004; Rajagopal *et al.*, 2007) show strong nuclear staining for phospho-Smad1/5, indicative of ongoing BMP signaling. Taken together, these findings are consistent with BMP affecting both early and late lens development via the canonical Smad 1/5 pathway, as is the case in many other organs. It was therefore surprising that Rajagopal *et al.* (2009) reported that conditional deletion of both *Smad1* and *Smad5* using *LoxP-Cre* does not block lens formation. The simplest interpretation of these results is that BMP-dependent Smads are not required for the earliest stages of lens development. This does not, however, appear to be the case for secondary fiber cell differentiation, given that images from *P3 LoxP-Cre Smad1/5* double-knockout mice published in Rajagopal *et al.* (2009) show a clearly abnormal lens, with retention of nuclei in fiber cells and what appears to be a monolayer of undifferentiated epithelial cells encircling the fiber cell mass. Both of these defects in secondary fiber differentiation are also present in

P14 mice that overexpress noggin in the lens (Boswell *et al.*, 2008b). The fact that epithelial cell proliferation is not obviously compromised in *Smad1/5* double-knockout (Rajagopal *et al.*, 2009) or noggin-overexpressing (Boswell *et al.*, 2008b) lenses is consistent with our finding in DCDMLs that FGF up-regulates cell proliferation in a BMP-independent manner (Figure 9B).

Model

We propose that in vivo, lens epithelial cells are continuously exposed to the low levels of BMP that they endogenously produce. One downstream effect of this autocrine/paracrine BMP signaling is to maintain lens cells in a state in which they can be optimally activated by high levels of FGF, most likely by promoting the function of FGFRs. Epithelial cells first encounter these levels of FGF at the lens equator, where they gain access to diffusible factors from the vitreous body (Schulz *et al.*, 1993; Le and Musil, 2001a). Vitreous-derived FGF then potentiates endogenous BMP signaling to the extent that expression of one or more critical BMP target genes is enhanced, leading to up-regulation of fiber differentiation and gap junctional coupling. This process is likely facilitated by enhanced expression of *BMP4* by FGF. Increased BMP-mediated transcription at the lens equator could explain why activated *Smad1* and its binding partner *Smad4*, localized in the cytosol in central epithelial cells, redistribute into the nucleus in elongating lens fiber cells in both mouse and chick lens (Rajagopal *et al.*, 2007). In this view, BMP signaling is as important as FGF signaling in normal secondary fiber cell differentiation and function. Our findings may also shed light on other important physiological processes in which BMP and FGFs act agonistically, such as nephrogenesis (Dudley *et al.*, 1999), specification of the ciliary body (Dias da Silva *et al.*, 2007), lens induction (Faber *et al.*, 2001), and primary lens fiber cell differentiation (Jarrin *et al.*, 2012).

MATERIALS AND METHODS

Materials

Recombinant bovine FGF2, human FGF9, human TGF β 1, mouse noggin/Fc chimera, human BMP4, human BMP6, and anti-BMP antibody (MAB3552) were from R&D Systems (Minneapolis, MN). The following antibodies were all purchased from Cell Signaling Technology (Danvers, MA): anti-phospho-p44/42 MAPK E10 mouse monoclonal (#9106), anti-total p44/42 MAPK (#9102), anti-phospho-p38 (#9211), anti-phospho-MEK1/2 (#9154), anti-phospho Raf-1 (#9427), anti-phospho-FRS2- α (Tyr-196) (#3864), and anti-phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465) (#9511). Other antibodies used were as follows: for CP49, rabbit anti-mouse CP49 polyclonal serum (#899 or #900; both generous gifts of Paul FitzGerald, University of California, Davis, CA); for phospho-tyrosine, 4G10 (a kind gift from Brian Druker, Oregon Health and Science University, Portland, OR); for luciferase, #G745A from Promega (Madison, WI); for GFP, JL-8 from Clontech (Mountain View, CA); for phospho-Smad3, ab51451 from Abcam (Cambridge, MA); for total Smad 1/5, ab75273 from Abcam; for total Raf-1, sc-7267 from Santa Cruz Biotechnology (Santa Cruz, CA); for total p38, sc-535 from Santa Cruz; and for total MEK, M17030 from Transduction Labs (Lexington, KY). UO126 (used at 15 μ M), PD173074 (100 nM), and dorsomorphin (5 μ M) were from Calbiochem (La Jolla, CA). All other reagents, including TPA, were from Sigma-Aldrich (St. Louis, MO).

Cell culture and treatments

Cultures were prepared from E10 chick lenses and plated at 1.2×10^5 cells/well onto laminin-coated 96-well tissue culture plates as previously described in Le and Musil (1998). Cells were cultured in

the absence of serum in M199 medium plus BOTS (2.5 mg/ml bovine serum albumin, 25 µg/ml ovotransferrin, 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 DCDMLs to distinguish them from related but functionally distinct systems such as central epithelial explants and immortalized lens-derived cell lines (Musil, 2012). Where indicated, DCDMLs were incubated with UO126, PD173074, or dorsomorphin for 45 min at 37°C before addition of growth factors. Anti-BMP antibodies were used at 40 µg/ml and noggin at 0.5 µg/ml.

Plasmids and transient transfection of lens cells

One day after plating, DCDML cultures were transfected in M199 medium without BOTS or antibiotics using Lipofectamine 2000 (Gibco-BRL, Grand Island, NY) following the manufacturer's suggested protocol. Each transfection experiment included both negative (no growth factor) and positive (BMP and/or FGF) controls, with the results presented as fold over the negative control in the same experiment for the same plasmid. Control experiments confirmed that the efficiency of transient transfection of DCDMLs is consistently ~70% (Boswell *et al.*, 2009). The DCR1- α A-promoter-EGFP reporter (Yang *et al.*, 2006) was a kind gift of Ales Cvekl, Albert Einstein College of Medicine (New York, NY). The BRE-Luc reporter construct (Korchynski and ten Dijke, 2002) was provided by Peter ten Dijke (Netherlands Cancer Institute, Amsterdam, Netherlands), and pID1SB-Luc (Nehlin *et al.*, 1997) was a generous gift from Judy Campisi, University of California (Berkeley, CA). The SBE4-Luc (Zawel *et al.*, 1998) and 5' Del 6 Id1-Luc (Tournay and Benezra, 1996) reporter constructs were provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD; Addgene [Cambridge, MA] plasmid 16495) and Robert Benezra (Memorial Sloan-Kettering Institute, New York NY; Addgene plasmid 16054), respectively. pEGFP-C2 was from Clontech.

Immunoblot analysis

For CP49, luciferase, and GFP, DCDML cultures were solubilized in lysis buffer as previously described (Le and Musil, 2001a). For all other proteins, cells were solubilized directly in SDS-PAGE sample buffer and boiled. Equal amounts of total protein were transferred to polyvinylidene fluoride membranes, and the blots were probed with primary antibodies. Immunoreactive proteins were detected using secondary antibodies conjugated to either IRDye800 (Rockland Immunochemicals, Limerick, PA) or Alexa Fluor 680 (Molecular Probes, Eugene, OR) and directly quantified using the LI-COR Biosciences Odyssey infrared imaging system (Lincoln, NE) and associated software.

[³⁵S]methionine metabolic labeling

DCDML cultures were labeled at 37°C with [³⁵S]methionine for 4 h in methionine- and serum- free DMEM (Gibco-BRL) and solubilized as previously described (Le and Musil, 1998; 2001a). [³⁵S]methionine incorporation into total cellular protein and into δ -crystallin was quantitated after SDS-PAGE using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and IPLab Gel software (Signal Analytics, Vienna, VA).

Immunofluorescence microscopy

DCDMLs grown on glass coverslips were fixed in 2% paraformaldehyde in phosphate-buffered saline and processed for immunocytochemical detection of luciferase (Le and Musil, 1998, 2001b). Immunofluorescence images were captured using a Leica (Wetzlar, Germany) DM LD photomicrography system and Scion Image 1.60 software (Scion, Frederick, MD).

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 previously described (Le and Musil, 2001b; Boswell *et al.*, 2008a). The distance that Lucifer yellow spreads into the monolayer is directly proportional to the number of coupled cells (Le and Musil, 2001b; Boswell *et al.*, 2008a). In Figure 9A, only the Lucifer yellow channel and only a portion of the right half of the scrape/load wound are shown.

MTT assay for cell proliferation

DCDMLs were plated at 0.7×10^5 cells/well onto laminin-coated, 96-well tissue culture plates. After 16 h, the subconfluent cultures were incubated with fresh M199/BOTS with or without additions for 48 h. Medium was replaced with M199/BOTS containing 0.8 mg/ml MTT and incubated for 2 h at 37°C. Conversion of MTT by living cells into a blue formazan dye was measured at 570 nM according to the manufacturer's protocol (kit TOX-1; Sigma-Aldrich) using an automated microtiter plate reader.

qRT-PCR (TaqMan)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and the manufacturer's protocol. RNA samples (1 µg) were treated with RNase-free DNase to avoid genomic DNA contamination and then reverse transcribed into cDNA using random primers and the SuperScript III first-strand synthesis system (Invitrogen). Real-time RT-PCR for the target (chicken *Id1*, *FGFR1*, *FGFR3*, *BMP7*, *BMP4*) and endogenous reference (*GAPDH*) genes was carried out in separate tubes in duplicate using the LightCycler 480 (Roche, Indianapolis, IN) sequence detection system and fluorescein amidite-labeled probes and primers. Negative control samples (no template or no enzyme in the reverse transcription reaction) were included. Thermal cycling conditions were as follows: 95°C for 10 min, 50 cycles of 95°C/10 s and 60°C/20 s, and 40°C for 30 s. PCRs contained twofold dilutions of cDNA template, TaqMan Universal PCR Master Mix, and TaqMan Assay Mix (for *Id1*, *FGFR1*, *FGFR3*, *BMP7*, *BMP4*, and *GAPDH*, assay IDs were, respectively, Gg03337774_g1, Gg03340351_m1, Gg03340329_m1, Gg03310499_m1, Gg03348675_m1, and Gg03346982_m1). All data were captured using LCS480 1.5.0.39 software and exported to Excel (Microsoft, Redmond, WA) worksheets for further analysis. Relative amounts of target cDNA (normalized to *GAPDH*) were calculated by the comparative CT (threshold cycle) method (User Bulletin #2; Applied Biosystems, Carlsbad, CA) and plotted as fold increase compared with untreated (or, in Table 1C, DRB only) samples in the same experiment. Standard curves confirmed that the efficiency of amplification of *GAPDH* and of target genes was approximately equal (≥ 0.95).

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