OSM supports this hypothesis (12, 13). Consequently, unamel mice, FGF18 is likely to directly regulate osteoblast and chondrocyte distribution of FGF18 and the phenotype of FGF18 null osteogenic fronts of membranous bones (8–11). Because of the developing endochondral bones as well as the periosteum and FGF18 is expressed in the perichondrial sheath and joints of is essential for bone and cartilage cell differentiation (8, 9). targeting studies in mice have clearly established that FGF18 identifying the key FGFs that control chondrocyte growth and are essential regulators of limb bud morphogenesis (4–7), but have been difficult. Many studies have shown that FGFs 8 and 10 polypeptide ligands (3). Given this large number of FGFs and these, FGFRs 1, 2, and 3, clearly contribute to the development of the skeleton (2). The FGFs are a family of more than 17 growth factor receptors (FGFRs) are important regulators of cartilage growth and development (1, 2). There are four members of the FGFR family of receptor tyrosine kinases. Three of these, FGFRs 1, 2, and 3, clearly contribute to the development of the skeleton (2). The FGFs are a family of more than 17 polypeptide ligands (3). Given this large number of FGFs and the partially overlapping expression patterns, the identification of the key regulators of chondrocyte differentiation has been difficult. Many studies have shown that FGFs 8 and 10 are essential regulators of limb bud morphogenesis (4–7), but identifying the key FGFs that control chondrocyte growth and differentiation has remained elusive. Recently, however, gene-targeting studies in mice have clearly established that FGF18 is essential for bone and cartilage cell differentiation (8, 9). FGF18 is expressed in the perichondrial sheath and joints of developing endochondral bones as well as the peristioem and osteogenic fronts of membranous bones (8–11). Because of the tissue distribution of FGF18 and the phenotype of FGF18 null mice, FGF18 is likely to directly regulate osteoblast and chondrocyte differentiation. Studies of cultured chondrocytes and osteoblasts support this hypothesis (12, 13). Consequently, understanding the pathways that regulate FGF18 gene expression should yield direct insight into principal controllers of chondrocyte and osteoblast differentiation.

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily that were first described based on their capacity to induce heterotopic bone (14). Subsequent studies have amply demonstrated that when applied to suitable target cells, BMPs rapidly stimulate osteoblast or chondrocyte differentiation (for review see Refs. 15–17). The biological action of BMPs is inhibited by a group of extracellular binding proteins including noggin (18), differential screening-selected gene abrative in neuroblasta, (18), cerberus (19), gremlin (20, 21), chordin (22), and sclerostin (23). Noggin binds the BMP family members BMP 2, 4, 6, and 7 in the extracellular milieu, and the noggin-BMP complex is unable to bind to or activate BMP receptors (24, 25). Noggin expression is rapidly induced following the activation of BMP receptors and therefore acts as an effective feedback antagonist (26, 27). Noggin is expressed in all developing cartilage and clearly has a vital role as an antagonist of BMP signaling in the regulation of skeletal development. This is evidenced by noggin null mice, which have expanded cartilage elements and joint fusions (28). Furthermore, mutations of the human noggin gene cause syndromes with varied joint fusions (29). Therefore, antagonism of BMP signaling by noggin is essential for joint development and determining the size of bone rudiments. Although noggin is induced by BMP signaling, the molecular pathways regulating noggin gene expression are largely uncharacterized.

Cytokines or growth factors like FGFs or BMPs and their inhibitors clearly have dramatic effects on chondrocyte gene expression and the development of cartilage. However, relatively little is understood of how these genes are induced or inhibited. Consequently, characterizing the pathways that stimulate growth factor expression will yield insight into the control of chondrogenesis. Many cytokines are induced following receptor-dependent calcium signaling. For example, we have shown that elevations of intracellular calcium induce BMP2 gene expression (30). This in turn promotes chondrocyte gene expression and chondrogenesis. To further understand the effects of calcium signaling on growth factor gene expression and chondrogenesis, we investigated the regulation of FGF18 expression. We show that calcium-calcineurin signaling induces FGF18 expression, as does the calcineurin substrate nuclear factor of activated T-cell 4 (NFAT4). Subsequently, FGF18 represses noggin gene expression and thereby facilitates BMP signaling and chondrogenesis. Significantly, these data demonstrate the diverse effects of FGFs on chondrocytes and their precursors. FGF signaling represses chondrocyte hypertrophy during endochondral ossification (31–33). However, at an earlier stage of cartilage development, FGFs may stimulate chondrogenesis by suppressing noggin at sites where FGF and BMP signaling overlap.
**EXPERIMENTAL PROCEDURES**

Cells—RCJ3.1C5.18 (RCJ) cells were obtained from Jane Aubin (Toronto, Canada) and maintained subconfluent in Dulbecco’s modified Eagle medium (Invitrogen) with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Limb bud mesenchymal cells were grown in 1:1 Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing the same supplements. Limb bud mesenchyme was derived from embryonic day 11.5 mouse embryos as previously described (34). Single cell suspensions were obtained from isolated mouse limb buds (C57Bl/6J) following digestion with 0.1% trypsin-EDTA (Invitrogen) and 2 ng/ml collagenase IA (Sigma) for 30 min at 37 °C with agitation. Proteolysis was inhibited with an equal volume of bovine serum, and the suspension was passed through a 70-μm cell strainer. Cell were washed twice with growth medium, suspended at 1–5 × 10^6 cells/ml, and plated at 20-μl aliquots. 4 h after plating, the wells were flooded with growth medium. Medium was changed every 3 days. In certain experiments, noggin was included at a concentration of 2 μg/ml. Alcian blue staining of formalin-fixed chondrocyte aggregates was done in a 3% acetic acid solution containing 5% Alcian blue (Sigma), pH 3.0. Unbound dye was removed by extensive washing with distilled water.

**Growth Factors and Antibodies and growth factors were obtained as follows:** FGF18 (PeproTech); BMP2, noggin, TGF-β1 (R&D Systems); anti-phosphoSmad1 (Upstate Laboratories); Cys3-goat anti-rabbit (Jackson Immunoresearch); and anti-phosphoysosome 4G10 (Upstate Laboratories). Anti-FGF1 antibody was prepared as described previously (35), and anti-FGF8 AB6 was a generous gift from A. Baird (Prism Pharmaceuticals).

**Indirect Immunofluorescence—**RCJ cells were cultured on glass coverslips in a 12-well cluster plate. The cells were stimulated with FGF18 (250 ng/ml) or BMP2 (50 ng/ml) for 30 min or 1 h in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The samples then were washed with phosphate-buffered saline, fixed 10 min in 4% paraformaldehyde, and again washed with phosphate-buffered saline. Phospho-Smad1 was detected using phosphate-buffered saline containing 0.1% Triton X-100, 5% goat serum, 10% fraction V bovine serum albumin, and a 1:500 dilution of anti-phosphoSmad1. Cys3 coupled goat anti-rabbit was used in the same blocking solution at a 1:800 dilution. Detection and deconvolution were performed using a Zeiss AxioSkope 2 and Axiovision software (Zeiss).

**Real Time Quantitative PCR—**Total RNA was extracted from cells using RNA-Bee (Tel-Test, Inc.) according to the manufacturer’s protocol with reverse transcription reagents with random hexamers according to the manufacturer’s protocol (Ambion). One microgram of DNA-free total RNA was reverse-transcribed in 25 μl at 42 °C for 2 h using TaqMan reverse transcription reagents with random hexamers according to the manufacturer’s instructions (Applied Biosystems). Quantitative PCR reactions were made of SYBR Green 2 PCR Master Mix (Applied Biosystems), 5 pmol of each forward and reverse primer, and 3 μl of cDNA. Cycling conditions were 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Primers were designed using Primer3 software (Whitehead Institute for Biomedical Research) with melting temperatures ranging from 59 to 61 °C and amplicons ranging from 130 to 162 base pairs. Primer pairs were tested previously (31). The noggin template for riboprobes synthesis was a gift from R. Harland (University of California). Probes synthesis and hybridization were done as described previously (35). The noggin template for riboprobes synthesis was a gift from R. Harland (University of California). The noggin template for riboprobes synthesis was a gift from R. Harland (University of California). The noggin template for riboprobes synthesis was a gift from R. Harland (University of California). The noggin template for riboprobes synthesis was a gift from R. Harland (University of California). The noggin template for riboprobes synthesis was a gift from R. Harland (University of California). The noggin template for riboprobes synthesis was a gift from R. Harland (University of California). The noggin template for riboprobes synthesis was a gift from R. Harland (University of California).

**Statistical Analyses—**Statistical comparisons were done using an unpaired Student’s t test. The null hypothesis was rejected for p < 0.05.

**RESULTS**

**Stimulation of Chondrogenesis by Elevation of Intracellular Calcium—** Cultures of mesenchymal cells derived from the limb buds of 11.5 day post-coitus mouse embryos undergo chondrogenesis when plated at a high cell density in vitro (38). To understand calcium-dependent signaling in this process, we treated limb bud cultures with the calcium ionophore, ionomycin. We found that ionomycin stimulated chondrogenesis in these cultures as evidenced by a dramatic increase in the number of Alcian blue-staining nodules (Fig. 1A). Additionally, the differentiation of immortalized chondrocytic cells (RCJ3.1C5.18) was stimulated by 1 μM ionomycin as shown by increased expression of the chondrocyte-specific gene, aggrecan (Fig. 1B). These data demonstrate that calcium-dependent
Fig. 2. Induction of FGF18 gene expression. FGF18 expression relative to ubiquitin was determined by quantitative PCR following reverse transcription of RNA isolated from RCJ cells. A, time course of FGF18 induction following treatment with 1 μM ionomycin (iono). B, time course of FGF18 induction following treatment with 1 μM ionomycin with or without 5 μg/ml cyclosporin A (CspA). C, FGF18 expression in RCJ cells following transduction of β-galactosidase (Adβ-gal) or activated calcineurin (AdΔCnA). D, FGF18 expression in RCJ cells following transduction of β-galactosidase or activated NFAT4 (AdΔNFAT4). Cells were infected with recombinant adenoviruses at a multiplicity of infection of 10. FGF18 expression is normalized to ubiquitin. Error bars represent mean ± S.D. E, FGF receptor phosphotyrosine Western blot. RCJ cells were stimulated with 1 μM ionomycin for 5 h in the presence or absence of 5 μg/ml cyclosporin A. Cell extracts were immunoprecipitated with a polyclonal anti-FGFR1 antibody, and Western blots were probed with anti-phosphotyrosine (p-Tyr) (top) or monoclonal pan anti-FGFR antibodies (bottom).
signals stimulate chondrocyte differentiation, and in other work (30), we show that the calcium-dependent pathway requires calcineurin. We showed that, when activated, calcineurin induces BMP2 gene expression and that differentiation in these culture systems proceeds via a BMP-dependent pathway (30).

Calcium-dependent Signaling Induces FGF18 Expression—
To further characterize the effects of calcium-dependent signals on chondrocyte differentiation and gene expression, we asked whether calcium signaling alters FGF18 gene expression. FGF18 is an essential regulator of chondrocyte differentiation during endochondral bone growth and thereby a potential target for the effects of calcium signaling on chondrocyte differentiation. Interestingly, we found that FGF18 expression is induced up to 12-fold by the calcium ionophore, ionomycin (Fig. 2A).

To investigate the signaling pathways required for the induction of FGF18 gene expression, we asked whether calcineurin is required. Calcineurin is a calcium-dependent serine-threonine phosphatase that is specifically inhibited by cyclosporin A. Interestingly, cyclosporin A completely inhibits the induction of FGF18 by ionomycin (Fig. 2B). Additionally, cyclosporin A represses the induction of FGF18 by ionomycin (Fig. 2B). In addition, the expression of an activated form of calcineurin A lacking the N-terminal regulatory domain (∆CnA) was sufficient to induce FGF18 expression in chondrocytic RCJ cells. Recombinant adenovirus Ad∆CnA induced FGF18 5-fold ($p = 0.01$) relative to a control virus transducing β-galactosidase (Fig. 2C). In addition, the transcription factor and calcineurin substrate, NFAT4, induced FGF18 expression. Adenoviral transduction of an activated form of NFAT4 (Fig. 2D) in RCJ cells induced FGF18 6-fold ($p = 0.05$) relative to a control virus. We conclude that the induction of FGF18 gene expression by elevated levels of intracellular calcium requires the calcium-dependent phosphatase, calcineurin.

To determine whether the activation of FGF18 expression by ionomycin stimulates FGF signaling, we assessed FGF receptor activation by phosphotyrosine Western blotting. Consistent with the activation of FGF18 expression, Fig. 2E shows that ionomycin stimulates tyrosine phosphorylation of the FGF receptor. Additionally, cyclosporin A represses the induction of FGF receptor autophosphorylation.

FGF Signaling Induces Chondrogenesis and Inhibits Noggin Expression—Because calcium-dependent signals induce both chondrocyte differentiation and FGF18 expression, we asked whether the induction of FGF18 and subsequent FGF receptor signaling contribute to calcium-induced chondrogenesis. FGF18 is a potent ligand for FGF receptor 3 (12). Therefore, we used a constitutively active FGFR3 as a substitute for FGF18 to assess for effects on chondrogenesis. Using a recombinant adenovirus, FGFR3 containing an activating mutation (FGFR3-R248C) was transduced into limb bud mesenchymal cultures. The results showing Alcian blue staining of differentiated cultures (Fig. 3, A and B) indicate that FGF receptor signaling strongly augments differentiation as evidenced by a substantial increase in the size of the cartilage nodules and a 50% increase ($p = 0.002$) in the number of cartilage nodules in FGFR3-R248C-transduced cultures relative to a control culture transduced with β-galactosidase. We conclude that FGF receptor signaling can enhance chondrogenesis and posit that calcium-calcineurin-dependent induction of FGF18 expression contributes to chondrocyte differentiation.

Similar to chondrogenesis in vivo, the limb bud differentiation assay is highly dependent on BMP signaling. Blocking BMP signaling with dominant negative receptors or BMP antagonists abolishes cartilage development (39, 40). Conversely, constitutive BMP receptors or excess BMP augment differentiation (41, 42). The finding that FGF signaling augments chondrogenesis (Fig. 3A) suggests that BMP signaling is enhanced by FGF. This could be achieved by several mechanisms including the induction of BMP gene expression or suppression...
Fig. 4. Regulation of noggin expression by FGF18. A, effect of FGF18 (250 ng/ml) on BMP2 expression. BMP2 expression was determined by quantitative PCR following reverse transcription of RNA isolated from RCJ cells at the indicated times. B, noggin expression following treatment with FGF18 (250 ng/ml), BMP2 (50 ng/ml), or both for 5 h. Expression was determined by quantitative PCR following reverse transcription of RNA isolated from RCJ cells. C, noggin expression following transduction of β-galactosidase (Adβ-gal) or activated FGF receptor 3 (AdFGFR3). Cells were transduced with recombinant adenovirus at a multiplicity of infection of 10. D, noggin expression in limb bud mesenchyme treated with FGF18 (250 ng/ml). Expression was determined by quantitative PCR following reverse transcription of total RNA. Expression levels were normalized to ubiquitin. Error bars represent mean ± S.D. E, noggin represses the induction of chondrogenesis by ionomycin (Iono). Limb bud cultures were stimulated or not with 1 μM ionomycin in the presence or absence of noggin (2 μg/ml). Cartilage nodules were stained with Alcian blue.
of BMP antagonists. FGF18 did not induce the expression of BMP2 (Fig. 4A), BMP4, or growth differentiation factor 5 (data not shown). However, FGF18 did repress the expression of the BMP antagonist, noggin. Noggin expression was induced in chondrocytic RCJ cells by stimulation with BMP2 in the presence or absence of FGF18. Noggin was induced 10-fold following the addition of BMP2 (Fig. 4B). However, noggin induction was substantially repressed by the addition of FGF18 (10-fold increase in the absence, 2-fold increase in the presence of FGF18). Similarly, chondrocytic RCJ cells transduced with an activated FGF receptor (FGFR3-R248C) showed a 92% (p = 0.02) reduction of noggin expression following stimulation with BMP2 compared with a control β-galactosidase adenovirus (Fig. 4C). Also, treatment of mouse limb bud mesenchymal cell cultures with FGF18 repressed noggin gene expression relative to vehicle-treated control samples (Fig. 4D). Furthermore, adding exogenous noggin to limb bud cultures wherein FGF18 is induced by ionomycin results in a repression of chondrogenesis (Fig. 4E). Lastly, we find that noggin expression is reduced in transgenic mice expressing an activated FGFR3 (FGFR3-G380R) in cartilage. Fig. 5 shows bright- and dark-field images of the epiphyseal growth plate from wild-type mice or transgenic mice expressing FGFR3-G380R driven by the type II collagen promoter (31). The dark-field in situ shows that noggin transcripts are substantially reduced in the cartilage of the

![Fig. 5. Comparison of noggin transcripts in the epiphyseal growth plate of wild-type (WT) and transgenic (G380R) mice. Top panels show bright-field images. Lower panels show the corresponding dark-field images of noggin transcripts detected with a radiolabeled riboprobe. The brackets highlight the cartilaginous portion of the growth plate (GP).](image)

![Fig. 6. Regulation of aggrecan expression by FGF18 and BMP signaling. A, effect of FGF18 (250 ng/ml), BMP2 (50 ng/ml), or both on aggrecan expression. Aggrecan expression was determined 5 h post-treatment by quantitative PCR following reverse transcription of RNA isolated from RCJ cells. B, effects of FGF18 (250 ng/ml) on the expression of aggrecan in cultured limb bud mesenchyme. Limb bud mesenchyme was cultured for 5 days in the presence or absence of FGF18, and aggrecan expression was determined by quantitative PCR following reverse transcription of total RNA. C, effect of adenoviruses β-galactosidase, ALK6KR, Smad6, or Smad7 on aggrecan expression in the presence or absence of FGF18 (250 ng/ml). RCJ cells infected by the indicated recombinant adenovirus were treated or not with FGF18 (250 ng/ml) for 6 h. Aggrecan expression was determined by quantitative PCR using reverse-transcribed total RNA. D, noggin represses the induction of aggrecan by FGF18. RCJ cells were stimulated or not with FGF18 (250 ng/ml) in the presence or absence of noggin (2 μg/ml). Aggrecan expression was determined by quantitative PCR using reverse-transcribed total RNA. Expression levels were normalized to ubiquitin. Error bars represent mean ± S.D.](image)
transgenic mice. These data support the conclusion that FGF18 can augment BMP signaling by suppressing the expression of the BMP antagonist, noggin. Additional evidence in support of this stems from results showing that (i) FGF18 augments the expression of aggrecan, a BMP-inducible gene, both in chondrocytic RCJ cells (Fig. 6A) and cultures of limb bud mesenchyme (Fig. 6B), (ii) the induction of aggrecan by FGF18 is repressed following adenoviral transduction of BMP signaling inhibitors including dominant negative BMP receptor Ib (ALK6KR) or inhibitory Smads 6 and 7 (Fig. 6C), and (iii) the induction of aggrecan by FGF18 is repressed following the addition of the BMP antagonist, noggin (Fig. 6D).

**FGF18 Does Not Repress SMAD1 Nuclear Translocation**—We showed that FGF18 suppressed the induction of noggin by BMP2. We hypothesize that FGF18 augments BMP signaling through this pathway and thereby augments differentiation. However, it is possible that FGF signaling suppresses noggin induction by directly inhibiting BMP signaling. In this case, we would expect FGF signaling to repress rather than augment differentiation. To investigate whether FGF signaling globally suppresses BMP signaling, the effect of FGF18 on Smad1 was assessed. BMP receptors directly phosphorylate the receptor-
dependent Smads 1 and 6 thereby activating nuclear translocation of Smads and subsequent gene expression (43). To determine whether FGF suppresses signaling through Smad1, we assessed the effect of FGF18 on the phosphorylation and nuclear localization of Smad1. RCJ cells were stimulated by BMP2 in the presence or absence of FGF18 and simultaneously assessed for phosphorylation and nuclear localization by immunofluorescence with an anti-phosphoSmad1 antibody. Fig. 7A shows that following a 30-min treatment with BMP2, nuclear localization of phospho-Smad1 was observed in nearly all of the cells. Neither phosphorylation nor nuclear translocation was observed in unstimulated or FGF18-treated cells. Co-treatment of cells with FGF18 and BMP2 does not inhibit phosphorylation and nuclear localization of Smad1. Similar results were obtained at 30 (Fig. 7, A and B) and 60 min (data not shown). These data support the conclusion that signaling pathways activated by FGF18 do not directly inhibit Smad1 phosphorylation or nuclear translocation. As additional evidence that FGF signaling does not globally repress BMP signaling or BMP-dependent gene expression, we examined the effect of FGF signaling on transcriptional targets of BMP signaling. As shown in Fig. 6, A and B, FGF18 stimulated rather than repressed the expression of aggrecan in BMP2-treated limb bud or RCJ cells. The expression of ID1, a direct transcriptional target of Smads (44), was partially inhibited in chondrocyte cells co-treated with FGF18 and BMP2 (Fig. 8A). However, there was no significant difference in the expression of ID1 following BMP2 treatment of RCJs infected with β-galactosidase or constitutive active FGF receptor 3 (FGFR3-R248C) recombinant adenoviruses (Fig. 8B). Therefore, these data support the conclusion that FGF signaling does not globally suppress BMP signaling.

**TGFB1, a Ligand That Can Induce FGF18 and BMP2—**

These data showed that elevations of intracellular calcium activate FGF18. In earlier work (30), we show that calcium-calmodulin, this observation suggests that TGF-β1 stimulates chondrogenesis and chondrocyte gene expression and is therefore an ideal candidate for a ligand that induces FGF18 and BMP2 gene expression. Interestingly, the treatment of RCJ cells with TGF-β1 significantly induced the expression of both FGF18 and BMP2 (Fig. 9A). Furthermore, the calcineurin antagonist, cyclosporin A, suppressed the induction of FGF18 by TGF-β1 (Fig. 9B). To test whether TGF-β1 stimulates FGF18 expression through the activation of calcium signaling cascades, we pre-loaded cells with the calcium chelator, BAPTA-AM. Interestingly and consistent with intracellular calcium contributing to the activation of FGF18 expression by TGF-β1, we found that pre-loading of cells with BAPTA-AM significantly inhibited the induction of FGF18 by TGF-β1 (Fig. 9C). These data suggest that TGF-β1 can activate a calcium/calmodulin-dependent pathway that induces FGF18 expression. The stimulation of RCJ cells with TGF-β1 produces a 2.4-fold increase in noggin gene expression (Fig. 9D). This compares with a 10-fold increase in noggin gene expression when cells are stimulated with BMP2 (Fig. 4B). This finding suggests that, although TGF-β1 induces noggin expression, the magnitude of noggin expression is diminished because FGF18 is simultaneously induced.

**DISCUSSION**

In previously published work (30), we show that BMP2 gene expression and chondrogenesis are induced following activation of calcineurin and the calcineurin substrate, NFAT4. Here we report that calcineurin also induces the expression of FGF18. In turn, activation of FGF receptor signaling suppressed noggin gene expression and thereby facilitated BMP signaling. These results are collectively schematized in Fig. 10. In this scheme, ligand-receptor complexes stimulate the elevations of intracellular calcium. In turn, calcineurin is activated and stimulates FGF18 and BMP2 expression. FGF18 and BMP2 then function cooperatively to stimulate chondrogenesis. Because the induction of FGF18 and BMP2 requires calcineurin, these results underscore the contribution of calcineurin to chondrogenesis and chondrocyte gene expression. The results imply that calcineurin substrates will also be vital regulators of chondrogenesis. Indeed, the calcineurin substrate and transcription factor, NFAT4, induces chondrogenesis and both FGF18 and BMP2. Other substrates of calcineurin may also contribute to the regulation of chondrogenesis.

Because FGF18 and BMP2 function together to induce chondrogenesis, ligands that induce the expression of FGF18 and BMP2 should be efficacious activators of chondrogenesis. TGF-β1 induced both FGF18 and BMP2, and as predicted, TGF-β1 is also a potent inducer of chondrogenesis both in limb bud chondrogenesis assays and when implanted in developing chicken limbs (45, 46). Cyclosporin A repressed the induction of FGF18 by TGF-β1, indicating a requirement for calcineurin. Because the activation of calcineurin requires an interaction with Ca²⁺-calmodulin, this observation suggests that TGF-β1...
stimulates calcium mobilization in RCJ cells. Interestingly, we find that chelation of intracellular calcium with BAPTA-AM strongly inhibited the induction of FGF18 by TGF-β1. These data support a model whereby TGF-β1 stimulates the elevations of intracellular calcium. Consistent with this finding, recent data show that increases in intracellular calcium are observed in cell populations treated with TGF-β1. Moreover, these calcium shifts were sufficient to activate calcineurin (47). Other published data show (48, 49) that TGF-β1 can either stimulate or inhibit calcium mobilization and calcium signaling. Hence the effects of TGF-β1 on calcium mobilization appear to be cell type-specific.

We have identified a calcium-dependent pathway that activates FGF18 expression and chondrocytic differentiation. Activation of FGF receptor signaling by FGF18 suppressed the expression of the BMP antagonist, noggin. Through this pathway, FGF18 facilitates differentiation that is BMP-dependent. Interestingly, augmented chondrogenesis is observed in mice with constitutive FGF signaling. Individuals with Apert or Pfeifer syndrome have activating mutations in the FGF receptors 1 or 2. These syndromes are characterized by digit abnormalities, including enlarged or fused digits, and premature fusion of cranial sutures. The enlarged or fused digits may result from expanded domains of chondrogenesis consequent to expanded domains wherein noggin expression is suppressed by FGF signaling. In a similar mechanism, FGF signaling was shown to suppress noggin expression in cranial sutures (50), thus explaining the premature fusion of cranial suture in individuals with activating mutations of FGF receptors 1 and 2. Experiments of chicken limb development have also shown that the application of FGF bound to beads can suppress noggin expression (51). These results highlight the interplay of BMP and FGF signaling that regulates both the osteoblast and chondrocyte lineage to shape both endochondral and membranous bones.

Mice expressing constitutively active FGF receptors also display digit abnormalities as well as fused joints. Transgenic mice expressing the activated forms of FGF receptors in cartilage have fused digits, and certain more proximal joints are absent (52). These data indicate the necessity of regulated FGF signaling for defining the boundaries and joint spaces of endochondral bones. Interestingly, the abnormal digits observed in these mice involve the penultimate phalange. The length and segmentation of this phalange is...
strongly controlled by interdependent BMP and FGF receptor signaling pathways (53). The results presented here demonstrate interdependence of BMP and FGF signaling are consistent with the model proposed by Sanz-Ezquerro and Tickle (53) wherein temporal/spatial control of apical ectodermal ridge-derived FGF interacts with BMP signaling to control the length and segmentation of the digits. Our data emphasize the significant interdependence of BMP/FGF signaling and extend this model by suggesting that local regulation of noggin expression by FGF signaling in digits as well as more proximal structures determines diarthrodial joints. We hypothesize that FGF signaling in chondrocytes or early chondroblasts locally suppresses noggin expression and that this contributes to cartilage and/or joint formation. Unregulated FGF signaling as in mice or humans with constitutively active FGF receptors may cause excessive inhibition of noggin expression and consequently develop abnormal digits or extremities. Noggin is widely expressed in condensing chondroblast mesenchyme and at the site of presumptive or developing joints (51). Therefore, local antagonism of BMP signaling by noggin may regulate the formation of joints and the shape of developing bone elements. Consistent with this observation, noggin−/− mice (28) show widespread failure of joint formation and enlarged cartilage elements. Therefore, the control of noggin expression by FGF signaling is anticipated to gauge the size and shape of bone rudiments.

Noggin expression is strongly induced by BMP signaling and is suppressed by FGF signaling. How does FGF signaling suppress the induction of noggin? One possible explanation is that FGF signaling globally suppresses BMP signaling. Evidence supports this possibility. Receptor tyrosine kinase activity can inhibit Smad activity through mitogen-activated protein kinase-dependent phosphorylation (54, 55) of the Smad linker domain. How mitogen-activated protein kinase phosphorylation suppresses Smad activity is not entirely clear. Data show that phosphorylation of the linker domain suppresses the nuclear import of Smads (54). However, we and others (56) did not observe inhibition of nuclear translocation of Smads in the presence of receptor tyrosine kinase signaling. Also, Nakayama et al. (57) show that receptor tyrosine kinase signaling can suppress the transactivating properties of a Gal4-Smad1 chimeric protein that is constitutively nuclear. Furthermore, we found that the BMP-dependent gene expression was not generally suppressed as might be expected if nuclear translocation of Smads was absent. For example, a gene directly induced by BMPs (45, 55), ID1, was not suppressed by a constitutive active FGF receptor. Aggrecan, another gene induced by BMP signaling (58–62), was also not inhibited by FGF signaling. Therefore, whereas it is possible that mitogen-activated protein kinase-dependent phosphorylation of Smads contributes to the reduction of the noggin gene expression, it appears that other inhibitory pathways are initiated by FGF receptors. Identification of these repressor pathways will require further characterization of the signaling and transcriptional elements that converge on the noggin gene promoter.

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Martina I. Reinhold, Makoto Abe, Ravi M. Kapadia, Zhixiang Liao and Michael C. Naski

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