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HSPG Modulation of BMP Signaling in Fibrodysplasia Ossificans Progressiva Cells

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Abstract Cell surface heparan sulfate proteoglycans (HSPGs) play important roles in morphogen gradient formation and cell signaling. Bone morphogenetic protein (BMP) signaling is dysregulated in fibrodysplasia ossificans progressiva (FOP), a disabling disorder of progressive heterotopic bone formation. Here, we investigated the role of HSPG glycosaminoglycan (GAG) side chains on BMP signaling and found increased total and HSPG-specific GAG chain levels and dysregulation in HSPG modulation of BMP signaling in FOP lymphoblastoid cells (LCLs). Specifically, HSPG profiling demonstrated abundant mRNA and protein levels of glypican 1 and syndecan 4 on control and FOP LCLs, with elevated core protein levels on FOP cells. Targeted downregulation of glypican 1 core protein synthesis by siRNA enhanced BMP signaling in control and FOP cells, while reduction of syndecan 4-core protein synthesis decreased BMP signaling in control, but not FOP cells. These results suggest that FOP cells are resistant to the stimulatory effects of cell surface HSPG GAG chains, but are susceptible to the inhibitory effects, as shown by downregulation of glypican 1. These data support that HSPG modulation of BMP signaling is altered in cells from patients with FOP and that altered HSPG-related BMP signaling may play a role in the pathogenesis of the disease. J. Cell. Biochem. 102: 1493–1503, 2007.

Key words: bone morphogenetic protein (BMP); heparan sulfate proteoglycans (HSPGs); heterotopic ossification; fibrodysplasia ossificans progressiva (FOP); multiple hereditary exostoses (MHE)

Fibrodysplasia ossificans progressiva (FOP; OMIM:135100) is a rare autosomal dominant genetic disorder, characterized by congenital malformations of the great toes and progressive heterotopic ossification of muscle and connective tissues [Kaplan et al., 2002, 2005]. Bone morphogenetic protein (BMP) 4 mRNA and protein are elevated in lesional tissue and cells from FOP patients [Shafritz et al., 1996; Gannon et al., 1998; Gannon et al., 1997; Shore et al., 2006]. Furthermore, BMP signaling is dysregulated and overactive in FOP lymphoblastoid cells (LCLs) resulting in increased ID mRNA expression [de la Pena et al., 2005; Fiori et al., 2006]. FOP is caused by a recurrent mutation in the *ACVR1* gene which encodes a BMP type I receptor [Shore et al., 2006]. Protein modeling studies predict constitutive activation of ACVR1 as the underlying cause of the ectopic chondrogenesis, osteogenesis, and joint fusion seen in FOP.

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In addition to toe malformations and heterotopic bone formation, FOP patients frequently form multiple osteochondromas (bony outgrowths with cartilaginous caps) [Kaplan et al., 2005]. In patients with the disorder multiple hereditary exostoses (MHE; OMIM:133700; 133701), multiple osteochondromas form near the growth plates of long bones and are caused by inactivating mutations in the *EXT1* or *EXT2* genes. Exostosin (EXT) proteins participate directly in heparan sulfate proteoglycan (HSPG) biosynthesis by catalyzing N-acetyl-glucosamine (GlcNAc) and glucoronic acid (GlcA) transfer to nascent glycosaminoglycan (GAG) chains in both syndecans and glypicans [Duncan et al., 2001]. Loss of heterozygosity at the *EXT* loci results in failure of GAG chain elongation and transformation of benign tumors into chondrosarcomas, suggesting that GAG chains act as tumor suppressors [Hecht et al., 1995]. Mice carrying mutations in *EXT1* exhibit elevated Indian hedgehog (Ihh) signaling during embryonic chondrogenesis due to loss of heparin sulfate binding. Mutations in *EXT* genes result in MHE, likely through effects on Ihh signaling in the perichondrium [Koziel et al., 2004]. However, the molecular pathophysiology of osteochondroma formation in FOP remains enigmatic, but plausibly related to HSPG function.

Two families of cell surface HSPGs, syndecans and glypicans, have been well characterized. Syndecan core proteins have transmembrane and cytoplasmic domains rendering them capable of signal transduction, while glypican core proteins are attached to cell membranes via a glycophosphatidylinositol (GPI) link [Bernfield et al., 1999]. Since glypicans have no direct contact with the intracellular environment, any glypican mediated signaling must be transduced by associated transmembrane molecules [De Cat and David, 2001]. The presence of two distinct types of cell surface HSPGs implies different roles in signaling, but distinct family-specific functions remain unconfirmed.

HSPGs are ubiquitously expressed, and consist of a core protein with GAG side chains that can interact with proteins including morphogens and their antagonists, cell surface adhesion molecules, protease inhibitors, growth factors, degradative enzymes, and extracellular matrix proteins [Zimmermann and David, 1999; Cadigan, 2002; Guimond and Turnbull, 2004; Kreuger et al., 2004]. HSPGs regulate cytokine and receptor signaling, including BMPs and the BMP antagonist Noggin [Paine-Saunders et al., 2002].

HSPGs have been shown to have multiple roles in B-cell growth, development, and maturation, but few studies have examined the effects of HSPG-mediated BMP signaling in lymphocytes. Activation of B-cell antigen receptor (BCR) and CD40 results in a strong transient expression of HSPGs on human tonsillar B-cells. Furthermore, HSPGs act as functional co-receptors, promoting cytokine signaling in B-cells, and suggesting a dynamic role for HSPGs in B-cell differentiation [van der Voort et al., 2000]. HSPGs have also been shown to influence B-cell chemokine (C-X-C motif) ligand 1 (CXCL1), paired box transcription factor (PAX), and extracellular regulated kinase (ERK) signaling [Wang et al., 2003].

Based on the known functions of HSPGs in BMP and hedgehog signaling [Baeg et al., 2001; Paine-Saunders et al., 2002; Irie et al., 2003; Takada et al., 2003; Yoon and Lyons, 2004; Kaplan et al., 2005] and osteochondroma formation [Duncan et al., 2001; Stickens et al., 2005], we investigated the effects of HSPG GAG chains and core proteins on BMP signaling in lymphocyte cell lines and further examined if HSPG modulation of BMP signaling was altered in cells from patients with FOP.

MATERIALS AND METHODS

Lymphoblastoid Cell Lines (LCLs)

FOP is diagnosed by two classic features: congenital malformations of the great toes and progressive heterotopic ossification in characteristic anatomic patterns. We recently identified a recurrent ACVR1 mutation (c.617G > A; R206H) in all classically affected patients with sporadic and familial FOP [Shore et al., 2006]. All of the cell lines used for this study were obtained from patients with both classic diagnostic features of FOP and have the ACVR1 missense mutation in codon 206. Peripheral blood samples were obtained following informed consent from FOP patients and unaffected individuals in accordance with institutional guidelines and following Institutional Review Board approval. LCLs were established by Epstein Barr Virus (EBV) transformation of peripheral blood mononuclear cells as described previously [Shafritz et al., 1996].

Tissue Culture and Labeling

Cells were maintained in RPMI 1640 (Invitrogen) containing 15% fetal bovine serum (FBS; HyClone) and 1% antibiotics/antimycotics (Invitrogen) at 37°C in a humidified environment of 5% CO_2. For all treatments, cells were washed with Hanks balanced salt solution (HBSS; Invitrogen) and plated at approximately 2×10^6 cells in six-well plates. Unless otherwise indicated, cells were treated with 200 ng/ml BMP4 (R&D) in RPMI containing 1.5% FBS for 1.5 h. For radioactive sulfation labeling, control and FOP cells were treated with $Na_2{}^{35}SO_4$ (PerkinElmer), the majority of which was incorporated into proteoglycan GAG chains on the cell surface. Cells were washed in phosphate buffered saline (PBS; Invitrogen), placed in DMEM (Invitrogen) containing 0.5% FBS and 100 μCi/ml of $Na_2{}^{35}SO_4$. Cells were then centrifuged (1,600 rpm, 5 min), washed and re-pelleted, and placed in 300 μl RIPA buffer (PBS, 1% NP-40, 0.5% Na-deoxycholate) containing Protease Inhibitor cocktail (Sigma), 10 μg/ml phenylmethylsulfonylfluoride (PMSF), 1 mM Na-Orthovanadate, and 0.1 mM NaF. RIPA samples were kept on ice and vortexed every 5 min for 30 min. Aliquots from each sample were then ethanol precipitated in 1 ml of 100% EtOH on ice for 30 min, collected on a microfiber filter, and counted in a liquid scintillation counter. The counts were normalized to protein levels, determined with a BCA protein assay (Pierce) using bovine serum albumin (BSA) as a standard.

Dimethylmethylene Blue (DMB)/GAG Assay

DMB binding assays were performed to quantify the levels of total GAG chain on the cell surface [de Jong et al., 1989]. Approximately, 1×10^6 cells were untreated or treated with 5 mU/ml heparinase III (Sigma) for 2 h, then resuspended in 100 μl PBS. DMB (125 μl/well) was added to 40 μl of samples and quantified at 520 nm using a Bio-Tek Synergy HT spectrophotometer and compared to a standard curve (0–50 μg/ml of chondroitin sulfate (C-6-S)).

Noggin Binding

Cells were grown in serum free media for 2 h and treated with 900 ng/ml Noggin (a gift of Regeneron Pharmaceuticals) or NogginΔB2 protein (heparan binding domain removed; Regeneron) for 30 min at room temperature. Cells were blocked on ice using PBSB (PBS, 1% BSA) for 45 min, and treated with a biotinylated anti-Noggin antibody (1:500; Regeneron) on ice for 2 h. Next, cells were incubated with Streptavidin-phycoerythrin (SA-PE) secondary antibody (1:1,000; Becton Dickinson) in PBSB for 45 min on ice, and then fixed with 0.3% formalin in PBS for 10 min. Cells were then analyzed by immunofluorescence and fluorescence activated cell sorting (FACS) analysis.

BMP4 Dose Response and Time Course

For dose response studies, cells were treated with 0–400 ng/ml of BMP4 for 1.5 h. For time course studies, cells were treated with 200 ng/ml BMP4 for 0–4 h.

GAG Removal by Enzymatic Digestion

Cells were pre-treated with 5 mU/ml heparinase III in serum free RPMI for 2 h, then treated with 200 ng/ml of BMP4 for 1.5 h. To confirm GAG cleavage, dimethylmethylene blue (DMB) binding assays were performed (see above). GAG cleavage was also assessed with anti-Δ-heparan antibody (1:100; Seikagaku), which specifically detects heparinase cleaved stubs. Primary antibody was detected with anti-mouse horse radish peroxidase (HRP) secondary antibody (1:1,000; Sigma), developed using tetramethylbenzidine (TMB; Sigma), and analyzed on a Bio-Rad 550 microplate spectrophotometer at 450 nm.

Immunofluorescence

Cells were washed with PBS, and blocked on ice in PBSB containing DAPI (1:5,000; Molecular Probes) for 30 min. Cells were incubated with primary antibodies against glypican 1 and syndecan 4 (final dilution 1:100; Santa Cruz) on ice for 1.5 h. Cells were incubated with biotinylated anti-IgG secondary antibodies (1:250, R&D) in PBSB on ice for 45 min, then incubated with SA-PE (1:1,000) in PBSB for 45 min on ice, fixed with 0.3% formalin in PBS for 10 min, and adhered to Thermo Shandon Cytoslides (Thermo Shandon) using a Shandon Cytospin 2. Cells were visualized with a Leica DMR microscope equipped with epi-fluorescence.

Fluorescence Activated Cell Sorting (FACS) Analysis

Cells were blocked in PBSB on ice for 30 min, incubated with glypican 1 or syndecan 4 primary antibodies (1:100) for 2 h followed by biotinylated anti-IgG secondary antibodies (1:250) for 45 min on ice. Cells were then incubated with SA-PE (1:1,000) for 45 min on ice, fixed in 0.3% formalin in PBS, and analyzed on a Becton Dickinson FACscan flow cytometer. Data analysis was performed using Cell Quest Pro (BD Biosciences) to determine the mean fluorescent intensity (MFI).

siRNA Transfection

Cells were placed in medium (RPMI, 15% FBS) lacking antibiotics for 24 h prior to transfection, then resuspended at 2×10^6 cells in 5 ml Optimem medium (Invitrogen). Control and FOP cells were transfected with siRNA master mix containing 20 nmol of each siRNA duplex (*glypican 1*, *glypican 5*, *syndecan 3*, *syndecan 4*, or scrambled (control, non-targeting)) in Lipofectamine 2000 (1:250; Invitrogen) for 4 h. An equal volume of RPMI containing 30% FBS was added and cells were grown for 72 h prior to treatment with 200 ng/ml of BMP4 for 1.5 h. Target gene transcription was determined by real time PCR.

RNA Extraction, cDNA Synthesis, and Real Time PCR

Total RNA was extracted from cells using Trizol (Invitrogen) reagent. Samples were DNase1 (Invitrogen) treated and cDNA was prepared from 5 μg total RNA using a SuperScript First-Strand cDNA Synthesis Kit (Invitrogen). Gene expression was quantified using the SYBR green method of real time PCR and mRNA levels were compared to standard curves and normalized to *GAPDH* mRNA. PCR reactions were performed in triplicate with 50 nM each primer. Primers were designed using Primer Express (ABI): *ID1*: forward, 5′-GGTGGAGATTCTCCAGCACG-3′ and reverse, 5′-TCCAACTGAAGGTCCCTG ATG-3′; *GAPDH*: forward, 5′-AGATCATCAGCAATGCCTCC-3′ and reverse, 5′-ATGGCAT GGACTGTGGTCATG-3′; *syndecan 3*: forward, 5′-GCTCAGACCCCAAC TCCAGA-3′ and reverse, 5′-TGGCTCATTCCGGATTGTG-3′; *syndecan 4*: forward, 5′-AGGCCGATACTTCT CCGGA-3′ and reverse, 5′-CATCCAGATCTCCAGAGCCA G-3′; *glypican 3*: forward, 5′-GGT TTTCCAAGAGGCCTTTG-3′ and reverse, 5′-CAAAAGCTTGTGGAGTCAGGCT-3′; and *glypican 5*: forward, 5′-TGAAGATCACA GACTGGATGCC-3′ and reverse, 5′-TCCTGCTCCT GTTGTGTCTAAAGT-3′. *Glypican 1* was commercially designed by SuperArray (propriety sequence). Real time PCR was performed on an ABI Prism 7000 sequence detection system using standard (default) conditions.

Statistical Analysis

For all data, statistical analysis was performed using ANOVA and Students *t*-Test. *P*-values are: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

GAG Chains Levels are Elevated on FOP Cells

To examine total GAG levels (HSPG and non HSPG-associated) on control and FOP cells, incorporation of ^{35}S into newly synthesized chains and detection of GAG chain surface levels using DMB assays were performed in the absence of BMP4 ligand. Newly synthesized GAG chains were detected by ^{35}S incorporation after 24 h. DMB assays detect total steady-state levels of GAG chains. FOP cells had increased DMB dye binding (Fig. 1A) and ^{35}S incorporation (Fig. 1B), demonstrating elevated GAG chain levels.

To determine HSPG-specific GAG levels, cells were serum starved and treated with Noggin protein, a BMP antagonist, and analyzed by FACS using Noggin antibodies (Fig. 1C). Noggin has HSPG binding sites and remains functionally active while bound to HSPGs [Paine-Saunders et al., 2002]. Additionally, binding of Noggin to the cell surface is highly selective for HSPGs, requiring specific structural motifs for their interaction [Viviano et al., 2004]. Therefore, in the absence of BMPs, Noggin should specifically bind to cell surface HSPGs. Consistent with ^{35}S and DMB results, FOP cells exhibited increased Noggin binding compared to control cells, indicating elevated HSPG-specific GAG levels. As a control, cells were treated with NogginΔB2, which lacks the heparan-binding domain and therefore cannot bind to HSPG GAG chains. As expected, decreased binding of NogginΔB2 (Fig. 1C) was observed on control and FOP cells. These results support that FOP cells exhibit elevated HSPG-specific GAG chains.

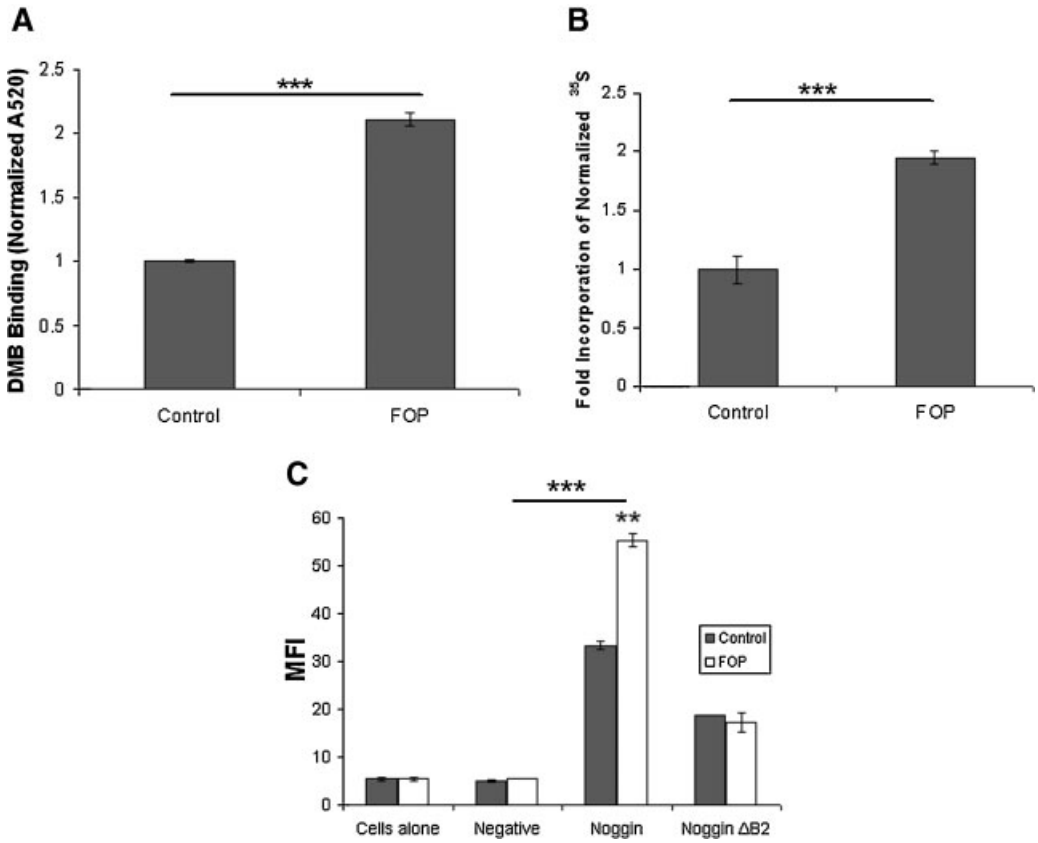

Fig. 1. GAG chains on LCLs. **A**: Control and FOP cells were incubated with DMB to detect total GAG chains and absorbance was measured at 520 nm. **B**: Control and FOP cells were grown in media containing ^{35}S and radioactive sulfate incorporation was normalized to total protein levels (CPM/μg protein). **C**: Control and FOP cells were treated with Noggin or NogginΔB2 (deleted heparin binding domain) for 30 min. Noggin binding was measured by FACS analysis and represented by mean fluorescent intensity (MFI). For all experiments, the results are presented as the average ± s.e.m. for three cell lines. Elevated GAG chain levels and Noggin binding were observed on FOP cells. $^{***}P < 0.001$.

ID1 mRNA is Reduced by GAG Chain Removal on Control, but not FOP Cells

To examine the effects of HSPGs on downstream BMP signaling, we examined mRNA expression of the *ID1* gene, a transcriptional target of BMP signaling [de la Pena et al., 2005; Fiori et al., 2006]. Treatment of control cells with BMP4 caused an upregulation of ID1 mRNA in a dose-dependent manner. At a concentration of 200 ng/ml, BMP4 increased ID1 mRNA expression approximately 20-fold (Fig. 2A). Time course studies showed maximal induction of ID1 mRNA 1.5 h after ligand treatment (Fig. 2B). BMP treatment induced ID1 mRNA in both control and FOP cells, and the level of induction was consistently higher in FOP cells, as previously reported [de la Pena et al., 2005; Fiori et al., 2006].

GAG chains are highly charged post-translational modifications of cell surface HSPG (glypican and syndecan) core proteins which can bind BMPs. To investigate the role of HSPG GAG chains in BMP signaling, all sulfated HSPG GAG chains were removed by heparinase III treatment, prior to BMP4 stimulation. Removal of GAG chains was confirmed using an antibody that recognizes heparinase cleaved stubs; increased antibody binding is directly correlated with heparinase III cleavage (Fig. 3A). Heparinase treatment alone (absence of ligand) had no effect on *ID1* gene expression. However, GAG chain removal reduced BMP4-stimulated *ID1* gene expression levels in control cells, but not in FOP cells (Fig. 3B).

HSPG mRNA Expression in LCLs

Since HSPG GAG chain removal affects BMP signaling in control cells, HSPG subtype expression profiling was performed in order to identify the specific HSPGs expressed by LCLs. RT-PCR for all glypicans and syndecans was performed

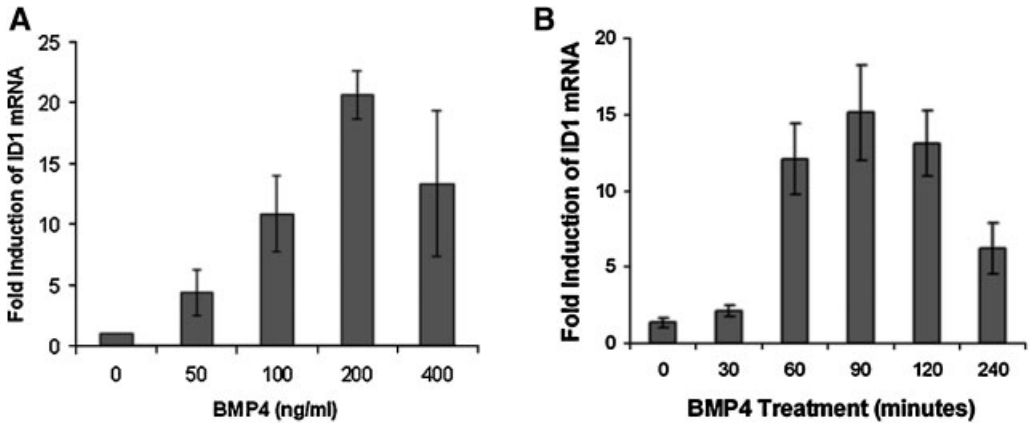

Fig. 2. ID1 Dose response and time course. **A**: For the dose response, control cells were treated with 0–400 ng/ml of BMP4 for 1.5 h and ID1 mRNA levels were quantified by real time PCR. **B**: For the time course, control cells were treated with 200 ng/ml BMP4 for 0–240 min and ID1 mRNA levels were quantified by real time PCR. The results of three different cell lines are presented as the average ± s.e.m. ID1 was maximally stimulated with 200 ng/ml of BMP4 at 1.5 h.

(data not shown). Syndecan 3, syndecan 4, glypican 1, and glypican 5 mRNAs were detected in control and FOP cells. However, the mRNA levels of glypican 1 and syndecan 4 were relatively high compared to glypican 5 and syndecan 3 as detected by real time PCR (Fig. 4A). No statistical differences in HSPG mRNA levels were observed between control and FOP cells.

Glypican 1 and Syndecan 4-Core Proteins are Elevated on FOP Cells

Based on the mRNA profiles of HSPGs, we examined glypican 1 and syndecan 4-core protein expression on the cell surface by immunofluorescence and demonstrated that both of these HSPGs were detected on control and FOP cells (Fig. 4B). Quantification of core protein levels by FACS analysis showed that glypican 1 and syndecan 4 levels were significantly increased (two-fold) on FOP LCLs compare to controls (Fig. 4C).

Glypican 1 and Syndecan 4 siRNA alter ID1 Expression

To determine the roles of specific HSPGs in BMP signal transduction, downregulation of individual HSPGs was performed using specific

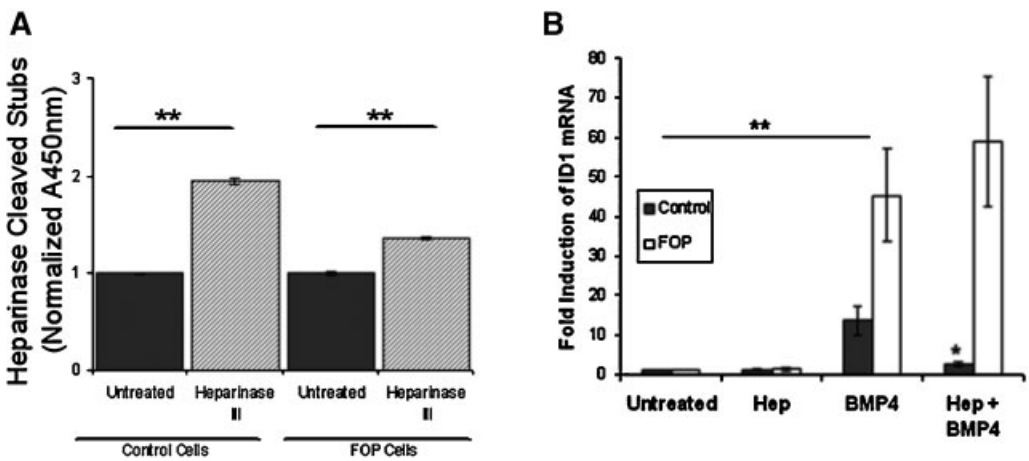

Fig. 3. Heparinase III cleavage and ID1 mRNA expression. **A**: Control and FOP cells were untreated or treated with heparinase III for 2 h. Heparinase III cleavage was confirmed using an anti-Δ-heparan antibody. Bound antibody was detected with an HRP conjugated secondary antibody, developed using TMB, and absorbance was measured at 450 nm. **B**: Control and FOP cells were treated with heparinase III (hep) for 2 h followed by BMP4 for 1.5 h (hep + BMP4), and ID1 mRNA was quantified by real time PCR. The results are presented as the average ± s.e.m. from three cell lines. GAG chain removal decreased ID1 mRNA in control cells, but not in FOP cells. $^{*}P < 0.05$, $^{**}P < 0.01$.

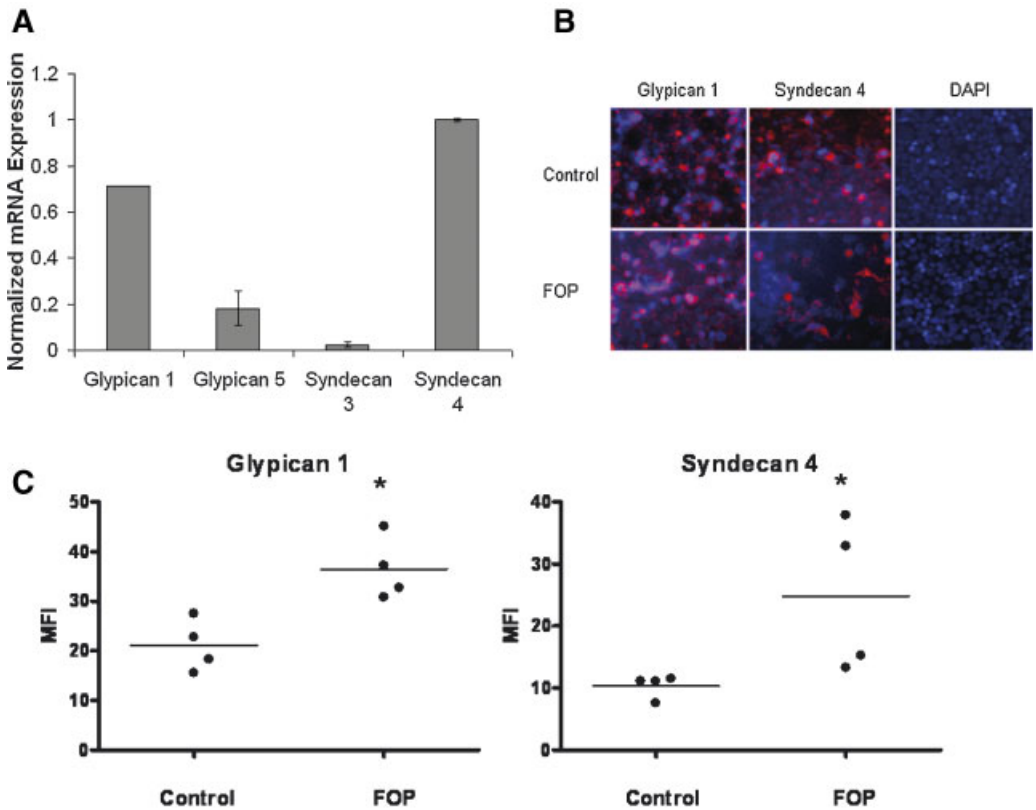

Fig. 4. HSPG expression in LCLs. **A**: HSPG mRNA was quantified by real time PCR. The results are presented as the average ± s.e.m. from three cell lines. **B**: Immunofluorescence analysis was performed using specific antibodies against glypican 1 and syndecan 4 (red) and counterstained with DAPI (blue). DAPI nuclear staining alone served as a negative control. **C:** Quantification of glypican 1 and syndecan 4 was performed on control and FOP cells by FACS analysis. The average linear values of relative quantification of glypican 1 and syndecan 4 on four control and four FOP cell lines is represented by the MFI. FOP cells exhibit increased levels of glypican 1 and syndecan 4-core protein. $^*P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

siRNAs. No differences in ID1 expression were observed following syndecan 3 or glypican 5 gene silencing in control or FOP cells (data not shown), suggesting that these HSPGs have little or no effect on BMP signaling in LCLs, perhaps due to their low mRNA levels (Fig. 4A).

Following glypican 1 or syndecan 4 siRNA treatment, the mRNA levels of these genes were reduced to 30–55% of the levels of cells transfected with scrambled (control) siRNA (Fig. 5A). BMP4 treatment of cells transfected with glypican 1 siRNA resulted in increased ID1 mRNA levels in both control and FOP cells (Fig. 5B), suggesting that glypican 1 normally plays a role in inhibiting BMP4-induced ID1 expression. In contrast, BMP4 treatment of cells transfected with syndecan 4 siRNA resulted in a 50% decrease in ID1 expression in control cells (Fig. 5C), but had no effect in FOP cells, suggesting that syndecan 4 enhances BMP signaling in control cells, but not in FOP cells.

DISCUSSION

Our study demonstrates that HSPG modulation of BMP signaling is altered in cells from FOP patients. FOP cells are resistant to the stimulatory effects of cell surface HSPGs, but are susceptible to the inhibitory effects, suggesting that the effects are secondary and adaptive to the underlying activating mutation in ACVR1. Further, the increased BMP signaling observed in FOP cells [de la Pena et al., 2005; Fiori et al., 2006] may diminish or abrogate the stimulatory roles of the HSPGs. HSPG GAG chain reduction does not decrease BMP signaling in FOP cells, as it does in control cells, and is likely resistant to the activity of the mutant ACVR1 receptor [Shore et al., 2006]. Increased HSPG levels are likely a secondary effect of the disease causing mutation in ACVR1.

Peripheral blood mononuclear cells (lymphocytes) are currently the only cell type available

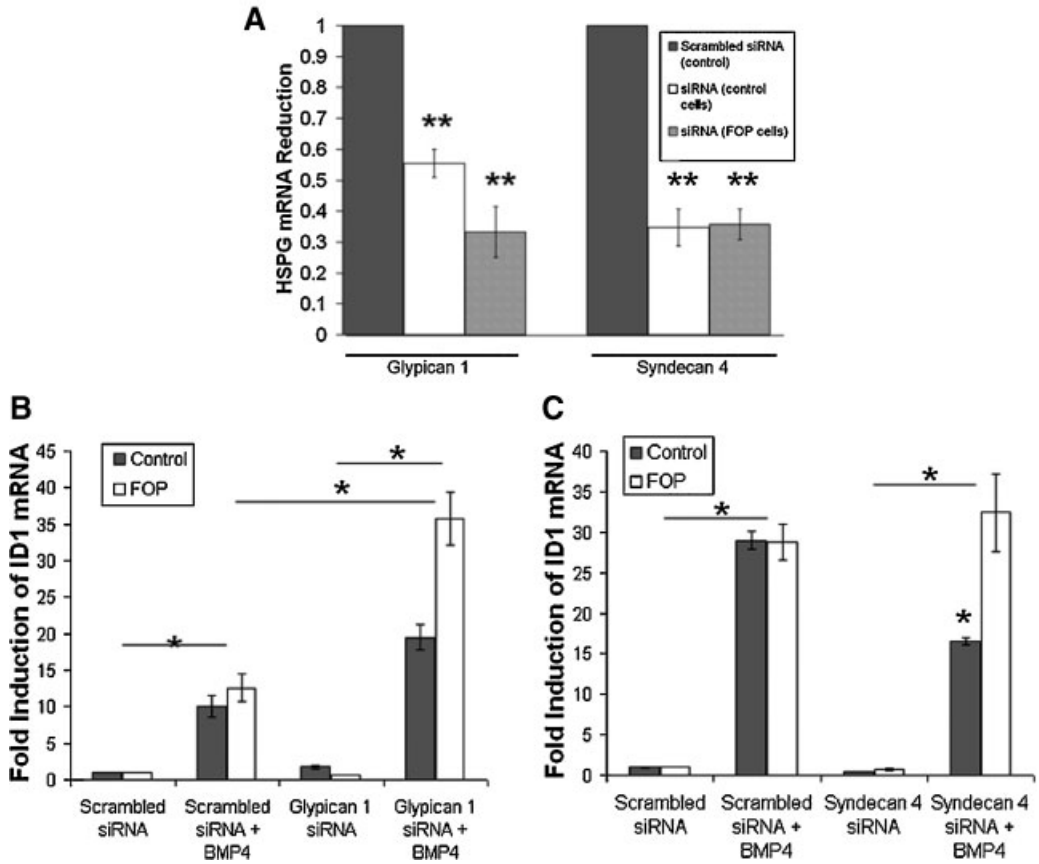

Fig. 5. Glypican 1 and syndecan 4 siRNA. **A**: Control and FOP cells were treated with glypican 1 or syndecan 4-specific siRNAs for 72 h. Glypican 1 and syndecan 4 mRNA levels were quantified by real time PCR. The results are presented as the average ± s.e.m. from three cell lines. Reduced levels of glypican 1 and syndecan 4 mRNA was detected following siRNA treatments in control and FOP cells. $^{**}P < 0.01$ **B**: Control and FOP cells were treated with glypican 1-specific siRNA or **C**: syndecan 4-specific siRNA for 72 h, followed by BMP4 for 1.5 h, and ID1 mRNA was quantified by real time PCR. The results are presented as the average ± s.e.m. from three cell lines. Glypican 1 gene silencing increased BMP4-stimulated ID1 expression in control and FOP cells. $^{*}P < 0.05$.

for the study of FOP. Any trauma to FOP patients, including biopsies, results in aggressive and catastrophically disabling new bone formation. A study of the effects of HSPGs in other cell types such as osteoblasts and chondrocytes is important; however, it is impossible to obtain these cell types from FOP patients without causing irreparable physical harm to the patients. Such primary tissue samples might ethically be obtained from willed post-mortem donations from FOP patients, but presently no such specimens are available. An alternative source might include primary tissue samples from FOP ACVR1 mutant knock-in mice, an animal model that is presently being developed.

Glypicans and syndecans are the major cell surface HSPGs involved in modulating cell signaling pathways. In LCLs, syndecan 4 and glypican 1 are the most abundantly expressed HSPG mRNAs. Syndecans are proline rich, resulting in an extended protein structure [Bernfield et al., 1999]. Selective reduction of syndecan 4-core protein synthesis by siRNA decreased BMP signaling in control cells, but not in FOP cells, consistent with GAG chain removal (Fig. 3). A reduction in BMP-induced *ID1* levels in control cells indicates that syndecan 4 normally enhances BMP signaling in LCLs. The enhancing effects of syndecan 4 may not be observed in FOP cells due to the already significantly increased levels of BMP signaling [Fiori et al., 2006]. In contrast to syndecans, glypicans have cysteine rich ectodomains capable of forming intramolecular disulphide bonds which result in the formation of large globular proteins that may prevent bulky molecules from interacting with GAG chains and cell surface receptors [Bernfield et al., 1999; De Cat and David, 2001; Filmus and Selleck, 2001].

Previous reports have shown that glypicans can be either stimulatory or inhibitory for different growth factors [Bonneh-Barkay et al., 1997; Iozzo and San Antonio, 2001]. For example, glypican 1 stimulated fibroblast growth factor 1 (FGF-1) activity while inhibiting FGF-7 [Berman et al., 1999]. Glypicans may be responsible for the extracellular distribution of growth factors and morphogens, and removal of glypicans could disrupt these gradients, thus impairing signaling [Baeg et al., 2001]. Interestingly, glypicans have also been shown to be associated with diseases of the skeleton such as Simpson-Golabi-Behmel (SGBS) syndrome, an X-linked condition characterized by overgrowth and skeletal anomalies resulting from glypican 3 mutations [Mariani et al., 2003]. Selective reduction of glypican 1 by siRNA treatment increased BMP signaling in both control and FOP cells as measured by elevated ID1 mRNA levels. These data suggest that glypican 1 normally has an inhibitory role in BMP signaling in LCLs, which is unaffected by dysregulated BMP signaling in FOP cells.

The effects of HSPG GAG chains on downstream BMP signaling were determined by quantifying the mRNA levels of *ID1*, a BMP early response gene. *ID1* is a member of a family of proteins that act as positive and negative regulators of cell proliferation and cell differentiation, respectively [Neuman et al., 1993; Ogata et al., 1993; Voronova and Lee, 1994; Deed et al., 1998; Norton et al., 1998]. Recent studies of *Id1* and *Id3* knockout mice demonstrated that these genes are positive factors for promoting bone formation [Lyden et al., 1999; Maeda et al., 2004]. Depletion of cell surface HSPG GAG chains on control cells resulted in decreased *ID1* mRNA levels, suggesting reduced BMP signaling. These data are consistent with previous studies in other cell types showing that GAG chain removal results in decreased Smad phosphorylation [Irie et al., 2003], and therefore decreased BMP signaling. Interestingly, in FOP cells, GAG chain removal by heparinase III had no effect on BMP signaling. It is possible that GAG chains on other non-HSPG proteoglycans are unaffected by heparinase III treatment and compensate for HSPG-specific GAG removal on FOP cells.

Many signaling molecules including Ihh, BMPs, Wnts, FGFs, and parathyroid hormone related protein (PTHrP) interact with one another in complex signaling networks to regulate specific steps in cell differentiation [Kronenberg, 2003]. Cell surface HSPGs can bind these factors and are well positioned to modulate their activity. For example, HSPGs bind Ihh in the extracellular space and act as negative regulators of signaling in a concentration-dependent manner [Koziel et al., 2004]. HSPGs play a central role in the pathogenesis of MHE, in which failure to synthesize GAG chains results in the formation of osteochondromas. Recent clinical investigations have shown that nearly all FOP patients have osteochondromas [Kaplan et al., 2005], although no mutations have been detected in the *EXT* genes in these patients (data not shown).

FOP is caused by a mutation in *ACVR1*, a type I BMP receptor [Shore et al., 2006], which is expressed in many tissues including skeletal muscle and chondrocytes. Constitutive activation of this receptor results in upregulation of BMP4, downregulation of BMP antagonists, and induction of alkaline phosphatase [Payne et al., 2001; Zhang et al., 2003]. The recurrent missense mutation in *ACVR1* in FOP patients suggests constitutive activation of the receptor, which is consistent with increased BMP signaling observed in FOP cells [de la Pena et al., 2005; Fiori et al., 2006; Shore et al., 2006]. An increase in BMP signaling through mutant *ACVR1* would increase Ihh signaling in the perichondrium and the formation of osteochondromas [Grimsrud et al., 2001; Minina et al., 2001; Zhang et al., 2003; Koziel et al., 2004]. Therefore, the osteochondromas observed in FOP patients may be a result of increased Ihh signaling caused by constitutive activation of ACVR1.

Observations from our laboratory [Fiori et al., 2006] support that FOP cells have a slight but consistent increase in basal BMP signaling in the absence of ligand, compared to control cells. We speculate that this is an effect of the mutant ACVR1 receptor. FOP cells have increased HSPG-bound Noggin; however, the effects may be abrogated as BMP signaling is enhanced in the absence of BMP4 and further with hyperstimulation by unantagonized BMP. In addition, increased levels of the BMPRIA receptor on FOP cells [de la Pena et al., 2005] provide additional binding sites for BMP4, thus, potentially increasing signal transduction. It is plausible that, due to the primary mutation in ACVR1, FOP cells are primed for signaling and upregulate proteins, including HSPGs that aid

the signaling process. More detailed investigations of the effects of mutated ACVR1 signaling are currently underway.

Based on our findings, we propose a hypothetical schema of GAG chain action on BMP signaling in FOP cells. Under normal conditions, BMP signaling is achieved either through direct ligand-receptor interactions or through HSPG-ligand-receptor interactions that enhance or inhibit signaling depending on HSPG structure. Syndecans have a proline rich extended structure allowing increased spatial binding to BMP ligand and potentially increasing ligand delivery to the receptor. Glypicans are cysteine rich with globular heads that may act as filters, trapping ligands and preventing their access to the receptor. However, on FOP cells, the levels of GAG chains as well as BMPRIA are increased on the cell surface. Due to this mass availability of binding sites for BMP ligand, FOP cells reach maximal signal saturation with additional binding sites still available, negating the stimulatory role of HSPGs (such as syndecan 4) seen in control cells (as shown in Fig. 5).

In conclusion, BMP signaling is modulated, in part, by the GAG side chains of HSPGs on LCLs. Given the recent findings, HSPG-associated BMP signal transduction may be disrupted in FOP cells due to a presumed activating mutation in ACVR1, as demonstrated in this study. However, alterations in the levels of cell surface HSPGs on FOP cells may also contribute to the dysregulation of BMP signaling seen in FOP LCLs [de la Pena et al., 2005; Fiori et al., 2006]. The enhanced BMP signaling observed in FOP cells could disrupt multiple signaling pathways downstream of the BMP pathway, contributing to the pathogenesis of the disease.

Our study suggests new avenues of investigation to better understand HSPG-mediated BMP signaling and the role of GAG chains in BMP binding and downstream signaling. These studies will provide important new insights into the extracellular distribution of morphogens that result in the induction of normal and ectopic osteogenesis.

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REFERENCES

Baeg GH, Lin X, Khare N, Baumgartner S, Perrimon N. 2001. Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. Development 128:87–94.

Berman B, Ostrovsky O, Shlissel M, Lang T, Regan D, Vlodavsky I, Ishai-Michaeli R, Ron D. 1999. Similarities and differences between the effects of heparin and glypican-1 on the bioactivity of acidic fibroblast growth factor and the keratinocyte growth factor. J Biol Chem 274:36132–36138.

Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. 1999. Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 68: 729–777.

Bonneh-Barkay D, Shlissel M, Berman B, Shaoul E, Admon A, Vlodavsky I, Carey DJ, Asundi VK, Reich-Slotky R, Ron D. 1997. Identification of glypican as a dual modulator of the biological activity of fibroblast growth factors. J Biol Chem 272:12415–12421.

Cadigan KM. 2002. Regulating morphogen gradients in the Drosophila wing. Semin Cell Dev Biol 13:83–90.

De Cat B, David G. 2001. Developmental roles of the glypicans. Semin Cell Dev Biol 12:117–125.

de Jong JG, Wevers RA, Laarakkers C, Poorthuis BJ. 1989. Dimethylmethaline blue-based spectrophotometry of glycosaminoglycans in untreated urine: a rapid screening procedure for mucopolysaccharidoses. Clin chem 35: 1472–1477.

de la Pena LS, Billings PC, Fiori JL, Ahn J, Kaplan FS, Shore EM. 2005. Fibrodysplasia ossificans progressiva (FOP), a disorder of ectopic osteogenesis, misregulates cell surface expression and trafficking of BMPRIA. J Bone Miner Res 20:1168–1176.

Deed RW, Jasiok M, Norton JD. 1998. Lymphoid-specific expression of the Id3 gene in hematopoietic cells. Selective antagonism of E2A basic helix-loop-helix protein associated with Id3-induced differentiation of erythroleukemia cells. J Biol Chem 273:8278–8286.

Duncan G, McCormick C, Tufaro F. 2001. The link between heparan sulfate and hereditary bone disease: Finding a function for the EXT family of putative tumor suppressor proteins. J Clin Invest 108:511–516.

Filmus J, Selleck SB. 2001. Glypicans: Proteoglycans with a surprise. J Clin Invest 108:497–501.

Fiori JL, Billings PC, de la Pena LS, Kaplan FS, Shore EM. 2006. Dysregulation of the BMP-p38 MAPK signaling pathway in fibrodysplasia ossificans progressiva (FOP) patients. J Bone Miner Res 21:902–909.

Gannon FH, Valentine BA, Shore EM, Zasloff MA, Kaplan FS. 1998. Acute lymphocytic infiltration in an extremely early lesion of fibrodysplasia ossificans progressiva. Clin Orthop Relat Res 364: 19–25.

Gannon FH, Kaplan FS, Olmsted E, Finkel GC, Zasloff MA, Shore E. 1997. Bone morphogenetic protein 2/4 in early fibromatous lesions of fibrodysplasia ossifican progressiva. Hum Pathol 28: 339–343.

Grimsrud CD, Romano PR, D'Souza M, Puzas JE, Schwarz EM, Reynolds PR, Roiser RN, O'Keefe RJ. 2001. BMP signaling stimulates chondrocyte maturation and the expression of Indian hedgehog. J Orthop Res 19: 18–25.

Guimond S, Turnbull JE. 2004. Proteoglycans make the grade-ient. Mol Cell 16:159–160.

Hecht JT, Hogue D, Strong LC, Hansen MF, Blanton SH, Wagner M. 1995. Hereditary multiple exostosis and chondrosarcoma: Linkage to chromosome II and loss of heterozygosity for EXT-linked markers on chromosomes II and 8. Am J Hum Genet 56:1125–1131.

Iozzo RV, San Antonio JD. 2001. Heparan sulfate proteoglycans: Heavy hitters in the angiogenesis arena. J Clin Invest 108:349–355.

Irie A, Habuchi H, Kimata K, Sanai Y. 2003. Heparan sulfate is required for bone morphogenetic protein-7 signaling. Biochem Biophys Res Commun 308: 858–865.

Kaplan FS, Glaser DL, Shore EM, Deirmengian GK, Gupta R, Delai P, Morhart R, Smith R, Le Merrer M, Rogers JG, Connor JM, Kitterman JA. 2005. The Phenotype of Fibrodysplasia Ossificans Progressiva. Clin Rev Bone Miner Metab 3: 183–188.

Kaplan FS, Shore EM, Conner JM. 2002. Fibrodysplasia ossificans progressiva. In: Royce PM, Steinmann B, editors. Connective tissue and its heritable disorders. NY: Wiley-Liss. pp 827–840.

Koziel L, Kunath M, Kelly OG, Vortkamp A. 2004. Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification. Dev Cell 6:801–813.

Kreuger J, Perez L, Giraldez AJ, Cohen SM. 2004. Opposing activities of Dally-like glypican at high and low levels of Wingless morphogen activity. Dev Cell 7: 503–512.

Kronenberg HM. 2003. Developmental regulation of the growth plate. Nature 423:332–336.

Kronenberg HM. 2003. Developmental regulation of the growth plate. Nature 423: 332–336.

Maeda Y, Tsuji K, Nifuji A, Noda M. 2004. Inhibitory helix-loop-helix transcription factors Id1/Id3 promote bone formation in vivo. J Cell Biochem 93:337–344.

Mariani S, Iughetti L, Bertorelli R, Coviello D, Pellegrini M, Forabosco A, Bernasconi S. 2003. Genotype/phenotype correlations of males affected by Simpson-Golabi-Behmel syndrome with GPC3 gene mutations: Patient report and review of the literature. J Pediatr Endocrinol Metab 16:225–232.

Minina E, Wenzel HM, Kreschel C, Karp S, Gaffield W, McMahon AP, Vortkamp A. 2001. BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. Development 128:4523–4534.

Neuman T, Keen A, Zuber MX, Kristjansson GI, Gruss P, Nornes HO. 1993. Neuronal expression of regulatory helix-loop-helix factor Id2 gene in mouse. Dev Biol 160: 186–195.

Norton JD, Deed RW, Craggs G, Sablitzky F. 1998. Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol 8:58–65.

Ogata T, Wozney JM, Benezra R, Noda M. 1993. Bone morphogenetic protein 2 transiently enhances expression of a gene, Id (inhibitor of differentiation), encoding a helix-loop-helix molecule in osteoblast-like cells. Proc Natl Acad Sci USA 90:9219–9222.

Paine-Saunders S, Viviano BL, Economides AN, Saunders S. 2002. Heparan sulfate proteoglycans retain Noggin at the cell surface: A potential mechanism for shaping bone morphogenetic protein gradients. J Biol Chem 277:2089–2096.

Payne TL, Postlethwait JH, Yelick PC. 2001. Functional characterization and genetic mapping of alk8. Mech Dev 100:275–289.

Shafritz AB, Shore EM, Gannon FH, Zasloff MA, Taub R, Muenke M, Kaplan FS. 1996. Overexpression of an osteogenic morphogen in fibrodysplasia ossificans progressiva. N Engl J Med 335:555–561.

Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Brown MA, Kaplan FS. 2006. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet 38:525–527.

Stickens D, Zak BM, Rougier N, Esko JD, Werb Z. 2005. Mice deficient in Ext2 lack heparan sulfate and develop exostoses. Development 132:5055–5068.

Takada T, Katagiri T, Ifuku M, Morimura N, Kobayashi M, Hasegawa K, Ogamo A, Kamijo R. 2003. Sulfated polysaccharides enhance the biological activities of bone morphogenetic proteins. J Biol Chem 278:43229–43235.

van der Voort R, Keehnen RM, Beuling EA, Spaargaren M, Pals ST, 2000. Regulation of cytokine signaling by B cell antigen receptor and CD40-controlled expression of heparan sulfate proteoglycans. J Exp Med 192: 1115–1124.

Viviano BL, Paine-Saunders S, Gasiunas N, Gallagher J, Saunders S. 2004. Domain-specific modification of heparan sulfate by Qsulf1 modulates the binding of the bone morphogenetic protein antagonist Noggin. J Biol Chem 279:5604–5611.

Voronova AF, Lee F. 1994. The E2A and tal-1 helix-loop-helix proteins associate in vivo and are modulated by Id proteins during interleukin 6-induced myeloid differentiation. Proc Natl Acad Sci USA 91:5952–5956.

Wang D, Sai J, Richmond A. 2003. Cell surface heparan sulfate participates in CXCL1-induced signaling. Biochemistry 42:1071–1077.

Yoon BS, Lyons KM. 2004. Multiple functions of BMPs in chondrogenesis. J Cell Biochem 93:93–103.

Zhang D, Schwarz EM, Rosier RN, Zuscik MJ, Puzas JE, O'Keefe RJ. 2003. ALK2 functions as a BMP type I receptor and induces Indian hedgehog in chondrocytes during skeletal development. J Bone Miner Res 18:1593–1604.

Zimmermann P, David G. 1999. The syndecans, tuners of transmembrane signaling. FASEB J 13(Suppl):S91–S100.