

Heparan Sulfate Proteoglycans (HSPGs) Modulate BMP2 Osteogenic Bioactivity in C2C12 Cells*

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Cell surface heparan sulfate proteoglycans (HSPGs) have been implicated in bone morphogenetic protein (BMP)-mediated morphogenesis by regulating BMP activity and gradient formation. However, the direct role of HSPGs in BMP signaling is poorly understood. Here we show that HSPGs directly regulate BMP2-mediated transdifferentiation of C2C12 myoblasts into osteoblasts. HSPGs sequester BMP2 at the cell surface and mediate BMP2 internalization. Depletion of cell surface HSPGs by heparinase III treatment or decreased glycosaminoglycan chain sulfation with sodium chlorate enhances BMP2 morphogenetic bioactivity. The addition of exogenous heparin, a widely used anticoagulant, reduced BMP2 signaling. Our results suggest that cell surface HSPGs mediate BMP2 internalization and modulate BMP2 osteogenic activity.

Cell surface heparan sulfate proteoglycans (HSPGs),² which include syndecans and glypicans, are expressed on most mammalian cell surfaces and consist of a core protein and highly sulfated glycosaminoglycan (GAG) side chains in the form of heparan sulfate (HS) (1). At least four syndecan family members, which are transmembrane HSPGs (type I integral membrane proteins), have been described. The glypican family consists of five members and differ from syndecans in that the core protein is attached to the cell surface via a COOH-terminal glycosylphosphatidyl-inositol anchor.

HSPGs bind to a litany of extracellular molecules, including growth factors, adhesion molecules, morphogens, and morphogen antagonists. Consequently, HSPGs are versatile coordinators of cellular function (2) and are emerging as important modulators of morphogen gradients during embryonic development (3).

Recent genetic studies implicate HSPGs in regulating multiple signaling pathways during embryogenesis and regeneration. For example, mutations in the *Drosophila* genes *sulfateless* and *sugarless*, encoding HS *N*-deacetylase/*N*-sulfotransferase and UDP-glucose dehydrogenase, respectively, exhibit abnormalities in Wingless (Wg)-mediated dorsal/ventral patterning (4, 5). A *Drosophila* mutant of *tout velu*, an HS co-polymerase gene, exhibits abnormal Wg, Hedgehog, and bone morphogenetic protein (BMP) activity (6). The clinical relevance of HSPG function is demonstrated by inactivating mutations in the tumor suppressor genes *EXT1* or *EXT2*, vertebrate homologues of *tout velu*, which cause hereditary multiple exostoses, an autosomal dominant disease in humans and mice characterized by benign orthotopic endochondral tumors, also known as osteochondromas, with malignant potential (7).

BMPs are secreted morphogens, playing crucial developmental roles in mesoderm formation, neural patterning, skeletal development, and limb formation (8). BMPs are heparin-binding proteins, and BMP-mediated developmental processes involve BMP-HSPG interactions (9). HS/heparan chains bind BMP4 and restrict the expression pattern of BMP4 in *Xenopus* embryos (10). *Drosophila* mutants of *dally*, a gene encoding the homologue of mammalian glypican-3, show abnormalities in modulating Decapentaplegic (Dpp), the homologue of BMP2/BMP4 (11). *dly*, a *dally*-like glypican, is also critical in Dpp gradient formation (12). In addition, the combined genetic deficiency for glypican-3 and BMP4 results in abnormal skeletal development in vertebrates (13). Mutations in the core protein of glypican-3, a member of the HSPG superfamily of molecules, are responsible for Simpson-Golabi-Behmel Syndrome, a genetic disease involving skeletal overgrowth in humans (14).

Despite compelling *in vivo* evidence, the direct role of HSPGs on BMP-mediated signal transduction remains poorly understood. Since BMP2 can induce transdifferentiation of C2C12 myoblasts into osteoblasts (15), we used this *in vitro* model to study involvement of HSPGs in BMP2-mediated osteogenic signaling. Our results suggest that HSPGs modulate BMP2 osteogenic activity by sequestering BMP2 at the cell surface and mediate its internalization.

MATERIALS AND METHODS

Cell Culture and Treatments—C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO₂ in air. For all treatments, cells were plated in 24-well plates at a

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² The abbreviations used are: HS, heparan sulfate; HSPG, HS proteoglycan; BMP, bone morphogenetic protein; BMPRIA, BMP receptor IA; GAG, glycosaminoglycan; DMB, dimethylmethylene blue; ALP, alkaline phosphatase.

density of 2×10^4 /well. Heparin (Sigma) and rhBMP2 (Genetics Institute, Cambridge, MA) were added simultaneously to the culture media.

To decrease GAG chain sulfation, cells were pretreated with 20 mM sodium chlorate (Sigma), a selective inhibitor of sulfation for 48 h. To selectively remove HSPG GAG chains, cells were treated with heparinase III (2 sigma units/ml) for 2 h, and then BMP2 and/or heparin were added in the continuous presence of sodium chlorate or heparinase. Cells were fed every 2 days with fresh medium supplemented with BMP2, heparinase III, or sodium chlorate as indicated.

GAG Chain Assessment—GAG chain sulfation levels were assessed with dimethylmethylene blue (DMB) dye binding, which interacts with sulfated GAG chains on all proteoglycans. For the dye binding assay, untreated heparinase III (2 sigma units/ml), or sodium chlorate (NaClO_3 ; 20 mM)-treated cells were washed and resuspended in 0.1 ml of phosphate-buffered saline. Thirty- μl aliquots of each sample were mixed with 125 μl of DMB and read in 96-well plates at 520 nm using a Bio-Tek Synergy HT microtiter plate spectrophotometer (16). Protein was determined by using a BCA protein assay (Pierce).

To confirm enzymatic cleavage of GAG chains, cells were treated with heparinase III, washed, and incubated with an anti- Δ -heparan mouse monoclonal antibody (antibody number 370260; Seikagaku America), which specifically detects heparinase cleaved stubs. The cells were washed and incubated with an anti-mouse horse radish peroxidase secondary antibody conjugate (Sigma). Samples were developed with tetramethylbenzidine substrate stopped by the addition of 0.1 N H_2SO_4 and read at 450 nm.

Anti-BMPRIA Antibody—Antibodies to BMP receptor IA (BMPRIA) (Entrez protein sequence number P36894) were made against a synthetic peptide GMKSDSDQKKSEN-C, comprising residues 34–46 in the extracellular domain of BMPRIA. Antibodies were prepared in rabbits by Cocalico Biologicals (Reamstown, PA) and purified by ammonium sulfate precipitation followed by affinity chromatography.

Alkaline Phosphatase (ALP) Assay—Cells were washed with Tris-buffered saline and incubated with insoluble substrates 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Moss, Inc. Pasadena, MD) for 10 min. Stained cells were fixed in 10% neutral buffered formalin for 5 min and air-dried. Alternatively, to quantify ALP activity, cell lysates were incubated with soluble substrate, *p*-nitrophenylphosphate (Sigma), for 30 min at room temperature. The reaction was stopped by adding an equal volume of 1 M NaOH, and color development was measured at 405 nm. ALP activity was normalized for total protein concentration, determined with a BCA protein assay.

Mineralization Assay—Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 mM L-ascorbate-2-phosphate, 10^{-6} M dexamethasone (Sigma), 5 mM β -glycerophosphate, and 300 ng/ml rhBMP2 and/or 2 mg/ml heparin for 4 weeks. Cells were stained with 1% Alizarin red (pH 4.2) for 10 min.

Real-time PCR—Total RNA was isolated using a RNeasy kit (Qiagen, Valencia, CA) and treated with DNase I (1 unit/5 μg of RNA) at 37 °C for 30 min. mRNA for osteoblast marker genes was detected by real-time PCR with ABI Prism 7000 sequence

detection system (Applied Biosystems, Foster City, CA) using β -actin as an endogenous control. Forward and reverse primer sequences were as follows: osteopontin, 5'-TTTGCTTTTGCCTGTTTGGC-3' and 5'-CAGTCACTTTCACCGGGAGG-3'; osteocalcin, 5'-CCGGGAGCAGTGTGAGCTTA-3' and 5'-TAGATGCGTTTGTAGGCGGTC-3'; collagen type I, 5'-GCATGGCCAAGAAGACATCC-3' and 5'-CCTCGGGTTTCCACGTCTC-3'; RUNX2, 5'-GTGCGGTGCAAACCTTCTCC-3' and 5'-AATGACTCGGTTGGTCTCGG-3'; alkaline phosphatase, 5'-GTGCCCTGACTGAGGCTGTC-3' and 5'-GGATCATCGTGTCTCTGCTCAC-3'; and β -actin, 5'-AAGATCATTGCTCCTCCTGAGC-3' and 5'-CATACTCCTGCTTGCTGATCC-3'.

Western Blots—Cells were lysed in radioimmune precipitation buffer (1% Nonidet P-40, 0.5% sodium deoxycholate in 1 \times phosphate-buffered saline) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 \times protease inhibitor mixture (Invitrogen) for 1 h on ice. The lysates were centrifuged at $10,000 \times g$ for 10 min at 4 °C. Twenty μg of protein were loaded in each lane, subjected to 12% SDS-PAGE electrophoresis, and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline, 0.1% Tween 20 for 2 h at room temperature and incubated with primary anti-phospho-Smad1 antibody (Upstate Biotechnology, Waltham, MA) at 4 °C overnight. The blots were washed and incubated with a horseradish peroxidase-conjugated secondary antibody and developed with chemiluminescence using ECL Plus (Amersham Biosciences) according to the manufacturer's instructions.

BMP2 Internalization Assay—BMP2 (12 μl of 400 ng/ml) was incubated with mouse anti-BMP2 monoclonal antibody (40 μl of 500 ng/ml) for 30 min at 37 °C. C2C12 cells were seeded in chamber slides and pretreated either with 20 mM sodium chlorate for 48 h and/or with antibody against BMPRIA for 2 h. Cells were next incubated with BMP2-anti-BMP2 complex (30 min, 4 °C) to allow complex binding to the cell surface. The cells were washed with ice-cold serum-free medium to remove unbound BMP2-anti-BMP2 complex and then incubated at 4 or 37 °C for 60 min to allow ligand internalization. Following incubation, the cells were washed with phosphate-buffered saline, fixed with acetone, and blocked with 5% goat serum for 20 min. For BMP2 staining, cells were incubated with a mixture of Alexa Fluor 488-labeled goat anti-mouse IgG (1:1000, Molecular Probes, Eugene, OR) and 4',6-diamidino-2-phenylindole (1:2000) for 30 min. Fluorescence was monitored with a Leica microscope equipped with epifluorescence, and images were analyzed with Openlab software (Improvision Inc, Lexington, MA).

Fluorescein Labeling—Carrier-free rhBMP2 (R&D systems, Minneapolis, MN) was fluorescein-labeled using an EZ-Label fluorescent labeling kit (Pierce) following the manufacturer's instructions. To assess ligand internalization, cells were grown on glass coverslips and incubated with FL-BMP2 in Dulbecco's modified Eagle's medium, 1% bovine serum albumin for 60 min on ice to allow ligand engagement onto its cognate receptor. The cells were transferred to 37 °C, and at defined time periods, samples were fixed in ice-cold acetone, air-dried, and mounted

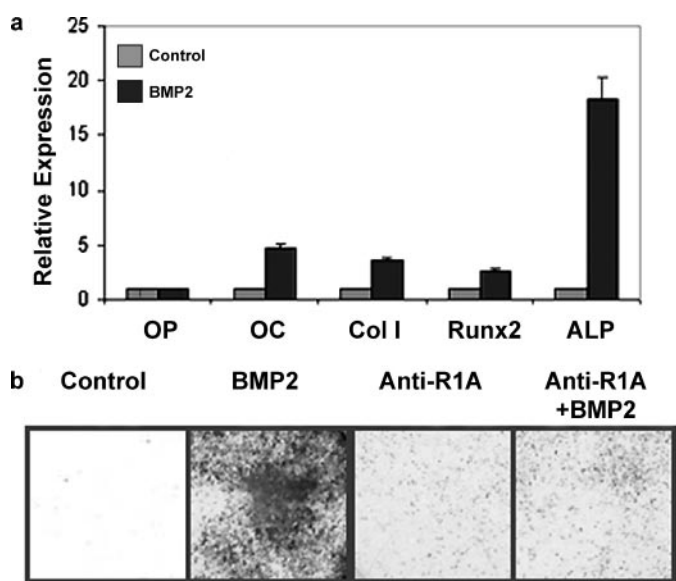


FIGURE 1. BMP2-induced expression of osteoblast markers in C2C12 cells. C2C12 cells were untreated or treated with anti-BMPRIA for 2 h and then grown in the presence of BMP2 (300 ng/ml) for 6 days. mRNA expression of osteoblast markers osteocalcin (OC), osteopontin (OP), Runx2, collagen type I (Col I), and ALP was measured by real-time reverse transcription-PCR (a). Data presented are mean \pm fold increase in gene expression from three experiments \pm S.E.; ALP activity was detected histochemically (b).

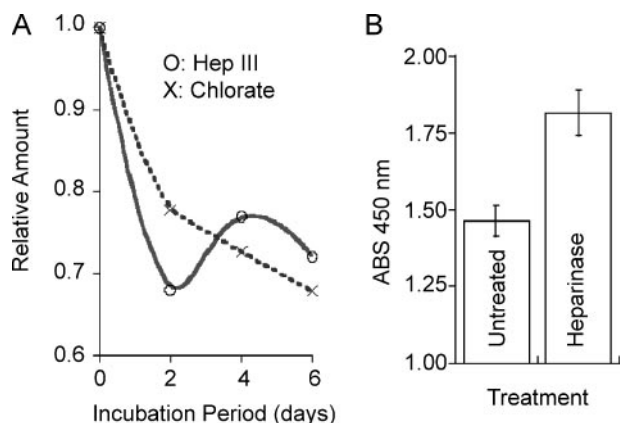


FIGURE 2. Determination of GAG chains. A, GAG Levels. C2C12 cells were treated with sodium chlorate or heparinase III (Hep III) for 0–6 days. At the termination of the assay, cells were isolated, incubated with DMB, and read at 520 nm. Samples were normalized to protein (absorbance, 520/ μ g of protein) and presented as the amount present relative to untreated controls. Results shown are those obtained from a representative experiment. B, assessment of heparinase cleavage. C2C12 cells were treated with heparinase III and subsequently incubated with anti- Δ -heparan antibody, which specifically detects the stubs remaining following heparinase cleavage; bound antibody was detected with a horseradish peroxidase secondary antibody conjugate. Samples were read at 450 nm. Note the increased antibody binding following heparinase III digestion. ABS, absorbance.

in ProLong Gold antifade reagent (Molecular Probes). To reduce surface HSPGs, cells were treated with heparinase III in serum-free medium (2 sigma units/ml) for 2 h at 37 °C, prior to incubation with FL-BMP2 ligand.

RESULTS

Reduction of Cell Surface HSPG Synthesis or GAG Chain Removal Enhances BMP2-induced ALP—First, we confirmed previous reports that BMP2 can direct C2C12 cell differentiation into an osteoblast lineage (15). When treated with 300

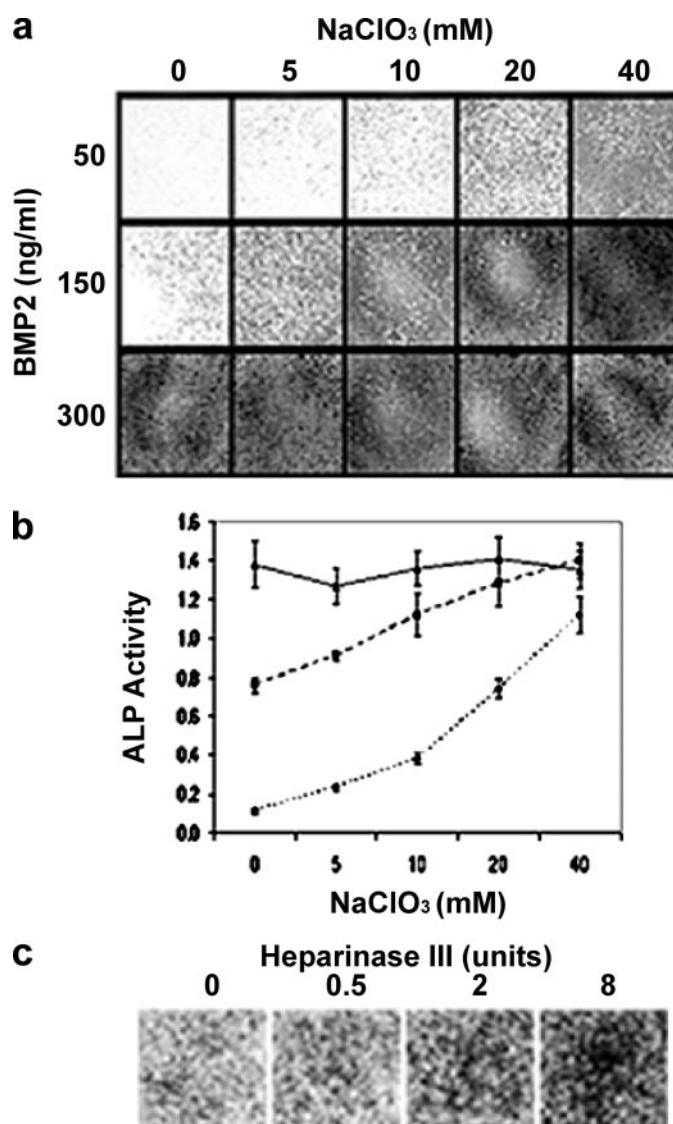


FIGURE 3. GAG chain removal enhances BMP2-induced ALP activity. C2C12 cells were pretreated with sodium chlorate for 48 h (a and b) or heparinase III for 2 h (c) and then incubated with BMP2 for 6 days in the continuous presence of sodium chlorate or heparinase III. Culture medium was replaced every 2 days, supplemented with BMP2, heparinase III, or sodium chlorate as indicated. ALP activity was detected histochemically (a and c) or quantitatively using soluble substrate *p*-nitrophenylphosphate (b, ▲, BMP2 300 ng/ml, ■, BMP2 150 ng/ml, ◆, BMP2 50 ng/ml, data represent mean \pm S.E.).

ng/ml BMP2 for 6 days, C2C12 cells expressed osteoblast markers including osteocalcin, collagen type I, Runx2, and ALP as measured by semiquantitative real-time reverse transcription-PCR (Fig. 1a). We observed no changes in osteopontin expression. BMP2 also induced robust ALP activity, as shown histochemically (Fig. 1b). BMPRIA has been reported to be critical for BMP2-mediated osteoblast differentiation in C2C12 cells (17, 18). When C2C12 cells were pretreated with antibodies against BMPRIA before the addition of BMP2, no ALP activity was detected, nor was any expression of osteoblast markers osteocalcin, collagen type I, Runx2, or ALP observed (Fig. 1b).

To determine the effect of GAG side chains on BMP2 signaling, cells were treated with sodium chlorate, which blocks sulfation of GAG chains on all cell surface proteoglycans including heparan sulfate, chondroitin sulfate, dermatan sulfate, and

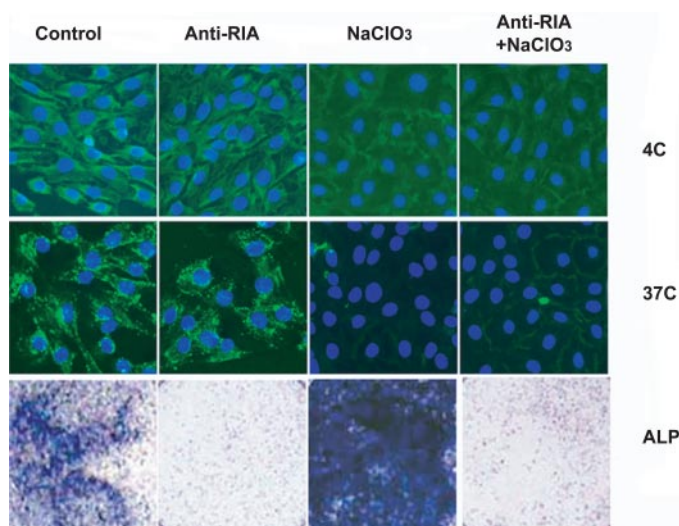


FIGURE 4. HSPGs bind BMP2 and mediate BMP2 internalization. C2C12 cells were untreated (control) or pretreated with 20 mM sodium chlorate for 48 h and/or anti-BMPRIA (*anti-RIA*) for 2 h. The cells were incubated with BMP2 prelabeled with mouse anti-BMP2 (30 min, on ice) and then fixed to detect surface bound BMP2 (*upper panel*) or transferred to 37 °C for 1 h (*middle panel*) to allow BMP2 internalization. BMP2 was detected with anti-mouse IgG (green), and cell nuclei were detected with 4',6-diamidino-2-phenylindole (blue). Subgroups of cells were grown for 6 days, and ALP activity was assessed histochemically (*bottom panel*).

keratan sulfate. Chlorate treatment reduced cell surface GAG chain sulfation levels over the 6-day treatment, as assessed by DMB binding (Fig. 2A).

Treatment of cells with heparinase III, which specifically cleaves HSPG GAG chains (18), reduced GAG chain levels as demonstrated by DMB staining. In these experiments, GAG chain levels were reduced at day 2 but exhibited a slight recovery at days 4 and 6 (Fig. 2A). Heparinase III cleavage was also established by immunological analysis (see "Materials and Methods"), which specifically detects heparinase cleaved stubs (Fig. 2B).

Reduction of GAG chain sulfation or HSPGs increased BMP2-induced ALP activity dose-dependently (Fig. 3, *a–c*). This indicated that GAG chain sulfation and cell surface HSPGs play a role in regulating ALP expression. However, ALP activity was dependent on BMP2 concentration. With BMP2 at 300 ng/ml, ALP activity remained stable, regardless of the concentration of sodium chlorate, *i.e.* regardless of the level of GAG chain sulfation at the cell surface.

HSPGs Mediate BMP2 Internalization—Since BMP2 has a heparin-binding domain, we investigated the consequence of BMP2 binding to HSPGs at the cell surface. C2C12 cells were pretreated with sodium chlorate and/or with antibodies against BMPRIA to block specific BMP receptors. The cells were subsequently treated with BMP2 preincubated with anti-BMP2 at 4 °C for 30 min to allow BMP2 binding to the cell surface. After unbound BMP2 was washed out, cells were incubated at 37 °C for 1 h to monitor BMP2 internalization. A subgroup of cells were grown for 6 days, and ALP activity was detected in those cells. When GAG chain *sulfation* was decreased by sodium chlorate, BMP2 binding capacity was reduced significantly, whereas there was no change in BMP2 binding when BMPRIA was blocked. These results suggest that the major component of

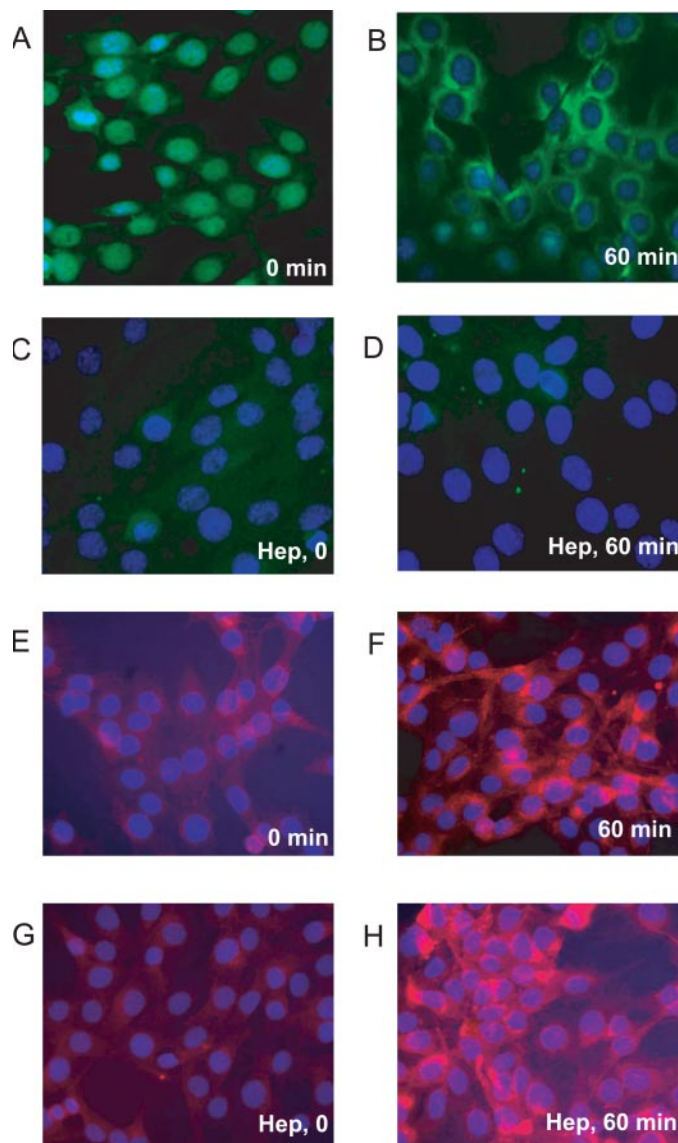


FIGURE 5. Internalization of BMP2 and Transferrin. C2C12 cells were untreated (A, B, E, and F) or treated with heparinase III (2 h, 37 °C; C, D, G, and H). The cells were incubated with FL-BMP2 (A–D; green fluorescence) or Alexa Fluor transferrin (E–H; red fluorescence) on ice, subsequently incubated for 0 or 60 min at 37 °C, and then viewed by fluorescence microscopy. Note that heparinase III digestion blocks BMP2 internalization (D) but has no effect on transferrin (H).

BMP2 binding capacity was due to GAG chain modification on HSPGs or other proteoglycans rather than BMP receptors. BMP2 binding capacity was further reduced to background levels when cells were treated with sodium chlorate and BMPRIA antibodies (Fig. 4). After 1 h at 37 °C, internalized BMP2 was discernable as cytoplasmic aggregates of various sizes. BMP2 internalization was seen only in cells with sulfated GAG chains intact, independent of receptor occupancy. In cells with reduced GAG chain sulfation, BMP2 internalization was not discernable (Fig. 4, *middle panel*). However, signaling was enhanced, as indicated by increased ALP induction (Fig. 4, *bottom panel*). BMP2 internalized through BMP2-receptor complexes, in the absence of GAG chain sulfation, was extremely low and undetectable by fluorescence microscopy, suggesting that BMP2 internalization may not be necessary for signaling.

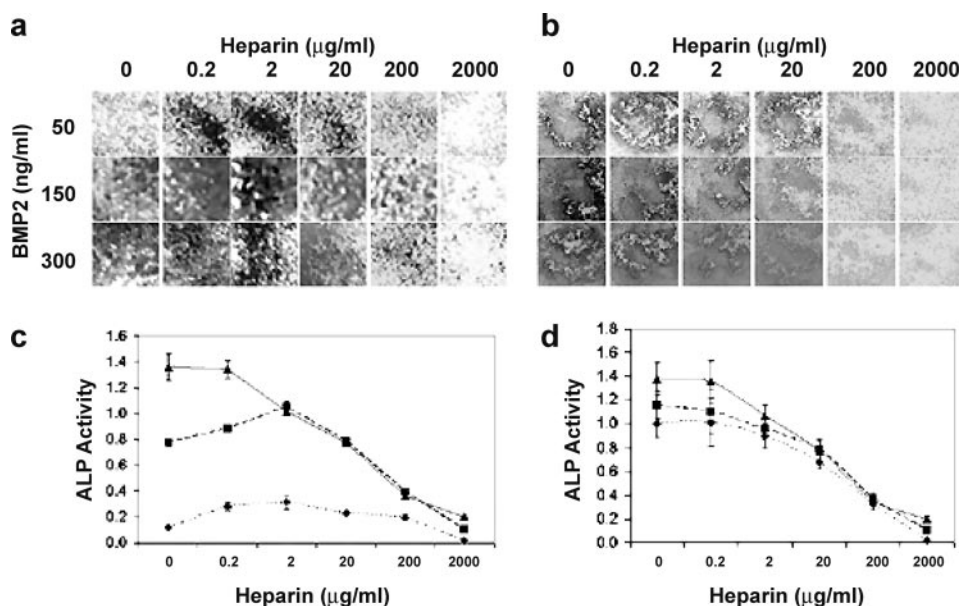


FIGURE 6. **Heparin modulates BMP2 osteogenic activity.** C2C12 cells were cultured for 6 days with cell surface HSPGs intact (*a* and *c*) or reduced by sodium chlorate treatment (*b* and *d*) in the presence of exogenous heparin (0–2000 $\mu\text{g/ml}$). ALP was measured at day 7 histochemically (*a* and *b*) or quantitatively using soluble substrate *p*-nitrophenylphosphate (*c* and *d*; \blacktriangle , BMP2 300 ng/ml; \blacksquare , BMP2 150 ng/ml; \blacklozenge , BMP2 50 ng/ml; data represent mean \pm S.E.).

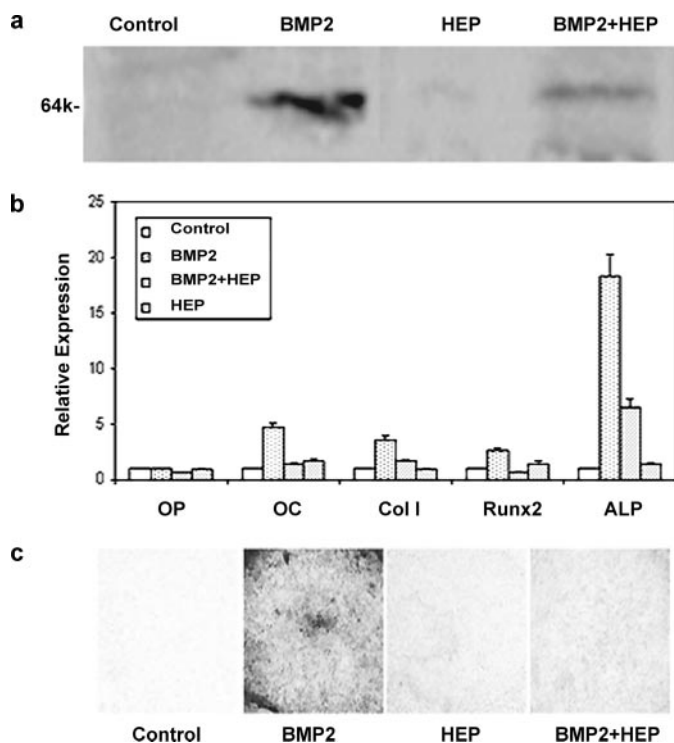


FIGURE 7. **High heparin concentrations inhibit BMP2 signaling.** C2C12 cells were incubated with BMP2 (300 ng/ml) in the absence or presence of soluble heparin (HEP; 2 mg/ml). Phosphorylated Smad1 (*pSmad1*) was detected on immunoblots after 1 h (*a*). OP, osteopontin; OC, osteocalcin. Expression of osteoblast markers was measured by real-time PCR after 6 days (*b*; data: mean \pm S.E.), whereas mineralization was assessed by Alizarin red staining at 4 weeks (*c*).

For the studies described above, BMP internalization was monitored by preincubating BMP2 with a specific BMP2 monoclonal antibody and followed by internalization of the BMP2-antibody complex. Native BMP2 is a dimer of molecular

mass of ~ 26 kDa, whereas the BMP2-antibody complex has a molecular mass of ~ 190 kDa ($26 + 160$). To confirm that internalization of the BMP2-antibody complex is the same as BMP2 alone, fluorescein-labeled BMP2 (BMP2-FL) was used (Fig. 5). The modified BMP2 was biologically active. To assess ligand internalization, cells were incubated with BMP2-FL on ice to allow ligand-receptor engagement and then incubated at 37°C for internalization. BMP2 localizes to the cell surface following incubation on ice. After incubation at 37°C , BMP2 localizes to small intracellular vesicles. Pretreatment of cells with heparinase III reduces BMP2 binding and internalization (Fig. 5, A–D), as observed with the BMP2-antibody complex (Fig. 4). As a positive control, cells were also incubated with fluorescent-tagged-

transferrin, an iron-binding protein whose internalization by cells has been well characterized (19). Transferrin binds and is internalized by C2C12 cells (Fig. 5, E and F). However, in contrast to BMP2, heparinase treatment has no effect on transferrin internalization (Fig. 5, G and H).

Exogenous Heparin Dose-dependently Modulates BMP2 Morphogenetic Bioactivity—We next examined the effects of exogenous heparin on BMP2 signaling with cell surface HSPGs intact. Heparin attenuated ALP expression at high concentrations (>2 $\mu\text{g/ml}$) with a minimal response at the lowest BMP concentration (50 ng/ml) tested (Fig. 6, *a* and *c*). In contrast, sodium chlorate treatment resulted in a similar reduction in BMP-induced ALP induction with increasing heparin concentration, regardless of the BMP2 concentration utilized for induction (Fig. 6, *b* and *d*). Heparin alone had no effect on ALP activity. In addition, treatment of cells with heparin or BMP2 did not have any significant effect on cell proliferation rate or cell viability, as demonstrated by a cell proliferation assay (data not shown).

The effects of HSPGs on BMP2 signaling were further investigated by examining activation of Smad1, a BMP2 signal transduction mediator. At a concentration of 2 mg/ml, heparin inhibited BMP2-mediated Smad1 phosphorylation (Fig. 7*a*) and blocked the increase of several osteoblast-specific markers including osteocalcin, Runx2, collagen type I, and ALP after 6 days (Fig. 7*b*), as well as mineralization after 4 weeks (Fig. 7*c*).

We postulate that at low concentrations, free heparin competes with cell surface HSPGs for BMP2 binding, whereas at higher concentrations (>2 $\mu\text{g/ml}$), it blocks BMP2-receptor interactions. On cells whose surface HSPGs have been depleted, heparin manifested only inhibitory effects in a dose-dependent manner (Fig. 6, *b* and *d*).

DISCUSSION

Although the role of BMPs as developmental morphogens is well established, the mechanism by which BMP activity gradients are generated is not well understood. HSPGs can bind BMPs and their secreted antagonists (20–22), and two models have been proposed for the role of HSPGs in BMP gradient formation. In one model, HSPGs move BMPs from cell to cell through restricted diffusion (12). In another model, HSPGs retain BMP antagonists such as noggin and establish inverse gradients of BMP activity (20). HSPGs have a relatively rapid turnover rate (23) and mediate internalization of a diverse array of extracellular ligands including FGF2 (24), Chordin (a BMP antagonist) (25), lipoproteins (26), and viral particles (27). Here we demonstrate that HSPGs can mediate the sequestration and internalization of BMP2, thus offering an additional mechanism by which HSPGs could regulate BMP morphogenetic gradients.

Our results suggest that HSPGs are abundant on the surface of C2C12 cells and, in addition to BMP signaling receptors, constitute a major part of the BMP2 binding capacity of the cells. When HSPG synthesis is reduced by sodium chlorate, the BMP2 binding capacity is decreased significantly, and no BMP2 internalization is detected. However, ALP activity is enhanced in the absence of HSPGs. These results suggest that HSPGs or other proteoglycans not only trap BMP2 on the surface but are also capable of mediating its internalization (Figs. 4 and 5). Following internalization, HSPGs are degraded rapidly by the lysosomal pathway or recycled back to the cell surface (23). It is not clear how BMP receptors mediate BMP internalization, but transforming growth factor- β receptors are reported to be internalized through both the lipid raft caveolar-mediated and clathrin-mediated early endosome pathways (18). Currently, we do not know which pathways mediate BMP2 internalization (28).

At low concentrations, exogenous heparin had little effect on BMP2 induction of ALP (Fig. 6). However, at higher concentrations, heparin exhibits an inhibitory effect as shown by decreased Smad1 phosphorylation and ALP induction (Fig. 7). This is consistent with previous reports demonstrating that heparin can inhibit BMP2 and BMP7 binding to its receptors (29, 30). Here we show an inhibitory effect of heparin on BMP2 signaling in C2C12 cells when surface HSPGs were reduced (Fig. 5). Similar effects of heparin have also been reported in osteoblast-like Saos-2 cells (31).

The methods used in our study to modify cell surface HSPGs were relatively nonspecific and did not allow us to determine whether the effects observed were due to the modification of all cell surface HSPGs or a consequence of specific changes within a single HSPG or a small subset of HSPGs. Recent work suggests that specific post-translational modifications of GAG side chains can lead to profound changes in the ability of HSPGs to modify morphogen gradients (20, 32). Further studies are needed to determine the specificity and sensitivity of HSPG modifications on BMP morphogen gradients and the role that endogenous enzymes, such as heparanases and sulfatases, play in this process.

In addition to insights into developmental regulation, our study has important clinical implications as well. We have dem-

onstrated that exogenous heparin, used at pharmacologic doses similar to those used in humans for the treatment of coagulopathies, profoundly inhibits BMP signaling. These data provide an explanation for the well known clinical observations that short term use of heparin (as a thromboprophylactic agent) after bone fracture can delay fracture healing (33) and that long term use of heparin can cause osteoporosis (34). Our findings also suggest that exogenous heparin or alteration of endogenous HSPGs could be exploited to modulate BMP-mediated heterotopic bone formation in conditions such as fibrodysplasia ossificans progressiva (FOP), a disabling genetic disease associated with a dysregulated BMP signaling pathway (35–37).

In summary, our results show that cell surface proteoglycans mediate BMP2 internalization and that a reduction of GAG chain sulfation and HSPGs increases BMP2 induction of osteogenic markers. Since HSPGs are spatially and temporally regulated during development (38), our findings imply that differentially expressed HSPGs play an important role in the formation of BMP activity gradients.

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