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# Dynamics and Cellular Localization of *Bmp2*, *Bmp4*, and *Noggin* Transcription in the Postnatal Mouse Skeleton

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## Abstract

Transcription of Bone Morphogenetic Proteins (BMPs) and their antagonists in precise spatiotemporal patterns is essential for proper skeletal development, maturation, maintenance, and repair. Nevertheless, transcriptional activity of these molecules in skeletal tissues beyond embryogenesis has not been well-characterized. In this study, we used several transgenic reporter mouse lines to define the transcriptional activity of two potent BMP ligands, *Bmp2* and *Bmp4*, and their antagonist *Noggin* in the postnatal skeleton. At 3–4 weeks of age, *Bmp4* and *Noggin* reporter activity was readily apparent in most cells of the osteogenic or chondrogenic lineages, respectively, while *Bmp2* reporter activity was strongest in terminally differentiated cells of both lineages. By 5–6 months, activity of the reporters had generally abated; however, the *Noggin* and *Bmp2* reporters remained remarkably active in articular chondrocytes and persisted there indefinitely. We further found that endogenous *Bmp2, Bmp4,* and *Noggin* transcript levels in postnatal bone and cartilage mirrored the activity of their respective reporters in these tissues. Finally, we found that the activity of the *Bmp2*, *Bmp4*, and *Noggin* reporters in bone and cartilage at 3–4 weeks could be recapitulated in both osteogenic and chondrogenic culture models. These results reveal that *Bmp2*, *Bmp4*, and *Noggin* transcription persists to varying degrees in skeletal tissues postnatally, with each gene exhibiting its own cell-type specific pattern of activity. Illuminating these patterns and their dynamics will guide future studies aimed at elucidating both the causes and consequences of aberrant BMP signaling in the postnatal skeleton.

### Keywords

*Bmp2*; *Bmp4*; *Noggin*; bone; cartilage

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Authors' roles

Study design and conduct: SKP. Data collection and analysis: SKP. Data interpretation: SKP and DPM. Drafting of manuscript: SKP. Revising and approving final version of manuscript: SKP and DPM. SKP and DPM share responsibility for the integrity of the data analysis.

Supplemental Data

This article includes supplemental methods, a supplemental table, and three supplemental figures.

## Introduction

Tight control of Bone Morphogenetic Protein (BMP) signaling is required for proper skeletal development, maturation, maintenance, and repair (1, 2). A fundamental means by which control over BMP signaling is achieved is the transcription of individual ligands in precise spatiotemporal patterns (3). This is well illustrated by the prototypical BMP ligands *Bmp2* and *Bmp4*, both of which are transcribed in widespread, yet distinct patterns during embryonic development. Their transcription, in turn, is governed by constellations of *cis*-regulatory sequences scattered across vast non-coding regions, also known as gene deserts (4). These regulatory sequences drive tissue-specific transcription of *Bmp2* and *Bmp4* in the skeleton, among other organs. Moreover, the consequences of their mis-regulation in skeletal tissues are severe (5–13).

Control of BMP signaling can also be achieved via extracellular ligand antagonists. *Noggin* is one such inhibitor, with specificity for *Bmp2*, *Bmp4* and closely related BMP family members. Similar to these two ligands, *Noggin* is transcribed in many tissues during embryogenesis, including those of the skeleton. Comparatively little is known about the transcriptional control of *Noggin*; however, similar to *Bmp2* and *Bmp4*, its mis-regulation in the skeleton also has devastating consequences (9, 12, 14–17).

Transgenic reporters are useful tools for characterizing the transcriptional output of specific genes in cellular detail over time. This is especially true of reporters that have been targeted to a native gene locus via homologous recombination in mouse embryonic stem cells. So-called “knock-in” reporters tend to faithfully recapitulate endogenous gene transcription *in vivo*, making it possible to follow cell-type specific transcription of a gene in any organ or tissue throughout development and beyond. Effective reporters for this purpose can also be generated through non-targeted insertion of modified bacterial artificial chromosomes (BACs). These typically include a reporter cassette in place of the gene of interest, along with up to several hundred kilobases of endogenous flanking sequence. In addition to highlighting transcriptional activity, BAC reporters are useful for mapping *cis*-regulatory sequences (4).

Knock-in or BAC-based transgenic reporters have been generated for several BMP ligands and their inhibitors, including *Bmp2*, *Bmp4*, and *Noggin* (14, 18–20). Their activity in the skeleton during embryogenesis has been well documented, providing important information regarding the spatial and temporal specifications of BMP signaling in early skeletal development, as well as the location of key *cis*-regulatory sequences. In contrast, activity of these same reporters in the postnatal skeleton has been characterized on a very limited basis (17, 21–25), despite the critical role of BMP signaling in skeletal maturation, maintenance, and repair, as well as the therapeutic benefits of manipulating BMP signaling in adults. We have assembled a collection of knock-in and BAC-based reporter transgenes for *Bmp2*, *Bmp4*, and *Noggin*, and here we characterize their activity in the postnatal skeleton.

## Materials and Methods

### Transgenic reporter mice

The *Bmp2* and *Bmp4* BAC reporter lines used in this study were generated in our lab (18, 19), while the *Bmp4* and *Noggin* knock-in reporter lines were generated elsewhere (14, 20). All of the lines were maintained on mixed genetic backgrounds, typically by breeding a heterozygous male with a wild-type CD1 or B6/D2-F1 female. Test-crosses and X-gal staining of resulting embryos confirmed the continuity of previously-reported expression patterns with each successive generation (Supplemental Fig. 1A). Only male offspring were used for these experiments. All animal usage was in accordance with policies and protocols established by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center.

### Isolation and culture of primary cells

All primary cells were harvested from femurs of male transgenic mice at 3–4 weeks of age. Chondrocytes were isolated from cartilage of the proximal epiphysis, which lacks a secondary ossification center (26). Briefly, the epiphysis was removed with a pair of tweezers and incubated in $Ca^{2+}$- and $Mg^{2+}$-free HBSS with 0.2% bacterial collagenase (Sigma, St. Louis) for 3–4 hours at 37°C to release chondrocytes from their extracellular matrix. BMSCs were isolated from femurs by cutting off both ends and flushing out the contents of the remaining diaphyseal shaft via brief high-speed centrifugation. Chondrocytes or BMSCs from multiple animals were pooled and plated at a density of $1 \times 10^5$ cells/$cm^2$ in 24-well tissue-culture-treated plates with complete media consisting of DMEM and the following components: 10% FBS, 4.5mg/ml D-glucose, 100 units/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml Fungizone (Life Technologies, Carlsbad), 2 mM L-alanyl-L-glutamine, and 2mM sodium pyruvate. Cultures were incubated at 37°C in a humidified chamber with 5% $CO_2$ and media was replaced every 2–3 days. Beginning at confluence, the complete media was supplemented with 100μg/ml ascorbate-2-phosphate and 2mM sodium phosphate (pH 7.4) to support mineralization.

### X-gal staining and histology

Bacterial β-galactosidase (*LacZ*) activity was detected in postnatal femurs and cultured cells via X-gal staining. Briefly, the cells or femurs were pre-fixed with 10% neutral-buffered formalin (NBF), equilibrated in wash buffer (100mM sodium phosphate, pH 7.4; 2mM magnesium chloride; 1% sodium deoxycholate; 1% Igepal CA630), and incubated with staining solution (wash buffer plus 0.5mg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactoside, 4mM potassium ferricyanide, and 4mM potassium ferrocyanide). Cultured cells were pre-fixed at room temperature for 10 minutes and stained overnight at 37°C, while femurs were pre-fixed for 1 hour at 4°C and stained overnight at room temperature or 4°C to increase the sensitivity or specificity of the assay, respectively.

Stained cultured cells and femurs were rinsed with PBS to remove all traces of staining solution and post-fixed. Cultured cells were fixed in 10% NBF for one hour at room temperature and femurs were fixed at 4°C for 48 hours in either 10% NBF or 100% ethanol. Femurs fixed in NBF were subsequently decalcified for two weeks in 10% EDTA, while

those fixed in ethanol were cleared for two weeks in 2% potassium hydroxide. Decalcified femurs were embedded in paraffin, sectioned at 10μm increments, and counter-stained with Nuclear Fast Red. Cultured cells were counter-stained in some cases with 0.1% Alizarin Red solution (pH 4.2) for 10 minutes at room temperature to highlight calcified nodules. Cells and cleared femurs were transferred to glycerol for imaging and permanent storage.

### Nucleic acid isolation and RT-qPCR

Total RNA and genomic DNA (gDNA) were collected from femurs and tails, respectively, of up to 10 male CD1 mice at 3–4 weeks or 5–6 months of age. Briefly, femurs were dissected in cold PBS, after which the proximal epiphyses were removed with a pair of forceps, pooled, and set aside. Next, the ends of the femurs were removed with a pair of scissors, the marrow was flushed out by high-speed centrifugation, and the remaining cortical bone pieces were pooled. The pooled cartilage and bone samples were then transferred to TriZol (Life Technologies, Carlsbad) and homogenized on ice using a rotor-stator homogenizer. After phase separation with chloroform, the crude RNA was further purified using the RNeasy Micro kit (Qiagen, Valencia). Genomic DNA was isolated from tail snips using the DNeasy Blood & Tissue kit (Qiagen). Quality and quantity of the nucleic acids were assessed via NanoDrop (Thermo Scientific, Waltham) and agarose gel electrophoresis. For each sample, 100 nanograms of total RNA were reverse-transcribed into cDNA using SuperScript VILO MasterMix (Life Technologies), according to the manufacturer's instructions. Matching no-RT control samples were generated in parallel. The cDNA samples, their corresponding controls, and approximately 100ng of gDNA were then each diluted 10-fold into 200μl of $H_20$.

Quantitative PCR was carried out using SsoAdvanced SYBR Green Supermix on a CFX96 Real-Time PCR Detection System (BioRad, Hercules) according to the manufacturer's instructions. Primers for *Bmp2*, *Bmp4*, *Noggin*, *LacZ*, *Polr1a*, and *Polr3b* (Supplemental Table 1) were designed using Primer3 (27) and validated on an 8-point standard curve generated with two-fold serial dilutions of gDNA. All primer sets yielded $R^2$ values greater than 0.9, with amplification efficiencies in the range of 95–105%; moreover, they all gave rise to a single amplicon as indicated by their melt curves. Each primer pair was used to amplify 5μl of gDNA, cDNA, and no-RT control from each sample in duplicate. Relative normalized expression was calculated from the resulting $C_t$ values as follows. First, the average $C_t$ value for each target in each cDNA sample was transformed via the $2^{\Delta Ct}$ method into a relative quantity using the average $C_t$ value from the corresponding gDNA sample as a reference. This step relates expression to genomic copy number, facilitating direct comparisons between different targets in the same cDNA sample. Next, the relative quantities of *Bmp2*, *Bmp4*, *Noggin*, and *LacZ* were normalized to the geometric mean of the relative quantities of *Polr1a* and *Polr3b* via the $2^{\Delta\Delta Ct}$ method, permitting comparisons across samples. All calculations, including standard deviation, were performed using the CFX Manager software (BioRad). Expression of a gene in a given cDNA sample was considered undetectable by our methods if the relative quantity did not differ by more than 10-fold from that of its corresponding no-RT control.

## Results

We assessed the transcription of *Bmp2*, *Bmp4*, and *Noggin* in the postnatal skeleton using six reporter transgenes (Fig. 1). Four of these were BAC-based constructs and two were targeted knock-in alleles. The BAC reporters contain the complete *Bmp2* or *Bmp4* gene, along with approximately 200 kilobases of non-coding flanking sequence in primarily the 5′ or the 3′ direction. This design captures the activities of various distant non-coding enhancers around either *Bmp2* or *Bmp4* (18, 19). The knock-in reporters for *Bmp4* and *Noggin* have been previously described (14, 20) and can be studied in heterozygous animals, which are viable in both cases and exhibit no overt skeletal abnormalities. For each reporter, the coding sequence of the endogenous gene has been disrupted by a bacterial *LacZ* cassette so that beta-galactosidase is expressed in place of functional *Bmp2*, *Bmp4*, or *Noggin*.

### Bmp2, Bmp4, and Noggin are persistently transcribed in the postnatal skeleton

To characterize reporter activity on a gross level, we performed whole-mount X-gal staining on various skeletal elements from transgenic mice at 3–4 weeks and 5–6 months of age (Fig. 2A and Supplemental Fig. 1B). At 3–4 weeks, the 5′ *Bmp2* BAC was strongly active in both bone and cartilage, while activity of the 3′ *Bmp2* BAC was completely undetectable in either tissue. The 5′ *Bmp4* BAC was likewise much more active postnatally than its 3′ counterpart, albeit with some important differences. Specifically, activity of the 5′ *Bmp4* BAC was restricted to bone (Fig. 2A), while activity of the 3′ *Bmp4* BAC was detectable in tendons up to their sites of insertion (Fig 2A and Supplemental Figure 1B). In agreement with the 5′ *Bmp4* BAC, the *Bmp4*-LacZ knock-in reporter was active in bone, but not cartilage. The *Nog*-LacZ reporter, by contrast, was highly active in cartilage compared to bone.

At 5–6 months of age, activity of the 5′ *Bmp2* BAC and *Nog*-LacZ reporters in cartilage was still remarkably strong (Fig. 2A and Supplemental Fig. 1C). In contrast, activity of the 5′ *Bmp2* BAC, the 5′ *Bmp4* BAC, and the *Bmp4*-LacZ reporters in bone was diminished. The 3′ *Bmp2* and *Bmp4* BACs continued to exhibit no activity in either skeletal tissue, although the 3′ *Bmp4* BAC remained highly active in tendons (Supplemental Fig. 1C). These patterns of reporter activity persisted in mice up to 1–2 years of age (data not shown). Taken together, these results reveal that *Bmp2*, *Bmp4*, and *Noggin* remain transcriptionally active in the skeleton beyond embryogenesis, with *Bmp2* and *Noggin* transcription persisting indefinitely in adult cartilage. Moreover, the inactivity of the 3′ *Bmp2* BAC – despite inclusion of several kilobases of promoter – indicates that *Bmp2* transcription in the postnatal skeleton requires distant upstream regulatory sequences present only in the 5′ BAC.

### Bmp2, Bmp4, and Noggin are transcribed in distinct cell-type specific patterns postnatally

To pinpoint the precise cell populations in which reporters for *Bmp2*, *Bmp4*, and *Noggin* are most active postnatally, we examined sections from whole-mount X-gal stained femurs. At 3–4 weeks of age, the *Bmp2*, *Bmp4*, and *Noggin* reporters each had cell-type-specific patterns of activity with varying degrees of overlap (Fig. 2B). The 5′ *Bmp2* BAC was active in hypertrophic sub-populations of chondrocytes in both growth plate and articular cartilage (Fig. 2B, *top row, left two panels*). The 5′ *Bmp2* BAC was also active in osteocytes

embedded in cortical bone, but not in osteoblasts within the primary spongiosa (Fig. 2B, *top row, right two panels*). The 5′ *Bmp4* BAC, in contrast, was active in both osteocytes and osteoblasts, but not chondrocytes (Fig. 2B, *middle row*). Finally, the *Noggin-LacZ* reporter was active in chondrocytes at various stages of maturation in both growth plate and articular cartilage, but not in osteoblasts or osteocytes (Fig. 2B, *bottom row*). These results indicate that transcription of *Bmp4* and *Noggin* in the skeleton during postnatal development is lineage-dependent, with *Bmp4* primarily transcribed by cells of the osteogenic lineage and *Noggin* primarily transcribed in cells of the chondrogenic lineage. In contrast, *Bmp2* transcription is linked to maturational status, with cells of both lineages exhibiting increased transcriptional activity as they progress towards terminal differentiation.

At 5–6 months of age, activity of the 5′ *Bmp2* BAC was restricted to chondrocytes at the articular surface (Fig. 2C), a sub-population from which it was excluded during earlier postnatal development (Fig. 2B, *top row, left panel*). This shift in activity from the deeper to the more superficial, permanent chondrocyte layers occurred between two and three months of age (data not shown), coinciding with the onset of skeletal maturity. The *Nog-LacZ* reporter was also active in this same cell population, albeit to a lesser extent (Fig. 2C). These patterns of activity persisted in mice up to 1–2 years of age with little alteration (data not shown). Thus, *Bmp2* and *Noggin* are transcribed throughout adulthood in a sub-population of articular chondrocytes distinct from that in which *Bmp2* is transcribed during postnatal development.

## Bmp2, Bmp4, and Noggin mRNA levels mirror activity of their reporters in postnatal skeletal tissues

Next, we examined endogenous *Bmp2*, *Bmp4*, and *Noggin* expression in postnatal skeletal tissues. Using total RNA isolated from epiphyseal cartilage or cortical bone derived from femurs of 3–4 week-old or 5–6 month-old wild-type mice, we measured relative transcript levels of the three genes by RT-qPCR. To facilitate direct comparisons between different transcripts, we normalized RT-qPCR data for each gene to qPCR results obtained with the same primers on a pool of genomic DNA isolated from the same mice, in which the 3 genes were present in equimolar amounts. The most abundant transcript in bone at 3–4 weeks of age was *Bmp2*, followed by *Bmp4*, which in turn was nearly an order of magnitude higher than *Noggin* (Fig. 3A). By 5–6 months, the transcript levels of all 3 genes had decreased by about 2-fold (Fig 3A). In cartilage at 3–4 weeks of age, *Bmp2* and *Noggin* transcripts were present in roughly equal amounts and were far more abundant than *Bmp4* transcripts, which were barely detectable (Fig. 3B). Transcript levels of all three genes remained about the same in cartilage between 3–4 weeks and 5–6 months of age (Fig. 3B). Collectively, these results confirm that *Bmp2, Bmp4, and Noggin* transcript levels mirror the activity of their reporters in the postnatal skeleton.

## Bmp2, Bmp4, and Noggin are transcribed in cultured skeletal lineage cells and their precursors

Finally, we assessed *Bmp2*, *Bmp4*, and *Noggin* reporter activity in primary cultures of committed skeletal-lineage cells and their precursors. To this end, we isolated epiphyseal chondrocytes and bone marrow stromal cells (BMSCs) from femurs of 3–4 week-old mice

harboring the 5′ *Bmp2* BAC, 5′ *Bmp4* BAC, or *Nog*-LacZ reporters. We then cultured these for up to three weeks in media supplemented with ascorbate and inorganic phosphate. Both cell types secrete and mineralize a thick extracellular matrix under these conditions and express markers generally consistent with terminal osteogenic or chondrogenic differentiation (28, 29), (Supplemental Figure 2). Thus, these systems provide us with an opportunity to follow the dynamics of reporter activity in differentiating cells of both lineages.

When assayed via X-gal staining, activity of the 5′ *Bmp2* BAC, 5′ *Bmp4* BAC, and *Nog-LacZ* reporters was undetectable in both culture models prior to supplementation (Fig. 4A, *Day 0*). After supplementation, and concomitant with mineralization, the 5′ *Bmp2* BAC and *Nog-LacZ* reporters both exhibited robust activity in chondrocyte cultures, while only the 5′ *Bmp2* BAC reporter appeared active in BMSCs (Fig. 4A, *Days 7–21*). Closer inspection revealed that both the *Nog-LacZ* and 5′ *Bmp4* BAC reporters were indeed active in BMSCs at modest levels (Fig. 4B, *middle and bottom panels on right*); however, there was no detectable activity in 5′ *Bmp4* BAC transgenic chondrocyte cultures (Fig. 4B, *middle panel on left*). Interestingly, activity of the *Nog-LacZ* and 5′ *Bmp4* BAC reporters was located primarily within mineralized nodules of the BMSC cultures, whereas activity of the 5′ *Bmp2* BAC was also present in flattened cells at the periphery of the nodules. A similar pattern of activity was seen in chondrocyte cultures derived from 5′ *Bmp2* BAC mice. Finally, *Nog-LacZ* reporter activity localized to the periphery of mineralized nodules in chondrocyte cultures, in complete contrast to its pattern in BMSCs.

These results reveal that *Bmp2*, *Bmp4*, and *Noggin* are transcribed in cells harvested directly from the postnatal skeleton and cultured in monolayers under mineralization-inducing conditions. In general, their patterns of transcriptional activity are reminiscent of those seen in skeletal lineage cells *in vivo*. The chondrocyte cultures, in particular, exhibit robust *Bmp2* and *Noggin*–but not *Bmp4*–transcriptional activity. BMSCs, on the other hand, transcribe all three genes at more modest levels, suggesting they may be a mixture of both chondrogenic and osteogenic cells.

## Discussion

The presence of *Bmp2* and *Bmp4* in the postnatal skeleton has been appreciated since their discovery more than two decades ago (30). Subsequent immunohistochemical studies have shed some light on their expression patterns (13, 31–34), (Supplemental Fig. 3). To our knowledge, this is the first study to characterize their transcriptional activity in skeletal tissues beyond embryogenesis. Our results reveal that while both genes are transcribed by osteocytes embedded in cortical bone, their transcription patterns in the growth plate and trabecular bone compartments are completely different. This striking divergence may help explain why limb-specific deletions of *Bmp2* or *Bmp4* result in different skeletal phenotypes (11, 35), despite their extremely high homology and generally similar signaling abilities (36).

In contrast to *Bmp2* and *Bmp4*, reports on *Noggin* protein localization in the postnatal skeleton are scarce. Our results indicate it is robustly transcribed by most cells of the

chondrogenic lineage. This, in turn, suggests that cartilage is the primary source of *Noggin* in the postnatal skeleton, and that *Noggin*-mediated antagonism of BMP signaling is required in this tissue. Interestingly, conditional knock-out of *Noggin* in the skeleton using a human *Osteocalcin*-Cre driver results in mild postnatal osteopenia and may reflect the importance of its expression by cells of the osteogenic lineage, albeit at low levels (15). Future studies aimed at determining the relative importance of *Noggin* expression by chondrogenic or osteogenic cells in the postnatal skeleton are thus warranted.

The robust and persistent transcription of *Bmp2* by adult articular chondrocytes is consistent with the requirement of BMP signaling for articular cartilage maintenance (37). Moreover, a cell-autonomous role for *Bmp2* in promoting cartilage anabolism is well-supported by *in vitro* studies (38). On the other hand, the concurrent transcriptional activity of *Noggin* suggests that BMP signaling is antagonized in articular cartilage. Interestingly, immunohistochemical studies have detected *Noggin* (39), but not *Bmp2* protein in normal adult articular cartilage (40–42). Absence of the latter may be due to a repressive post-transcriptional regulatory mechanism (43, 44). Taken together, these results suggest that the potent anabolic activity of *Bmp2* must be carefully balanced in adult articular cartilage. Indeed, joint pathologies such as osteoarthritis may reflect a disruption of this delicate balance (45).

Our previous work demonstrated that the 3′ *Bmp2* BAC is active in bone during embryogenesis, mediated by a distant downstream enhancer termed ECR1 (19). Surprisingly, we were unable to detect activity of the 3′ *Bmp2* BAC reporter in the skeleton from 3–4 weeks of age and beyond, or in cells cultured from postnatal skeletal tissues (Fig. 2A and data not shown). Thus, the influence of ECR1 on *Bmp2* transcription in bone appears to be a transient embryonic phenomenon. Moreover, it is not recapitulated in primary osteoblast culture models derived from postnatal skeletal tissues. In contrast, the 5′ *Bmp2* BAC exhibits robust and persistent activity in chondrocytes during embryogenesis and throughout postnatal life. It is also active in osteogenic cells during postnatal development. Finally, the 5′ *Bmp2* BAC is active in differentiating cells of both lineages *in vitro*, thus making it amenable to studies aimed at dissecting distant upstream transcriptional regulatory mechanisms.

In conclusion, we have used some of the best available reporters for *Bmp2*, *Bmp4*, and *Noggin* to illuminate both the dynamics and cellular localization of their transcription in the postnatal skeleton. By pinpointing specific cell populations in which these three genes are transcribed, our results will guide future studies aimed at further elucidating the causes and consequences of BMP signaling in the skeleton during postnatal development and beyond.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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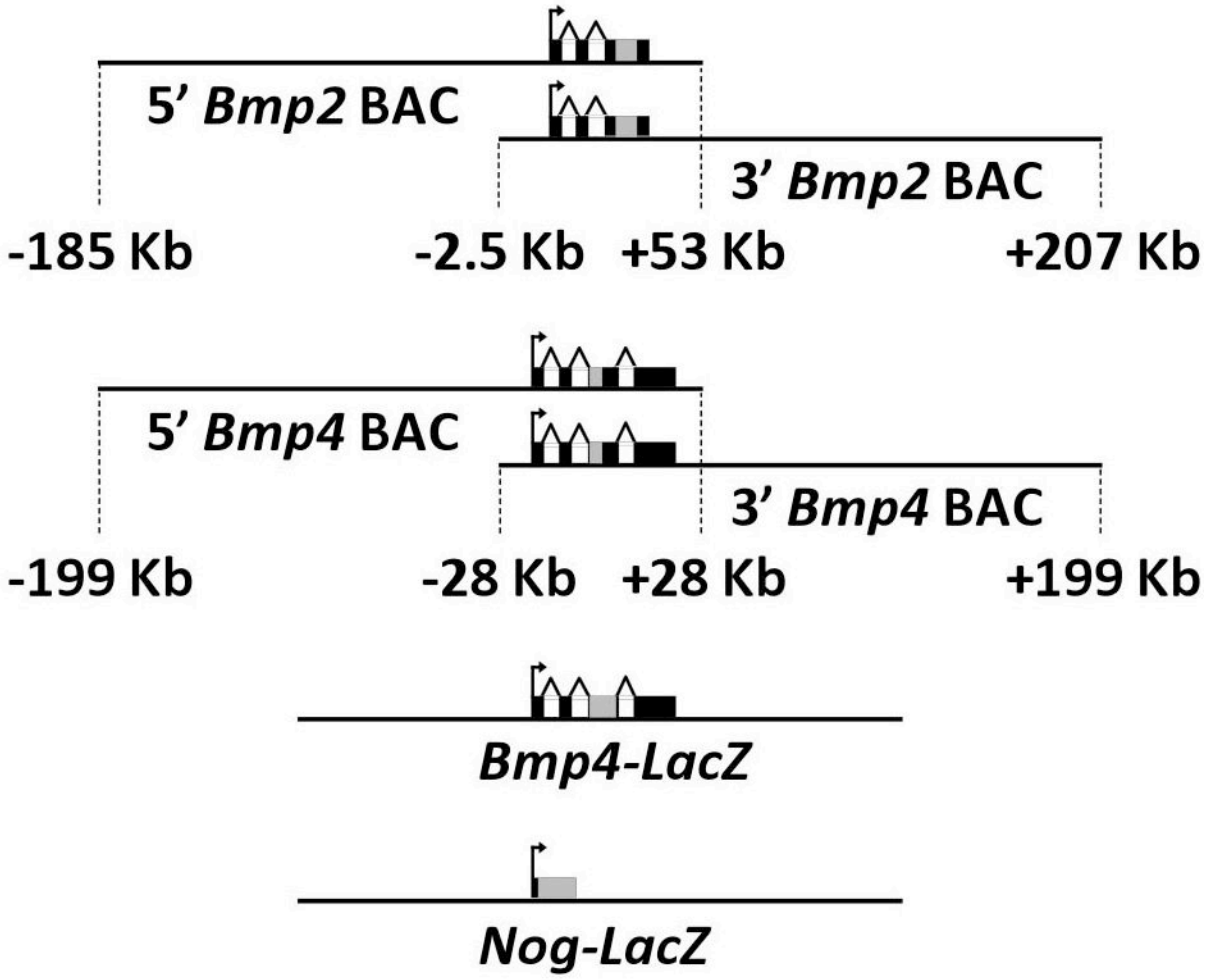


**Figure 1. Bmp2, Bmp4, and Noggin transgenic reporters**

Schematic diagrams of the six reporter transgenes used in this study are shown, including the location of the *LacZ* cassette (*gray rectangle*) with respect to the endogenous exons (*black rectangles*). For the BAC reporters, relative amounts of flanking non-coding sequence contained by the constructs are indicated.

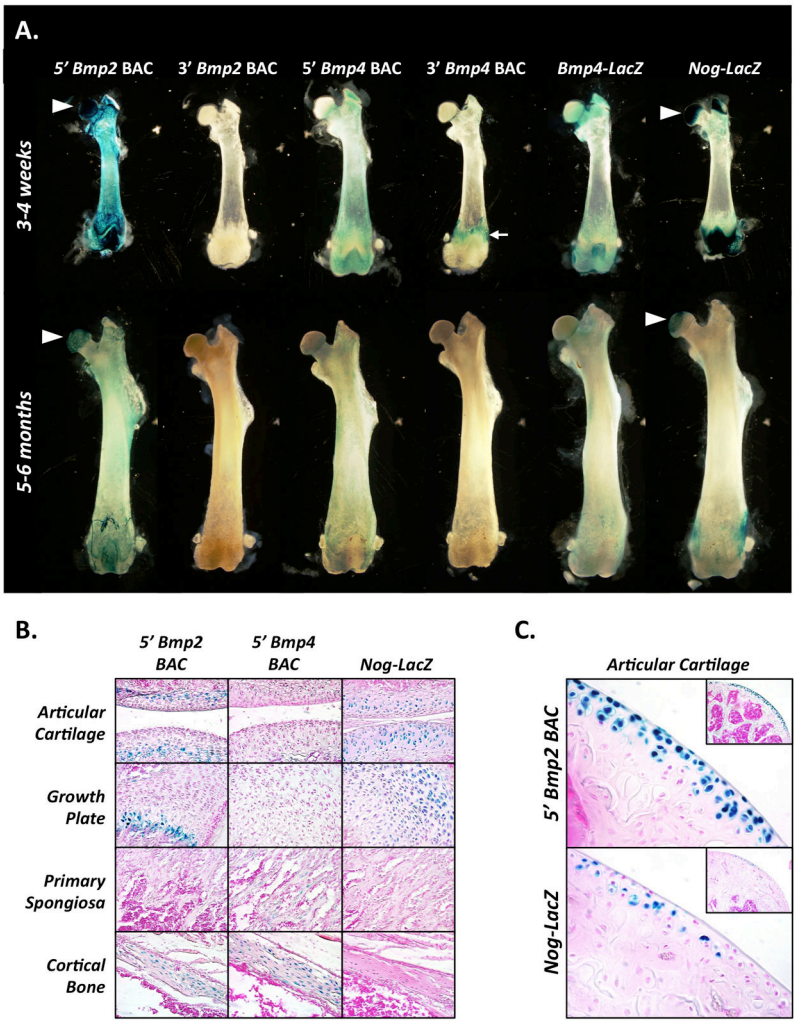


**Figure 2. Bmp2, Bmp4, and Noggin are dynamically transcribed in distinct cell-type specific patterns in the postnatal skeleton**

(A) Whole-mount X-gal-stained femurs from each of the six transgenic lines at points of peak postnatal growth (*3–4 weeks*) and maturity (*5–6 months*) are shown. Arrowheads indicate intensely-stained femoral heads of the 5′ *Bmp2* BAC and *Nog-LacZ* reporter lines. Tendon insertion staining in the 3′ *Bmp4* BAC reporter line is indicated by a white arrow. (B) Sections taken from whole-mount X-gal stained femurs of 5′ *Bmp2* BAC, 5′ *Bmp4* BAC, or *Nog-LacZ* mice at 3–4 weeks are shown with key skeletal cell populations highlighted, including chondrocytes (*articular cartilage, growth plate*), osteoblasts (*primary spongiosa*), and osteocytes (*cortical bone*). (C) Activity of the 5′ *Bmp2* BAC and the *Nog-LacZ* reporters in articular cartilage of the proximal femur at 5–6 months (*shown at low magnification in insets*).

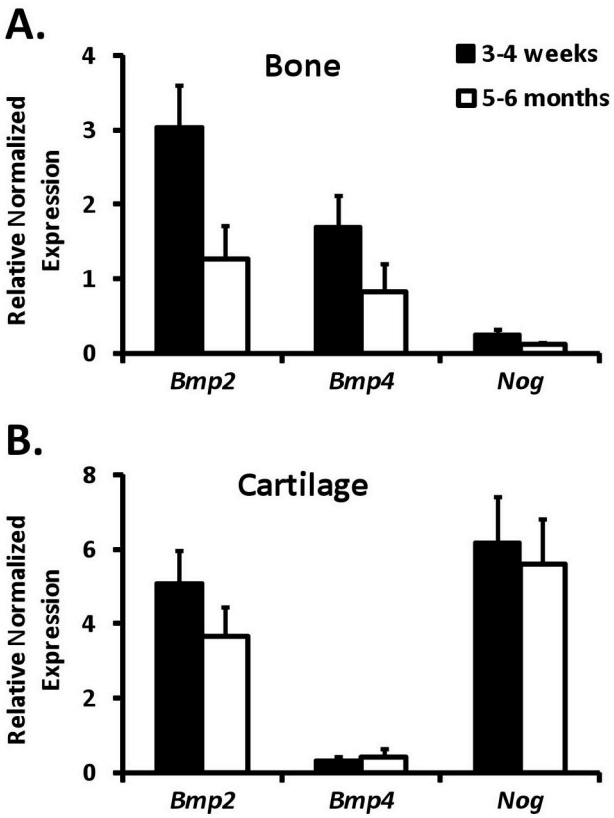


**Figure 3. Expression of endogenous Bmp2, Bmp4, and Noggin mRNA in postnatal skeletal tissues**

Relative transcript levels of *Bmp2*, *Bmp4*, and *Noggin* were measured by RT-qPCR using total RNA isolated from cortical bone (A) or epiphyseal cartilage (B) of femurs from wild-type mice at 3–4 weeks (*black bars*) or 5–6 months (*white bars*) of age. Graphed values represent the mean (± SD, n=3) expression level calculated for each transcript via the $2^{\Delta\Delta Ct}$ method, with genomic DNA isolated from the same mice serving as reference samples and *Polr1a* and *Polr3b* serving as internal reference genes.

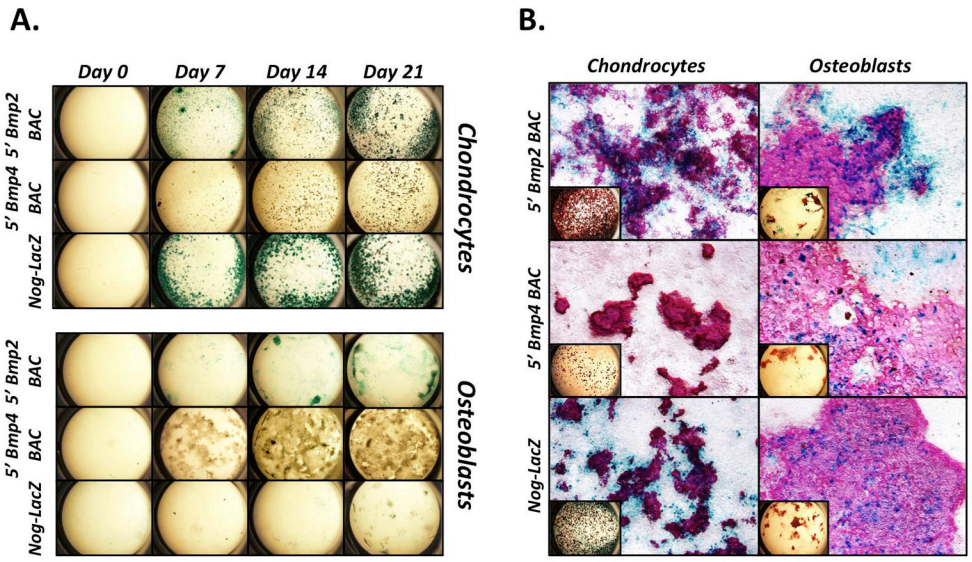


**Figure 4. Characterization of Bmp2, Bmp4, and Noggin transcription in cultured skeletal lineage cells and their precursors**

(A and B) Epiphyseal chondrocytes or BMSCs (*osteoblasts*) were isolated from the femurs of 3–4 week-old 5′ *Bmp2* BAC, 5′ *Bmp4* BAC, or *Nog-LacZ* mice, cultured with ascorbate and inorganic phosphate for up to 3 weeks, and stained with X-gal. (A) Time course showing a week-by-week progression of staining, beginning at the point when mineralization-supporting media was first added to the cultures (*Day 0*). (B) Day 21 cultures counter-stained with Alizarin Red to highlight mineralized nodules (*shown at low magnification in insets*).