Gradients in bone morphogenetic protein-related gene expression across the growth plate

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Abstract

In the growth plate, stem-like cells in the resting zone differentiate into rapidly dividing chondrocytes of the proliferative zone and then terminally differentiate into the non-dividing chondrocytes of the hypertrophic zone. To explore the molecular switches responsible for this two-step differentiation program, we developed a microdissection method to isolate RNA from the resting (RZ), proliferative (PZ), and hypertrophic zones (HZ) of 7-day-old male rats. Expression of approximately 29 000 genes was analyzed by microarray and selected genes verified by real-time PCR. The analysis identified genes whose expression changed dramatically during the differentiation program, including multiple genes functionally related to bone morphogenetic proteins (BMPs). BMP-2 and BMP-6 were upregulated in HZ compared with RZ and PZ (30-fold each, $P < 0.01$ and 0.001 respectively). In contrast, BMP signaling inhibitors were expressed early in the differentiation pathway; BMP-3 and gremlin were differentially

expressed in RZ (100- and 80-fold, compared with PZ, $P < 0.001$ and 0.005 respectively) and growth differentiation factor (GDF)-10 in PZ (160-fold compared with HZ, $P < 0.001$). Our findings suggest a BMP signaling gradient across the growth plate, which is established by differential expression of multiple BMPs and BMP inhibitors in specific zones. Since BMPs can stimulate both proliferation and hypertrophic differentiation of growth plate chondrocytes, these findings suggest that low levels of BMP signaling in the resting zone may help maintain these cells in a quiescent state. In the lower RZ, greater BMP signaling may help induce differentiation to proliferative chondrocytes. Farther down the growth plate, even greater BMP signaling may help induce hypertrophic differentiation. Thus, BMP signaling gradients may be a key mechanism responsible for spatial regulation of chondrocyte proliferation and differentiation in growth plate cartilage.

Journal of Endocrinology (2007) **193**, 75–84

Introduction

In mammals, longitudinal bone growth occurs primarily at the growth plate. The growth plate contains chondrocytes spatially organized in three distinct zones according to defined stages of differentiation. The resting zone, located closest to the epiphysis, contains stem-like cells which replicate slowly (Schrier *et al.* 2006) and generate the chondrocyte clones of the proliferative zone (Abad *et al.* 2002). In the proliferative zone, cells replicate at a high rate and the newly formed cells line up along the long axis of the bone, thus forming columns of cells (Kember & Walker 1971). In the lower part of the proliferative zone, cells stop dividing, and then increase in size and modify the extracellular matrix to form the hypertrophic zone. The hypertrophic cartilage is invaded by endothelial cells and bone cells from the metaphysis and subsequently remodeled into bone.

Thus, cells in the growth plate must differentiate from resting chondrocytes to become proliferative chondrocytes and then to become hypertrophic chondrocytes. To explore the molecular switches responsible for this two-step differentiation program,

we first developed a microdissection method that allowed for extraction of high-quality RNA from the resting (RZ), proliferative (PZ), proliferative–hypertrophic transition zone, and hypertrophic zone (HZ) of 7-day-old male rats. Expression of approximately 29 000 genes was analyzed in each zone by microarray.

This analysis identified genes whose expression levels changed dramatically during the differentiation program, including multiple genes functionally related to bone morphogenetic proteins (BMPs). BMPs belong to the transforming growth factor- β superfamily and were identified by their ability to induce ectopic bone formation (Urist 1965, Reddi 1981). They were later shown to be part of a complex system of paracrine factors required for the development of multiple organs, including heart, lungs, kidneys, gonads, eyes, and skeleton (Zhao 2003). Mice deficient in both BMP receptor-1a and -1b in cartilage lack the majority of skeletal elements that form through endochondral ossification (Yoon *et al.* 2005), thus demonstrating the importance of BMP signaling in skeletal development. Modulation of BMP signaling by BMP inhibitors is critical during early cartilage

formation as well as for correct patterning and outgrowth of limbs. In particular, mice deficient in *noggin* have multiple skeletal abnormalities including enlarged growth plates, presumably due to loss of opposition to BMP signaling (Brunet *et al.* 1998). Furthermore, loss of BMP antagonism by gremlin leads to defective patterning and outgrowth of the limbs (Khokha *et al.* 2003). In the growth plate, BMP signaling not only appears to promote hypertrophic differentiation (De Luca *et al.* 2001, zur Nieden *et al.* 2005), but may also promote earlier stages of chondrocyte differentiation (Kobayashi *et al.* 2005).

Since multiple BMP-related genes were identified by the microarray screening analysis and since the BMP system has important effects on growth plate chondrocytes, we further quantified the mRNA expression patterns of BMP-2, -3, -4, -6, -7, GDF-10, gremlin, chordin, BMP receptor-1a, -1b, -2 in the different zones of the growth plate, and the surrounding perichondrium and bone using quantitative real-time PCR.

Materials and Methods

Animal procedures and tissue processing

Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were maintained and used in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2003). All animals received standard rodent chow (Zeigler Bros, Gardners, PA, USA) and water *ad libitum*. The protocol was approved by the Animal Care and Use Committee, National Institutes of Child and Human Development, National Institutes of Health. Seven-day-old male rats ($n=5$ and $n=5-7$ for microarray and real-time PCR respectively) were killed by carbon dioxide inhalation, and proximal tibial epiphyses were rapidly excised, embedded in Tissue-Tek O.C.T. Compound (Electron Microscopy Sciences, Hatfield, PA, USA) and stored at -80°C for subsequent processing.

Growth plate microdissection

We chose to study 7-day-old animals because, at this age, the growth plate is relatively tall, facilitating microdissection into individual zones. Frozen longitudinal sections ($60\ \mu\text{m}$) of proximal tibial epiphyses were mounted on Superfrost Plus slides (Fisher Scientific, Chicago, IL, USA). Slides were thawed for 15 s and then placed in 70% ethanol, fixed in 100% methanol, washed in 95% ethanol, and stained in eosin (0.2% eosin, 0.5% acetic acid, 75% ethanol). Stained slides were washed in 70% ethanol, dehydrated in 100% ethanol, and then placed in xylene (each step for 1 min, at room temperature). Using an inverted microscope, razor blades, and hypodermic needles, growth plate sections, under a xylene droplet, were separated, based on histological hallmarks, into: epiphyseal cartilage, resting zone, proliferative zone, proliferative–hypertrophic transition zone, and

hypertrophic zone (Fig. 1). Epiphyseal cartilage contains single cells that are round and randomly oriented. RZ cartilage contains predominantly single chondrocytes that are flat and oriented in the same direction as the chondrocytes in the proliferative columns of PZ. In order to minimize cross-contamination between RZ and PZ, the uppermost part of the proliferative columns and the lowest part of RZ was discarded (Fig. 1). HZ chondrocytes were distinguished from PZ chondrocytes by their larger size. In addition, perichondrium and metaphyseal bone were collected from the same sections. Metaphyseal bone was obtained from a region of trabecular bone beginning approximately $100\ \mu\text{m}$ distal to the hypertrophic zone. Cortical bone was excluded from the dissected sample. For each zone, tissue dissected from both proximal tibias of one animal (21–33 sections) was pooled prior to RNA isolation. RNA isolation was performed as previously described except that one-tenth of every volume was used (Heinrichs *et al.* 1994). The final pellet was resuspended in $9\ \mu\text{l}$ diethylpyrocarbonate (DEPC)-treated water. For each animal, approximately 30–200 ng total RNA were extracted from every zone of the growth plate. The 28S/18S ratio was typically between 1.7 and 2.0 as assessed by a Bioanalyzer 2100 using RNA Pico Chips and version A.02.12 of the Bio Sizing software according to the manufacturer's instructions (Agilent Biotechnologies, Inc., Palo Alto, CA, USA).

Microarray analysis

For microarray analysis, 30–50 ng total RNA extracted from epiphyseal cartilage ($n=4$), RZ ($n=5$), PZ ($n=5$), proliferative–hypertrophic transition zone ($n=5$), HZ ($n=5$), and

Figure 1 Growth plate microdissection. Photomicrograph of a frozen longitudinal section ($60\ \mu\text{m}$) of proximal tibial epiphyses from 7-day-old rats used for microdissection. The section has been cut with a blade to separate the sample into epiphyseal cartilage, resting zone, proliferative zone, proliferative–hypertrophic transition zone, and hypertrophic zone. To minimize cross-contamination between the resting zone and the proliferative zone, we discarded a segment of cartilage containing the transition from resting to proliferative zone.

perichondrium ($n=5$) were amplified and labeled using the Two-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA P/N 900494) according to Expression Analysis Technical Manual, version 701021 Rev.5 (Affymetrix). Briefly, total RNA (30–50 ng) and diluted poly-A spike in controls (Affymetrix, P/N 900433) were denatured in the presence of T7-tagged oligo dT primers (Affymetrix, P/N 900480) and then reverse transcribed using Superscript II (Invitrogen) for 1 h. The second strand was synthesized by the addition of DNA polymerase I, *Escherichia coli* DNA ligase, RNase H, and incubation at 16 °C for 2 h. Anti-sense RNA was synthesized by incubation of double-stranded cDNA with nucleotides and T7 RNA polymerase (MEGAscript high yield transcription kit, Ambion, Inc., Austin, TX, USA) at 37 °C for 16 h. Unincorporated nucleotides were removed using the sample clean-up module (Affymetrix, P/N 900371). For the second round of cDNA synthesis, cRNA was denatured in the presence of diluted random primers at 70 °C for 10 min and then reverse transcribed using Superscript II (Invitrogen) for 1 h at 42 °C. The cRNA strand was removed by RNase H at 37 °C for 20 min. RNase H was then deactivated at 95 °C for 5 min. The cDNA was denatured in the presence of T7-Oligo-dT primers at 70 °C for 6 min and second strand synthesis was performed with *E. coli* DNA polymerase I at 16 °C for 2 h and T4 DNA polymerase at 16 °C for 10 min. Unincorporated nucleotides were removed using spin columns (sample clean-up module, Affymetrix). The synthesized cDNA was used for *in vitro* transcription using an *in vitro* transcription labeling kit (Affymetrix, P/N 900499). Fluorescently labeled cRNA was purified with sample clean-up module (sample clean-up module, Affymetrix) and fragmented before hybridization.

Rat Genome 230 2.0 GeneChip Arrays (Affymetrix; in total 29 arrays) were prehybridized at 45 °C for 10 min and hybridized at 45 °C for 16 h with a hybridization cocktail containing 15 µg fragmented and labeled cRNA in a hybridization oven (Affymetrix) at 60 r.p.m. for 16 h. After hybridization, the arrays were washed and stained in a Fluidics Station FS-400 (Affymetrix) using the EukGE-WS2v5 protocol and scanned using a GeneChip Scanner 3000.

Microarray signal values were background corrected and normalized (scaled) according to the microarray suite (MAS) 5 statistical algorithm using GeneChip Operating Software (Affymetrix). Gene lists of differentially expressed genes were generated using BRB-Array Tools Version 3.3.0 and revealed steep gradients across the growth plate for a number of genes related to BMP signaling according to the Kyoto encyclopedia of genes and genomes (KEGG) pathway database (http://www.genome.jp/dbget-bin/www_bget?path:rno04350). BMP-related genes that appeared to be expressed in zone-specific patterns by microarray analysis were further studied using real-time PCR. With one exception (BMP11b), genes that were not represented on the arrays (e.g. BMP-11, GDF-2, -5, -7, and SMAD-5, -7, -8) or did not have a majority of present calls, according to the MAS 5 statistical algorithm, in any of the zones/tissues (including BMP-5, -15, GDF-6, -8,

-9, -11, and -15) were excluded from further analysis. BMP-1 is not a true BMP and was therefore not included in the analysis (Kessler *et al.* 1996).

Real-time quantitative RT-PCR

For real-time PCR, 30–200 ng total RNA extracted from epiphyseal cartilage, RZ, PZ, proliferative–hypertrophic transition zone, HZ, and perichondrium and metaphyseal bone ($n=5–7$) were reverse transcribed using 200 U Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. The resulting cDNA solution was diluted 10–25 times and stored at –20 °C until used for real-time quantitative PCR.

Real-time quantitative PCR was performed using pre-prepared assays containing primers and specific intron-spanning FAM-labeled TaqMan probes provided by Applied Biosystems (Foster City, CA, USA): Alkaline phosphatase (ALP): Rn00564931_m1; BMP-2: Rn00567818; BMP-3: Rn00567346_m1; BMP-6: Rn00432095_m1; BMP-7: Rn01528885_m1; GDF-10: Rn00577682_m1; BMPR-1a: Rn00821213_g1; BMPR-2: Rn01437209_m1. For the remaining assays, intron-spanning primers (designed using Primer Express 2.0; Applied Biosystems) and SYBR green were used for the PCRs (Table 1). All SYBR green assays were confirmed to generate a single band of the expected size by gel electrophoresis before the start of the study and continuously validated by dissociation curve analysis during the study. Reactions were performed in triplicate using 1 µl cDNA solution, 2× TaqMan Universal PCR Master Mix or SYBR green Master mix (Applied Biosystems), primers and probes (Applied Biosystems) in a 24 µl final reaction volume, using the ABI prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The cDNA was amplified using the following thermal cycling conditions: one cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 41 cycles of 15 s at 95 °C and 1 min at 60 °C. In order to account for variability in the initial concentration and quality of total RNA as well as in the conversion efficiency of RNA into cDNA, the relative amounts of transcripts were normalized to a calibrator gene, 18S ribosomal RNA. The relative quantity of each transcript was calculated relative to the amount of starting cDNA (using the calibrator gene 18S rRNA), taking into account the efficiency of the respective PCRs (E), using the formula

$$\text{Relative expression}_i = \left(\frac{(E_r)^{CT_r}}{(E_i)^{CT_i}} \right) \times 10^6$$

where r represents 18S ribosomal RNA, i represents the gene of interest, and CT represents the threshold cycle. Relative expression values were multiplied by 10^6 to produce more convenient numbers. Serial tenfold dilutions of bone, liver, or spleen cDNA were used to determine the efficiencies of the PCRs (Pfaffl 2001). The quantitative PCR was performed with cDNA from a different set of animals ($n=5–7$) than

Table 1 Background corrected and normalized microarray signal values of bone morphogenetic protein (BMP)-related mRNA in specific zones of growth plate and perichondrium of 1-week-old rats (mean ± s.e.m.)

	Epiphyseal cartilage	Resting zone	Proliferative zone	PZ–HZ transitional zone	Hypertrophic zone	Peri-chondrium	Probe Id
Gene							
BMP-2	1730 ± 170	513 ± 36	1090 ± 72 [†]	5000 ± 390	7510 ± 960 ^{†,§}	1290 ± 88	1398270_at
BMP-3	1420 ± 260	2110 ± 290	63 ± 8 [†]	63 ± 11	283 ± 170*	1680 ± 210	1369773_at
BMP-4	111 ± 15	138 ± 25	83 ± 22	126 ± 18	166 ± 31	1020 ± 150	1387232_at
BMP-6	761 ± 160	274 ± 47	335 ± 53	2080 ± 180	4120 ± 450 ^{†,§}	267 ± 14	1388920_at
BMP-7	202 ± 58	446 ± 52	687 ± 84*	396 ± 42	311 ± 39*,§	379 ± 42	1389403_at
GDF-10	88 ± 16	325 ± 38	5090 ± 420 [†]	1180 ± 430	75 ± 23 [§]	59 ± 5	1368459_at
GREMLIN	3070 ± 390	1790 ± 250	31 ± 16 [†]	55 ± 17	203 ± 42 ^{†,‡}	122 ± 22	1369113_at
CHORDIN	765 ± 104	708 ± 85	236 ± 25*	189 ± 25	272 ± 62*	370 ± 6	1380285_at
NOGGIN	4150 ± 790	2610 ± 380	3860 ± 410*	3870 ± 180	4372 ± 630	167 ± 27	1371052_at
BMPR-1a	526 ± 77	650 ± 57	1020 ± 180	784 ± 81	568 ± 32	770 ± 61	1368899_at
BMPR-2	572 ± 31	588 ± 26	535 ± 60	729 ± 44	709 ± 49	1490 ± 160	1383201_at
ACVR-2a	412 ± 31	527 ± 69	398 ± 60	300 ± 29	280 ± 49	363 ± 38	1381220_at
ACVR-2b	310 ± 64	226 ± 36	224 ± 37	120 ± 26	193 ± 36	131 ± 14	1388179_at
SMAD-1	900 ± 63	708 ± 22	2300 ± 260 [†]	1780 ± 160	1130 ± 120*,‡	2310 ± 230	1389373_at
SMAD-2	722 ± 100	675 ± 98	846 ± 67	831 ± 23	818 ± 65	1320 ± 68	1368214_at
SMAD-3	421 ± 61	294 ± 27	250 ± 29	281 ± 29	532 ± 44 ^{†,§}	412 ± 60	1390827_at
SMAD-4	1790 ± 170	2020 ± 68	2800 ± 210*	2760 ± 240	3440 ± 93	2200 ± 30	1386984_at
SMAD-6	98 ± 14	75 ± 15	51 ± 12	73 ± 12	115 ± 23 ^{†,‡}	315 ± 43	1394025_at

**P* < 0.05 vs RZ, [†]*P* < 0.01 vs RZ, [‡]*P* < 0.05 vs PZ, [§]*P* < 0.01 vs PZ.

those used for microarray and the reactions were carried out in triplicate.

Statistical analysis

All expression data were log-transformed before analysis to obtain a normal distribution. Statistical analysis was performed on relative expression data from microarray and real-time PCR analysis using paired *t*-test comparing RZ, PZ, and HZ. The Holm–Sidak method was used to correct multiple comparison.

Results

Validation of microdissection

The accuracy of the microdissection technique was validated by assessment of type X collagen and alkaline phosphatase mRNA abundance in pooled cartilage samples. Using real-time PCR, type X collagen and alkaline phosphatase mRNA were detected at approximately 250- and 100-fold higher levels in HZ than in PZ respectively (Fig. 2). Some expression of type X collagen and alkaline phosphatase was observed in the epiphyseal cartilage, presumably representing the hypertrophic differentiation that is beginning to occur in the center of the proximal tibial epiphysis, which will eventually produce the secondary ossification center.

Microarray findings for BMP-related genes

Microarray analysis revealed dramatic gradients across the growth plate for a number of genes related to BMP signaling

according to the KEGG pathway database (Table 1). The genes that appeared to be expressed in zone-specific patterns by microarray analysis were further studied using real-time PCR. In general, the real-time PCR results agreed closely with the microarray results.

Expression of BMPs in growth plate cartilage

BMP-2 mRNA was detected by real-time PCR in HZ at levels approximately 30-fold higher than in RZ and PZ. Similar to BMP-2, BMP-6 mRNA was also detected at approximately 30-fold higher levels in the HZ than in RZ and PZ (Fig. 3). In contrast, BMP-7 was expressed at the highest level in PZ, approximately sixfold higher than in HZ and twofold higher than in RZ. Furthermore, BMP-2, -6, and -7 mRNA levels in the growth plate zone with highest expression were similar to or greater than levels in the surrounding metaphyseal bone and perichondrium (Fig. 3). BMP-4 expression in growth plate was not detected by microarray analysis or real-time PCR analysis (data not shown).

Expression of BMP antagonists in growth plate cartilage

Inhibitors of BMP signaling, BMP-3, GDF-10, gremlin, and chordin were expressed early in the differentiation pathway. BMP-3, gremlin, and chordin were specifically expressed in RZ (100-, 80-, and 5-fold, compared with PZ, *P* < 0.001, 0.001, and 0.01 respectively; Fig. 4). GDF-10 was primarily expressed in PZ (160-fold compared with HZ, *P* < 0.01; Fig. 4). By microarray, noggin was found to be expressed at similar levels throughout the growth plate at levels

Figure 2 Validation of growth plate microdissection. Relative expression of type X collagen and alkaline phosphatase mRNA in microdissected growth plate cartilage was determined using microarray analysis ($n=5$) and real-time PCR ($n=7$). Microarray signal values (white bars, left y-axis) were background corrected and normalized. Relative expression values (hatched bars, right y-axis) generated by real-time PCR were normalized to 18S rRNA. N. S., not significant.

approximately 20-fold higher than in perichondrium (Table 1).

Expression of BMP receptors in growth plate cartilage

In the growth plate, BMPR-1a and BMPR-1b mRNA appeared to be expressed in all zones of the growth plate, but

in slightly different patterns (Fig. 5). BMPR 1a was detected at similar levels throughout the growth plate, whereas BMPR-1b was found to be expressed approximately eightfold higher in the HZ than in the PZ ($P<0.05$) (Fig. 5). BMPR-2 was detected at similar levels in all the zones of the growth plate and at levels three- to fourfold lower than in metaphyseal bone and perichondrium (Fig. 5).

Figure 3 BMP-2, -6 and -7 expression in growth plate and surrounding tissues. Growth plate cartilage was microdissected and relative expression of BMP-2, -6 and -7 was determined using microarray analysis ($n=5$) and real-time PCR ($n=7$). Microarray signal values (white bars, left y-axis) were background corrected and normalized. Relative expression values (hatched bars, right y-axis) generated by real-time PCR were normalized to 18S rRNA. N. S., not significant.

Figure 4 Expression of BMP-3, GDF-10, Gremlin, and Chordin in growth plate and surrounding tissues. Relative expression was determined in microdissected growth plate cartilage, surrounding perichondrium and metaphyseal bone. Microarray signal values (white bars, left y-axis, $n=5$) were background corrected and normalized. Relative expression values (hatched bars, right y-axis, $n=7$) generated by real-time PCR were normalized to 18S rRNA. N. S., not significant.

Discussion

Microarray analysis revealed large differences in BMP-related mRNA expression among different zones of the growth plate from 7-day-old rats. Real-time PCR analysis confirmed these

findings; BMP-2 and -6 were primarily expressed in HZ whereas gremlin, chordin, and BMP-3, which act as antagonists of BMP action, were detected at higher levels in RZ. GDF-10, which also can antagonize BMP action and BMP-7 were expressed primarily in the proliferative zone.

Figure 5 Relative expression of BMPR-1a, BMPR-1b, and BMPR-2 in growth plate and surrounding tissues. Relative expression was determined using microarray analysis (only BMPR1a and -1b; $n=5$) and real-time PCR ($n=5-7$). Microarray signal values (white bars, left y-axis) were background corrected and normalized. Relative expression values (hatched bars, right y-axis) generated by real-time PCR were normalized to 18S rRNA. N. S., not significant.

Isolation of each growth plate zone was accomplished by manual microdissection. This technique, like laser microdissection and laser-capture microdissection, allows for visual inspection of dissected cartilage and thus allows for dissection of individual growth plate zones. However, manual dissection, unlike the other techniques does not require expensive equipment, does not use lasers that may heat up and damage the tissue, and also allows for the use of thick sections, thus decreasing the required number of dissections. The method produced intact RNA with a yield sufficient for microarray and real-time PCR analysis. Using this approach, we found that type X collagen mRNA levels were approximately 250-fold higher in hypertrophic zone than in proliferative zone, suggesting that the method provides an accurate separation of these zones. In addition, we found that analysis by microarray and by real-time PCR provided similar results. Since these two analyses were performed using tissue from different dissections, this agreement provides further evidence for the consistency of this approach. Thus, manual microdissection, combined with microarray analysis and real-time PCR, appears to provide a powerful method to study mRNA expression in growth plate cartilage quantitatively.

In order to minimize cross-contamination between different zones of the growth plate, a buffer zone was discarded between RZ and PZ, and a transitional zone was collected between PZ and HZ. Even with every precaution taken, some low-grade cross-contamination may occur. However, any small amount of cross-contamination would tend to decrease differences in mRNA levels between adjacent zones. Therefore, the actual differences in mRNA expression between adjacent zones and tissues might be even greater than the observed differences.

In the growth plate, we found that BMP-2 and -6 are expressed primarily in the hypertrophic zone at levels similar to or greater than those found in the trabecular bone of the metaphysis. In contrast, BMP-7, previously shown to inhibit growth plate chondrocyte differentiation in cultured fetal metatarsal bones (Haaijman *et al.* 1999), was found to be expressed at the highest levels in PZ. These quantitative findings are consistent with earlier non-quantitative studies using *in situ* hybridization in embryonic mice (Chung *et al.* 2001, Kugimiya *et al.* 2005, Minina *et al.* 2005), but not with some studies using immunohistochemistry (Anderson *et al.* 2000, Ngo *et al.* 2006). The apparent discrepancies among these previous studies could be due to differences in developmental stage, a real difference in distribution of mRNAs and proteins, and/or a difference in the sensitivity and specificity of the methods employed. Similarly, our finding that BMP-4 mRNA expression was minimal in all the zones of the growth plate confirms earlier *in situ* hybridization studies (Kugimiya *et al.* 2005, Minina *et al.* 2005).

In addition, we found other gradients in BMP-related gene expression that, to our knowledge, have not been reported previously. Expression of gremlin, chordin, and BMP-3, all inhibitors of BMP-induced bone formation and/or dorsalization of *Xenopus laevis* embryos (Daluiski *et al.* 2001, Zhang *et al.* 2002, Khokha *et al.* 2003), are upregulated in RZ and epiphyseal cartilage compared with PZ and HZ. The magnitude of these gradients was striking. For example, expression of BMP-3 and gremlin was approximately 100- and 80-fold greater in RZ than in PZ respectively. GDF-10 mRNA, which has an amino acid sequence similar to BMP-3 (Cunningham *et al.* 1995) was detected at substantially higher levels in PZ than in any other zone of the growth plate (160-fold higher than HZ).

Our findings suggest that there is a strong gradient in BMP-signaling across the growth plate created by expression of BMP agonists primarily in HZ and expression of BMP antagonists in epiphyseal cartilage, RZ, and PZ (Fig. 6). This pattern suggests that increases in BMP signaling could contribute to both major steps in chondrocyte differentiation within the growth plate. Thus, low levels of BMP signaling in the resting zone, accomplished by localized expression of BMP antagonists, may help maintain these cells in a quiescent state. In the lower RZ, greater BMP signaling may induce differentiation to proliferative chondrocytes. Farther down the growth plate, even greater BMP signaling may induce terminal differentiation to hypertrophic chondrocytes (Fig. 6). Indeed, previous studies are consistent with this model. Immunolocalization of phosphorylated SMAD-1, -5, and -8 increases from top to bottom of the growth plate, with the highest percentage of immunopositive cells in the lower proliferative and early hypertrophic zones (Yoon *et al.* 2006). BMPs can stimulate chondrocyte differentiation at multiple steps (Kobayashi *et al.* 2005, Ryoo *et al.* 2006). In organ culture, BMP-2 stimulates resting zone chondrocytes to proliferate and stimulates proliferative zone chondrocytes to hypertrophy (De Luca *et al.* 2001). With higher concentrations, there is widespread hypertrophic differentiation in the growth plate (De Luca *et al.* 2001). Conversely, in this organ culture system, treatment with

Noggin, a BMP antagonist, inhibits proliferation of resting zone chondrocytes and hypertrophy of proliferative zone chondrocytes (De Luca *et al.* 2001). Overexpression of a constitutively active BMPR1a in mice accelerates hypertrophic differentiation, but has no effect on proliferation (Kobayashi *et al.* 2005). The model may also help explain the finding that explant cultures of fetal metatarsal bones show accelerated hypertrophic differentiation when the distal ends of the bone rudiments are removed (Haaijman *et al.* 1999).

Chondrocyte proliferation and differentiation are also regulated by PTHrP and Ihh, and there is evidence for complex interaction between the BMP system and the Ihh-PTHrP regulatory systems (Minina *et al.* 2001). However, there is also evidence that BMPs may regulate chondrocyte differentiation by mechanisms independent of the Ihh-PTHrP system (Kobayashi *et al.* 2005). Thus, multiple studies suggest that BMPs have important effects on growth plate chondrocyte differentiation, and therefore the gradients in BMP-related gene expression observed in the current study may provide a key mechanism responsible for the spatial regulation of chondrocyte proliferation and differentiation during chondrogenesis at the growth plate.

BMP receptors, BMPR1a, -1b, and -2, were found to be expressed in all zones of the growth plate. The finding that BMPR1b was expressed at significantly higher levels in HZ

Figure 6 BMP-gradient hypothesis. Microarray and real-time PCR analysis of microdissected growth plate cartilage detected differential expression of BMP-related genes in specific regions of the growth plate. BMP agonists were expressed primarily in the HZ, whereas BMP antagonists were expressed primarily in the resting zone. BMP-7 is listed as a possible antagonist because it reportedly inhibits chondrocyte hypertrophic differentiation (Haaijman *et al.* 1999). These findings suggest that there is a BMP signaling gradient across the growth plate that may be important for spatial control of chondrocyte differentiation within the growth plate.

than in RZ and PZ may suggest that BMPR1a may mediate the effects of BMPs earlier in the differentiation pathway, especially in the transition from resting to proliferative phenotype, whereas BMPR1b may preferentially mediate the effects of BMPs on hypertrophic differentiation. However, development of most skeletal elements are normal in BMPR1a and BMPR1b null mice, whereas mice lacking both BMPR1a and BMPR1b lack all skeletal elements that form through endochondral bone formation, thus suggesting that BMPR1a and -1b have redundant roles in skeletal development (Kobayashi *et al.* 2005, Yoon *et al.* 2005).

In summary, we have developed a method of manual microdissection followed by microarray analysis and real-time PCR that allows quantitative study of mRNA expression in growth plate cartilage. Using this method, we detected dramatic differences in expression of BMP-related genes in different regions of the growth plate. BMP agonists were expressed primarily in HZ, whereas BMP antagonists were expressed primarily in the resting zone. These findings suggest that there is a strong BMP signaling gradient across the growth plate. Previous studies suggest that BMPs can promote growth plate chondrocyte proliferation and differentiation. Therefore, the gradients in BMP action observed in the current study may provide a key mechanism responsible for spatial regulation of chondrocyte proliferation and differentiation in growth plate cartilage, contributing to the two-step program by which resting zone chondrocytes differentiate into proliferative zone chondrocytes and then into hypertrophic zone chondrocytes.

Acknowledgements

This research was supported by the Intramural Research Program of the National Institute of Child Health and Human Development, NIH. O N was supported by grants from the Swedish Research Council (K2003-72PK-15191-01A) and the Swedish Society for Medical Research. BRB-Array Tools Version 3.3.0 was developed by Dr Richard Simon and Amy Peng at the Biometric Research Branch, Division of Cancer Treatment and Diagnostics, the National Cancer Institute, Bethesda, MD, USA. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 19 December 2006

Accepted 10 January 2007

Made available online as an Accepted Preprint
19 January 2007