Journal of Cellular Biochemistry 90:1149–1165 (2003)

Transcriptional Characterization of Bone Morphogenetic Proteins (BMPs)-Mediated Osteogenic Signaling

Ying Peng,[1,2] Quan Kang,[1,3] Hongwei Cheng,[1] Xinmin Li,[4] Michael H. Sun,[1] Wei Jiang,[1] Hue H. Luu,[1] Jae Yoon Park,[1] Rex C. Haydon,[1] and Tong-Chuan He[1,2]*

[1]Molecular Oncology Laboratory, Department of Surgery, The University of Chicago Medical Center, Chicago, Illinois 60637
[2]Committee on Genetics, The University of Chicago, Chicago, Illinois 60637
[3]The Children's Hospital of Chongqing University of Medical Sciences, Chongqing 400016, China
[4]The Functional Genomics Facility of The University of Chicago, Illinois 60637

Abstract Bone formation is presumably a complex and well-orchestrated process of osteoblast lineage-specific differentiation. As members of the TGFβ superfamily, bone morphogenetic proteins (BMPs) play an important role in regulating osteoblast differentiation and subsequent bone formation. Several BMPs are able to induce de novo bone formation. Although significant progress has recently been made about the transcriptional control of osteoblast differentiation, detailed molecular events underlying the osteogenic process remain to be elucidated. In order to identify potentially important signaling mediators activated by osteogenic BMPs but not by non-osteogenic BMPs, we sought to determine the transcriptional differences between three osteogenic BMPs (i.e., BMP-2, BMP-6, and BMP-9) and two inhibitory/non-osteogenic BMPs (i.e., BMP-3 and BMP-12). Through the microarray analysis of approximately 12,000 genes in pre-osteoblast progenitor cells, we found that expression level of 203 genes (105 up-regulated and 98 down-regulated) was altered >2-fold upon osteogenic BMP stimulation. Gene ontology analysis revealed that osteogenic BMPs, but not inhibitory/non-osteogenic BMPs, activate genes involved in the proliferation of pre-osteoblast progenitor cells towards osteoblastic differentiation, and simultaneously inhibit myoblast-specific gene expression. BMP-regulated expression of the selected target genes was confirmed by RT-PCR, as well as by the CodeLink Bioarray analysis. Our findings are consistent with the notion that osteogenesis and myogenesis are two divergent processes. Further functional characterization of these downstream target genes should provide important insights into the molecular mechanisms behind BMP-mediated bone formation. J. Cell. Biochem. 90: 1149–1165, 2003.

Key words: bone formation; expression profiling; osteoblast differentiation; osteogenesis; signal transduction

Bone is a highly mineralized tissue and undergoes continuous remodeling throughout life [Reddi, 1998; Olsen et al., 2000]. Although its exact mechanisms remain to be defined, bone formation is presumably a complex and well-orchestrated process of osteoblast lineage-specific differentiation [Aubin, 1998]. Derived from mesenchymal stem cells, which also serve as progenitor cells for myocytes, adipocytes, and chondrocytes, osteoblasts are specialized cells capable of producing an authentic bone matrix. During osteogenesis, pluripotent stem cells undergo successive stages of differentiation with a decreasing proliferation potential, giving rise to committed pre-osteoblasts. Subsequently, pre-osteoblasts differentiate into mature osteoblasts that deposit the necessary components to form bone matrix, followed by mineralization [Aubin, 1998]. Although significant progress has been made about the transcriptional control of osteoblast differentiation [Lian et al., 1998; Aubin, 2001], detailed molecular events underlying the osteogenic process remain to be elucidated.

Bone morphogenetic proteins (BMPs) play an important role in regulating osteoblast

Grant sponsor: Aircast Foundation; Grant sponsor: Brinson Foundation; Grant sponsor: Orthopaedic Research and Education Foundation.

*Correspondence to: Tong-Chuan He, MD, PhD, Assistant Professor and Director, Molecular Oncology Laboratory, Department of Surgery, The University of Chicago Medical Center, 5841 S. Maryland Avenue, MC3079, Room J-611, Chicago, IL 60637. E-mail: tche@surgery.bsd.uchicago.edu

Received 12 August 2003; Accepted 16 September 2003
DOI 10.1002/jcb.10744

differentiation and subsequent bone formation [Urist et al., 1987; Wozney et al., 1988]. The identification of BMPs has generated great interest due to their potential use in bone regeneration [Reddi, 1998; Sun et al., 2003]. A plethora of studies demonstrated the ability of several BMPs, notably BMP-2 and BMP-7 (a.k.a., OP-1), in promoting osteogenesis [Reddi, 1998; Baltzer et al., 2000; Sun et al., 2003]. We have recently conducted a comprehensive comparative analysis of the relative osteogenic activity of the 14 types of human BMPs. Our findings demonstrate that, in addition to BMP-2 (BMP-7 to a lesser extent), BMP-6 and BMP-9 are the most potent osteogenic BMPs of 14 BMPs [Cheng et al., 2003], strongly suggesting that these BMPs may transduce a distinct set of signaling during osteogenesis.

BMPs belong to the TGFβ superfamily and play an important role in development and bone formation [Hogan, 1996; Urist, 1997; Zou et al., 1997]. This family also includes TGF-βs, activins and mullerian-inhibiting substance [Massague, 1998]. At least 15 types of BMPs have been identified in humans [Hogan, 1996; Reddi, 1997; Hoffmann and Gross, 2001]. Genetic disruptions of BMPs have resulted in various skeletal and extraskeletal abnormalities during development [Zhao, 2003]. Interestingly, only BMP-3 deletion leads to a relatively skeleton-specific phenotype of increased bone density [Daluiski et al., 2001]. BMPs fulfill their signaling activity by interacting with the heterodimeric complex of two transmembrane serine/threonine kinase receptors, BMPR type I and BMPR type II [Massague, 1998]. The activated receptor kinases phosphorylate the transcription factors Smads 1, 5, and/or 8. The phosphorylated Smads then form a heterodimeric complex with Smad 4 in the nucleus and activate the expression of target genes in concert with other co-activators [Heldin et al., 1997; Massague, 1998; Itoh et al., 2000; Wrana, 2000].

Recently several expression profiling studies have been performed on different stages of osteoblast maturation [Beck et al., 2001; Garcia et al., 2002; Raouf and Seth, 2002; Vaes et al., 2002; Roman-Roman et al., 2003] and BMP-2-treated mesenchymal derived cells [Harris and Harris, 2001; Locklin et al., 2001; Balint et al., 2003; Kang et al., 2003]. However, much remains to be learned about how BMPs transduce their osteogenic signaling. For example, although most, if not all, BMPs presumably function through the Smad signaling network, it is unclear why and how only a few BMPs exert potent osteogenic activity [Cheng et al., 2003]. In this study, we sought to determine potentially important downstream mediators of BMP-mediated bone formation by comparing the gene expression profiles induced by osteogenic BMPs (i.e., BMP-2, BMP-6, and BMP-9) but not by non-osteogenic BMP controls (i.e., BMP-3, BMP-12, and GFP). Using microarray approach, we analyzed the BMP-regulated expression of ~12,000 genes in the pre-osteoblast progenitor C2C12 cells. Significance analysis of microarrays (SAM) revealed that 203 genes, 105 up-regulated and 98 down-regulated genes, exhibited >2-fold changes in expression upon osteogenic BMP stimulation. Gene ontology/pathway analysis suggests that osteogenic BMPs (i.e., BMP-2, BMP-6, and BMP-9), but not negative/non-osteogenic BMPs (i.e., BMP-3 and BMP-12), promote osteoblastic differentiation, and simultaneously inhibit myoblast-specific gene expression. BMP-regulated expression of the selected target genes was verified by RT-PCR and an independent CodeLink microarray platform. These findings are consistent with the notion that osteoblastic differentiation and myogenesis are two divergent processes [Murray et al., 1993; Katagiri et al., 1994]. Further functional characterization of these downstream targets should provide important insights into the molecular mechanisms behind BMP-mediated bone formation.

MATERIALS AND METHODS

Cell Culture and Chemicals

Human embryonic kidney HEK 293 cell line and mouse pluripotent mesenchymal precursor line C2C12 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HEK 293 and C2C12 cells were maintained in complete DMEM supplemented with 10% fetal calf serum (FCS, Mediatech, Herndon, VA), 100 U of penicillin, and 100 μg of streptomycin at 37°C in 5% CO_2. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Construction of Recombinant Adenoviral Vectors Expressing BMPs

The cDNA clones for human BMP-2, -3 (a.k.a., osteogenin), -6, -9 (a.k.a., GDF-2), and -12

(a.k.a., GDF-7 or CDMP-3) were kindly provided by the Genetics Institute (Cambridge, MA). The coding regions of above BMPs were subcloned into pAdTrack-CMV, resulting in pAdTrack-BMPs, and recombinant adenoviruses expressing BMPs (i.e., AdBMPs) were subsequently generated as previously described [He et al., 1998b]. For a control, we used an analogous adenovirus only expressing GFP (i.e., AdGFP) as previously described [He et al., 1998a]. Details on vector constructions are available upon request.

Orthotopic Bone Formation in Athymic Nude Mice

The use of animals was approved by the Institutional Animal Care and Use Committee. Young athymic nude mice (male, 5–6 months, Frederick Cancer Research Center) were used in this study. Each experimental group had four animals. For the injection with adenovirus-transduced C2C12 cells, subconfluent C2C12 cells were infected with AdBMPs or AdGFP at pre-optimized titers (MOIs ~50–100). At 15 h after infection, cells were collected and resuspended in PBS at an approximate density of 1×10^8 cells/ml. Fifty microliters of the cell suspension (approx. 5×10^6 cell) were used for the intramuscular injection of right quadriceps. Injected animals resumed activities immediately without any restrains on food and drinks. At 3 and 5 weeks after injections, animals were sacrificed and subjected to X-ray radiography. The injected sites were harvested for histological evaluation. Representative results from three independent batches of experiments are shown.

Histochemical Staining of Alkaline Phosphatase Activity

Exponentially growing C2C12 cells were seeded in 48-well cell culture plates, and infected with AdBMPs and AdGFP. The induction of alkaline phosphatase expression was detected at 4 days after infection using histochemical staining assays. Briefly, infected cells were fixed with 0.05% (v/v) glutaraldehyde (Sigma-Aldrich) at room temperature for 10 min. After being washed with PBS, cells were stained by using a mixture of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt (Sigma-Aldrich). Histochemical staining was recorded using bright field microscopy.

Isolation of Total RNA

Subconfluent C2C12 cells were seeded in 25 cm^2 cell culture flasks for 12 h in complete DMEM medium supplemented with 0.5% FCS, and infected with an optimal and compatible titer of AdBMP-2, AdBMP-3, AdBMP-6, AdBMP-9, AdBMP-12, or AdGFP. At 30 h after infection, total RNA was isolated using RNAgent Total RNA Isolation kit (Promega, Madison, WI) according to the manufacturer's instructions.

Microarray Hybridizations and Initial Data Processing

Total RNA from each sample was used for microarray hybridizations after the purity and integrity of RNA was affirmed using an Agilent 2100 Bioanalyzer and a GeneSpec III. Fully characterized RNA samples were used for target preparation and then subjected to hybridizations to Affymetrix mouse genechip U74Av2 (containing ~12,000 known genes and ESTs). The target preparation protocol followed the Affymetrix GeneChip Expression Analysis Manual (Santa Clara, CA) with minor modifications, and was conducted at the Functional Genomics Facility of The University of Chicago. The target preparation and hybridizations to CodeLink Uniset mouse I Bioarray (Amersham Biosciences, Piscataway, NJ) were also carried out at the Functional Genomics Facility of The University of Chicago by following the manufacturer's manual. The acquisition and initial quantification of array images were performed using the Affymetrix Microarray Suite Version 5.0 (MAS 5.0) with the default analytic parameters (Alpha 1, 0.04; Alpha 2, 0.06; Tau, 0.015; Global scaling target signal, 500). The quality of hybridizations was initially evaluated by examining the MAS 5.0 Report file for housekeeping gene hybridization, Spike control hybridization, percentage of genes called present, 5′ to 3′ ratio, signal to background ratio and scale factor ratio, and then by using DNA-Chip Analyzer (dCHIP) analysis for regional image contamination and/or sample contamination.

The acquired microarray raw data were further filtered and normalized to remove noise while retaining true biological information by using a two-step filtration strategy. The first step was to filter genes with signal intensity in all samples $\leq$100 intensity units. The rationale for choosing 100 as the first-step cutoff intensity

was based on our observation that the final concentration of spike control BioB in the hybridization mix was 1.5 pM, which was equivalent to 1–3 RNA molecules per cell, but the signal intensity of BioB was normally above 100 when the global scaling target signal was set as 500 (Affymetrix Microarray Suit default setting). Furthermore, over 99% of the genes with signal intensity $\leq$100 were called absent by MAS 5.0. The second step filtration was to remove the genes that receive an "absent" call for all chips. Data filtration ensured that only genes that were considered as significantly present at least in one of the samples were used for further analysis. All data were scaled to a target signal of 500 and therefore, they were comparable among samples. In addition, we adopted an alternative normalization strategy, i.e., normalize the data to medium per chip and per gene before high-level data analysis.

SAM

SAM was initially developed by Tusher et al. [2001]. For each gene (i) in the array, SAM computes the T-statistics (di), a score derived from the changes of gene expression in relation to the SD of repeated measurements for that gene. A SAM threshold tuning parameter of Δ was set on the basis of di to identify potentially significant changes in gene expression, which can be adjusted on the basis of an associated false discovery rate (FDR) value. In our studies, each sample was normalized to medium signal intensity before SAM analysis. The SAM Microsoft Excel add-in was used to generate a significant gene list at a delta value of 1.2 with a FDR of $<$2.

Hierarchical Clustering Analysis

Hierarchical clustering is a technique to build clusters of genes with similar patterns of expression. This is done by iteratively grouping together genes that are highly correlated in terms of their expression measurements, then continuing the process with the groups themselves. We performed a model-based expression analysis using dCHIP with the *.CEL files obtained from MAS 5.0 [Li and Wong, 2001]. The model-based approach allowed probe-level analysis and facilitated automatic probe selection in the analysis stage to reduce errors caused by outliers, cross-hybridizing probes, and image contamination. In this experiment, we selected 200 most differentially expressed genes using dCHIP's filter function and then performed cluster analysis with this group of genes.

Gene Ontology/Pathway Analysis

This was conducted using GenMAPP/MAPPFinder (http://www.genmapp.org), publicly available software designed to visualize gene expression data on maps representing biological pathways and functionally related groups of genes. The GenMAPP 1.0 database included 51 mouse GenMAPPs and 958 mouse Gene Ontology MAPPs, which facilitated the analysis of the large amounts of data produced in our microarray experiments and allowed visualizing gene expression data in a biological context with the graphical and more intuitive format of MAPPs. In this analysis, 1.5-fold up- or down-regulation was defined as change. Percentage of change on each ontology term was calculated using MAPPFinder.

Reverse Transcriptase (RT)-PCR

Ten micrograms of total RNA were used to generate cDNA templates for RT-PCR. The first strand cDNA synthesis was performed using a hexamer (Promega carlsbad) and Superscript II RT (Invitrogen, Carlsbad, CA). The first strand cDNA products were further diluted 20- to 50-fold and used as PCR templates. Expression level was determined by touchdown PCR analysis using respective pairs of oligonucleotides to amplify the 3′-end of the selected genes. A complete list of PCR primers used in this study is posted at www. boneandcancer.org/c2c12arrays.htm. Touchdown PCR was performed by using the following program: 94°C $\times$ 2 min for 1 cycle, 12 cycles at 92°C $\times$ 20 s, 68°C $\times$ 30 s, and 70°C $\times$ 45 s with a decrease of one degree per cycle, and 35 cycles at 92°C $\times$ 20 s, 55°C $\times$ 30 s, and 70°C $\times$ 45 s. The amplified products were resolved on 1% agarose gels, and visualized under ultraviolet light after ethidium bromide staining.

RESULTS

Distinct Osteogenic Signaling Activity Mediated by BMPs

Although several BMPs, notably BMP-2 and BMP-7, have been extensively investigated for their osteogenic activity, our recent studies demonstrate that BMP-6 and BMP-9 exert

equally, if not more, potent osteogenic activity (than that of BMP-2) [Cheng et al., 2003]. As illustrated in Figure 1, three osteogenic BMPs, i.e., BMP-2, BMP-6, and BMP-9, were shown to induce a significant increase in alkaline phosphatase activity, a well-established early osteogenic marker, at 4 days after adenoviral vector-mediated expression of these BMPs in C2C12 cells, while the GFP mock and BMP-3 infection did not induced any detectable alkaline phosphatase activity (Fig. 1A). As a very weak or non-osteogenic factor, BMP-12 expression led to marginal or sparsely positive staining of alkaline phosphatase activity (Fig. 1A).

Accordingly, the ability of the above-mentioned five BMPs to induce alkaline phosphatase activity in vitro is correlated well with their in vivo osteoinductive capability. When AdBMP-transduced C2C12 cells were injected into the quadriceps of athymic mice, orthotopic ossification was radiographically evident in the animals injected with AdBMP-2, AdBMP-6, and AdBMP-9-transduced cells at 3 weeks post injection (Fig. 1B). However, no radiographic evidence of bone formation was observed in BMP-3, BMP-12, and the GFP control groups. Histological examination confirmed the above findings from X-ray radiography. BMP-2, BMP-6, and BMP-9 demonstrated varying degrees of ossification, and had multiple foci of immature woven trabecular bone (Fig. 1C). Conversely, the injection sites recovered from BMP-3, BMP-12, and the GFP control animals exhibited no evidence of osteogenesis and only contained highly proliferative C2C12 cell masses. These findings were reproducible in three batches of experiments, and an increased maturation with more mature osteoid matrix and trabecular bone-like structures were observed in BMP-2, BMP-6, and BMP-9-treated animals at 5 weeks after injections (data not shown). These in vitro and in vivo results further confirm the osteogenic ability of BMP-2, BMP-6, and BMP-9.

Microarray Expression Profiling of Osteogenic BMPs Versus Non-Osteogenic BMPs

In order to gain insights into the molecular basis of BMP-mediated osteogenesis, we carried out an expression profile analysis of genes whose expression was affected by osteogenic BMPs. For the past few years, microarray technology has emerged as an efficient and robust method to analyze differential gene expression [Butte, 2002]. We were particularly interested in elucidating the early signaling events upon BMP stimulation. Briefly, exponentially growing C2C12 cells were infected with three osteogenic BMP viruses (AdBMP-2, AdBMP-6, and AdBMP-9), along with AdGFP (mock infection), AdBMP-3 (a negative BMP), and AdBMP-12 (a low or non-osteogenic BMP). At 30 h after infection, total RNA was isolated and subjected to microarray hybridization analysis using the Affymetrix's mouse genechip U74Av2. Each chip contained approximately 12,000 known mouse genes and ESTs. The microarray hybridization and data analyzes were performed at The University of Chicago Functional Genomics Facility. Overall, the microarray hybridization data were of good quality and consistent with most of the differential expression analyzes performed in our previous studies [He et al., 1998a].

Hierarchical Clustering Analysis of Gene Expression Pattern Regulated by Osteogenic BMPs Versus Non-Osteogenic BMPs

A commonly used method to determine gene expression pattern is to conduct gene clustering analysis of microarray data. Hierarchical clustering is a commonly used technique to build clusters of genes with similar patterns of expression, and is particularly advantageous in visualizing overall similarities in expression patterns. The resultant hierarchical clustering is usually visualized as dendrograms, which represent all genes as leaves of a large branching tree. As shown in Figure 2, the three osteogenic BMPs (i.e., BMP-2, BMP-6, and BMP-9) induced a similar overall expression pattern that was distinct from that of BMP-3, BMP-12, and the GFP control, suggesting that BMP-3 and BMP-12 behaved differently from that of the three osteogenic BMPs. Among the three osteogenic BMPs, BMP-2 induced a more closely related expression pattern to BMP-9 than to BMP-6. This is intriguing because the biological functions of BMP-9 are poorly understood. Nevertheless, there were several subclusters that were regulated by all three osteogenic BMPs (Fig. 2). The similarities in gene expression pattern among the three osteogenic BMPs may underscore a fundamental mechanism behind bone formation. Similar clustering results were obtained when GeneSpring software was used for the hierarchical clustering analysis (data not shown).

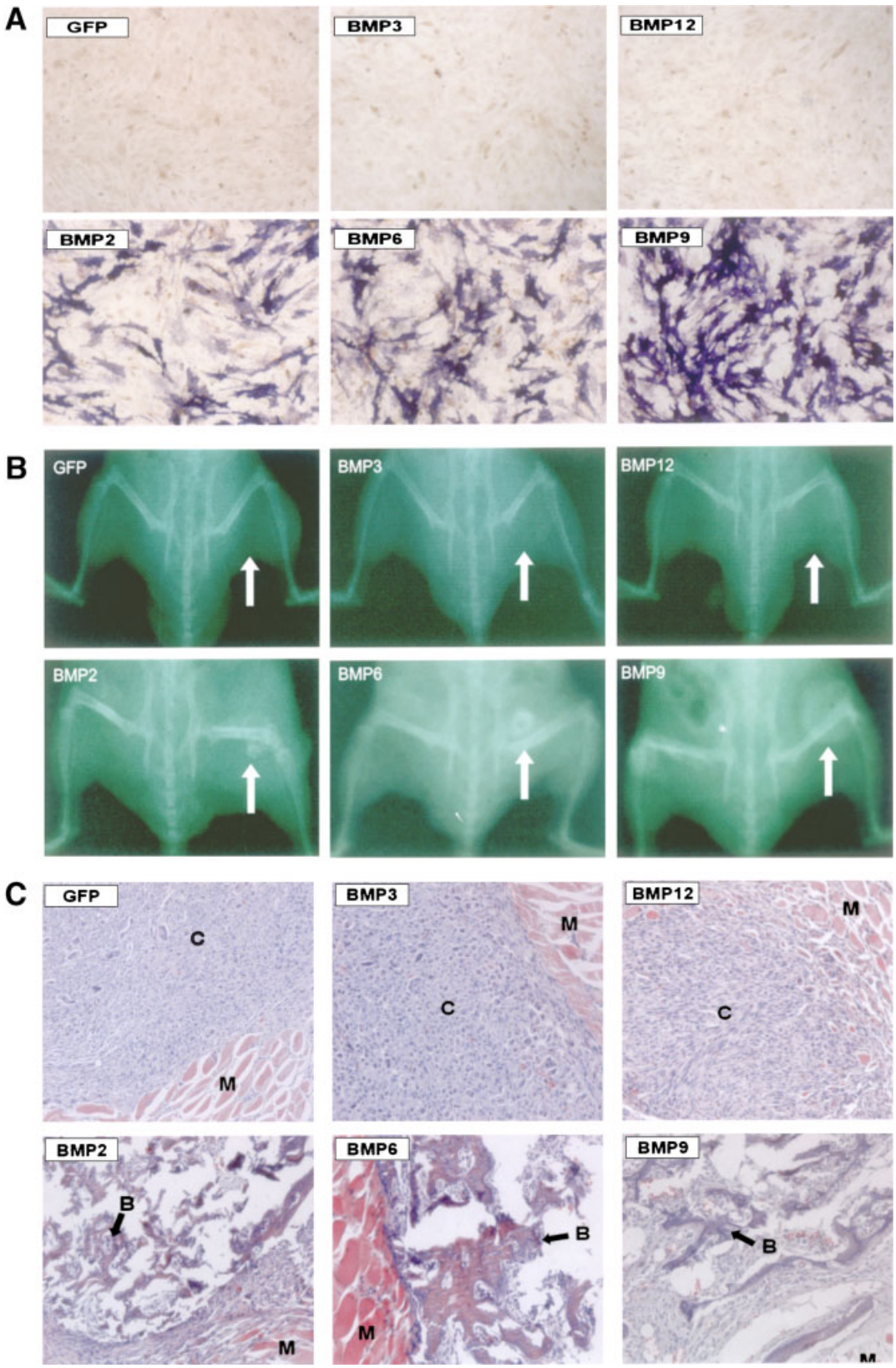
A
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Fig. 1.

SAM Analysis of Gene Expression Regulated by Osteogenic BMPs Versus Non-Osteogenic BMPs

Results from the clustering analysis indicate that the overall expression patterns of three osteogenic BMPs (i.e., BMP-2, BMP-6, and BMP-9) were similar to each other, but were distinct from those of BMP-3, BMP-12, and the GFP control. We next grouped the filtered microarray data derived from three osteogenic BMPs (as osteogenic BMP dataset) and from BMP-3, BMP-12, and GFP (as non-osteogenic dataset), and conducted the SAM to identify differentially expressed genes [Tusher et al., 2001]. A significant gene list was generated by cutting off the ranked list at a given threshold of Δ (approx. 1.2, and FDR value $<2\%$) when we analyzed the datasets for osteogenic BMPs (i.e., BMP-2, BMP-6, and BMP-9) versus non-osteogenic BMPs (i.e., GFP, BMP-3, and BMP-12) (Fig. 3). Under this analysis condition, there were 203 genes whose expression changed by at least 2-fold, 105 up-regulated and 98 down-regulated by osteogenic BMPs (Table I). A complete list of significant genes (>2-fold) from the SAM analysis is available at www.boneandcancer.org/c2c12arrays.htm.

Downstream Mediators of Osteogenic BMP Signaling

The acquired microarray data provided important insights into the early signaling events of osteogenesis. Several known TGFβ/BMP target genes were induced upon BMP stimulation. For instance, the two inhibitory Smads, Smad 6 and Smad 7, which are known to be induced as a part of the negative feedback inhibition of BMP signaling [Imamura et al., 1997; Nakao et al., 1997; Afrakhte et al., 1998; Takase et al., 1998], were induced by three osteogenic BMPs (e.g., 7- to17-fold for Smad 6 and 2- to 4-fold for Smad 7, respectively). Another known Smad target gene, *junB*, was induced approximately 3-fold. Other significantly induced known target genes included transcription factor GIF, latent TGFβ binding protein 2 (LTBP2), plasminogen activator inhibitor (PAI-1), and Cbfa1/Osf2/Runx2. Further, two known BMP antagonists, noggin and follistatin, were also induced 2- to 6-fold by osteogenic BMPs. In general, above target genes were induced only by osteogenic BMPs (i.e., BMP-2, BMP-6, and BMP-9), and BMP-2 and BMP-9 induced a much higher expression level of these target genes than BMP-6. These results suggest some of these known target genes of TGFβ/BMP signaling may play a role in regulating early steps of osteogenesis.

SAM analysis identified nearly 200 genes whose expression was differentially regulated by osteogenic BMPs at least 2-fold; approximately half of those were up-regulated. Among the top 30 up-regulated genes (Table IA), several transcription regulators were significantly induced. These included inhibitor of DNA binding (Id)-1, Id-2, Id-3, nuclear receptor PPARγ, GIF (a.k.a., TIEG1), junB, and homeobox genes (*Dlx-3* and *Prx2*). Significantly up-regulated genes also included several growth factors/cytokines (e.g., hepatocyte growth factor, brain derived neurotrophic factor, and connective tissue growth factor), signaling molecules (e.g., Grb10, receptor type protein tyrosine phosphatase M, and interferon activated gene 202). It is notably intriguing that nearly half of the top 30 negative significant genes were ESTs (Table IB). One of the top genes on the list was TNF receptor associated factor 4 (TRAF4). Other notable down-regulated targets were transmembrane protein Wfs1, integrin α7, and several muscular/cytoskeleton structure proteins (e.g., myosin binding protein H, M-protein, microtubule-associated protein tau, α-actinin-3, myosin alkali light chain, and α-actinin-2 associated LIM protein). Overall,

Fig. 1. The in vitro and in vivo osteogenic activity of the selected bone morphogenetic proteins (BMPs). **A**: Induction of alkaline phosphatase activity by BMPs in C2C12 cells. Subconfluent C2C12 (**B**) cells were infected with AdBMPs or AdGFP. At 4 days after infection, alkaline phosphatase activity (stained as purple blue) was determined histochemically using naphthol AS-MX/fast blue BB mix as a substrate. Representative results from at least three independent experiments are shown. B: Orthotopic bone formation induced by AdBMP-transduced C2C12 in athymic nude mice. Exponentially growing C2C12 cells were infected with AdBMPs or the control AdGFP for 15 h. Approximately 5×10^6 of the infected cells were injected into the right quadriceps of athymic nude mice (indicated as arrows). At 3 weeks after injections, mice were sacrificed and subjected to X-ray radiography. Each experimental group contained four mice and representative radiographies from three batches of experiments were shown. **C**: H&E staining and histological evaluation of AdBMP-induced orthotopic ossification of the injection sites at 3 weeks. B, osteoid matrix (indicated by arrows); C, injected C2C12 cells; and M, muscle cells. Magnification, 200×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

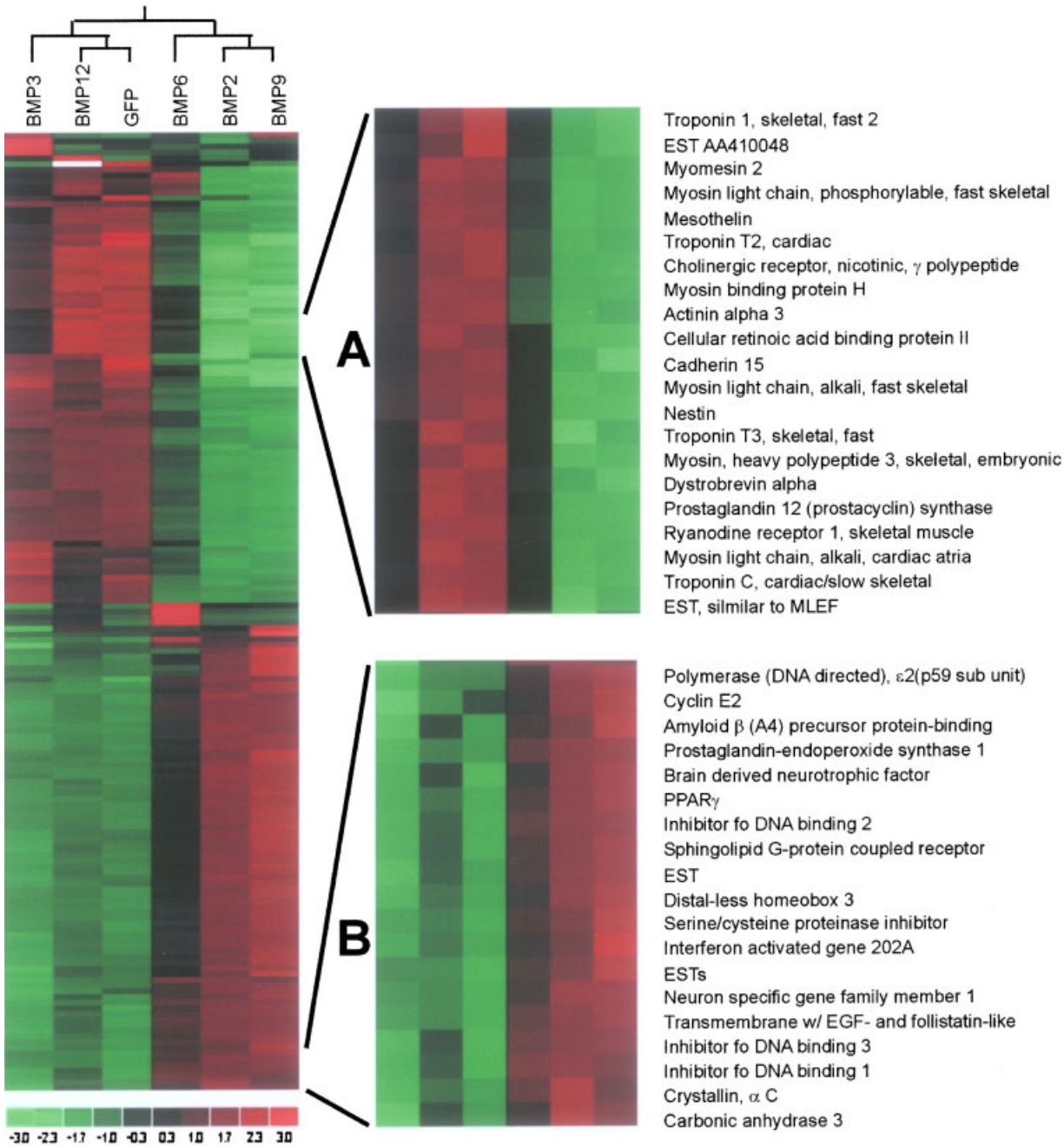

Fig. 2. Hierarchical clustering of BMP microarray data. Approximately 200 genes were filtered out using DNA-Chip Analyzer (dCHIP) data filtration default settings and were used for hierarchical clustering analysis by dCHIP. The expression level matrix is shown in a log ratio representing normalized values from −3 (green, below the mean) to +3 (red, above the mean). The mean (0 value) is represented by the black color. Two representative sub-hierarchical clusters of down-regulated genes (**A**) and up-regulated genes (**B**) are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

these up- and down-regulated target genes may suggest a general trend in the early stage of BMP-mediated osteogenic signaling: an increase in osteoblast differentiation and a decrease in myogenesis.

Gene Ontology/Pathway Analysis of Gene Expression Regulated by Osteogenic BMPs

We next analyzed how different cellular pathways/gene ontologies were affected by the

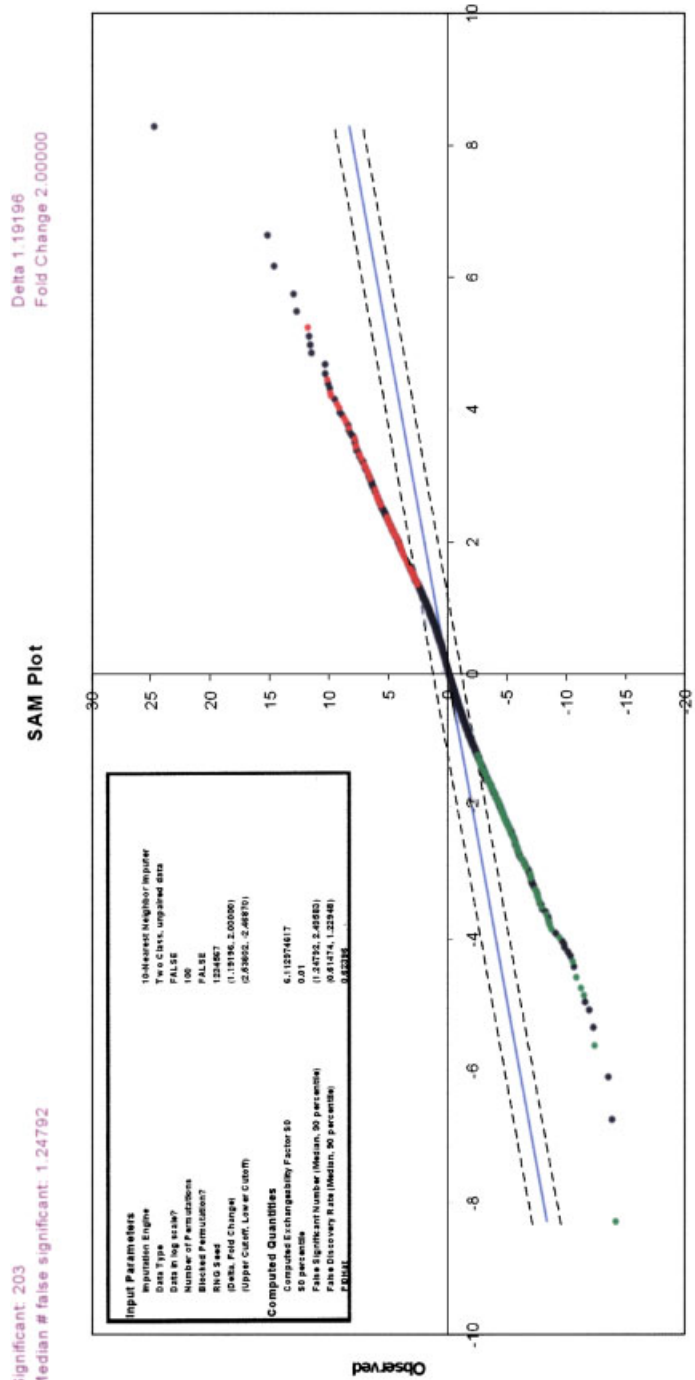

Fig. 3. Significance analysis of microarrays (SAM) of the positive and negative significant genes for the osteogenic BMPs. Scatter plot of the observed relative difference $d(i)$ versus the expected relative difference $d_E(i)$. The solid line indicates the line when $d(i) = d_E(i)$, and the dotted lines are drawn at a distance $\Delta = 1.2$ from the solid line. The 203 positive and negative significant genes (>2-fold) are those lied outside the dotted lines. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Significance Analysis of Microarrays (SAM) of Bone Morphogenetic Protein (BMP)-Regulated Genes

Genbank accession	Description	Score (d)	Numerator (r)	Denominator (s + s0)	Fold change	q-Value (%)
A: Top 30 of the 105 significantly up-regulated genes						
M34141	Prostaglandin-endoperoxide synthase 1 (PTGS1)	11.8090	2214.733	187.546	2.21	0.3167303
AF077861	Inhibitor of DNA binding 2	10.1600	4395.800	432.658	9.04	0.3167303
X72307	Hepatocyte growth factor	9.8747	305.600	30.948	2.36	0.3167303
AI848798	Similar to HSP22	9.8397	2279.033	231.616	2.26	0.3167303
U10374	PPAR gamma	9.3284	312.267	33.475	2.80	0.3167303
M31885	Inhibitor of DNA binding 1	9.1580	1524.733	166.493	5.63	0.3167303
AI851750	EST	8.7627	157.167	17.936	2.89	0.3167303
M60523	Inhibitor of DNA binding 3	8.5752	4120.700	480.538	8.00	0.3167303
D10837	Lysyl oxidase	8.3350	3012.167	361.389	2.28	0.3167303
M14223	Ribonudeotide reductase M2 subunit	7.8619	3937.933	500.888	3.78	0.3167303
AW121294	EST	7.7553	1122.167	144.696	2.01	0.3167303
U20735	Transcription factor jun B (junB)	7.7416	480.100	62.016	2.95	0.3167303
X52875	Homeobox gene *Prx2*	7.4602	3859.533	517.349	5.26	0.3167303
U79738	Homeodomain protein Dlx-3	7.3159	866.567	118.450	3.86	0.3167303
AF064088	Transcription factor GIF mRNA	7.0508	748.633	106.178	2.79	0.3167303
D50086	Neuropilin, complete cds	6.9885	1135.933	162.543	2.07	0.3167303
U20735	Transcription factor junB (*junB*) gene	6.8074	524.233	77.010	2.40	0.3167303
M31418	Interferon activated gene 202	6.7187	556.833	82.878	2.03	0.3167303
M63335	Lipoprotein lipase gene, partial cds	6.5505	559.900	85.475	3.40	0.3167303
AF004874	Latent TGF beta binding protein 2 (LTBP2)	6.1539	903.700	146.849	4.13	0.3167303
L32838	Germline interleukin 1 receptor antagonist (IL-1m)	6.0345	202.867	33.618	2.01	0.3167303
X58287	Protein tyrosine phosphatase, receptor-type. M	6.0046	281.633	46.903	2.30	0.3167303
AF020313	Proline-rich protein 48 mRNA (Grb 1C)	6.0045	805.433	134.139	2.62	0.3167303
X55573	Brain derived neurotrophic factor	5.9535	791.933	133.020	2.24	0.3167303
X13335	A disintegrin and metalloprotease domain (ADAM) 8	5.9201	198.233	33.485	4.95	0.3167303
M98530	Neuron specific gene family member 1	5.8950	167.300	28.380	7.10	0.3167303
AJ001418	Pyruvate dehydrogenase kinase-like protein	5.7829	173.433	29.991	2.31	0.3167303
AF033350	CDCREL-1 homolog (Cdcrel-1), partial cds	5.6643	406.367	71.742	2.09	0.3167303
AA726364	Lipoprotein lipase	5.6626	1313.200	231.906	3.08	0.3167303
M70642	Connective tissue growth factor (FISP-12)	5.1705	3243.100	627.233	7.80	0.3167303
B: Top 30 to the 98 significantly down-regulated genes						
AV109962	EST	−14.1061	−2050.533	145.36	0.25	0.3167303
AF068748	Sphingosine kinase (SPHK1a)	−12.3569	−217.567	17.61	0.17	0.3167303
X92346	TNF receptor associated factor 4	−11.4908	−610.933	53.17	0.49	0.3167303
AF084482	Transmembrane protein (Wfs1)	−11.2019	−1631.967	145.69	0.35	0.3167303
AI006228	EST	−10.8132	−517.600	47.87	0.24	0.3167303
AA656550	EST	−10.5355	−517.533	49.12	0.40	0.3167303
AJ001038	mRNA for M-protein	−9.3738	−604.900	64.53	0.37	0.3167303
L23423	Integrin alpha 7	−8.7007	−2609.700	299.94	0.38	0.3167303
U68267	Myosin binding protein H (MyBP-H)	−8.5627	−1259.167	147.05	0.29	0.3167303
M18775	Microtubule-associated protein tau	−8.4793	−159.367	18.79	0.08	0.3167303
AW125453	EST	−8.2608	−1444.933	174.91	0.49	0.3167303
AA611766	EST	−7.8900	−291.367	36.93	0.40	0.3167303

AI836718	EST	−7.6944	−2251.567	292.63	0.32	0.3167303
AF093775	α-Actinin-3 (Actn3)	−7.6330	−877.067	114.91	0.30	0.3167303
AW125442	EST	−7.5854	−1271.233	167.59	0.27	0.3167303
X03818	mRNA for nicotinic acetylcholine receptor gamma	−7.5303	−812.600	107.91	0.35	0.3167303
AF002283	α-Actinin-2 associated LIM protein mRNA	−7.4972	−1464.733	195.37	0.22	0.3167303
X75316	seb4 mRNA	−7.3414	−164.133	22.36	0.50	0.3167303
AV093331	EST	−6.9629	−2448.033	351.58	0.32	0.3167303
AA726223	EST	−6.9033	−1219.000	176.58	0.49	0.3167303
AI152789	EST	−6.5928	−247.733	37.58	0.28	0.3167303
AW122030	EST	−6.5169	−1582.800	242.88	0.41	0.3167303
M82831	Macrophage metalloelastase	−6.3951	−492.500	77.01	0.24	0.3167303
X83202	11Beta-hydroxysteroid dehydrogenase	−6.3294	−205.333	32.44	0.31	0.3167303
AV359510	EST	−6.1152	−221.267	36.18	0.39	0.3167303
AW123564	EST	−5.9123	−191.133	32.33	0.43	0.3167303
AI844911	EST	−5.8490	−717.533	122.68	0.35	0.3167303
L10076	Nicotinic acetylcholine receptor delta 1-subunit	−5.8014	−338.833	58.41	0.23	0.3167303
X12973	*MLC1F/MLC3F* gene for myosin alkali light chain	−5.7622	−4476.800	776.93	0.44	0.3167303
D86370	Megakaryocyte potentiating factor	−5.6731	−586.567	103.39	0.35	0.3167303

three osteogenic BMPs using GenMAPP/MAPPFinder. After filtration of the acquired microarray data, expression of 358 genes was shown to increase >1.5-fold in the osteogenic BMP-treated samples, and 229 of them represented distinct genes and were used to calculate the z scores. On top of the up-regulated gene ontology terms are growth factors, cytokines, and extracellular matrix structural components, while genes involved in chemoattractants, transcriptional regulation, signal transduction, and cell proliferation were also up-regulated upon osteogenic BMP stimulation (Table IIA). Conversely, 310 genes were down-regulated by >33% (or <0.66-fold), 182 of which represented distinct genes and were used for the z score calculation. Not surprisingly, genes involved in muscle development and muscle structural components were the top list of gene ontology terms, i.e., the expression of those genes was inhibited upon osteogenic BMP stimulation (Table IIB). Other notable down-regulated pathways were cell motility, cytoskeleton, calcium binding proteins, and two of the integrin complex. A complete list of the gene ontology analysis is available at www.boneandcancer.org/c2c12arrays.htm. Taken together, the above findings are consistent with the notion that osteoblastic differentiation and myogenesis are two divergent processes [Murray et al., 1993; Katagiri et al., 1994]. Osteogenic BMPs promote osteoblastic differentiation, and simultaneously inhibit myoblast-specific gene expression.

Verification of Osteogenic BMP-Regulated Expression of Target Genes

We next sought to verify the expression of target genes upon BMP stimulation. We employed two independent methods to investigate BMP-regulated expression of candidate target genes from SAM analysis (Fig. 3). First approach was to conduct RT-PCR. Experimentally, exponentially growing C2C12 cells were infected with AdBMP-2, AdBMP-3, AdBMP-6, AdBMP-9, and AdGFP. At 30 h after infection, total RNA was isolated for RT-PCR analysis using gene-specific primers. Representative results from more than 15 up-regulated genes and 10 down-regulated genes were shown in Figure 4. All three osteogenic BMPs were shown to induce expression of the selected target genes (Fig. 4A), which was consistent with their increased signal intensity on Affymetrix genechip

TABLE II. Gene Ontology/Pathway Analysis Using GenMAPP

GO name	Go type	Number changed in hierarchy	Number measured in hierarchy	Percent changed in hierarchy	z Score
A: Partial list of the gene ontology analysis of >1.5-fold genes[a]					
Growth factor	F	16	35	45.7	8.664
Cytokine	F	13	31	41.9	7.344
Extracellular matrix structural constituent	F	11	27	40.7	6.612
Heparin binding	F	8	16	50	6.494
Chemoattractant	F	5	7	71.4	6.449
Regulation of transcription	P	45	313	14.4	4.917
Signal transducer	F	51	372	13.7	4.896
Cell proliferation	P	8	29	27.6	4.145
Skeletal development	P	5	15	33.3	3.824
Morphogenesis	P	23	166	13.9	3.226
Cellular_component	C	166	2,226	7.5	0.002
Biological_process	P	188	2,324	8.1	2.354
Molecular_function	F	206	2,704	7.6	0.925
Gene ontology	R	229	3,071	7.5	0
B: Partial list of the gene ontology analysis of <0.66-fold genes[b]					
Muscle contraction	P	14	18	77.8	12.946
Muscle fiber	C	11	14	78.6	11.536
Muscle development	P	14	30	46.7	9.495
Cell motility	P	15	36	41.7	9.134
Myosin	C	4	6	66.7	6.306
Cytoskeleton	C	24	148	16.2	5.433
Morphogenesis	P	25	166	15.1	5.124
Calcium ion binding	F	14	98	14.3	3.561
Signal transducer	F	35	372	9.4	3.034
Integrin complex	C	2	8	25	2.287
Cellular_component	C	132	2,226	5.9	0.013
Biological_process	P	145	2,324	6.2	1.295
Molecular_function	F	156	2,704	5.8	−1.001
Gene ontology	r	182	3,071	5.9	0

[a]Three hundred fifty eight genes met the (fold) >1.5 criteria; 25 genes did not link to a MGI term; 104 genes did not link to a GO term; 254 genes were used to calculate the results shown below; the z score is based on an N of 3,071 and a R of 229 distinct genes.
[b]Three hundred ten genes met the (fold) <0.66 criteria; 26 genes did not link to a MGI term; 102 genes did not link to a GO term; 208 genes were used to a calculate the results shown below; the z score is based on an N of 3,071 and a R of 182 distinct genes.

hybridizations (Fig. 4B). Conversely, BMP-2 and BMP-9 (to a lesser extent, BMP-6) were able to inhibit the expression of Actn3, MyBP-H, myogenin, and integrin α7 (Fig. 4C). These inhibitions were correlated well with the decreased signal intensity on microarray hybridizations (Fig. 4D). It should be pointed out that one of the 15 selected up-regulated genes (Accession no. AF020313) and one of the 10 selected down-regulated genes (Accession no. D86370) were not confirmed. We do not have explanations although we could not rule out possible artifacts during microarray hybridizations or the RT-PCR primers were not optimal. Nevertheless, the vast majority of the selected candidate genes from SAM analysis were readily verified as targets of osteogenic BMPs.

The second approach was to conduct microarray analysis using the CodeLink platform. CodeLink bioarrays represent a similar platform to Affymetrix's oligonucleotide arrays. Unlike Affymetrix chips, CodeLink bioarrays are arrayed with pre-synthesized oligos with one validated probe per gene (vs. multiple probes per gene for Affymetrix's chip). Theoretically, CodeLink bioarrays should provide a better sensitivity, especially for low abundance transcripts. We carried out CodeLink bioarray hybridization (mouse chips, approx. 10,000 genes per chip) in the same fashion as that for Affymetrix chips using the same set of RNA samples. The acquired microarray data were filtered, normalized, and analyzed in a similar fashion. Because the probe sets were different in the two platforms, we were not able to embark a direct comparison on the microarray data between Affymetrix's and CodeLink's. However, for most of the candidate genes identified by SAM analysis there was a strong correlation on the signal intensity between these two microarray platforms. Representative results of comparing four up-regulated target genes (Fig. 5A) and four down-regulated genes (Fig. 5B) were shown. Thus, these findings, along with the RT-PCR results, demonstrate that the most, if not all, of the target genes identified by microarray analysis may represent genuine downstream mediators of osteogenic signaling of

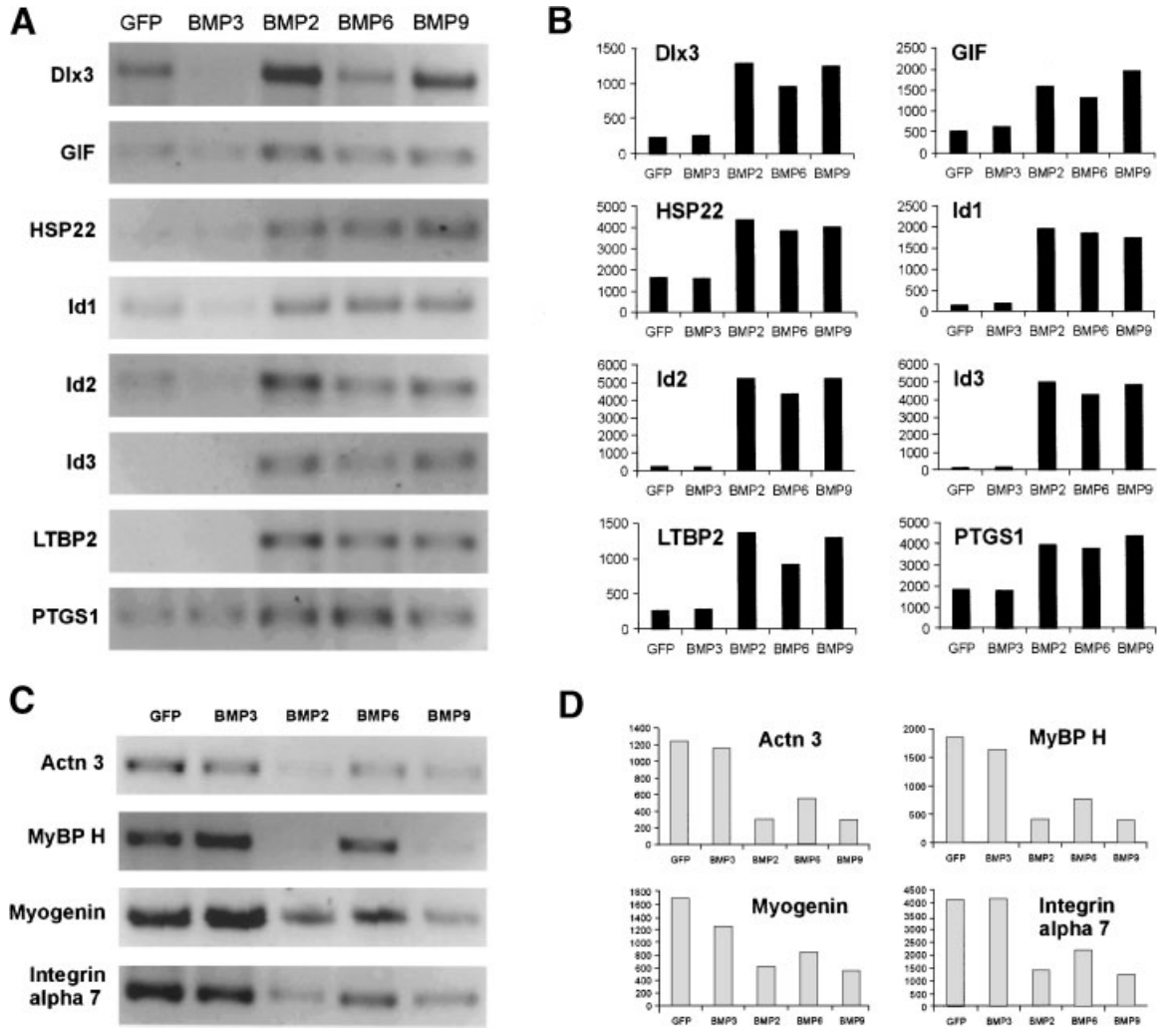

Fig. 4. Osteogenic BMPs-regulated expression of target genes. C2C12 cells were infected with AdBMPs or AdGFP. At 30 h after infection, total RNA was isolated for reverse transcriptase (RT)-PCR. **A**: Representative RT-PCR analysis of eight up-regulated genes from SAM analysis were shown. **B**: Normalized signal levels from microarray hybridizations for the same eight genes listed in (A). **C**: Representative RT-PCR analysis of four down-regulated genes from SAM analysis were shown. **D**: Normalized signal levels from microarray hybridizations for the same four genes listed in (C). See text for details.

BMPs although further characterization of these target genes are warranted.

DISCUSSION

Although several BMPs, notably BMP-2 and BMP-7, have been widely studied in various bone formation models, our recent studies have demonstrated that BMP-6 and BMP-9 exert equally, if not more, potent osteogenic activity [Cheng et al., 2003]. Genetic disruption studies have demonstrated that BMP-3 functions as an inhibitor of bone density [Daluiski et al., 2001]. It is assumed that BMPs bind to their receptors and activate BMP R-Smads, which subsequently trigger the signaling cascades [Heldin et al., 1997; Massague, 1998; Wrana, 2000]. However, little is known about how osteogenic signaling is transduced through the BMP receptor/Smad network, and what downstream mediators are specifically regulated by osteogenic BMPs, and not by non-osteogenic BMPs.

In order to determine the early mediators of osteogenic BMPs, we conducted a comparative expression profiling analysis of three osteogenic BMPs (i.e., BMP-2, BMP-6, and BMP-9) and two inhibitory/non-osteogenic BMPs (i.e., BMP-3 and BMP-12)-stimulated C2C12 cells. Using unsupervised hierarchical clustering and SAM analysis of global gene expression, we have

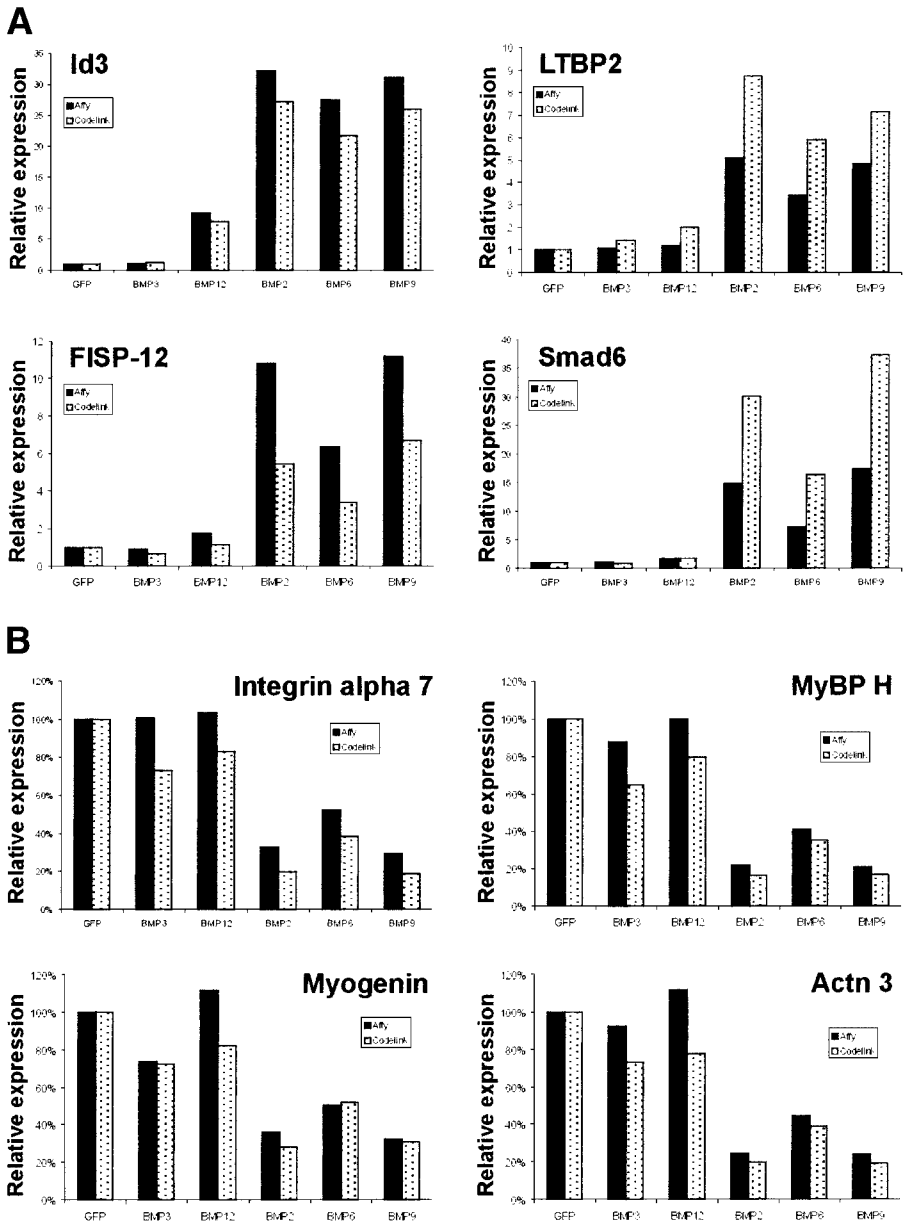

Fig. 5. Verification of target gene expression using CodeLink microarray analysis. The total RNA samples were prepared in the same fashion as those used for Affymetrix microarray analysis. Probe preparation and CodeLink genechip hybridizations were performed according to manufacturer's manual. Normalized data were compared with Affymetrix's. **A**: Relative expression of four representative up-regulated genes was compared between Affymetrix (solid bars) and CodeLink (dotted bars) microarrays. Relative expression (ratio) was calculated using the signal levels of GFP-treated samples as one unit. **B**: Relative expression of four down-regulated genes was compared between Affymetrix (solid bars) and CodeLink microarrays (dotted bars), using the signal levels of GFP-treated samples as 100%.

identified approximately 200 genes whose expression is regulated by osteogenic BMPs. Gene ontology/pathway analysis has revealed that these BMPs play a role in regulating genes involved in cell growth, transcriptional control, cell architecture, cell adhesion, and extracellular matrix formation. The most striking expression trend during the early stage of osteogenic BMP signaling is the induction of various growth factors and transcription factors, and the inhibition of myogenesis.

It is notably intriguing that multiple members of two gene families were significantly induced by osteogenic BMPs. One group was three of the Id helix-loop-helix proteins, Id1, Id2, and Id3, which were significantly induced

by BMP-2, BMP-6, and BMP-9 (up to 30-fold), and marginally affected by BMP-12 (<3-fold), but not altered by BMP-3 at all. *Id* genes have been reported as BMP-2 targets [Ogata et al., 1993; Katagiri et al., 1994; Hollnagel et al., 1999; Locklin et al., 2001; Kang et al., 2003], but their actual roles in bone formation are unknown. Since Id proteins are key negative regulators of bHLH transcription factors of myogenic differentiation [Kreider et al., 1992], they may function through inhibiting myogenesis and promoting proliferation of other lineages, including osteoblast lineage [Kreider et al., 1992; Norton, 2000]. Another group was three members of the distal-less homeobox proteins, Dlx-1, Dlx-2, and Dlx-3, which were also up-regulated by the three osteogenic BMPs, but not by BMP-3 and BMP-12. *Dlx* genes comprise a highly conserved family of homeobox genes, and are involved in the development of the forebrain, branchial arches, sensory organs, and limbs [Merlo et al., 2000; Panganiban and Rubenstein, 2002]. Mice lacking *Dlx* genes have defects in skeleton [Qiu et al., 1997]. However, possible roles of *Dlx* genes in BMP-mediated osteogenic signaling pathway remain to be defined. Nevertheless, these findings suggest that the above-mentioned transcriptional regulators may play a role in regulating osteoblastic differentiation of mesenchymal progenitor cells.

Our results indicate that osteogenic BMPs significantly inhibit the expression of a group of genes conferring muscle phenotype. These genes include actinin α2 associated LIM protein, cadherin 15, myosin heavy chain, actinin α3, tropomyosin 2β, myosin light chain, myomesin 2, and myosin binding protein H. Several genes involved in regulating myogenesis, such as myogenin and MyoD, are also inhibited by osteogenic BMPs. These results are consistent with the notion that osteogenesis and myogenesis are opposite and divergent processes [Murray et al., 1993; Katagiri et al., 1994].

Gene expression profiling has been recently performed on different stages of osteoblast maturation [Beck et al., 2001; Garcia et al., 2002; Raouf and Seth, 2002; Vaes et al., 2002; Roman-Roman et al., 2003] and BMP-2-treated mesenchymal derived cells [Harris and Harris, 2001; Locklin et al., 2001; Balint et al., 2003; Kang et al., 2003]. Not surprisingly, a portion of the downstream targets of osteoblast differentiation from these reports overlaps with some of those identified in this study. However, by comparing expression profiles between osteogenic BMPs (i.e., BMP-2, BMP-6, and BMP-9) versus inhibitory/non-osteogenic BMPs (i.e., BMP-3 and BMP-12), we may identify targets that are more specifically regulated by osteogenic BMPs. Moreover, our studies will allow us to conduct further analyzes of the signaling differences between osteogenic BMPs (e.g., BMP-2 vs. BMP-9 or BMP-2 vs. BMP-6), or osteogenic BMPs and BMP-3 (e.g., BMP-2 vs. BMP-3 or BMP-9 vs. BMP-3). These cross-section analyzes may illuminate a common osteogenic signaling pathway, while revealing the functional distinctions among different BMPs. Future studies should be devoted to functional characterization of the identified signaling mediators of osteogenic BMPs. Ultimately this line of investigation could provide important insights into the molecular basis of BMP-mediated bone formation.

ACKNOWLEDGMENTS

The authors thank the Genetics Institute of Cambridge, MA, for providing human BMP cDNAs.

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