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Phenotype Discovery by Gene Expression Profiling: Mapping of Biological Processes Linked to BMP-2-Mediated Osteoblast Differentiation

Eva Balint,[1] David Lapointe,[1,3] Hicham Drissi,[1] Caroline van der Meijden,[1] Daniel W. Young,[1] Andre J. van Wijnen,[1,2] Janet L. Stein,[1,2] Gary S. Stein,[1,2] and Jane B. Lian[1,2]*

[1]Department of Cell Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655-0106
[2]Cancer Center, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655
[3]Information Services, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655

Abstract Understanding physiological control of osteoblast differentiation necessitates characterization of the regulatory signals that initiate the events directing a cell to lineage commitment and establishing competency for bone formation. The bone morphogenetic protein, BMP-2, a member of the TGFβ superfamily, induces osteoblast differentiation and functions through the Smad signal transduction pathway during in vivo bone formation. However, the molecular targets of BMP-mediated gene transcription during the process of osteoblast differentiation have not been comprehensively identified. In the present study, BMP-2 responsive factors involved in the early stages of commitment and differentiation to the osteoblast phenotype were analyzed by microarray gene expression profiling in samples ranging from 1 to 24 h following BMP-2 dependent differentiation of C2C12 premyoblasts into the osteogenic lineage. A total of 1,800 genes were responsive to BMP-2 and expression was modulated from 3- to 14-fold for less than 100 genes during the time course. Approximately 50% of these 100 genes are either up- or downregulated. Major events associated with phenotypic changes towards the osteogenic lineage were identified from hierarchical and functional clustering analyses. BMP-2 immediately responsive genes (1–4 h), which exhibited either transient or sustained expression, reflect activation and repression of non-osseous BMP-2 developmental systems. This initial response was followed by waves of expression of nuclear proteins and developmental regulatory factors including inhibitors of DNA binding, Runx2, C/EBP, Zn finger binding proteins, forkhead, and numerous homeobox proteins (e.g., CDP/cut, paired, distaless, Hox) which are expressed at characterized stages during osteoblast differentiation. A sequential profile of genes mediating changes in cell morphology, cell growth, and basement membrane formation is observed as a secondary transient early response (2–8 h). Commitment to the osteogenic phenotype is recognized by 8 h, reflected by downregulation of most myogenic-related genes and induction of a spectrum of signaling proteins and enzymes facilitating synthesis and assembly of an extracellular skeletal environment. These genes included collagens Type I and VI and the small leucine rich repeat family of proteoglycans (e.g., decorin, biglycan, osteomodulin, fibromodulin, and osteoadherin/osteoglycin) that reached peak expression at 24 h. With extracellular matrix development, the bone phenotype was further established from 16 to 24 h by induction of genes for cell adhesion and communication and enzymes that organize the bone ECM. Our microarray analysis resulted in the discovery of a class of genes, initially described in relation to differentiation of astrocytes and oligodendrocytes that are functionally coupled to signals for cellular extensions. They include nexin, neuropilin, latexin, neuroglian, neuron specific gene 1, and Ulip; suggesting novel roles for these genes in the bone microenvironment. This global analysis identified a multistage molecular and cellular cascade that supports BMP-2-mediated osteoblast differentiation. J. Cell. Biochem. 89: 401–426, 2003.

Key words: C2C12 cells; neuronal differentiation; BMP-2; osteogenesis; small leucine rich proteins; signal transduction; transcription factors; genetic circuits

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*Correspondence to: Jane B. Lian, Department of Cell Biology and Cancer Center, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655-0106. E-mail: jane.lian@umassmed.edu

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Comprehensively defining the genetic network involved in commitment to a cellular phenotype and subsequent growth and differentiation is pre-requisite for understanding osteogenic signaling mechanisms that control bone specific gene expression. Conventional analysis of gene expression has identified a temporal sequence of phenotypic genes that characterize stages of maturation of the osteoblast phenotype (proliferation, matrix maturation, mineralization). However, key regulatory events that are essential for the initial stages of skeletogenesis in response to physiologic regulatory signals remain to be established.

To date, only a few factors have been identified in the commitment towards the osteoblast phenotype and established as central components for bone formation through genetic models. BMP-2 is a member of the TGF-β superfamily and plays a key regulatory role as a paracrine/autocrine-signaling molecule in bone and limb development. BMP-2 can induce ectopic bone formation when injected subcutaneously or intramuscularly into rats, mimicking the normal developmental events of endochondral bone formation [Reddi and Anderson, 1976; Wozney et al., 1988]. Recombinant BMP-2 can repair or regenerate bone defects [Bostrom et al., 1999], and systemic administration of BMP-2 can reverse osteopenia [Turgeman et al., 2002]. These clinical applications underscore the importance of understanding BMP-2-mediated gene expression. Mutations in BMP-2/4 are lethal soon after gastrulation [Zhang and Bradley, 1996]. Thus, a complete understanding of BMP-2-mediated functions in the skeleton has not been achieved.

Several BMP responsive genes have been identified by analysis of either candidate or differentially displayed genes [Bachner et al., 1998; Gori et al., 2001]. Important BMP responsive genes include developmental signaling factors (sonic hedgehog, follistatin), BMP-2 antagonists (chordin, noggin), and extracellular matrix proteins (fibromodulin) [Gori et al., 2001; Reddi, 2001; Balemans and van Hul, 2002]. Transcription factors that have been identified as BMP-2 responsive for skeletogenesis include Sox9, which is crucial for chondrogenesis and Cbfa1/Runx2 and Osterix that have both been shown by null mouse models to be essential for bone formation [Komori et al., 1997; Otto et al., 1997; Nakashima et al., 2002]. Hence, characterization of the genetic regulatory networks involved in phenotype differentiation is required to define the biological mechanisms that control the progressive expression of stage-specific genes. The key factors that are necessary for bone formation and the known spectrum of BMP-2-induced proteins are each insufficient to induce bone formation; the interrelationships between the osteogenic and BMP-2 responsive regulatory cues remain to be established.

BMPs are known to induce various mesenchymal cell lines to differentiate to osteoblast and chondroblast lineage cells [Rosen et al., 1996]. The C2C12 premyoblast cell line provides a biologically relevant model to delineate components of the regulatory cascade of genes responsive to BMP-2 that mediate development of the osteoblast phenotype [Katagiri et al., 1994]. In this study, we used unsupervised hierarchical and functional clustering analysis of global gene expression to identify processes that contribute to BMP-2-mediated osteoblast differentiation of C2C12 pre-myoblasts. Our findings show that cellular responses of this bipotential mesenchymal cell line to BMP-2 involve a cascade of progressive events which is characterized by four key stages: (1) early *activation and repression* (1–4 h) of non-osteogenic BMP-2-related developmental systems (angiogenesis, myogenesis, neurogenesis); (2) a period of *remodeling cell architecture* (4–8 h), reflected by changes in cytoskeletal-related genes and synthesis of a transitional extracellular environment; (3) *commitment* to the osteogenic pathway (8–12 h) through induction of signaling-related genes (kinases, phosphatase, receptors); and (4) *establishment* of the bone phenotype (16–24 h) with peak expression of genes related to assembly of an ECM, cellular adhesion, and cellular communication. Our analyses resulted in the discovery of novel gene regulatory programs related to osteoblast differentiation that were originally described in relation to neural migration and extension of astrocyte and oligodendrocyte cell processes. Major hierarchical and functional clusters of BMP-2 responsive genes suggested co-regulation of genes involved in modulation of cell architecture, transcriptional control, growth control, basement membrane formation, cell adhesion and communication, and extracellular matrix formation. These studies have defined a sequence of molecular and cellular events

associated with the BMP-2-mediated cascade for osteoblast differentiation.

MATERIALS AND METHODS

Cell Culture

The murine pre-myoblastic cell line C2C12 was purchased from ATCC (American Tissue Culture, Manassas, VA). Cells were maintained in regular growth medium (DMEM 10% FBS and antibiotics; Life Technologies, Inc., Gaithersburg, MD) at 37°C in a humidified atmosphere in 5% CO_2 as previously described [Katagiri et al., 1994; Lee et al., 1999]. Cells (1×10^6) were plated into 100-mm tissue culture dishes under serum-free conditions, because different concentrations of serum were previously shown to induce Runx-2 expression in C2C12 cells [Banerjee et al., 2001]. At 24 h after plating, regular growth medium was changed to 0.25% albumin (Calbiochem, San Diego, CA) in DMEM and cells were maintained for an additional 24 h. Cells were treated 48 h after plating with either 300 ng/ml recombinant human BMP-2 (Wyeth Research Genetics Institute, Cambridge MA) or 2 ng/ml of recombinant human TGF-β (R&D Systems, Inc., Minneapolis, MN) or vehicle in albumin containing media. Cultures are approximately 50% confluent at the beginning of serum starvation and about 90% confluent at the time of treatment (data not shown). Cells were harvested at 0 and 1, 2, 4, 6, 8, 12, 16, 20, and 24 h following BMP-2 or vehicle treatments from two independent temporally separated experiments. Serum starvation conditions did not significantly alter cell survival or myotube formation (data not shown).

Gene Expression Profiling Analysis

Total cellular RNA was isolated from control- and BMP-2-treated groups at every time point using the RNeasy RNA isolation kit (Qiagen, Inc., Valencia, CA), according to the manufacturer's recommendations. The concentration and quality of RNA samples were examined by both spectrophotometry and gel electrophoresis. After Northern blot analysis for phenotypic gene expression, duplicate samples from each independent experiment were selected for gene chip microarray analysis at 1, 2, 4, 8, 12, and 16 h of BMP-2 treatment and for the 0 and 16 control groups. The 24-h control and the 1- and 24-h treatment groups were single samples. Samples of total RNA (5 ng) were processed for microarray analysis using the *Mu11k A* and *B* gene chip set containing 13,179 genes (Affymetrix, Inc., Santa Clara, CA) following the manufacturer's instructions. Briefly, cDNA synthesis was performed using Custom SuperScript Double-Stranded cDNA Synthesis Kit (Life Technologies, Inc., Gaithersburg, MD). Biotin-labeled antisense cRNA target was produced using the BioArray HighYield RNA Transcript Labeling Kit (Enzo Diagnostics, Inc., Farmingdale, NY). Hybridizations were performed with each chip type (A and B) for every sample using the GeneChip Fluidics Station (Affymetrix, Inc.) of the University of Massachusetts Medical School Genomics Core Facility (Worcester, MA). Chip performance, background levels and presence/absence calls were assessed using the Microarray Suite software (Affymetrix, Inc.), which recorded 1,800 genes changing in response to BMP-2. Further expression analysis was performed on microarray data exported as scanned data files (CEL and CDF) to dChip software [Li and Wong, 2001]. Each chip in a given set was normalized by adjusting the probe intensities so that the median probe intensity for each chip in the set for all samples was equal. Absence and presence calls were re-evaluated and intensity normalization was performed across the multiple arrays. Following this adjustment, the expression values for each gene were calculated using model expression analysis [Li and Wong, 2001]. Expression values for all of the genes for the two chip types were combined (13,179 genes) and imported into dChip for advanced analysis (filtering and comparisons of hierarchical and functional clustering). Data was filtered such that genes included in the advanced analyses met at least one of the following criteria (a) expression values showing at least 1.8-fold change across the multiple arrays and/or (b) a ratio of the standard deviation to the mean of the expression values across all the time points for a given gene (coefficient of variation) was between 0.20 and 10 resulting in 784 BMP-2 responsive genes or 0.35 and 10, which resulted in 104 genes as indicated. These standard filters enriched for BMP-2 responsive genes change throughout the time course.

Advanced Analyses of Microarray Data

Advanced analysis included hierarchical and functional clustering, which was performed using the dChip1.1 software [Li and Wong,

2001]. Hierarchical clustering is an unsupervised clustering method that groups genes according to similarities in their expression patterns. Before clustering, the expression values for genes across the various samples were standardized to have a mean of 0 and standard deviation of 1 (distance method correlation matrix). These standardized values were used for calculating correlations between either genes within the time course or between control and treated groups. These values served as the basis for merging nodes using the average linkage method to construct the hierarchical clusters. In this article, visualization of the expression levels for each gene is shown in a blue and red matrix. Pure red represents a log ratio of expression levels that are 3 or higher above the mean, white is the mean, and pure blue represent −3 or lower than the mean across all time points for a given gene.

Data were also analyzed by grouping together genes with similar expression patterns using Microsoft Excel for the purpose of identifying cellular functions at specific times during the osteoblast differentiation process. For graphical illustration of expression levels, from hierarchical clustering analysis or Excel gene grouping profiles, relative ratios were obtained from the intensity value at each time point/the average intensity across the time course. These relative expression values were plotted as a $\log_2$ of the ratio to facilitate visualization of the quantitative changes between the time points.

Based on the hierarchical clustering, enrichment for gene functions was also performed using the dChip program. Information for functional category clustering was derived from public databases and genes were classified according to molecular function, biological process, and cellular components using the Gene Ontology Consortium terms (as of June 2002; http://www.geneontology.org) [Ashburner et al., 2000]. This process identifies clusters enriched for genes sharing a particular function with nominal P values computed from the hypergeometric distribution (Fisher's exact test) [Siegel, 1956].

Supplemental Information Online

The entire data set of nearly 1800 BMP-2 responsive genes (from the *Mu11K* gene chip set) can be found on our website: www.umassmed.edu/cellbio/labs/Steinlab. The gene sets described in this manuscript are displayed in an Excel format of the intensity values (average of duplicate samples) for each gene across the time course (T0, T1, T2, T4, T8, T12, T16, T24) and control groups together with, probe identification numbers, GenBank accession numbers, and gene ontology categories.

RESULTS AND DISCUSSION

Validation of the Experimental Paradigms With Microarray Data for Identifying BMP-2 Responsive Genes

Treatment of the pre-myoblast C2C12 cell line with BMP-2 is a well-established model for studying osteoblast differentiation [Katagiri et al., 1994; Chalaux et al., 1998; Fujii et al., 1999; Lee et al., 1999]. We used this system to identify major events associated with the commitment of mesenchymal cells towards the osteoblast phenotype. Under the serum free experimental conditions used here to maximize the initial expression of BMP-2 responsive genes [Banerjee et al., 2001], we first evaluated whether commitment to the osteogenic phenotype was evident between 0 and 24 h. By phase contrast microscopy, untreated cells initiated myotube formation; while the BMP-2-treated cells developed a rounded morphology by 24 h (data not shown). Myogenin and myoD mRNAs were downregulated in the BMP-treated samples by 24 h, while induction of Runx 2, alkaline phosphatase, and osteocalcin mRNAs was observed at 12, 16, and 20 h, respectively in two independent experiments by Northern analysis (data not shown). Based on this temporal expression pattern of phenotypic genes in all control and treated samples, time points and control groups from two independent time courses were selected for microarray analyses (shown in Fig. 1A). We then validated the microarray expression profile of these previously characterized genes in BMP-2-treated C2C12 cells (Fig. 1B,C). For microarray analyses, RNA samples representing BMP-2 treatments of 0-1-2-4-8-12-16-24 h were compared with controls at 0, 16, and 24 h (vehicle only) that represent the myogenic pathway.

Gene chip hybridization expression patterns (Fig. 1B,C) were found to be consistent with our Northern expression profile. We observed MyoD to be transiently upregulated from 2 to 4 h in response to BMP-2, and then downregulated from 8 to 24 h. Osteocalcin was detected by 16 h in Northern analysis, while the microarray de-

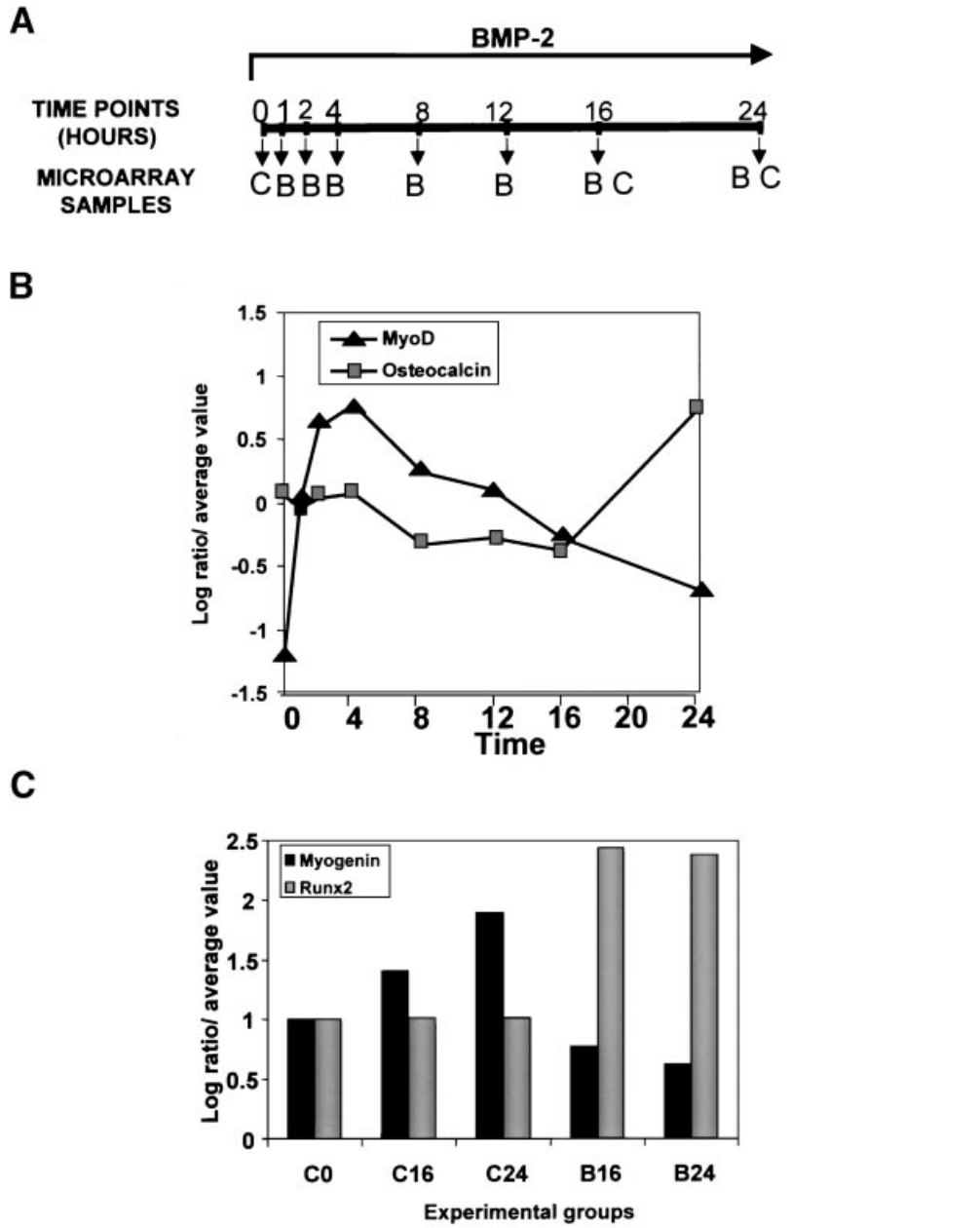

Fig. 1. Experimental validity of microarray expressed genes for BMP-2-mediated osteogenic differentiation of C2C12 Cells. **A**: Schematic illustration of samples selected for microarray analyses following BMP-2 treatment of C2C12 cells at times collected after addition of 300 ng/ml BMP-2 (B) or vehicle control groups (C) (see Materials and Methods). **B**: Expression levels of phenotypic genes, myoD and osteocalcin, during the differentiation time course detected by gene chip microarray values obtained from the combined expression data of the duplicate experiments and microarrays. The relative expression values that are plotted equal the $\log_2$ [expression value at each time point/the average value across the time course] to emphasize the pattern of expressed genes. **C**: Expression levels of phenotypic genes, myogenin and Runx2, between BMP-2 and control groups at 0 time and the end points, 16 and 24 h of treatment with vehicle (C) and BMP-2 (B). Values plotted represent a ratio for each sample of intensity value/time 0 to emphasize that Runx2 is induced only in the BMP-2 groups and myogenin increases only in the control groups.

tected osteocalcin only at 24 h (Fig. 1B). The osteogenic phenotype was confirmed by induction of robust Runx2 expression in BMP-2-treated C2C12 cells, but not in the control cell groups (Fig. 1C). In the controls, myogenin was expressed and increased during the time course (Fig. 1C). Thus, the microarray expression profile detects changes in marker gene expression

consistent with biochemical and molecular analyses, although absolute expression levels vary due to the very distinct hybridization conditions of the two methodologies.

Global Analysis of BMP-2-Treated C2C12 Cells Shows Temporal Processes Related to Osteoblast Differentiation

Of the 13,179 total gene sets on the Affymetrix *Mu11K* chips, 8,555 were present in one or more samples of the time course and 1,791 were present and changing in response to BMP-2 based on a filtering criteria of a minimum average intensity of 100 and a 1.5-fold change between the minimum and maximum values. We initially examined a large subset of 784 genes obtained using filtering criteria that included: (a) a 0.2–10 coefficient of sample variation and (b) a requirement for the presence of a given gene in at least 13% of the samples. These parameters resulted in genes exhibiting a 2.0 or greater fold change. The expression profile of this group of genes characterizing the BMP-2-induced phenotypic changes during osteoblast differentiation is displayed in Figure 2A and the BMP-2 responsive genes are compared to the controls at 0 time and at the end points (16 and 24 h) (Fig. 2B). Hierarchical clustering analysis resulted in two main gene expression patterns within the time course: genes that are upregulated from 12 to 24 h (Fig. 2, group 1), and genes that are maximally expressed at 0 time, then downregulated by 4 h (group 2). A subset of the downregulated genes are first induced (1 h), and then acutely repressed at 4 h (group 3). This altered gene expression is largely a response to BMP-2 and not simply a consequence of in vitro culture as demonstrated by comparison to vehicle-treated controls at 16 and 24 h (Fig. 2B). Only a very small subset of genes is found in both control and BMP-2 groups at 24 h.

In order to identify global categories of genes involved in the BMP-2-induced phenotypic conversion, a functional clustering method was employed using the dChip program. Functional clustering analysis is based on GeneOntology annotations of genes. Among the total of 13,179 genes on the Affymetrix mouse array, 4,991 are annotated with at least one category. In the 784 gene hierarchical cluster, annotated genes form several functional clusters marked with orange bars (Fig. 2A). A functional cluster is recognized when the occurrence of a GeneOntology category in a particular hierarchical cluster is significantly enriched compared to the total number of genes with the same annotation among the entire pool of 13,179 genes examined. As the functional groups may span fairly large hierarchical clusters, many different gene expression patterns could be included within the same functional cluster (e.g., the extracellular matrix, cell adhesion, cell motility, etc.). Regardless of their expression pattern, enrichment of genes in a functional cluster indicates a general involvement of genes with these functions during BMP-2-mediated osteoblast differentiation.

Representative annotated genes in selected functional clusters are listed in Table I, which shows genes that are common to several functional clusters. These genes are either upregulated, downregulated, or transiently expressed during osteoblast differentiation. We also identified one functional cluster that inhibited a 23% enrichment in functionally related genes, not in any one hierarchical cluster, but in the entire 784-gene array (Fig. 2A and Table I, column 1, rows 1 and 2). This cluster includes 333 genes encompassing extracellular matrix (ECM), basement membrane, cell motility, cell adhesion, and cell communication functions (Fig. 2A). The majority of the upregulated and transiently induced genes represent skeletal-related ECM components, and the downregulated genes encode cytoskeletal, signaling, and adhesion proteins for non-osseous cell systems (Table I). Recent genetic evidence also indicates the importance of extracellular matrix fibrillar components in providing signals for limb development. Noteworthy is fibrillin 1, the major structural component of extracellular microfibrils [Handford, 2000]. Mutations in the human fibrillin genes are the basis for manifestations of Marfan syndrome that involves skeletal, ocular, and cardiovascular abnormalities [Ramirez, 1996].

Functional clustering revealed categories of genes that were similar in function and also displayed similar expression patterns. For example, plasma membrane and intracellular trafficking genes represents 21 and 9%, respectively, of genes within their functional categories and similar temporal expression patterns (Fig. 2A). These genes show a marked induction at 1 h and are repressed at 4 h, reflecting mechanisms mediating changes in cellular architecture, protein synthesis, trafficking to

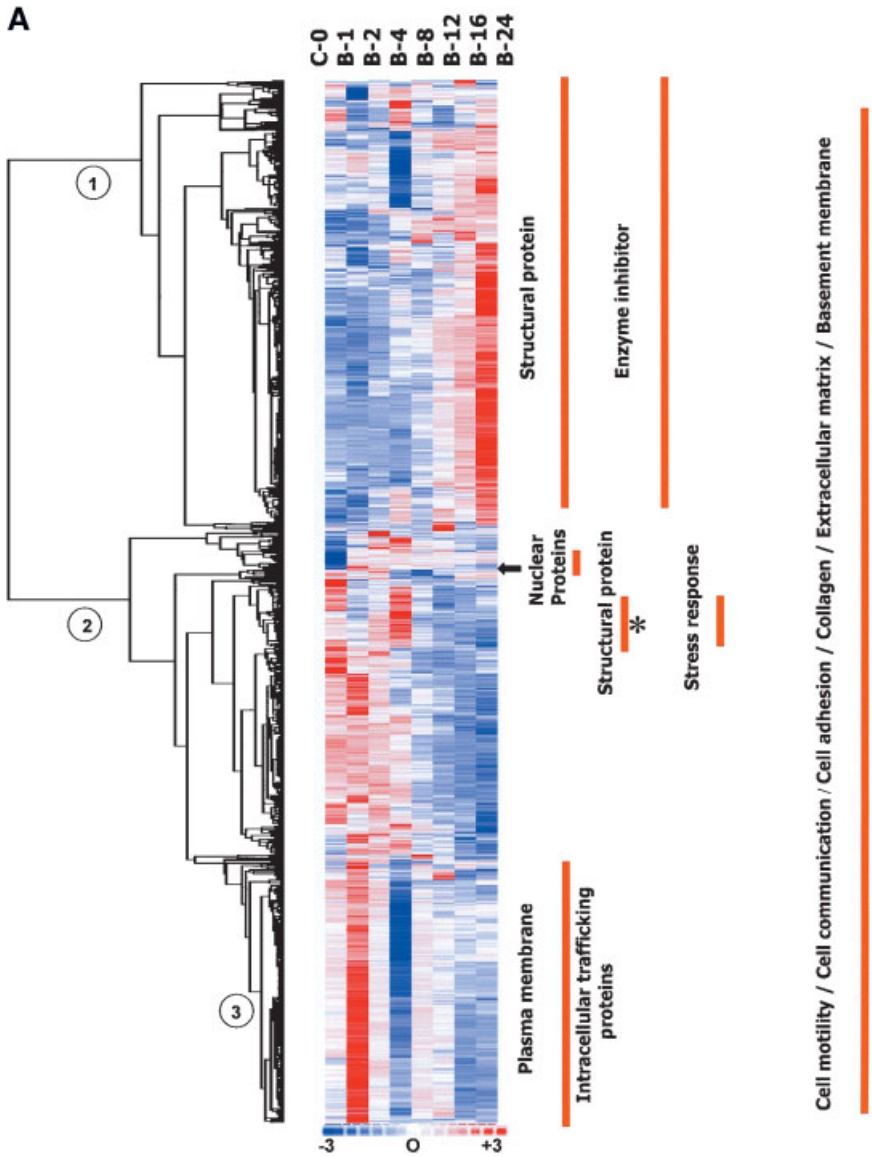

Fig. 2. Hierarchical and functional clustering of BMP-2 responsive genes show cellular modifications during requirements for osteoblast differentiation of C2C12 cells. **A**: Microarray expression profile in a group of 784 genes. This set was defined by filtering criteria to include genes changing during the time course in at least 2 time points with a 1.8-fold or greater response. The expression level matrix is shown representing normalized values from −3 (blue, below the mean) to +3 (red, above the mean). The mean (0 value) is represented by the white color. Rows correspond to different genes, and the columns represent the various time points (time 0 and 1, 2, 4, 8, 12, 16, and 24 h of BMP-2 treatment from left to right, respectively). The dendogram on the left and the horizontal distance between the tree nodes represent statistical similarities between the neighboring genes and clusters. Three major hierarchical clusters representing the genes induced (1), downregulated (2), or transiently up- and downregulated (3) are indicated. Functional clusters spanning specific regions of the hierarchical tree are marked in orange on the right. An arrow indicates a nuclear cluster that is magnified in Figure 3A, the asterisk indicates a structural protein cluster expanded in C. These data were obtained by dChip analysis. **B**: Control samples at 0 (C0), 16 (C16), and 24 h (C24) are compared to BMP-2-treated samples at 16 and 24 h to further establish specificity of BMP-2-modified genes in **Panel A**. Filtering criteria and the blue and red matrix of expressed genes are as described in (A). The dendogram on top illustrates the final clustering tree among the experimental groups, which further confirms the distinction between BMP-2 groups and all vehicle control groups. Brackets indicate the few genes expressed in the control and BMP-2 groups at 24 h. **C**: Enlargement of a structural protein cluster expressed transiently during the time course in a small hierarchical cluster is shown. Annotated genes are shown in blue print and their intensity values of the functionally related genes are shown in the graph to the right (**D**). **E**: Graphical illustration of selected enzyme inhibitors in a functional cluster that spans a large hierarchical cluster (several hundred genes) as indicated in panel A.

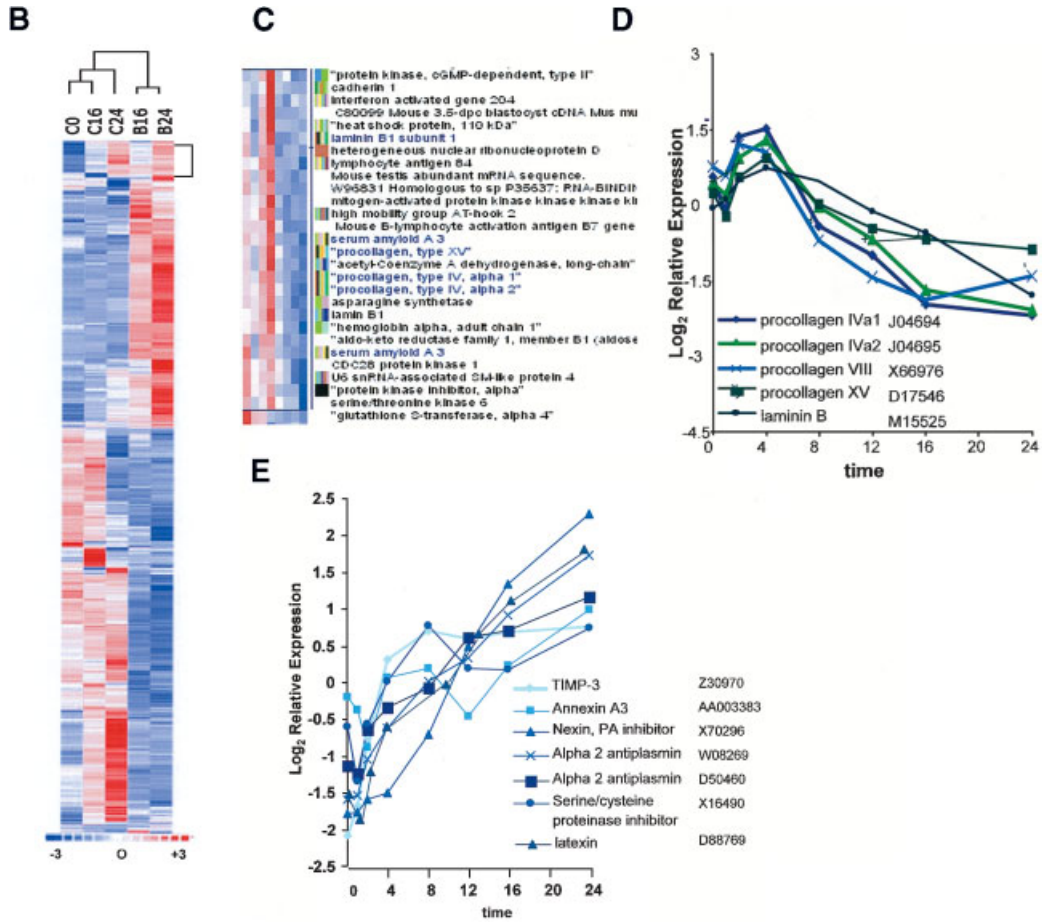

Fig. 2. (*Continued*)

Golgi, and processing of proteins necessary for differentiation towards the osteoblast phenotype (Table I). A significant number of the structural and enzyme inhibitor genes show a general pattern of late induction (16–24 h) (Fig. 2A). However, a small cluster of structural protein genes (designated in Fig. 2A and magnified in Fig. 2C with annotated genes displayed in blue) is transiently upregulated at 4–8 h and has related functions, suggesting coordinated activities in this biological model. The procollagens Types IV and XV contribute to the molecular architecture of basement membranes. Additionally, they become degraded during embryonic development by matrix metalloproteinases and cathepsins (which are also induced from 16 to 24 h) producing functional fragments that are anti-angiogenic, e.g., endostatin [Ortega and Werb, 2002]. Consistent with a transient expression of basement membrane-related genes is the downregulation of nidogens (Table I), which are adhesive proteins for basement membrane components [Salmivirta et al., 2002]. Selected genes annotated in the enzyme inhibitor cluster are graphically illustrated in Figure 2E. One of these upregulated genes, *TIMP 3*, maintains the stability of connective tissue ECMs, including bone [Mizutani et al., 2001].

Taken together, these expression waves of functional genes, provide an indication of the stages of BMP-2-induced osteogenesis including modifications in cellular architecture, formation of a scaffolding matrix, and subsequent organization and stabilization of an extracellular matrix.

Genes Maximally Induced in Response to BMP-2 Reflect ECM Modifications for Phenotypic Conversion to Committed Osteoblasts

We utilized stringent filtering criteria to further evaluate the main patterns of BMP-2-modified genes. These criteria included the requirement for presence of a given gene in at least 20% of samples and variation across the samples of 0.35–10 SD/mean, which resulted in

TABLE I. Expression of Genes in Functional Clusters Modified During BMP-2-Induced Osteoblast Differentiation

Functional clusters	Genes upregulated	Genes downregulated	Genes transiently altered
Cell motility Cluster size: 333 In cluster/total: 13/76	Troponin T3, skeletal, fast Myosin, heavy polypeptide 3, skeletal muscle	Actinin alpha 3 Small inducible cytokine A2 Lymphocyte specific 1 Troponin T2, cardiac	Small inducible cytokine A7 Chemokine orphan receptor 1 Chemokine (C-X-C) receptor 4
Cluster size for all: 333 Cell communication In cluster/total: 49/478 Cell adhesion In cluster/total: 25/202 Extracellular matrix In cluster/total: 24/90 Extracellular In cluster/total: 24/105 Basement membrane In cluster/total: 9/21	Laminin beta 3 Guanin nucleotide binding protein beta 4 Neural cell adhesion molecule Procollagen type VI alpha 1 Procollagen type VI alpha 2 Gap junction membrane channel protein alpha 1 Procollagen type I alpha 2 Fibroblast growth factor receptor 1 Neuropilin Kinesin-associated protein 3 Laminin alpha 2 Thrombospondin 1 Decorin Perlecan (heparan sulfate proteoglycan 2) Chondroitin sulfate proteoglycan 2 Biglycan Laminin alpha 2 Osteomodulin Fibrillin 1	Nidogen 1 Nidogen 2 Vascular cell adhesion molecule 1 Small inducible cytokine A2 Phosphoprotein enriched in astrocytes 15 Small inducible cytokine B subfamily member 5 Cadherin 15 Zyxin Vinculin Gap junction membrane channel protein alpha 7 CD44 antigen Activin receptor interacting protein 1 Nicotinic receptor, beta polypeptide 1 (muscle) Integrin alpha 7 Procollagen type VIII alpha 1 ect2 oncogene	Inhibin beta C Fibroblast growth factor 2 Tenascin C Small inducible cytokine A7 Thrombospondin 1 Lymphoid blast crisis-like 1 Integrin alpha 7 Cadherin 1 Laminin B1 subunit 1 Procollagen type XV Procollagen type IV alpha 1 Procollagen type IV alpha 2 Protein kinase, mitogen activated eightinteracting protein Adrenergic receptor beta 2 Interleukin 1 receptor antagonist Syntaxin 3 GABA-A receptor, Subunit gamma 2
Structural protein Cluster size: 140 In cluster/total: 21/367 Structural protein Cluster size: 9 In cluster/total: 6/367	Troponin T3, skeletal muscle, fast Laminin beta 3 Laminin alpha 2 Procollagen type VI alpha 1 Procollagen type VI alpha 2 Procollagen type I alpha 2 Decorin Keratin complex 1, acidic, gene 14 Chondroitin sulfate proteoglycan 2 Biglycan Osteoglycin Gelsolin Perlecan (heparan sulfate proteoglycan 2) Syndecan 2 Osteomodulin Ribosomal protein L7	CD44 antigen Glypican 1 Procollagen type VIII alpha 1	Laminin B1 subunit 1 Serum amyloid A3 Procollagen type XV Procollagen type IV alpha 1 Procollagen type IV alpha 2 Ribosomal protein L7 Tubulin beta 2 Troponin T2, cardiac Glypican 4 Ribosomal protein L13
Stress response Cluster size: 26 In cluster/total: 7/404	B lymphoma Mo-MLV insertion region 1 Lymphocyte antigen 6 complex, locus C Orosomucoid 1	GRO-1 oncogene Small inducibe cytokine A2 Lymphocyte specific 1 CD44 antigen Heat shock protein, DNAJ-like 3	Heat-shock protein cognate 7 Small inducible cytokine A7 Telometric repeat binding factor 1 Interferon activated gene 202A Chemokine orphan receptor 1 Chemokine (C-X-C) receptor 4 Lymphocyte antigen 6 complex, locus E Fc receptor, IgE, high affinity I, gamma polypeptide Mannan-binding lectin serine protease 1

(*Continued*)

TABLE I. (*Continued*)

Functional clusters	Genes upregulated	Genes downregulated	Genes transiently altered
Intracellular trafficking	ATPase, Na^+/K^+ beta 3 polypeptide	Lymphocyte antigen 84	Neural cell adhesion molecule
Cluster size: 57	Gap junction membrane channel protein alpha 1	Facilitated glucose transporter	Solute carrier family 12, member 2
In cluster/total: 5/98	Lymphocyte antigen 6 complex, locus C	Vascular cell adhesion molecule	Glypican 1
		CD82 antigen	Lymphocyte antigen 6 complex, locus E
		Gap junction membrane channel protein alpha 7	Adrenergic receptor beta 2
Plasma membrane		CD44 antigen	Adaptor protein complex AP-2, alpha 2 subunit
Cluster size: 57		Glypican 1	Monocarboxylic acid transporters, member 2
In cluster/total: 12/438		Solute carrier family 20, member 1	Fc receptor, IgE, high affinity I, gamma polypeptide
			Interleukin 1 receptor antagonist
			Very low density lipoprotein receptor
			Huntingtin interacting protein 1 related
			GABA-A receptor, subunit gamma 2
			Nucleoprotein 50
			Adaptor protein complex AP-3, delta subunit
			RAB23, member RAS oncogene family

Genes in the functional clusters marked in orange in Figure 2A are listed. Cluster size refers to the number of genes in the hierarchical cluster(s) of our 784-gene microarray in which the functional genes are enriched. The "cluster/total" represents the number of genes with the particular annotation within the hierarchical cluster versus the total number of genes with the same annotation within the entire gene ontology database. Several functional clusters are shown together because of the overlap in genes within the annotated groups. The *P* value signifying the enrichment of all the clusters is $0.001 < P.000$.

104 genes (1% of the total genes in the array), displayed in Figure 3. Genes in this group are either repressed by 4 h or induced from 16 to 24 h with 3- to 8.7-fold modification in expression. Hierarchical clustering analysis displays retention of the two principal gene expression patterns (downregulated and upregulated with a small subset transiently induced, then repressed) within the time course observed in the larger array of 784 genes in Figure 2. Within the 104-gene array is a high representation of phenotypic genes (~60%). Transcription factors, enzymes, and other signaling proteins and factors related to cell growth account for approximately 28% of the displayed genes. The remaining 15% of this group include ESTs and proteins unexpected in the differentiating osteoblasts (e.g., a plasma selenoprotein and a keratin complex).

Comparison of the muscle-related genes in this group, as well as other myogenic genes in the 784-gene pool demonstrates the progression of inhibited muscle differentiation in relation to appearance of osteogenic markers (Fig. 4A). Cadherin 15, an important cell–cell interacting protein for the developing myotube and myosin heavy chain, rapidly declines after 1 h, while most other muscle-related genes fall below 0 (the average value across the time course) by 4–8 h (e.g., troponin, alpha 2 actinin, alpha 3 actinin, myosin, tropomyosin).

Skeletal-related extracellular genes, representing the greatest fold changes displayed in the 104-gene microarray, exhibited induction by BMP-2 as early as 4 h and continued to increase in expression during osteogenic differentiation with the greatest fold change from 16 to 24 h (Fig. 4B). A histone deacetylase enzyme (HDAC 5) is induced from 8 to 16 h, reflecting chromatin inactivation, then becomes downregulated at 24 h indicating de-repression of chromatin remodeling, consistent with the requirement to downregulate myogenic genes and induce osteogenic-related genes. The highly induced matrix genes include the major collagen of the bone extracellular matrix (Type I), Type VI procollagen, fibromodulin, osteomodulin, and osteoblast specific factor 2 (OSF2). Collagen VI, which is found in articular cartilage [Eyre, 2002], bone and dentin [Becker et al., 1986], and also associated with ECM development during adipocyte differentiation [Nakajima et al., 2002], has multiple adhesion domains for cells and matrix components that

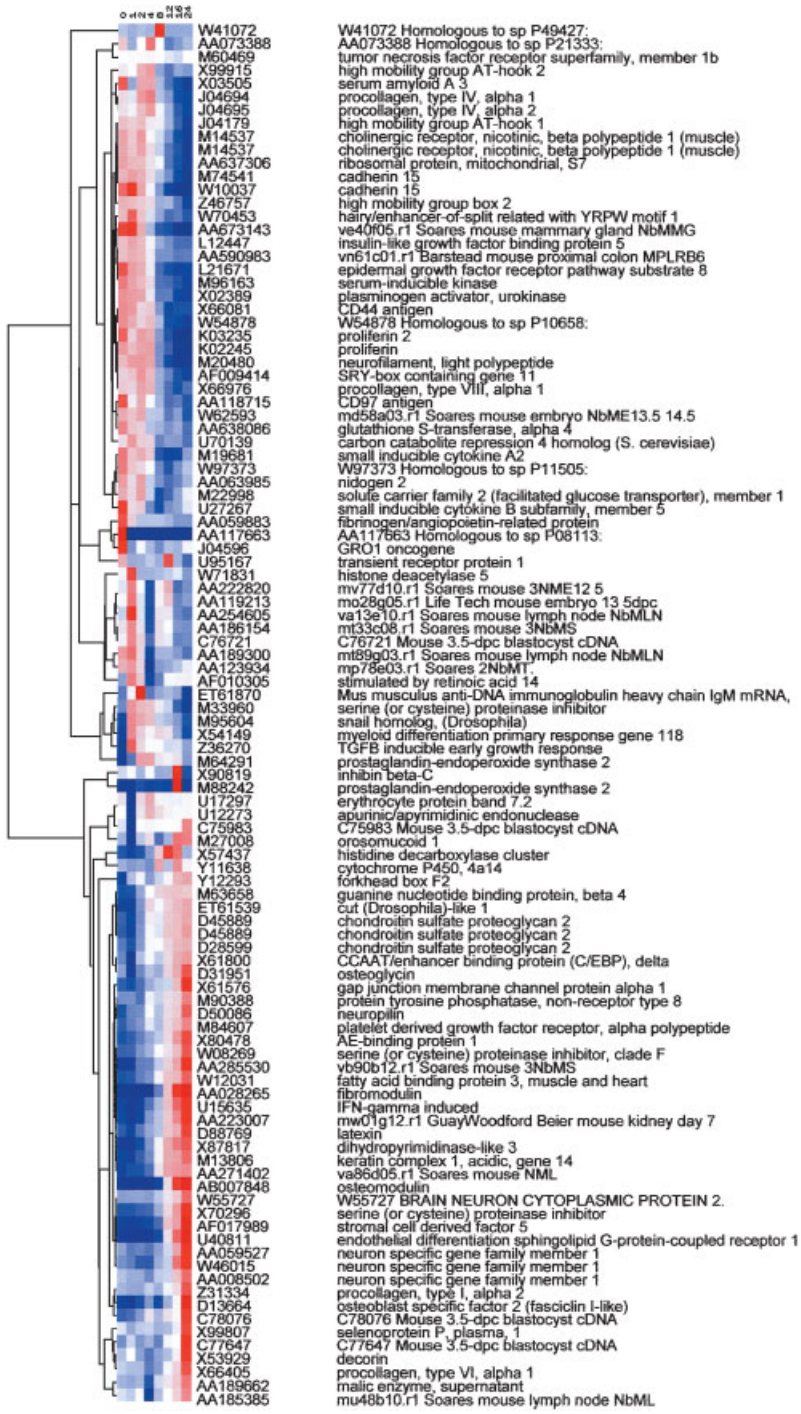

Fig. 3. Hierarchical clustering of BMP-2 responsive genes exhibiting greater than 3-fold changes show signal transduction cascades. Microarray of 104 genes resulting from the applied filtering criteria in which the ratio of the standard deviation to the mean expression value across the time course was between 0.35 and 10. Rows correspond to different genes and the columns represent the various time points (time 0 and 1, 2, 4, 8, 12, 16, and 24 h of BMP-2 treatment from left to right, respectively). The dendogram on the left illustrates the final clustering tree; and the horizontal distance between the tree nodes represent statistical similarities in expression pattern between the neighboring genes and clusters. The two main expression patterns of induction and inhibition occur relatively late (12–24 h) during the BMP-2 time course. Accession numbers and names are shown. Note some of the 104 genes appear duplicated by name, but have different accession numbers and their expression profiles are nearly identical.

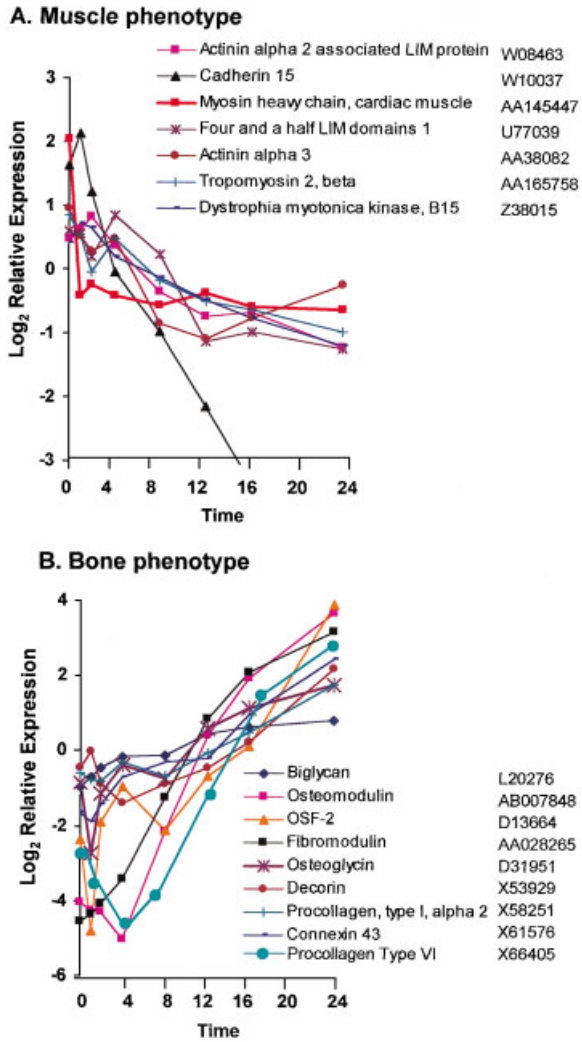

Fig. 4. Phenotypic matrix-related genes are among the highest fold expressed genes during BMP-2-induced osteoblast differentiation. **A**: Muscle-related genes that are downregulated in Figure 3A. Graph also includes selected genes from Figure 2A. **B**: Osteoblast-related phenotypic genes that are upregulated. These are collagens I and VI and the SLRP family of related genes present in the 104-gene microarray (Fig. 3) which have the highest fold induction late in the time course. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

signal rearrangement of the actin skeleton for extension of lamellipodia [Tillet et al., 2002]. OSF-2, also called periostin, has a structure similar to fasciclin I, a cell–cell adhesion molecular expressed in the CNS of insects. OSF-2 was isolated initially as an osteoblast-specific factor, preferentially expressed in periosteum, perichondrium, and periodontal ligament. It supports the attachment and spreading of osteoblasts [Horiuchi et al., 1999]. Connexin 43 (the gap junction membrane channel) is a requirement for osteoblast communication [Lecanda et al., 2000]. Thus, by 24 h of BMP-2 treatment, genes that support an osteoblast syncytium have been induced.

Also highly represented in this significantly induced group at 24 h in response to BMP-2 are genes in the class of the small leucine-rich repeat proteoglycans (SLRP)s, decorin, osteoglycin, biglycan, osteomodulin, chondroitin sulphate proteoglycan 2, and fibromodulin [Matsushima et al., 2000] (Fig. 4B). Notably, the SLRP lumican (AF1013262), isolated as a corneal keratin proteoglycan [Funderburgh et al., 1997] is downregulated 4.3-fold in our time course, indicating specificity of the induced SLRPs for the skeletal environment. Fibromodulin, osteoglycin, and biglycan have been previously characterized as BMP-2 responsive [Takagi et al., 1999; Scott et al., 2000; Gori et al., 2001]. The importance of biglycan and the closely related decorin proteoglycans in bone formation has been demonstrated in null mice models [Chen et al., 2002] and human diseases [Ameye and Young, 2002; Corsi et al., 2002]. Osteomodulin (shown to be the same protein as osteoadherin, a keratin sulfate proteoglycan) was first described in bone as promoting αVβ3 integrin-mediated cell binding [Wendel et al., 1998]. In situ hybridization shows its presence in mature osteoblasts located superficially on trabecular bone [Sommarin et al., 1998]. Thus, the genes maximally induced by BMP-2, which occur at 24 h, are all functionally linked to the SLRP family, which regulate tissue organization and cell–matrix interactions [Wiberg et al., 2002]. In our study, the coordinate increase in collagen VI and biglycan (which uniquely organizes collagen VI into hexagonal-like networks), and other SLRP genes during BMP-2-induced osteogenic differentiation, highlights the importance of BMP-2-mediated fibrillar organization of the bone ECM. There is increasing evidence for the involvement of extracellular matrix proteoglycans also in modulating growth factor activity [Christian, 2000]. Our findings, therefore, suggest that BMP-2 is a central regulator of ECM development at an early stage of osteoblastogenesis through induction of SLRP genes.

In conclusion, our analyses of the 104 BMP-2 maximally responsive genes shows that a directional change in expression of myogenic and osteogenic genes is established and amplified during the time course of BMP-2 treatment of

C2C12 cells. Significant decreases in muscle-related genes and increases in expression of osteogenic-related genes are observed by 8 h, although most curves cross the 0 value (average value across the samples) at 12 h. This 8–12 h window may mark the point of osteoblast phenotypic determination. After this point, a significant upregulation of ECM matrix genes representing an early stage in organization of a bone extracellular matrix and osteoblast differentiation is evident.

Transcriptional Control of Osteoblast Differentiation

Much of our laboratories' work is focused on understanding the mechanisms by which cells are committed to the osteoblast lineage. We thus wanted to investigate transcription factors responsive to BMP-2. One nucleus gene cluster (arrow in Fig. 2A) contains 15 annotated genes, of which 8 are known transcriptional regulators that are immediately responsive to BMP-2 and induced over 3.5-fold within 1 h (Fig. 5A). This functional cluster of nuclear genes was also identified as a hierarchical cluster by analysis of expression patterns and exhibits sustained expression (Fig. 5B). The group includes three inhibitors of DNA binding proteins (Id-1, Id-2, Id-3), which are key negative regulators of basic helix-loop-helix (bHLH) transcription factors that promote the differentiation of many cell types. Id genes are direct targets of the BMP superfamily, and notably BMP-2 [Hollnagel et al., 1999; Lopez-Rovira et al., 2002]. Functions indicated for the Id family include inhibition of developmental programs, expansion of progenitor cell populations, and induction of apoptosis or cell survival depending on the cellular context [Yokota and Mori, 2002]. JunB, which is also rapidly induced, is involved in BMP-2-mediated inhibition of myogenesis [Jonk et al., 1998; Rauch et al., 2002], as well as enhancement of osteogenesis pathways [Chalaux et al., 1998]. JunB expression is retained at significant levels in differentiated osteoblasts [McCabe et al., 1996]. Also in this cluster are three early response genes, a myeloid differentiation primary response gene, TGFβ inducible early growth response gene (TIEG), and the connective tissue growth factor (CTGF); encoded by an immediate early gene and member of the CCN family. TIEG and CTGF have been studied in osteoblasts [Xu et al., 2000; Yamashiro et al., 2001; Johnsen et al., 2002]. The fact that these genes are clustered together by two methods (Fig. 5A,B) raises the possibility that they are functionally linked to the osteogenic process.

We next selected transcriptional regulatory proteins from the 784-gene array ($n = 62$) and applied hierarchical clustering in order to identify their expression patterns in relation to the process of BMP-2-induced osteoblast differentiation. We find two main patterns in response to BMP-2; induction and inhibition of genes through the time course (Fig. 5C). However, in contrast to the phenotypic genes displayed in the larger arrays (Figs. 2A and 3A), transcriptional regulators exhibit narrow windows of altered expression to BMP-2. Approximately 70% of this group of transcription factors exhibits dramatic changes in expression levels within 1–4 h, 12% are expressed transiently between 4 and 8 h, and 18% are not induced until 16 h. The group of early downregulated genes include those transcription factors which regulate development of non-osseous systems; e.g., neuro differentiation factor 2, the four and a half LIM domain, and hairy and enhancer of split (HES) proteins, which are both involved in myogenesis, and Notch, a BMP-2 antagonist.

We also find a sequential expression of members of transcription factor families representing forkhead, SRY box proteins, homeobox, zinc fingers, and Wnt-related proteins. Figure 5A shows forkhead box C2 (FHC2) (also known as MFH-1) in the cluster of immediate responsive genes (1 h). Notably, FHC2, which is expressed in the embryo in the paraxial mesoderm and somites that give rise to skeletal elements, has been characterized as a requirement for BMP-2-induced osteoblast differentiation in the C2C12 cell line, and in skeletal precursor cells [Yang et al., 2000; Nifuji et al., 2001]. MFH-1 null mice display defects in craniofacial bone formation in vivo, supporting its essential function in skeletal development [Iida et al., 1997]. In contrast to FHC2, forkhead (FH) box F2, previously reported to be a transactivator in lung, mesoderm, sclerotome, skeletal precursor cells, and perichondrium, shows late BMP-2 responsiveness from 16–24 h [Miura et al., 1998; Furumoto et al., 1999; Nifuji et al., 2001].

SRY box genes are so named to represent the SOX subfamily of HMG box containing genes; the mammalian sex-determining gene on the Y chromosome (SRY) is the prototypic member of

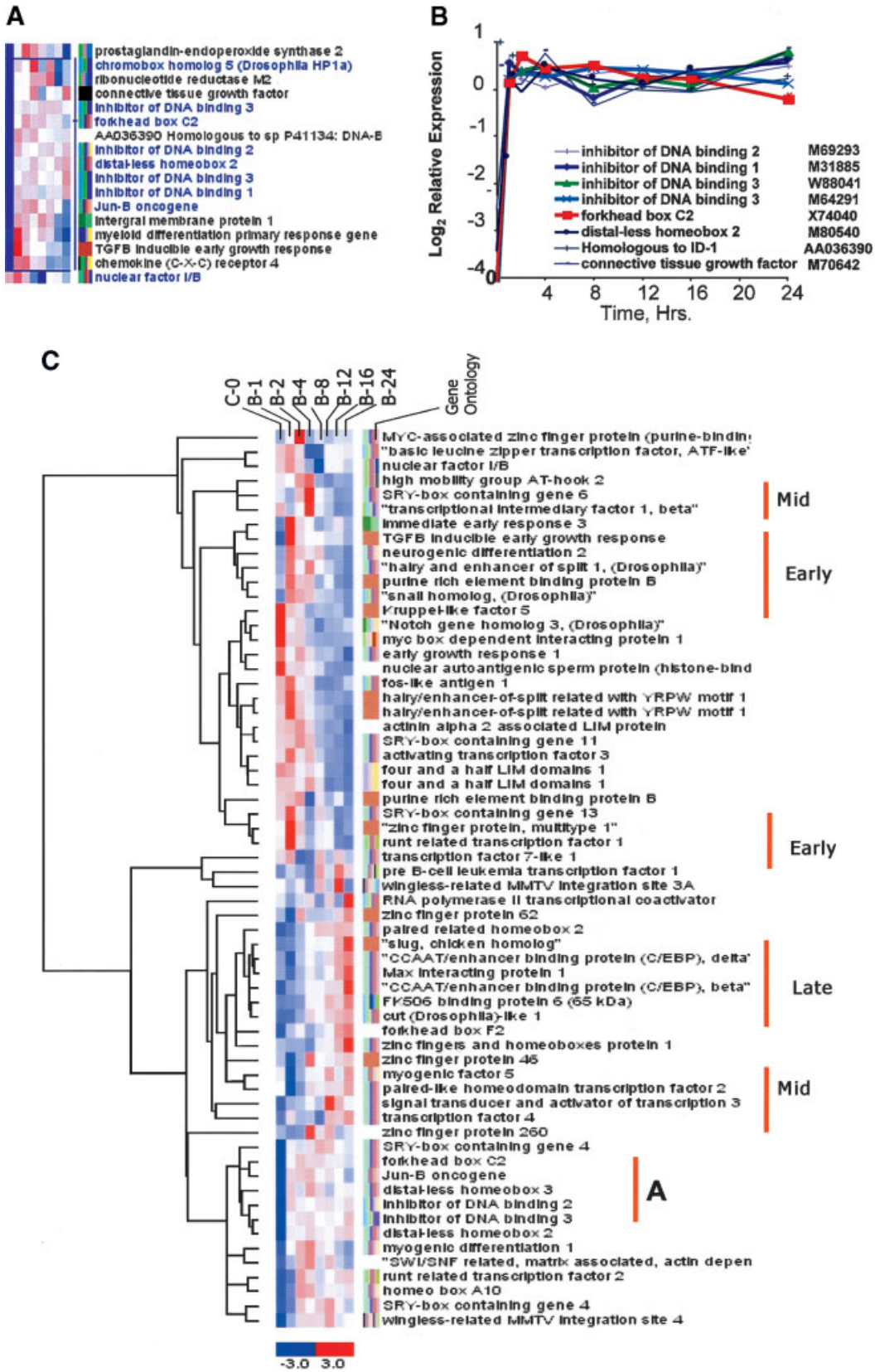
A
prostaglandin-endoperoxide synthase 2
chromobox homolog 5 (Drosophila HP1a)
ribonucleotide reductase M2
connective tissue growth factor
inhibitor of DNA binding 3
forkhead box C2
AA036390 Homologous to sp P41134: DNA-B
inhibitor of DNA binding 2
distal-less homeobox 2
inhibitor of DNA binding 3
inhibitor of DNA binding 1
Jun-B oncogene
intergral membrane protein 1
myeloid differentiation primary response gene
TGFB inducible early growth response
chemokine (C-X-C) receptor 4
nuclear factor I/B
B
Log2 Relative Expression
Time, Hrs.
inhibitor of DNA binding 2 M69293
inhibitor of DNA binding 1 M31885
inhibitor of DNA binding 3 W88041
inhibitor of DNA binding 3 M64291
forkhead box C2 X74040
distal-less homeobox 2 M80540
Homologous to ID-1 AA036390
connective tissue growth factor M70642
C
C-0
B-1
B-2
B-4
B-8
B-12
B-16
B-24
Gene Ontology
MYC-associated zinc finger protein (purine-bindin
"basic leucine zipper transcription factor, ATF-like"
nuclear factor I/B
high mobility group AT-hook 2
SRY-box containing gene 6
"transcriptional intermediary factor 1, beta"
immediate early response 3
TGFB inducible early growth response
neurogenic differentiation 2
"hairy and enhancer of split 1, (Drosophila)"
purine rich element binding protein B
"snail homolog, (Drosophila)"
Kruppel-like factor 5
"Notch gene homolog 3, (Drosophila)"
myc box dependent interacting protein 1
early growth response 1
nuclear autoantigenic sperm protein (histone-bind
fos-like antigen 1
hairy/enhancer-of-split related with YRPW motif 1
hairy/enhancer-of-split related with YRPW motif 1
actinin alpha 2 associated LIM protein
SRY-box containing gene 11
activating transcription factor 3
four and a half LIM domains 1
four and a half LIM domains 1
purine rich element binding protein B
SRY-box containing gene 13
"zinc finger protein, multitype 1"
runt related transcription factor 1
transcription factor 7-like 1
pre B-cell leukemia transcription factor 1
wingless-related MMTV integration site 3A
RNA polymerase II transcriptional coactivator
zinc finger protein 62
paired related homeobox 2
"slug, chicken homolog"
"CCAAT/enhancer binding protein (C/EBP), delta"
Max interacting protein 1
"CCAAT/enhancer binding protein (C/EBP), beta"
FK506 binding protein 6 (65 kDa)
cut (Drosophila)-like 1
forkhead box F2
zinc fingers and homeoboxes protein 1
zinc finger protein 45
myogenic factor 5
paired-like homeodomain transcription factor 2
signal transducer and activator of transcription 3
transcription factor 4
zinc finger protein 260
SRY-box containing gene 4
forkhead box C2
Jun-B oncogene
distal-less homeobox 3
inhibitor of DNA binding 2
inhibitor of DNA binding 3
distal-less homeobox 2
myogenic differentiation 1
"SWI/SNF related, matrix associated, actin depen
runt related transcription factor 2
homeo box A10
SRY-box containing gene 4
wingless-related MMTV integration site 4
Mid
Early
Early
Late
Mid
A
-3.0 3.0

Fig. 5.

the SOX subfamily [Nagai, 2001]. The SOX proteins are involved in various differentiation processes in mammals [Schepers et al., 2002]. Our data show early induction (by 4 h) and repression of SRY genes 6, 11, and 13, which regulate neuronal development [Uwanogho et al., 1995] and lymphocyte differentiation [Schilham and Clevers, 1998]. In contrast, the SRY box gene 4 (*SOX4*) increases by 2-fold from 8 to 12 h. SOX 4, which is important for B cell development, has been characterized in the embryonic growth plate and is regulated by PTHRP in osteoblast like cells [Reppe et al., 2000].

A significant number of homeobox proteins are transiently expressed during the osteoblast differentiation process, for example paired-related homeobox 1 and 2 genes, Hox A10, Msx2, Dlx-2, Dlx-3, and CDP/Cut. Mice lacking these genes have defects in hair and the skeleton [Qiu et al., 1997; Price et al., 1998; ten Berge et al., 2001; Wilkie et al., 2001; Luong et al., 2002]. Both BMPs and homeodomain proteins are implicated in specifying the apoptotic fate of mesenchymal cells and programmed cell death pathways during limb and tooth development [Hogan, 1996; Jernvall and Thesleff, 2000]. The CCAAT displacement protein, CDP/Cut, is maximally expressed at 24 h, following Dlx2 expression. Homeobox factors Msx2, Dlx5, and CDP/cut have been shown to downregulate the osteocalcin gene in osteoblasts [Hoffmann et al., 1994; Towler et al., 1994; van Gurp et al., 1999]. Thus, our studies have identified several homeobox-containing proteins expressed at different times that may provide a continuum of activity during the osteogenic process to regulate phenotypic genes at the appropriate differentiation stages.

The induction of Runx2 by 2 h (see Fig. 5C) suggests signaling for commitment to the osteogenic phenotype has initiated, although the microarray cannot distinguish the bone-related isoforms from the ubiquitous Runx2 isoforms expressed in mesenchyme [Banerjee et al., 2001]. A later increase at 12 h may represent the bone-related isoforms, which we detected by Northern blot analysis with specific probes. Of particular interest is the pattern of downregulated expression after 4 h of the hematopoietic factor Runx1 and Hes1 transcription factor, which is a co-regulatory inhibitor protein of Runx activity (Fig. 5C). This profile is consistent with the current understanding of the regulation of Runx activity in cells [Javed et al., 2000; McLarren et al., 2001].

Several of the transcription factors induced after 8–12 h with emergence of the osteoblast phenotype become maximally expressed at 24 h. These include a protein in the chromatin remodeling SWI/SNF complex, which promotes gene activation and cellular differentiation [de la Serna et al., 2001] and two members of the CAAT enhancer binding proteins (C/EBPβ and C/EBPδ). C/EBPβ and C/EBPδ have been previously reported to support the transcription of bone-related genes in mature osteoblasts. They are increased during differentiation of fetal rat calvarial osteoblasts and synergize with Runx2 to activate osteocalcin and IGF1 expression [McCarthy and Centrella, 2001; Gutierrez et al., 2002; Pereira et al., 2002]. A slug-related factor, not previously studied in relation to osteoblastogenesis, is induced after 16 h. In vivo slug is expressed in mesenchymal cells undergoing chondrogenic differentiation [Savagner et al., 1998] and it has recently been reported to function as an antiapoptotic factor in hematopoietic progenitor cells [Inoue et al., 2002]. Insummary, the significant representation of transcription factors (17% in the 784-gene microarray), coupled with the observation that these are largely responsive to BMP-2 within the first 4 h or at the final 24 h, suggests that BMP-2 influences phenotype differentiation primarily at the level of transcriptional control.

Fig. 5. Expression of transcriptional regulatory proteins during osteoblast differentiation. **A**: The nucleus functional cluster (indicated by the arrow in Fig. 2A) is enlarged from the time course hierarchical cluster. Rows correspond to different genes named on the right and the columns represent the various time points (BMP-2 treatment at 0-1-2-4-8-12-16 h from left to right, respectively). Genes with known ''nucleus'' annotations are highlighted in blue. **B**: A pattern of genes analyzed by Microsoft Excel resulted in the same genes profiled in the hierarchical cluster shown in **panel A**. This group represented genes immediately responsive to BMP-2 with at least a 4-fold induction within the first hour; expression then remains constitutive. **C**: A selected group of transcription factors (n = 62) in a hierarchical cluster. The red, white, and blue matrix corresponds to different genes (rows) and the various time points (columns) as described in Figure 2A. The orange line highlights significant clusters exhibiting a marked induction at 1 or 2 h (early), 4 h (mid), and 16–24 h (late) during BMP-2-mediated osteoblast differentiation. The hierarchical cluster marked A included the same genes profiled by functional clustering (Figs. 2A and 3A) and using Excel (Fig. 3B).

Signal Transduction Gene Expression in Preparation for BMP-2-Mediated Osteoblast Differentiation

Analysis of the 784 genes responsive to BMP-2, shown in Figure 2, demonstrates a range of different hierarchical clusters based on their temporal pattern of expression. We examined several distinct temporal patterns of expression to assess classes of BMP-2 responsive genes that may reflect functional pathways leading to osteogenic differentiation. We show in Figure 6, several selected profiles that identify signaling pathways and modifications in cellular processes.

Three downregulated gene profiles are presented (Fig. 6A–C). Immediately responsive genes are either downregulated several fold (by 1 h) and remain repressed throughout the entire time course (Fig. 6A), or are rapidly induced (approximately 4-fold), before a steady decline (Fig. 6A). A third group of genes declines continuously (Fig. 6B) with initiation of BMP-2 treatment. By 8 h, the expression values of downregulated genes have already fallen below their average value across the time course (panels A,B) or begin to decline (panel C). These downregulated genes include cytoskeletal proteins (tubulins, actinin), regulatory growth factors (Gro1 oncogene, epiregulin, platelet derived growth factor), proliferin-1 and proliferin-related protein-2 which stimulate and inhibit angiogenesis respectively, developmental and transcriptional regulators (e.g., hairy enhancer of split, four and a half LIM, SRY box gene 11, Snail) and genes that support differentiation of non-osseous tissues (myogenic differentiation 1, myeloid differentiation response gene, nestin, fibrinogen/angiopoietin-related protein). Among the downregulated genes mediating signaling are the BMP antagonist Notch, the activin receptor interacting protein, and the TGFβ inducible early growth response gene (TIEG). These findings are consistent with BMP-2-mediated downregulation of non-osseous-related genes.

Distinct temporal patterns of induced genes, which are elevated in the 784-microarray included a significant number of genes mediating signaling (phosphatase, kinases, receptors) shown in Figure 6D–F. Genes that increased during a 0–8 h window are developmental regulators (RAR receptor, paired-related homeobox 2), enzymes and genes related to growth control, or cytoarchitecture (Fig. 6D). Genes upregulated between 4 and 12 h with a 3–4-fold induction (Fig. 6E) include those that provide protection against cell death, enzymes involved in signaling through phosphorylation (PKC, MAPK, tyrosine phosphatases), transcription factors (STAT3, FH box F2), cell adhesion proteins, and notably a follistatin-like gene, which was found to inhibit BMP-2-induced transcriptional response [Tsuchida et al., 2000]. Follistatin, an activin binding protein, is expressed in chondrocytes and osteoblasts and appears to regulate the transition from cartilage to bone [Inoue et al., 1994; Funaba et al., 1996]. In this window, protein kinase C, IGF-BP7, the FGF receptor and TGFβ1, become upregulated. The IGF binding protein family (reviewed in Firth and Baxter, 2002), are regulators of IGF-1, important for bone formation. IGF-BPs provide both positive and negative regulation of cellular growth and differentiation. IGF-BP7 (Fig. 6E) is upregulated in contrast to IGF-BP5 (Fig. 6B) and IGF-BP2 (Fig. 6B), which are downregulated at 8 h. By 12 h and continuing to increase to 24 h, a 3–4-fold induction of several matrix proteoglycans (PG) (chondroitan sulfate 2, syndecan, and perlecan), enzymes, growth factors (stromal cell derived factor), and receptors related to skeletal tissues (PDGFα, the platelet derived growth factor and TGFβ3R) can be identified (Fig. 6F). Syndecan, a heparin sulfate PG, which begins to increase in response to BMP-2 by 2 h is expressed at the surface of osteoblastic cells [Modrowski et al., 1997]. Thus, the induction of signaling pathways mediating expression of skeletal-related matrix components from 16 to 24 h, as well as inhibitory signals of BMP-2 transcriptional control of non-osteogenic pathways (Fig. 6A–C) appears highly coordinated. These findings suggest a complex network of regulatory signals to achieve an appropriate biological response from the C2C12 cells for osteoblast differentiation.

A classical reciprocal expression of proliferation versus growth inhibitory genes was observed in response to BMP-2 consistent with phenotype differentiation. Several regulators of cell growth are downregulated either immediately, as the GRO1 oncogene, or after the first 4 h, as proliferin 1 and proliferin 2, and a G0/G1 switch gene declined after 12 h (Fig. 6A,B,D). Therefore, we examined the pattern of several cell cycle-related genes that changed in

response to BMP-2 during the time course of osteoblast differentiation (Fig. 7A,B). Cyclin D1, which rapidly and continuously declines, controls the G0/G1 transition, consistent with exit from the cell cycle in response to BMP-2. Two cell proliferation markers, DNA primase and Histone H2 are downregulated in stages that indicates a population of cycling cells up to

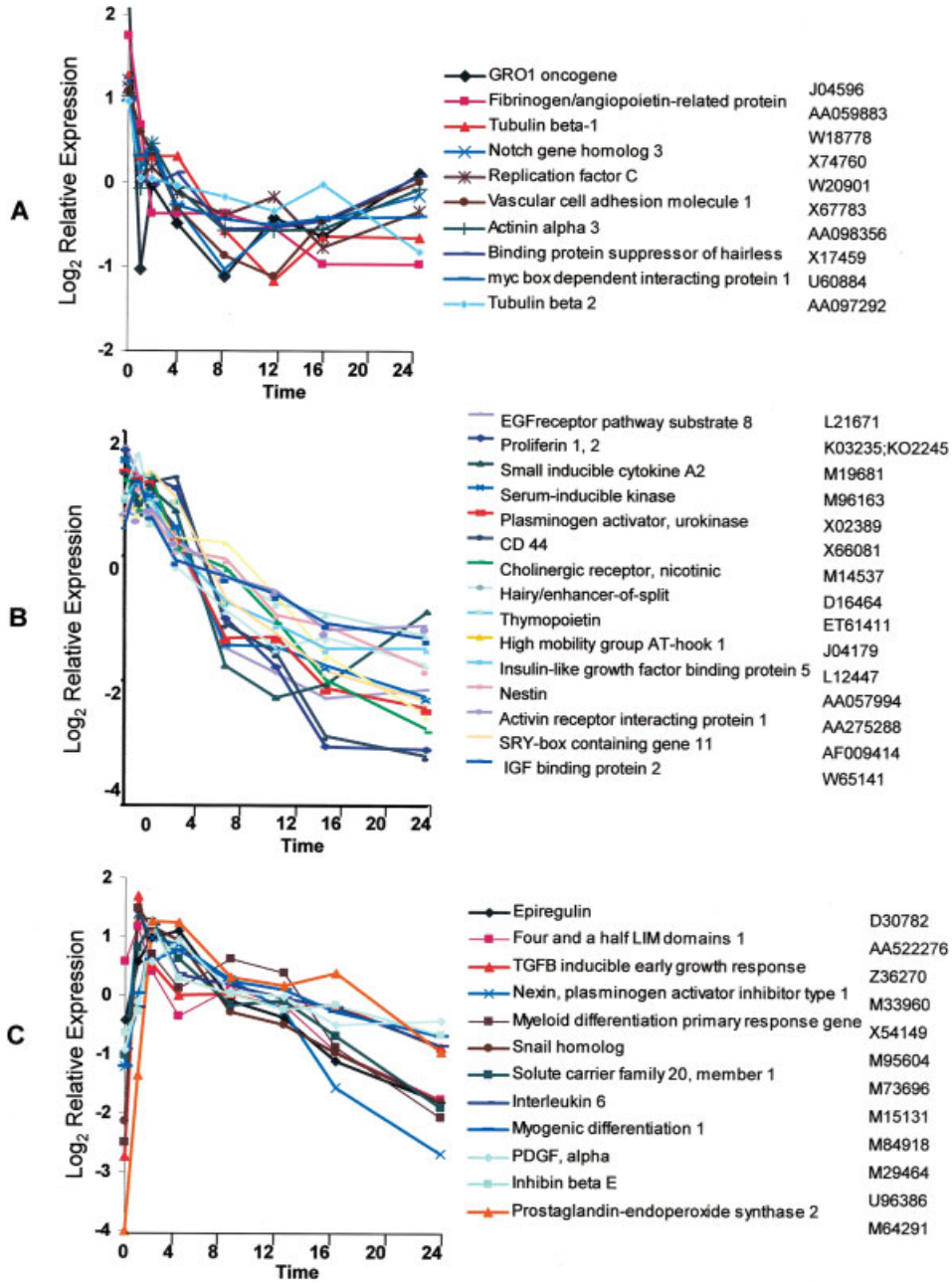

Fig. 6. Signal transduction cascades are indicated by temporal expression profiles. Groups of genes having similar expression patterns are displayed. **Panels A–C** show three selected down-regulated gene profiles. Panel A: Immediate responsive genes are downregulated 3-fold in 1 h and remain suppressed; Panel B includes genes that show a marked reduction from 4 to 8 h, then continuously decline with overall 4–5-fold changes; and Panel C shows genes upregulated 4–5-fold within the first hour before decreasing 4–5-fold by 24 h. **Panels D–F** are examples of genes upregulated with different profiles during BMP-2-induced osteoblast differentiation. Panel D profile shows genes increased between 0 and 8 h in a fold range from 1.8 to 2.5 with maintained expression. Panel E includes genes that increased from 2- to 3-fold between 4 and 12 h. Panel F shows genes continuously increasing from 3- to 6-fold during osteoblast differentiation with expression values above the mean at the later 12–24 h period. Accession numbers corresponding to each gene are shown.

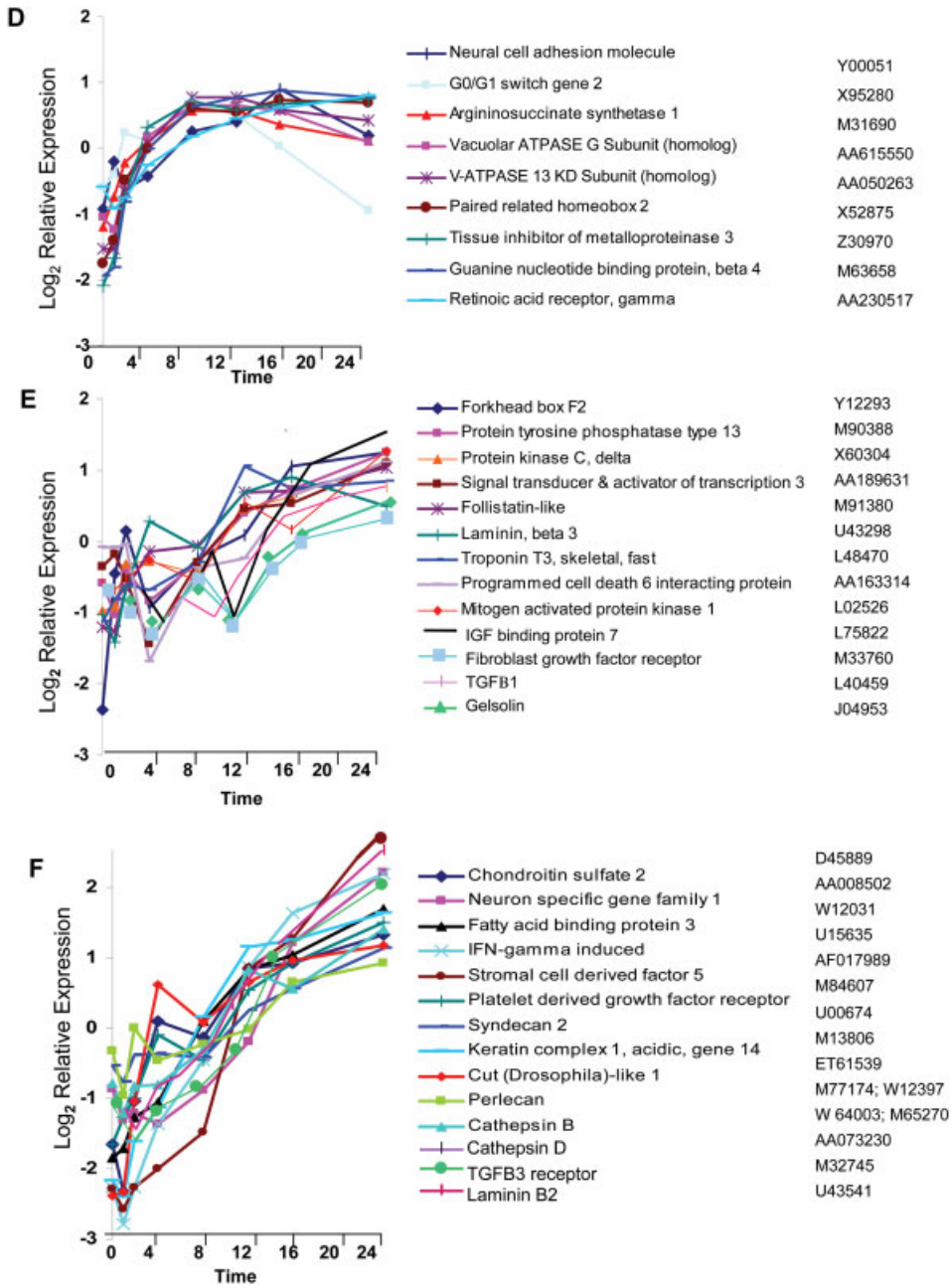

Fig. 6. (*Continued*)

16 h [Sherr, 1996]. By 8 h, tumor suppressor genes p53 and Rb and the growth arrest specific gene 6 (Gas6), are induced to support the post-mitotic state of the cells for differentiation (Fig. 7B). Gas6 is induced in the 12–24 h time period with the onset of significant increases in expression of bone matrix-related genes. This analysis reflects general growth inhibition and post-mitotic differentiation that normally characterizes osteoblast maturation [Lian and Stein, 1998].

Taken together, the temporal coordination of different functional gene categories provides insight into the complexity of cellular processes involved in differentiation to the osteogenic phenotype. These analyses of expression patterns of BMP-2 responsive genes demonstrate a cascade of molecular events that first mediate an initial activation of BMP-2-regulated genes in non-osseous developmental systems, followed by downregulation of these genes via activation of transcriptional repressors in preparation

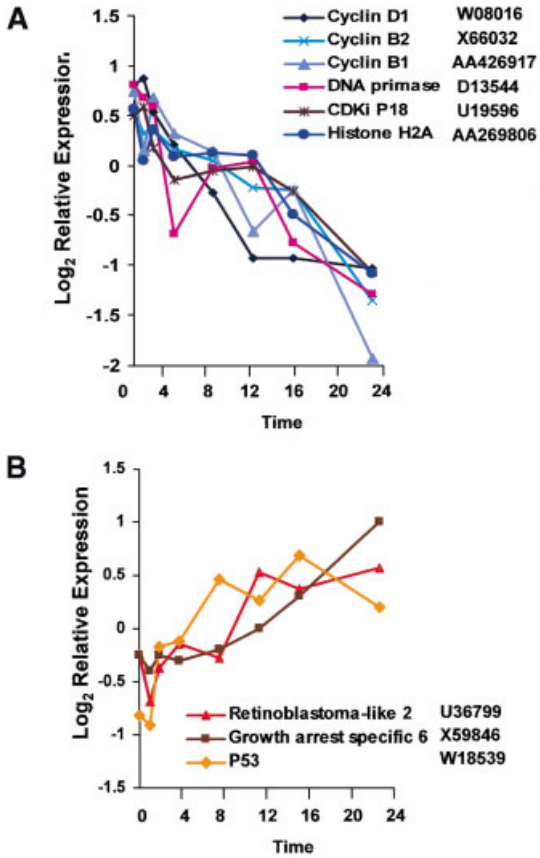

Fig. 7. Expression of proliferation-related genes during BMP-2-mediated osteoblasts differentiation. Genes were selected by GeneOntology categories (cell cycle, cell growth) from the microarray shown in Figure 2A. **A**: Downregulated genes include proteins reflecting cell growth and exiting the cell cycle. **B**: Upregulated growth control-related genes during the time course reflect inhibition of proliferation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

for osteoblast differentiation. The upregulated genes demonstrate the ongoing modifications in cell metabolism and adhesion, the induction of enzymes mediating signaling pathways, and of secreted proteins for extracellular matrix formation. Thus, the C2C12 cell model responds to BMP-2 signaling in a broad context of gene modifications that reveal the multiple mechanisms required for differentiation from a premyoblastic phenotype to the morphologically distinct osteoblast.

BMP-2-Treated C2C12 Cells Show Modifications of Genes Representing Non-Osseous Phenotypes: Potentially Novel BMP-2 Responsive Genes in the Bone Microenvironment

A striking change in expression of genes representing neurogenesis is observed during BMP-2-induced osteoblast differentiation of C2C12 cells. There are 118 annotated genes related to neural function and 56% of these are changing in our global profile (784 genes) in response to BMP-2. BMP-2 inhibits neurogenesis by upregulating the expression of HLH genes (Id 1, Id 2, Id 3, and Hes 5) that are negative regulators of transcription [Nakashima et al., 2001] (shown in Fig. 5A). A marked decrease in neuronal phenotypic genes was evident by 2 h (shown in Fig. 8A), as for example, nestin, which is a marker of undifferentiated neural precursor cells [Lendahl et al., 1990] (in Fig. 4B), and nexin-1, a serine protease inhibitor which promotes neurite growth [Meins et al., 2001]. Other downregulated neuronal cell markers include neurofilament light peptide, which is an intermediate filament of the internal scaffold of nerve cells, a neutrotrophic factor and pentraxin 1, which is induced by synaptic activity [Omeis et al., 1996]. Thus, these downregulated genes reflect an inhibition of early neurogenesis in the C2C12 model.

A group of nerve-related genes were strikingly increased in expression after establishment of the bone phenotype when expression of osteogenic markers became evident from 16 to 24 h (Fig. 8B). These include the highest fold-induced genes (from 2.1 to 4.1), latexin, which is a carboxy peptidase A inhibitor expressed in many tissues (shown in the enzyme inhibitor cluster in Fig. 2E) and is a marker of glutamatergic projection neurons [Liu et al., 2000; Takiguchi-Hayashi, 2001]. A brain enriched GTPase p21-activating protein [Baba et al., 1995] increases during BMP-2-induced osteoblast differentiation. Nexin 2, like nexin 1 (in Fig. 8A), is also a cerebral anti-coagulant and functions as an axonal guidance receptor [Blasi and Carmeliet, 2002]. We noted earlier the transient expression of the basement membrane collagen type IV (Fig. 2D). The NC1 domains of collagen IV promote axonal growth [Lein et al., 1991]. Other genes in Figure 8 include neuropilin, which is a receptor for ligands that promote axonal guidance [He et al., 2002] and the Ulip protein, which is one of a family of intracellular phosphorylation proteins also implicated in axon guidance and outgrowth [Byk et al., 1998; Quinn et al., 1999]. In addition to neuroglian, a cell adhesion protein that leads to cell aggregation [Hortsch et al., 1998], several other neural cell adhesion molecules were observed to be increased several fold in the larger

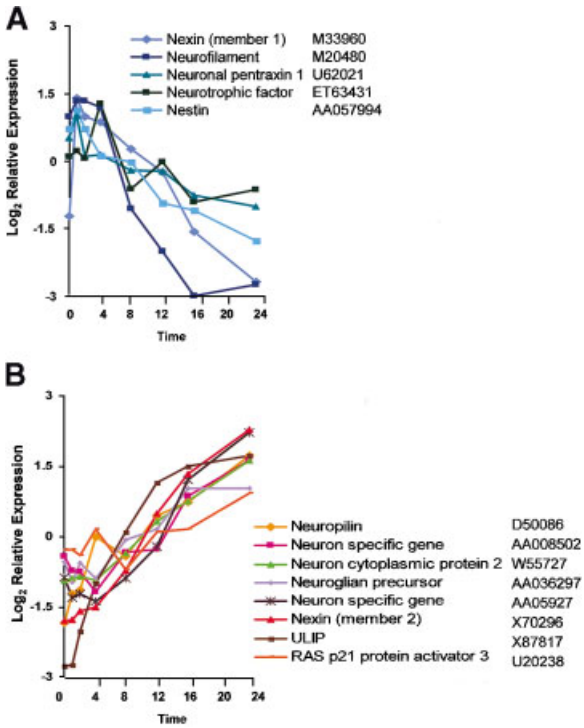

Fig. 8. Novel neural-related genes potentially expressed in the bone environment. Graphical illustration of selected genes from the 784- and 104-gene microarray profiles that are modified over 3-fold. A significant number of neural-related genes were either downregulated (**panel A**), reflecting inhibition of neurogenesis by BMP-2 or continuously increasing during osteoblasts differentiation (**panel B**), representing genes associated with axonal guidance and projections of oligodendrocytes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

array; for example, neural cell adhesion molecules (NCAM Y00051; X15052, see Fig. 6D). Two neuron specific genes encode cytoplasmic proteins of unknown function. Thus, these genes expressed at the late stage (16–24 h) are characteristic of neurite growth, cell migration, and cell adhesion [Arimatsu et al., 1999; Nakashima et al., 2001]. The role of these genes in the bone environment is unknown, but they may be involved in extension and adhesion of lamellipodia of osteocytic processes.

Like neuronal cells, osteocytes form a functional syncytium created by a network of gap junctions that contribute to extensive cellular interconnections. Connexin 43, which is increased over 8-fold from 12 to 24 h (shown in Fig. 4B) maintains extensive gap junctions in osteoblasts/osteocytes [Civitelli et al., 1993], and also supports BMP-2-mediated astrocyte differentiation [Bani-Yaghoub et al., 2000]. Connexin 30 is a major gap junction protein of nerve cells and is upregulated in astrocytes [Condorelli et al., 2002], but is not expressed in our study, although present in the microarray chip sets. Thus, similarly related functional genes expressed in the supporting cells of nerves are selectively induced during the osteogenic phase of BMP-2-treated C2C12 cells. The fact that the microarray displays specificity for BMP-2-mediated downregulation of neurogenesis, suggests that the upregulated neural genes are involved in the bone microenvironment mediating extension of cellular processes and cellular connectivity of the osteoblast/osteocyte. Notably, many similarities between osteocytes and neural cell networks have been described [Turner et al., 2002]. Thus the global analysis of microarray gene expression profiling in this in vitro system can both uncover broad biological effects and lead to identification of genes novel to the bone microenvironment.

The commitment of a cell to the osteogenic lineage and subsequent differentiation induced by BMP-2 is characterized by a sequential cascade of several key developmental periods. Our microarray profile of BMP-2 responsive genes provides a template of potential molecular targets for the regulatory events associated with the osteogenic process(es). The transient induction and repression of growth factors, transcriptional and developmental regulatory proteins, and extracellular matrix proteins reflect the mechanisms functionally linked to regulation of BMP-2-mediated osteoblastogenesis. The modifications in expression of cell motility, cell adhesion, and cell–cell communication genes during the first 24 h of BMP-2 treatment, indicates the complexity of the cellular requirements for development of the osteoblast phenotype. The cascade of BMP-2-mediated gene regulatory programs demonstrated by our global analysis of C2C12 cell differentiation to osteoblasts is summarized in Figure 9.

The immediate responses to BMP-2 can be viewed as a period of *activation and repression* of non-osteogenic BMP-2-related developmental systems encompassing induction of BMP-2-regulated genes controlling early development, with suppressor genes activated to limit the cell's options for differentiation (Fig. 9). The second phase from 4 to 8 h is a stage of *remodeling cell architecture*. Downregulation of neurogenesis and myogenesis occurs rapidly. By 8 h, *commitment* to the osteogenic lineage is indicated by transcriptional repression of non-osseous BMP-2 respon-

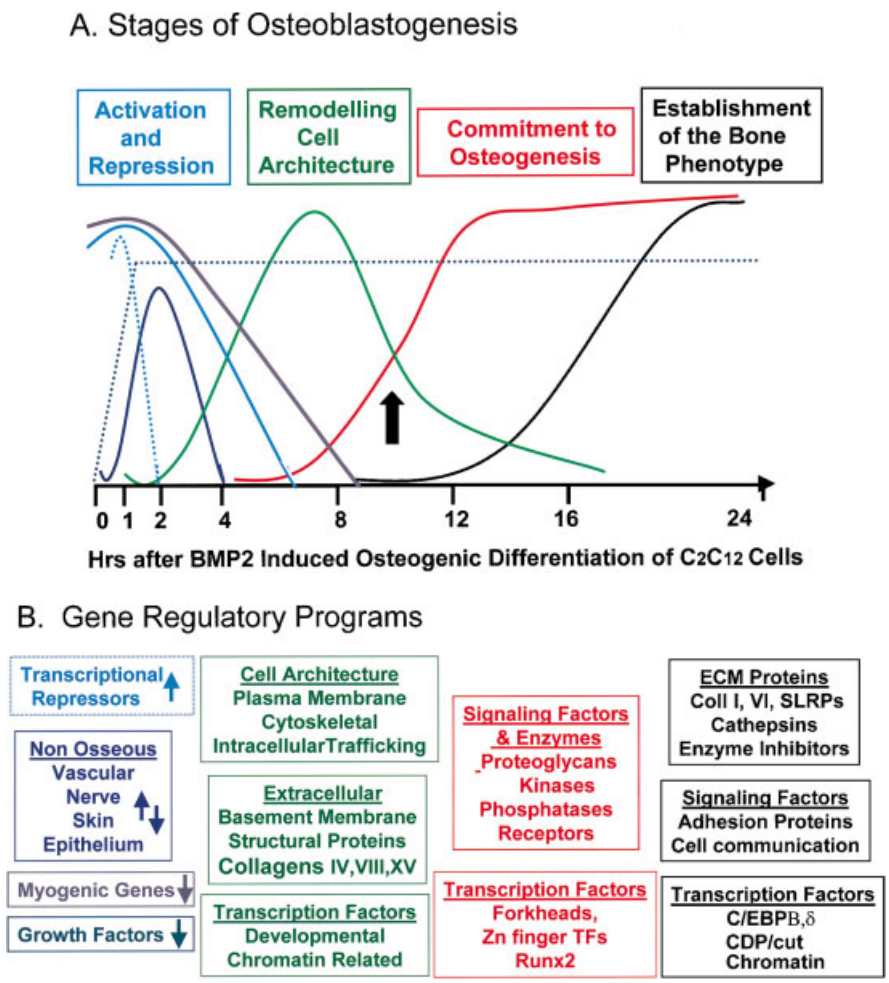

Fig. 9. Biological processes associated with BMP-2-mediated cellular differentiation. **A**: Schematic illustration of the initial stages of osteoblast differentiation reflected by BMP-2-modified gene expression in C2C12-treated cells from 0 to 24 h. Waves of activated and repressed genes are shown. Dotted lines represent immediate and sustained responses. **B**: Proposed model of gene regulatory programs required for BMP-2 induction of the osteoblast phenotype in C2C12 cells. Functional classes of genes changing in response to BMP-2 at specific stages indicated in **panel A** are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

sive genes and activation of bone-related transcriptional regulators (e.g., Runx 2 and other developmental factors) [Umayahara et al., 1999; Gutierrez et al., 2000]. The stage of development of the osteogenic phenotype is recognized between 8 and 12 h by a reciprocal pattern of growth and growth inhibitory-related genes together with an induction of cell adhesion proteins, enzyme inhibitors, receptors, kinases, and skeletal-related matrix components. Thus, the period from 8 to 12 h appears to be a point of bone phenotypic determination. A period of *establishment of the osteoblast* phenotype is indicated by upregulation of genes reflecting synthesis, assembly, and organization of an osteogenic matrix by 16 h. At this time, a coordinated increase occurs in specific collagens Type I, VI, and the SLRP family of matrix proteoglycans that reach peak expression at 24 h.

The novel identification of neural-related genes involved in cell migration and extension of cellular process may reflect the role of BMP as a morphogen in the development of the multiple tissues necessary for formation of the whole limb, which includes blood vessels, nerves, tendons, and surrounding muscle. Notably, for example, we observe increases in several skeletal muscle genes [troponin (L49470) and myosin heavy chain (M74753)] at 24 h, while the cardiac muscle genes [e.g., troponin (X67140)] are downregulated earlier. Alternatively and more plausibly, expression of the neural-related

genes involved primarily in axonal guidance and extension of cellular processes, may support a related function in the differentiating osteoblast. In a broader context, our gene expression profiling studies have shown that the C2C12 model is not restricted to the activation of bone phenotypic genes and have demonstrated a sequence of molecular and cellular activities associated with the BMP-2-mediated cascade for osteoblast differentiation.

Recent applications of gene expression microarrays have shown that common themes are reiterated in BMP responsive genes independent of the osteogenic model and the microarray system. Gene expression profiling has been performed using osteoblasts at distinct stages of maturation [Beck et al., 2001; Garcia et al., 2002; Raouf and Seth, 2002; Vaes et al., 2002] and BMP-2 treatment of mesenchymal derived cells [Locklin et al., 2001; Harris et al., 2002]. In one study using either a 600 or 5,000 gene microarray with an immortalized osteoblastic 2T3 mouse cell line [Harris and Harris, 2001], the transcription factors induced within 8 h of BMP-2 treatment are similar to those we observe in C2C12 cells treated with BMP-2. Microarray analysis of C2C12 cells [Vaes et al., 2002] and human marrow stromal cells treated with BMP-2 to induce the osteoblast phenotype [Locklin et al., 2001], also showed similar modifications in nuclear genes, including ID-2 and ID-3, Jun B, HES-1, STAT-1, Dlx-2, Sox 4, and forkhead C2 at 24 h. In contrast, because our genome wide gene expression studies focused on early events of BMP-2 regulation (1, 2, 4, 8, 12 h), many of the genes that we find induced within 24 h were not reported or declined after 48 h in other studies. Thus, our study has demonstrated initial changes in cell architecture and signal transduction cascades that characterize early events in the osteogenic process. The application of both hierarchal and functional clustering approaches has identified the biological processes involved in early events of commitment and differentiation towards the osteoblast phenotype-mediated by BMP-2 at the initial stages of osteogenesis.

Recognizing that BMP transcriptional modifications occur through SMAD intermediates that form co-regulatory complexes with DNA binding proteins, it is difficult to identify direct downstream targets of BMPs without further analyses. Nonetheless, the transcriptional regulatory genes responsive to BMP-2 from 0 to 4 h in our array should include novel candidate BMP-2-regulated genes. How BMP-2 exerts the many diverse effects through the same Smad pathway in all cell types has been a question of intense investigation at the molecular level. Numerous studies have identified the interaction of the BMP signaling pathway with other pathways at the cell surface as well as the interaction of Smad with intracellular and nuclear coregulatory factors to provide specificity for target gene regulation [von Bubnoff and Cho, 2001]. Cross-talk between BMP/Smad and either calcium calmodulin signaling, Wnt/calcium signaling, Erk-Mapk, and Stat pathways has been identified. Genes representing these pathways are significantly modified in our 784-gene array. Recently the convergence of BMP–Smad complexes with the bone-related Runx2 transcription factor, essential for osteogenesis, has been identified [Zhang et al., 2000; Bae et al., 2001; Zaidi et al., 2002]. Further analyses of these microarray data for novel genes that cluster with the expression patterns of known factors may lead to further understanding of the specificity of BMP-2 signaling.

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