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Transcriptional Profiling of Human Osteoblast Differentiation

J. Billiard,[1]* R.A. Moran,[1] M.Z. Whitley,[2] M. Chatterjee-Kishore,[2] K. Gillis,[2] E.L. Brown,[2] B.S. Komm,[1] and P.V.N. Bodine[1]

[1]Women's Health Research Institute, Wyeth Research, Collegeville, Pennsylvania
[2]Expression Profiling Sciences, Wyeth Research, Cambridge, Massachusetts

Abstract Osteoblast differentiation is a key aspect of bone formation and remodeling. To further our understanding of the differentiation process, we have developed a collection of conditionally immortalized adult human osteoblast cell lines representing discrete stages of differentiation. To evaluate changes in gene expression associated with differentiation, polyA$^{(+)}$ RNA from pre-osteoblasts, early and late osteoblasts, and pre-osteocytes was subjected to gene chip analysis using the Affymetrix Hu6800 chip in conjunction with an Affymetrix custom chip enriched in bone and cartilage cDNAs. Overall, the expression of 47 genes was found to change threefold or more on both chips between the pre-osteoblastic and pre-osteocytic stages of differentiation. Many of the observed differences, including down-regulation of collagen type I and collagen-processing enzymes, reflect expected patterns and support the relevance of our results. Other changes have not been reported and offer new insight into the osteoblast differentiation process. Thus, we observed regulation of factors controlling cell cycle and proliferation, reflecting decreased proliferation, and increased apoptosis in pre-osteocytic cells. Elements maintaining the cytoskeleton, extracellular matrix, and cell–cell adhesion also changed with differentiation reflecting profound alterations in cell architecture associated with the differentiation process. We also saw dramatic down-regulation of several components of complement and other immune response factors that may be involved in recruitment and differentiation of osteoclasts. The decrease in this group of genes may provide a mechanism for controlling bone remodeling of newly formed bone. Our screen also identified several signaling proteins that may control osteoblast differentiation. These include an orphan nuclear receptor DAX1 and a small ras-related GTPase associated with diabetes, both of which increased with increasing differentiation, as well as a high mobility group-box transcription factor, SOX4, that was down-regulated during differentiation. In summary, our study provides a comprehensive transcriptional profile of human osteoblast differentiation and identifies several genes of potential importance in controlling differentiation of osteoblasts. J. Cell. Biochem. 89: 389–400, 2003.

Key words: osteoblasts; bone; differentiation; transcription; gene expression

Osteoblasts synthesize and mineralize the bone matrix [Gehron Robey et al., 1992; Aubin et al., 1993; Rodan and Rodan, 1995]. They develop from mesenchymal stem cells and undergo further differentiation to either lining cells or osteocytes [Gehron Robey et al., 1992; Aubin et al., 1993; Parfitt, 1994; Manolagas and Jilka, 1995; Rodan and Rodan, 1995]. Based on in vitro studies with primary fetal rat calvarial osteoblasts, Stein and Lian [1993] have divided osteoblast differentiation into three phases: proliferation, maturation, and mineralization. We have devised a similar model for human osteoblast differentiation which consists of four stages: pre-osteoblast, mature osteoblast, pre-osteocyte, and mature osteocyte [Bodine and Komm, 2002]. Each stage is characterized by expression of distinct protein markers as well as by individual morphological features. This model is based on in situ studies of others [Gehron Robey et al., 1992] as well as our work with a collection of adult human bone cell lines (HOB cells) that represent different stages of osteoblast differentiation.

The HOB cells were immortalized with a temperature-sensitive mutant form of large T-antigen [Martin and Chou, 1975] and express a transformed phenotype at the permissive temperature of 34°C but revert to a non-trans-

*Correspondence to: Julia Billiard, PhD, Wyeth Research, 500 Arcola Rd., Collegeville, PA 19426-3930.
E-mail: billiaj@wyeth.com

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formed phenotype at the non-permissive temperature of 37–40°C. We developed cells representing all four stages of osteoblast differentiation [Bodine et al., 1996a,b, 1997, 1999; Bodine and Komm, 1999; Prince et al., 2001]. Our rational for assigning the HOB lines to various stages of differentiation has been summarized before [Bodine and Komm, 2002] and is presented in Results and Discussion. For this study, we used representatives of three stages: proliferative-stage pre-osteoblasts (HOB-03-C5); mature osteoblasts (early mature osteoblasts HOB-03-CE6 and late mature osteoblasts HOB-02-C1); and matrix mineralization-stage pre-osteocytes (HOB-01-C1). The mRNA from mature osteocytic stage (HOB-05-T1) was only assayed on one of the two chips and these data were not included in the analysis.

The microarray and gene chip technologies allow analysis of changes in mRNA expression of a large number of genes at the same time. These approaches have been used most extensively to investigate genetic profiles of osteoblast differentiation in mouse models. Several groups have followed changes in gene expression in the murine MC3T3-E1 cell line differentiated to an osteoblast-like phenotype by induction with ascorbic acid and β-glycerophosphate [Seth et al., 2000; Beck et al., 2001; Raouf and Seth, 2002]. Comprehensive microarray analyses of the bone morphogenetic protein-2 (BMP-2)-induced osteoblast differentiation of murine C2C12 mesenchymal progenitor cell line have been reported in this issue [Balint et al., in press] and previously [de Jong et al., 2002; Vaes et al., 2002]. Fewer genome-wide screens have been performed on human osteoblast differentiation, mostly due to lack of adequate human models. Locklin et al. [2001] used gene chips to assess gene regulation during pre-osteoblast differentiation of the conditionally immortalized human marrow stromal cells treated with BMP-2 for up to 72 h. Doi et al. [2002] performed a cDNA microarray screening during osteogenic differentiation of pluripotent primary human mesenchymal stem cells (hMSC).

The current experiments were initiated to assess the transcriptional profile of human osteoblast differentiation starting at the committed pre-osteoblastic stage and following the cells into pre-osteocytic matrix mineralization stage. The polyA$^{(+)}$ RNA was isolated from HOB-03-C5, HOB-03-CE6, HOB-02-C1, and HOB-01-C1 cell lines and subjected to gene chip analysis using the Affymetrix Hu6800 chip (from Affymetrix, West Sacramento, CA) with 7,070 genes in conjunction with the Affymetrix custom-made GIHuman1a chip enriched in bone and cartilage cDNAs with 3,378 genes. Overall, the expression of 47 genes was found to change in a consistent pattern between the HOB cell lines in both experiments. Many of the observed changes followed expected patterns and further validate the HOB cell collection as a model for osteoblast differentiation. Other changes have not been previously reported and offer new insights into the differentiation process. These include genes regulating cell proliferation, cell motility, and migration; genes involved in maintaining the cytoskeleton and extracellular matrix; as well as several genes whose role in the differentiation process is uncertain. The readers need to keep in mind that our results are obtained using transformed stable cell lines and will ultimately need to be validated in unmodified osteoblasts and in animal models. However, this study presents a comprehensive profile of changes in gene expression during human osteoblast differentiation and offers new avenues for investigation of this key aspect of bone remodeling.

MATERIALS AND METHODS

Cell Culture and RNA Isolation

The HOB cell lines were maintained at 34°C in DMEM/F-12 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 1% (v/v) penicillin–streptomycin, and 2 mM glutaMAX-I (all from Invitrogen, Carlsbad, CA). Cells were plated at 9.8×10^4 cells/cm^2, allowed to attach overnight at 34°C, and transferred to 39°C in DMEM/F-12 medium containing 0.25% (wt/v) bovine serum albumin (Serologicals Proteins, Inc., Kankakee, IL), 1% (v/v) penicillin–streptomycin, 2 mM glutaMAX-I, 50 μg/ml ascorbate-2-phosphate (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 10 nM menadione sodium bisulfite (vitamin K_3, Sigma, St. Louis, MO). Forty eight hours later, the cells were washed in phosphate-buffered saline and the whole-cell RNA was isolated with TRIzol reagent (Invitrogen) per manufacturer's protocol. Subsequently, the polyA$^{(+)}$ RNA fraction was extracted using Oligotex mRNA Maxi Kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

Gene Chip Analysis

The polyA$^{(+)}$ RNA samples were analyzed on the Hu6800 chip and the custom-made GIHuman1a chip enriched in bone and cartilage cDNAs (both from Affymetrix). Target complementary RNA (cRNA) preparation and hybridization to Affymetrix GeneChips were done essentially as described [Hill et al., 2000, 2001]. Eleven biotin-labeled control cRNA transcripts were spiked into hybridization solution at known concentrations and used to generate a calibration curve between average difference (AD) values and picomolarity. Picomolarity values were converted to frequency per million (FPM) based on the assumption that the average cRNA length is 1 kb. Chips were scanned and analyzed using the Affymetrix MAS 4.0 software and AD values for each probe set were calculated per Affymetrix instructions. AD values were then used to derive FPM values for each probe set using the calibration curve obtained from control transcripts.

Real-Time RT-PCR Analysis

Changes in expression of selected genes were confirmed by real-time RT-PCR. The same polyA$^{(+)}$ RNA samples used for gene chip analysis, were subjected to the real-time RT-PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) per manufacturer's instructions. Genes chosen for confirmation as well as sequences of primers and probes used are listed in Table I. All probes were obtained from Applied Biosystems and labeled with the reporter fluorescent dye FAM. Primers and probe labeled with the reporter fluorescent dye VIC, specific for 18S rRNA, were purchased from Applied Biosystems and included in the reactions as an internal control. The RT step was performed at 48°C for 30 min and the cDNA was amplified for 40 cycles at the following conditions: 95°C for 15 s and 60°C for 1 min. The mRNA amount for each gene was calculated using the Standard Curve Method described in User Bulletin #2 (Applied Biosystems) and normalized to the expression of 18S rRNA.

Data Analysis

The data from the Hu6800 and the GIHuman1a chip were analyzed separately. The frequency of expression obtained for a given gene at the end of differentiation (pre-osteocytic cells, HOB-01-C1) was divided by its frequency of expression at the beginning of differentiation (pre-osteoblastic cells, HOB-03-C5). The genes whose expression increased or decreased threefold or more on both chips were selected as changing with differentiation.

RESULTS AND DISCUSSION

We have previously developed a collection of conditionally immortalized adult HOB cell lines representing different stages of osteoblast differentiation [Bodine et al., 1996a,b, 1997, 1999; Bodine and Komm, 1999; Prince et al., 2001]. Evidence demonstrating that the HOB cell lines are in distinct stages of differentiation has been summarized before [Bodine and Komm, 2002]. Figure 1 compares the relative levels of expression at non-permissive temperature of two osteoblastic/osteocytic markers for the HOB cell lines used in this study. Expression of alkaline phosphatase, the marker of the proliferative and early matrix maturation stages of human osteoblast differentiation, is highest in the HOB-03-C5 and HOB-03-CE6 cells. Expression of a mineral binding protein osteopontin that marks the pre-osteocytic stage of extracellular matrix mineralization, peaks in HOB-01-C1 cells. In addition, the HOB-03-C5 cells continue to proliferate at non-permissive temperature and secrete the most type I collagen [Bodine and Komm, 1999] and thus represent the proliferative/pre-osteoblastic stage of differentiation. All the other HOB cell lines cease to proliferate at non-permissive temperature. The HOB-03-CE6 cells express less type I collagen, but

TABLE I. Primers and Probes Used in the Real-Time PCR Analysis

Human OB-cadherin, accession no. NM_001797	
Forward primer (686–707)	5′-AGGAGGAGTACCACGTGGTGAT-3′
Reverse primer (763–741)	5′-TCACTTTGGTTGTCCCTGAGAGT-3′
Probe (reverse complement, 731–709)	5′-TGTCCACCCATGTCCTTGGCCTG-3′
Human TWIST, accession no. NM_000474	
Forward primer (419–439)	5′-CGGACAAGCTGAGCAAGATTC-3′
Reverse primer (516–496)	5′-CATCTTGGAGTCCAGCTCGTC-3′
Probe (451–474)	5′-CTGGCGGCCAGGTACATCGACTTC-3′

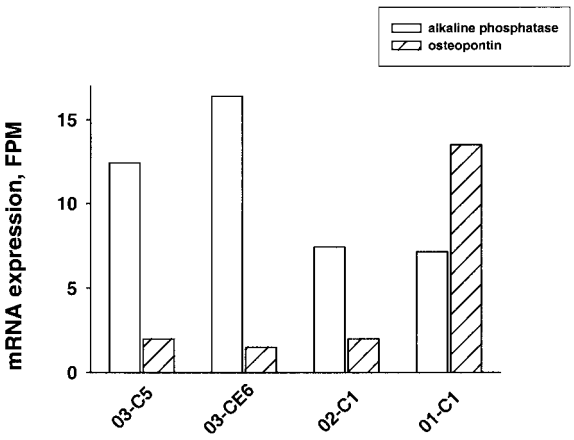

Fig. 1. Expression of osteoblastic markers by human bone (HOB) cell lines. PolyA$^{(+)}$ RNA from the indicated cell lines was analyzed on the Hu6800 (alkaline phosphatase) and the GIHuman1a (osteopontin) chips as described in Materials and Methods. The levels of expression are plotted as frequency per million (FPM). Cell lines: 03-C5–HOB-03-C5, pre-osteoblasts; 03-CE6–HOB-03-CE6, early osteoblasts; 02-C1–HOB-02-C1, mature osteoblasts; 01-C1–HOB-01-C1, pre-osteocytes.

produce more osteocalcin in response to vitamin D_3 than does the HOB-03-C5 line [Bodine et al., 1997, 1999; Prince et al., 2001]. The HOB-03-CE6 cells are classified as early mature osteoblasts. The HOB-02-C1 [Bodine et al., 1996b] and HOB-01-C1 [Bodine et al., 1996a] cells secrete the most osteocalcin in response to vitamin D_3 treatment. In addition, the HOB-01-C1 cells form the greatest amount of mineralized matrix in the presence of ascorbic acid (when normalized to cell protein) and exhibit the most robust regulation of cAMP by PTH treatment [Bodine et al., 1996a]. These cells also exhibit a morphology that resembles pre-osteocytes in vivo. Therefore, the HOB-02-C1 line is classified as post-proliferative mature osteoblasts; and the HOB-01-C1 line, as post-proliferative pre-osteocytes. Under the experimental conditions employed here, all of the HOB lines maintained their phenotype for the duration of the experiment.

To assess the expression profile of human osteoblast differentiation, we isolated two independent sets of polyA$^{(+)}$ RNA from pre-osteoblasts (HOB-03-C5), early osteoblasts (HOB-03-CE6), late osteoblasts (HOB-02-C1), and pre-osteocytes (HOB-01-C1). The first set of RNA was analyzed on the Affymetrix Hu6800 chip with 7,070 genes and the second set, on the custom-made Affymetrix GIHuman1a chip enriched in bone and cartilage cDNAs with 3,378 genes. The frequencies of expression for every given gene varied between the two chips due to differences in the number and the hybridization efficiency of the probes used, but the trends of the observed changes were very similar. The data from the two chips were analyzed separately to identify genes whose expression on both chips changed significantly between pre-osteoblastic and pre-osteocytic stages of differentiation. Since there was no replicas of identical probe sets on the same chip to perform statistical analysis of the data, we had to select a cut-off rate for changes to be considered significant. In our past experience, changes of threefold and higher observed in gene chip experiments were consistently reproducible by other methods of RNA analysis, i.e., by TaqMan and, therefore, the cut-off was set at threefold. It is important to note that whereas this cut-off rate led to identification of a reliable set of data, it may have discounted some smaller but possibly physiologically relevant changes (see later). Another limitation of our experiments was due to the fact that probes for some transcripts were only present on one chip, but not the other, and those transcripts were not included in the analysis. Finally, our analysis was not designed to identify those transcripts that changed in the intermediate stages of differentiation but returned to their pre-osteoblastic levels in pre-osteocytes. Among such genes, with more complex expression patterns, was insulin-like growth factor binding protein-6 that peaked in early osteoblasts, but came back down in pre-osteocytes.

We found a total of 47 genes whose expression on both chips changed threefold or more between pre-osteoblastic (HOB-03-C5) and pre-osteocytic (HOB-01-C1) stages of differentiation. The expressional profiles for these genes are listed in Table II. These genes are divided into seven functional groups, including matrix and matrix-associated factors, metabolism control factors, cell cycle and proliferation control factors, cell structure and movement control elements, immune response factors, signaling molecules, and enzymes and enzyme inhibitors. The expression profiles of many of the matrix and matrix-associated factors follow previously described patterns and support the physiological relevance of our results. However, most of the genes in other groups have not been previously implicated in the control of osteoblast differentiation and reflect the novelty of our approach. Indeed, the majority of genome-wide

TABLE II. Genes That Change During Osteoblast Differentiation*

Gene name	Accession no.	03-C5[a]	03-CE6[a]	02-C1[a]	01-C1[a]	01-C1/03-C5 (fold change)	Direction
Matrix and matrix-associated factors							
Collagen, type I, alpha 1	NM_000088	287.8	202.1	117.2	74.1	0.23	Down
		78.2	64.2	21.3	15.8		
Collagen, type III, alpha 1	NM_000090	213.2	118.0	99.2	61.1	0.19	Down
		57.3	85.6	11.3	5.3		
Collagen, type VIII, alpha 1	NM_001850	80.6	60.1	17.9	2.4	0.14	Down
		65.0	74.2	35.3	15.7		
Lumican	NM_002345	369.5	404.00	163.5	70.0	0.11	Down
		88.3	72.8	11.2	2.2		
Lysyl oxidase	NM_002317	125.0	213.2	165.2	21.0	0.17	Down
		32.0	22.8	24.4	5.7		
Lysyl oxidase-like 2	NM_002309	189.1	67.7	53.7	43.3	0.21	Down
		86.2	39.8	29.1	15.5		
Matrix metalloproteinase 2 (72 kDa type IV collagenase)	NM_004530	359.6	167.8	330.9	99.4	0.21	Down
		259.1	176.4	121.7	36.9		
OB-cadherin (cadherin 11, type 2)	NM_001797	76.6	17.6	23.2	11.7	0.12	Down
		33.2	9.2	2.1	2.6		
Osteoprotegerin (TNF receptor superfamily, member 11b)	NM_002546	212.0	78.1	34.8	7.2	0.12	Down
		15.6	7.1	4.2	3.1		
Procollagen C-endopeptidase enhancer	NM_002593	132.2	61.8	47.1	27.8	0.19	Down
		115.8	75.1	22.5	19.0		
Matrix metalloproteinase 1 (interstitial collagenase)	NM_002421	2.6	6.2	28.4	30.8	17.20	Up
		2.1	1.7	1.2	47.4		
Plasminogen activator, urokinase	NM_002658	3.6	19.7	11.6	49.1	10.34	Up
		3.4	15.8	3.4	23.1		
Cell cycle and proliferation control factors							
Connective tissue growth factor	NM_001901	120.0	261.5	143.0	23.0	0.23	Down
		101.4	125.6	50.1	26.2		
Follistatin	NM_006350	67.5	38.5	10.5	12.0	0.15	Down
		25.1	19.4	7.5	2.8		
Platelet-derived growth factor receptor, alpha polypeptide	NM_006206	138.5	54.0	53.5	7.0	0.75	Down
		71.8	31.8	26.7	7.5		
Platelet-derived growth factor receptor, beta polypeptide	NM_002609	31.5	10.0	7.5	9.5	0.25	Down
		64.7	17.9	8.5	12.8		
Pleiotrophin (neurite growth-promoting factor 1)	NM_002825	25.0	21.5	7.0	5.5	0.25	Down
		14.9	8.5	6.0	4.2		
Tumor necrosis factor (ligand) superfamily, member 10	NM_001252	16.0	3.5	6.0	4.0	0.28	Down
		6.9	2.6	2.0	2.1		
CDC20 (cell division cycle 20, *S. cerevisiae*, homolog)	NM_001255	2.5	6.5	5.0	29.0	8.21	Up
		5.5	9.9	7.2	26.6		
H2A histone family, member O	NM_003516	44.5	36.0	24.0	197.0	3.75	Up
		7.9	10.8	3.7	24.2		
S100 calcium-binding protein A4	NM_002961	2.6	11.0	5.2	15.0	23.17	Up
		3.5	4.5	7.0	141.5		
Survivin (baculoviral IAP repeat-containing 5)	NM_001168	6.0	29.0	12.5	20.0	3.66	Up
		13.9	43.2	9.1	55.4		
Tumor necrosis factor (ligand) superfamily, member 7	NM_001252	2.0	13.5	4.0	54.0	15.14	Up
		2.8	5.9	2.4	9.1		
Metabolism control factors							
Fatty acid binding protein 5 (psoriasis-associated)	NM_001444	4.0	23.0	5.5	19.5	5.13	Up
		1.7	5.8	1.5	9.7		
Metallothionein 1H	NM_005951	225.0	368.5	325.5	683.0	4.21	Up
		50.9	109	110.4	273.6		
Cell structure and movement control elements							
Ankyrin 3, node of Ranvier (ankyrin G)	NM_001149	28.0	1.50	2.5	4.5	0.24	Down
		5.7	1.7	1.3	1.8		
Stromal cell-derived factor 1	NM_000609	258.0	52.50	138.5	58.0	0.17	Down
		30.4	15.7	24.8	3.3		
Thrombospondin 2	NM_003247	115.5	22.00	31.0	7.0	0.06	Down
		50.4	18.6	3.3	2.4		
Vascular cell adhesion molecule 1	NM_001078	47.0	5.50	10.5	4.0	0.14	Down
		13.4	5.1	1.6	2.6		
Actin filament capping protein (gelsolin-like)	NM_001747	17.5	70	50.5	119	5.23	Up
		8.6	28.1	25.9	31.4		
Midkine (neurite growth-promoting factor 2)	NM_002391	30.5	109.00	87.0	106	5.82	Up
		2.4	32.5	38.3	19.7		
Tubulin, alpha 1 (testis specific)	NM_006000	2.0	7.50	1.5	22.5	10.00	Up
		1.7	4.2	1.5	15.0		
Immune response factors							
Complement component 1, r subcomponent	NM_001733	348.5	198.5	115.0	103.0	0.24	Down
		74.5	48.8	26.8	13.5		
Complement component 1, s subcomponent	NM_001734	435.0	248.5	126.5	119.5	0.21	Down
		128.0	103.6	40.5	19.6		

(*Continued*)

TABLE II. (*Continued*)

Gene name	Accession no.	03-C5[a]	03-CE6[a]	02-C1[a]	01-C1[a]	01-C1/03-C5 (fold change)	Direction
H factor (complement)-like 1	NM_000186	103.5	40.0	28.0	16.0	0.13	Down
		29.0	14.6	5.9	3.3		
Pentaxin-related gene, rapidly induced by IL-1 beta	NM_002852	510.5	284.5	94.0	46.5	0.10	Down
		146.8	385.8	67.7	14.9		
Interferon, alpha-inducible protein 27	X67325	160.5	482.0	707.0	657.5	3.56	Up
		59.9	153.6	249.0	181.0		
Signaling molecules							
Oxytocin receptor	NM_000916	10.5	4.5	2.5	3.0	0.29	Down
		7.9	3.3	1.7	2.2		
SOX4, SRY (sex determining region Y)-box 4	NM_003107	19.0	8.0	9.0	4.5	0.19	Down
		13.6	7.4	1.4	1.7		
TWIST	X99268	83.5	40.5	33.5	38.0	0.37	Down
		7.6	3.1	2.2	2.2		
Coagulation factor III (thromboplastin, tissue factor)	NM_001993	2.5	10.5	22.0	26.0	6.76	Up
		2.7	2.3	10.0	8.3		
DAX1, nuclear receptor sub-family 0, group B, member 1	NM_000475	9.5	14.5	10.5	30.0	4.30	Up
		2.5	4.2	2.1	13.5		
Enzymes and enzyme inhibitors							
Serine (or cystein) proteinase inhibitor, clade G, member 1	NM_000062	92.0	63.0	26.0	4.5	0.08	Down
		48.5	48.3	15.7	5.3		
Galactose-4-epimerase, UDP	NM_000403	8.5	13.0	8.0	39.5	4.11	Up
		4.0	4.1	5.4	14.4		
Microsomal glutathione S-transferase 1	NM_020300	56.0	122.5	240.5	369.0	10.27	Up
		7.0	27.8	45.6	97.9		
Ral guanine nucleotide dissociation stimulator	AF295773	3.0	3.0	1.5	11.0	4.02	Up
		4.2	12.7	3.9	18.2		
Ras-related associated with diabetes	NM_004165	37.5	54.0	53.5	163.0	13.4	Up
		1.7	1.9	14.9	38.6		

*Gene chip experiments were performed as described in Materials and Methods on GIHuman1a chip (top lines) and Hu6800 chip (bottom lines). Cell lines: 03-C5–HOB-03-C5, pre-osteoblasts; 03-CE6–HOB-03-CE6, early osteoblasts; 02-C1–HOB-02-C1, mature osteoblasts; 01-C1–HOB-01-C1, pre-osteocytes. Fold and direction of change between mRNA expression in HOB-03-C5 and HOB-01-C1 are shown as an average between the two chips. Only those genes that changed threefold or more on both chips are listed.
[a]Frequency per million.

screenings to date were done in murine models of osteoblast differentiation. Studies using the human models started with non-committed pluripotent cells and followed them either through the initial stages of pre-osteoblastic differentiation [Locklin et al., 2001] or up to fully differentiated stage characterized by the highest levels of alkaline phosphatase and collagen type I expression [Doi et al., 2002]. Our studies can be interpreted as starting where these reports left off and analyzing differentiation into the pre-osteocytic stage.

The expression profiles from the GIHuman1a chip listed in Table II are also presented graphically in Figure 2 for several matrix and matrix-associated factors. Many of these profiles follow previously documented expression patterns and support the HOB cell lines as a valid model of human osteoblast differentiation. In agreement with previous studies [Bortell et al., 1990; Owen et al., 1990; Stein and Lian, 1993], we observed down-regulation of collagen types I, III, and VIII (Fig. 2A) as well as parallel down-regulation of the collagen processing enzyme, lysyl oxidase, and a related enzyme, lysyl oxidase-like 2 (Fig. 2B). Decrease in procollagen C-peptidase enhancer is expected since it prepares collagen for fibril organization [Miyahara et al., 1982; Peltonen et al., 1985]. Up-regulation of matrix metalloproteinases and plasminogen activator, urokinase (Fig. 2C) has been well documented [Meikle et al., 1992; Panagakos and Kumar, 1995; Filanti et al., 2000; Mizutani et al., 2001]. Figure 2C also shows differentiation-induced suppression of the osteoblast-specific member of the TNF receptor superfamily, osteoprotegerin. In agreement with previous reports [Gori et al., 2000; Doi et al., 2002], osteoprotegerin is expressed highly in the pre-osteoblasts and early osteoblasts that need to interact with the marrow environment for regulation of osteoclastogenesis. To our knowledge, ours is the first observation that osteoprotegerin is suppressed in preosteocytes that are moving into lacunae away from the marrow. Figure 2D shows differentiation-induced suppression of OB-cadherin found in the gene chip experiments (solid line) and confirmed by the real-time RT-PCR analysis (open bars). The relative abundance of cadherins defines the differentiation pathway of mesenchymal precursors to specific lineages with OB-cadherin increasing in osteoblastic

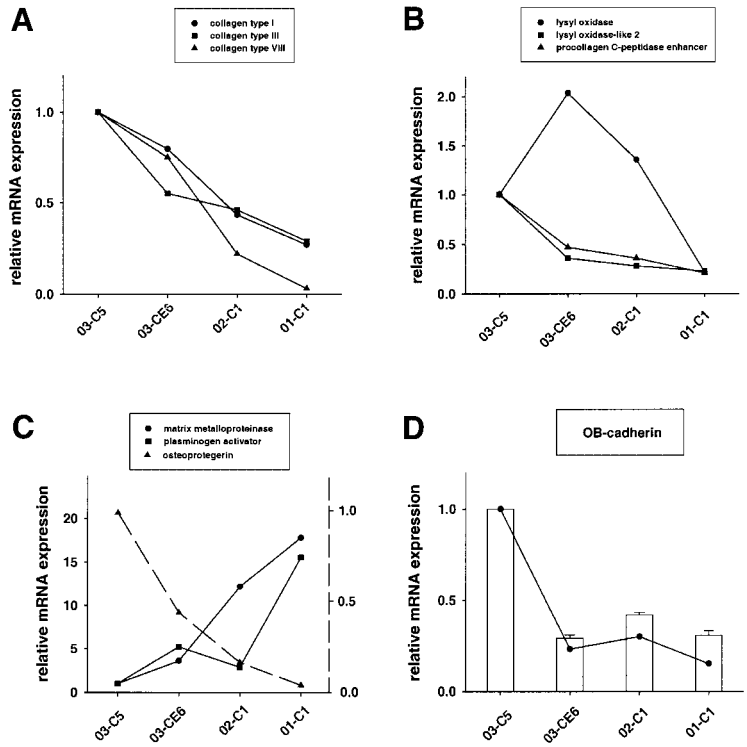

Fig. 2. Expression patterns of matrix and matrix-associated factors during osteoblast differentiation. **A**, **B**, **C**: Data from the GIHuman1a chip listed in Table II are normalized to the values obtained in HOB-03-C5 cells and plotted as a function of differentiation for the indicated matrix and matrix-associated genes. Data for osteoprotegerin (in C) are normalized to the value obtained in HOB-01-C1 cells. **D**: Gene chip analysis on GIHuman1a chip (solid line) and real-time RT-PCR results (open bars) for the OB-cadherin transcript. For both methods, the relative mRNA expression in HOB-03-C5 was set at one. Real-time RT-PCR was performed as described in Materials and Methods using probes and primers listed in Table I. The levels of mRNA were normalized to the expression of 18S rRNA in each sample. Means ± SEM of three RT-PCR reactions per cell line. Cell line abbreviations: see Figure 1.

cells [Shin et al., 2000]. OB-cadherin is also up-regulated during BMP-2-induced osteogenesis in murine cell lines with the highest expression observed in alkaline phosphatase-expressing cells [Okazaki et al., 1994; Shin et al., 2000]. In our study, the highest level of OB-cadherin mRNA was also found in cells that express the most alkaline phosphatase and declined in later stages of differentiation characterized by decreasing levels of this enzyme.

Our experiments also identified many other changes that offer new insights into the osteoblast differentiation process. As cells progress from pre-osteoblasts into mature osteoblasts and then to pre-osteocytes, they cease to proliferate, withdraw from cell cycle, and undergo increased apoptosis. Therefore, it is not surprising that we saw changes in genes involved in cell cycle and proliferation control (see Table II). These novel observations are presented graphically for both Hu6800 and GIHuman1a chips in Figure 3. Connective tissue growth factor and pleiotrophin, growth factors promoting cell proliferation, were strongly suppressed in pre-osteocytic cells. The two components of the platelet-derived growth factor receptor were also down-regulated. By contrast, increased expression of the inhibitor of apoptosis, survivin, and of the metastasis-promoting S100A4 calcium-binding protein may represent a negative-feedback mechanism counteracting cell death. Interestingly, another member of the S100 family of small calcium-binding proteins, S100A10, was found to be up-regulated in hMSC treated with osteogenic supplements [Doi et al., 2002].

Follistatin is an activin-binding protein that neutralizes diverse activin effects [Nakamura et al., 1990; Mather et al., 1997]. Since activin promotes osteoblast proliferation [Centrella et al., 1991; Hashimoto et al., 1992; Gaddy-Kurten et al., 2002], the observed decrease in follistatin expression would result in increased cell proliferation and may again be a part of

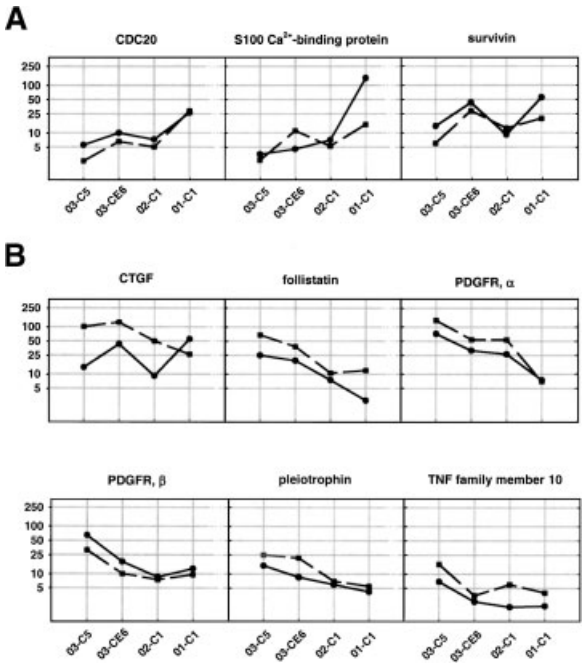

Fig. 3. Genes involved in cell cycle and proliferation control change during osteoblast differentiation. Data from both the Hu6800 (solid lines, circles) and the GIHuman1a (dashed lines, squares) chips listed in Table II are plotted for the indicated cell cycle (**A**) and proliferation control (**B**) genes. CDC20: cell division cycle 20; CTGF: connective tissue growth factor; PDGFR, α and β: platelet-derived growth factor receptor, alpha and beta polypeptides. Cell line abbreviations: see Figure 1.

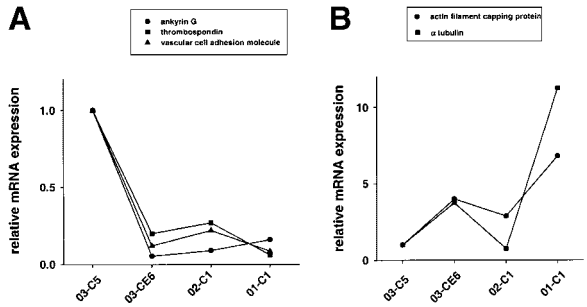

Fig. 4. Cell structure and movement control elements change during osteoblast differentiation. Data from the GIHuman1a chip listed in Table II are normalized to the values obtained in HOB-03-C5 cells and plotted for the indicated cell structure and movement control genes decreasing (**A**) or increasing (**B**) with differentiation. Cell line abbreviations: see Figure 1.

the negative-feedback mechanism counteracting cell death. Follistatin has also been shown to inhibit osteoclast formation [Murase et al., 2001], and its decrease in the late stages of osteoblast differentiation may represent an important mechanism enhancing bone turnover. Figure 3 also illustrates the remarkable agreement between the data obtained with independent sets of polyA$^{(+)}$ RNA on two different chips and, therefore, increases confidence in our observations.

Another set of genes newly identified in this report consists of seven cell structure and movement control elements (see Fig. 4 for graphic representation). Architectural proteins are the necessary component of the cell cycle progression, and their expression may decrease following cell cycle arrest that accompanies osteoblast differentiation. Indeed, we observed down-regulation of ankyrin, a protein linking the cytoskeleton to a variety of membrane proteins. Additionally, as osteocytes become imbedded in the bone matrix, their cell–cell adhesion mechanisms must undergo significant modifications. These modifications are reflected in down-regulation of thrombospondin 2 and vascular cell adhesion molecule. Vascular cell adhesion molecule was previously found to be down-regulated during differentiation of human marrow stromal cells into pre-osteoblasts [Locklin et al., 2001], so that this protein shows continuous down-regulation throughout the time course of differentiation. We also observed differentiation-induced increases in expression of actin filament capping protein that regulates actin-based motility and in levels of α tubulin. These proteins may help generate multiple actin-rich cellular projections characteristic of mature osteocytes.

We also found four transcripts in the immune response pathway that dramatically decreased during osteoblast maturation and differentiation (see Fig. 5 for graphic representation). Osteoblasts have been previously shown to produce the third component of complement (C3) in response to vitamin D [Hong et al., 1991; Sato et al., 1991]. Stimulated immune complexes and components of complement have been shown to potentiate bone resorption [Torbinejad et al., 1979; Sato et al., 1993]. Addition of the antibody specific for C3 has been demonstrated to inhibit differentiation of bone marrow cells into osteoclasts [Sato et al., 1993] indicating that C3 can potentiate osteoclast development. In addition, deposition of a component of complement onto hydroxyapatite crystals can recruit mononuclear osteoclasts to that site [Mangham et al., 1993]. The decrease in components of complement and other immune response factors observed during osteoblast differentiation may result in suppressed osteoclast formation and

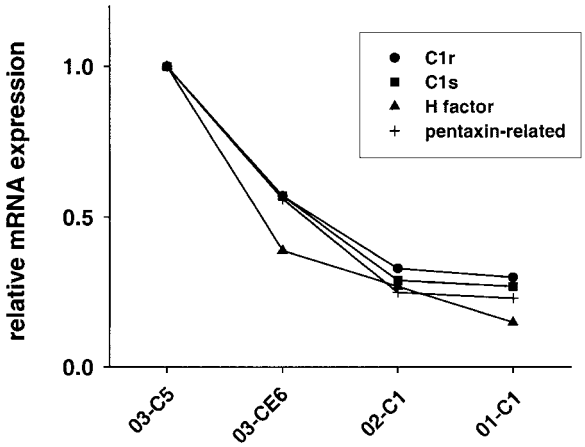

Fig. 5. Several immune response factors decrease during osteoblast differentiation. Data from the GIHuman1a chip listed in Table II are normalized to the values obtained in HOB-03-C5 cells and plotted for the indicated genes decreasing with differentiation. C1r: complement component 1, r subcomponent; C1s: complement component 1, s subcomponent; H factor: H factor (complement)-like 1; pentaxin-related: pentaxin-related gene, rapidly induced by IL-1 beta. Cell line abbreviations: see Figure 1.

thereby provide a mechanism for inhibition of bone resorption at the site of the new bone formation.

Our screen also identified several signaling molecules that may control osteoblast differentiation (Table II and Fig. 6). These include an orphan nuclear receptor DAX1 and a small ras-related GTPase associated with diabetes (Rad), both of which increased with differentiation, as well as a high mobility group-box transcription factor, SOX4, that was down-regulated. DAX1 is a co-repressor of activated estrogen receptor (ER) [Zhang et al., 2000] and may increase with osteoblast differentiation to counterbalance increasing levels of ER [Arts et al., 1997; Bodine et al., 1998]. Rad has been discovered as a protein controlling glucose metabolism [Reynet and Kahn, 1993; Moyers et al., 1996], but has also been shown to play a role in controlling tumor cell growth [Zhu et al., 1999]. Genes of the SOX family have been identified as sex determining region Y (SRY)-related transcription factors involved in sex determination [Gubbay et al., 1990; Sinclair et al., 1990], but alternative functions have long been speculated [Pevny and Lovell-Badge, 1997]. The decrease of SOX4 with osteoblast differentiation suggests a possible function for it in bone remodeling.

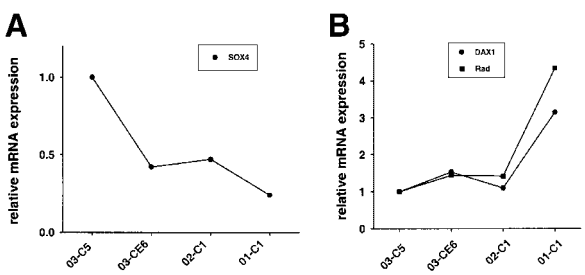

Fig. 6. Cell signaling molecules that may control osteoblast differentiation. Data from the GIHuman1a chip listed in Table II are normalized to the values obtained in HOB-03-C5 cells plotted for the indicated genes decreasing (**A**) or increasing (**B**) with differentiation. SOX4: sex determining region Y-box 4; DAX1: nuclear receptor subfamily 0, group B, member 1; Rad: Ras-related GTPase associated with diabetes. Cell line abbreviations: see Figure 1.

It is important to note that whereas the threefold cut-off for the expression changes used in our analysis produced high-confidence data, it may have discounted some smaller, yet possibly physiologically relevant changes. Thus, we did not pick up the steady decrease in expression of the basic helix–loop–helix transcription factor TWIST during osteoblast differentiation. Indeed, TWIST expression decreased over threefold on the Hu6800 chip, but only twofold on the GIHuman1a chip. Mutations in human TWIST cause Saethre-Chotzen syndrome, a bone disease characterized by premature fusion of the cranial sutures [el Ghouzzi et al., 1997; Howard et al., 1997]. Overexpression of TWIST in human osteoblast HSaOS-2 cells leads to reduced levels of alkaline phosphatase expression whereas TWIST antisense-overexpressing cells exhibit increased levels of alkaline phosphatase and type I collagen mRNA [Lee et al., 1999]. Because of its importance to bone biology, we confirmed the decrease in TWIST expression by real-time PCR (Fig. 7) and observed a threefold decrease in TWIST mRNA level between pre-osteoblasts and pre-osteocytes. This observation supports the hypothesis that high levels of TWIST delay osteoblast differentiation or cause de-differentiation of osteoblasts.

Several important tendencies in gene expression became apparent from our investigation. Thus, many of changes between the early stage cells and the late stage cells occurred in the growth factor and signaling molecule categories. Further, a number of transcripts in different functional categories increased or decreased steadily with differentiation (i.e., several types of collagen; osteoprotegerin; a number of growth factors; and immune response elements). Such steady patterns reflect the continuum of differentiation among the four cell lines, further

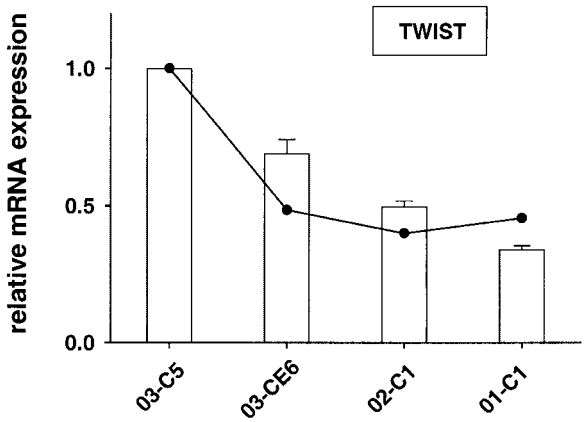

Fig. 7. Real-time RT-PCR confirms expression profile of TWIST during osteoblast differentiation. Gene chip analysis on GIHuman1a chip (solid line) and real-time RT-PCR results (open bars). For both methods, the relative mRNA expression in HOB-03-C5 was set at one. Real-time RT-PCR was performed as described in Materials and Methods using probes and primers listed in Table I. The levels of mRNA were normalized to the expression of 18S rRNA in each sample. Means ± SEM of three RT-PCR reactions per cell line. Cell line abbreviations: see Figure 1.

validating their usefulness as a model to study the molecular mechanisms governing the progression through specific stages of osteoblast maturation. Finally, taken together with the previous reports on genes associated with human osteoblast differentiation, our study identified several transcripts that are specifically expressed in early osteoblasts. Some of these transcripts are well-known osteoblastic markers, such as collagen types I and III and procollagen C-endopeptidase enhancer. They were up-regulated as hMSC differentiated into osteoblasts [Doi et al., 2002] and subsequently declined during further differentiation to osteocyte (Fig. 2, this study). In addition, osteoprotegerin was strongly up-regulated at day 15 of hMSC treatment with osteogenic agents, but started declining by day 27 [Doi et al., 2002] and declined steadily as differentiation progressed in our study (Fig. 2C). SOX4 was stimulated during the first 6 h of BMP-2 treatment of human marrow stromal cells [Locklin et al., 2001] and declined sharply between proliferative-stage pre-osteoblasts and early mature osteoblasts (Fig. 6A, this study). Based on their expression pattern, we propose that osteoprotegerin and SOX4 can be used as markers of early proliferative-stage pre-osteoblasts.

In summary, this study provides a comprehensive profile of gene expression during human osteoblast differentiation. Importantly, it follows the differentiation process into the osteocytic stage, past the point of the highest alkaline phosphatase activity. Another novelty of our approach is that we do not examine a time course during osteoblastic differentiation of a pluripotent cell line, but use independent cell lines that represent distinct stages of differentiation. This approach should make follow-up investigations easier and more reliable. Gene chip analysis is a powerful tool permitting us to associate transcription changes with osteoblast physiology. With this information, the role of the discrete steps in osteoblast differentiation in the bone remodeling process will become more completely characterized.

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