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Differential Display of Human Marrow Stromal Cells Reveals Unique mRNA Expression Patterns in Response to Dexamethasone

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Abstract Human bone marrow stromal cells (hBMSC) are pluripotent cells that have the ability to differentiate into bone, cartilage, hematopoietic-supportive stroma, and adipocytes in a process modulated by dexamethasone (DEX). To characterize changes in hBMSC in response to DEX, we carried out differential display experiments using hBMSC cultured for 1 week in the presence or absence of 10^{-8} M DEX. When RNA from these cells was used for differential display, numerous cDNA bands were identified that were up-regulated and down-regulated by DEX. The cDNA bands were reamplified by PCR and directly used to screen an hBMSC cDNA library. Seven clones were isolated and characterized by DNA sequencing and found to encode the following genes: transforming growth factor-β-induced gene product (βig-h3), calphobindin II, cytosolic thyroid-binding protein, 22-kDA smooth muscle protein (SM22), and the extracellular matrix proteins osteonectin/SPARC, type III collagen, and fibronectin. To confirm that these genes were regulated by DEX, the cells were treated continuously with this hormone for periods ranging from 2 to 30 days, and steady-state mRNA levels were measured by Northern blot analysis. All genes showed some level of regulation by DEX. The most profound regulation by DEX was observed in the βig-h3 gene, which showed a relative 10-fold decrease in mRNA levels after 6 days of treatment. Interestingly, βig-h3 expression was not altered by DEX in fibroblasts from other human tissues, including thymus stromal fibroblasts, spleen stromal fibroblasts, and foreskin fibroblasts. In summary, differential display of DEX-treated hBMSC revealed unique patterns of gene expression and has provided new information about phenotypic changes that accompany the differentiation of hBMSC toward osteogenesis. J. Cell. Biochem. 76:231–243, 1999. Published 1999 Wiley-Liss, Inc.†

Key words: differential display; human bone marrow stroma; dexamethasone; βig-h3

Bone marrow stroma contains cells that are pluripotenti, with the capacity to differentiate into multiple cell phenotypes, including bone, cartilage, hematopoiesis-supporting reticular stromal cells and associated adipocytes, and fibrous tissue [Ashton et al., 1980; Bennett et al., 1991; Friedenstein, 1990; Kutzsetsov et al., 1989; Owen, 1988; Patt et al., 1982]. Treatment with dexamethasone (DEX) dramatically alters the differentiation capacity of bone marrow stromal cells (BMSC), promoting a shift toward osteogenic differentiation in vitro. Signs of differentiation induced by DEX include morphological changes from an elongated to a more cuboidal shape, an increase in alkaline phosphatase activity, and cyclic adenosine monophosphate (cAMP) response to parathormone (PTH) and prostaglandin E_2, and are essential for mineralization of the extracellular matrix.

Gene expression by differentiating osteoblasts shows an extending list of extracellular matrix components in bone that serve as biochemical markers for osteogenesis, including collagens, proteoglycans such as biglycan and decorin, glycoproteins including osteopontin, osteonectin, bone sialoprotein, and γ-carboxyglutamic acid-containing proteins (i.e., osteocal-

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cin) [reviewed in Young et al., 1992, 1993; also Gehron Robey and Boskey, 1996]. Regulatory pathways that drive differentiation of BMSC into osteoblasts are of significant interest for the study of osteogenesis. In an attempt to characterize changes in gene expression that underlie DEX-induced differentiation, we have carried out a series of differential display analyses using human bone marrow stromal cells (hBMSC) cultured in the presence or absence of DEX. Seven complementary DNA (cDNA) clones were isolated that encoded the messenger RNA (mRNA) of transforming growth factor-β (TGF-β)-induced gene product (βig-h3), calphobindin II, cytosolic thyroid-binding protein, 22-kDA smooth muscle protein (SM22), and the extracellular matrix proteins osteonectin/SPARC, type III collagen, and fibronectin. To confirm that these genes were regulated by DEX in HMSC, Northern blot analysis was performed on cell cultures treated for 2–30 days with DEX. The most profound regulation was observed in the βig-h3 gene, which was expressed in substantial amounts in untreated hBMSC. DEX-induced changes of βig-h3 appeared to be marrow stromal cell type specific, indicating a potential role as a negative regulator for this abundant matrix gene in DEX-induced osteogenic differentiation in vitro.

MATERIALS AND METHODS

Surgical specimens of normal human bone with bone marrow was obtained from 8- and 11-year-old females (NIH protocol 94-D-0188). To study the effect of DEX on hBMSC proliferation and morphology, marrow cells were released, pipetted, filtered through 70-μm mesh, and plated at 5×10^7 nucleated cells per 75-cm^2 flask. The cells were cultured in α-minimum essential medium (α-MEM) with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/μl streptomycin sulfate (Biofluids, Rockville, MD) with or without 10^{-8} M DEX (Sigma, Chemical Co., St. Louis, MO) with 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (AscP, Wako, Osaka, Japan). After 11 days, when the adherent cells were approaching confluence, the cells were released by trypsin, and the cell number determined. For differential display analysis, hBMSC of the second passage were plated at 1×10^6 cells per 100-mm dish and cultured in medium with and without 10^{-8} M DEX for 1 week. RNA was extracted from hBMSF, using RNA STAT-60 (Tel-Test B, Friendswood, TX). For comparison, total RNA (tRNA) was isolated from 7-day-old subconfluent cultures of various cell types, including human thymus stromal fibroblasts, spleen stromal fibroblasts, bone marrow stromal cells (all harvested at the second passage), and foreskin fibroblasts (harvested at the fourteenth passage), and treated with or without 10^{-8} M DEX. To study βig-h3 induction, some hBMSC cultures were treated with 5 ng/ml human recombinant TGF-β1 (R&D Systems) for 0–72 h.

Differential Display

tRNA (1 μg) was subjected to differential display with five separate sets of commercially prepared primers designated AP-1 to AP-5 that were components of the RNAimage™ kit of GenHunter Corporation (Brookline, MA). Differential display was carried out following the recommendations of the manufacturer, using [α-S^{35}]ATP as tracer. The resulting differentially displayed products were separated using 6% acrylamide DNA sequencing gels, dried, and exposed to x-ray film for 1 week.

Characterization of Differentially Displayed Products

Development of the exposed x-ray film demonstrated that multiple mRNA species were clearly distinct in intensity between hBMSC cultured with and without DEX (Fig. 1B). cDNA bands that showed the most obvious regulation were isolated, reamplified by polymerase chain reaction (PCR), and subcloned into a TA™ cloning vector (Invitrogen) for DNA sequence analysis. Based on the sequences derived from the 3′ end of the molecules, no matches with previously described genes were noted, using a BLAST search with the Wisconsin DNA sequence analysis program. For more extensive sequence analysis, more 5′ DNA from the differential display products was obtained by constructing a cDNA library from hBMSC. This library was prepared from a heterogeneous mixture of hBMSC (i.e., DEX treated and untreated) and theorized to contain genes both up- or down-regulated by this hormone. The cDNA library was inserted into a λ UniZap™ vector (Stratagene), and plated using conventional procedures and screened separately using several differentially displayed PCR products as probes. Seven clones were purified to homogeneity, converted to plasmids using a Zap™ procedure (Stratagene), and

then sequenced directly using an automated DNA sequencer. All DNA sequences were analyzed using the GCG Wisconsin Program.

Northern Blot Analysis and Quantitation of Differential Regulation by DEX

To confirm that the genes isolated from the differential display procedure were regulated by DEX, hBMSC cultures were treated continuously with or without 10^{-8} M DEX, for periods of 2–30 days. RNA was extracted after 2, 6, 12, 20, and 30 days of treatment. RNA was separated using formaldehyde containing agarose gels, transferred to Zeta probe nylon™ membranes (Bio-Rad), and immobilized onto the membrane by treatment with ultraviolet (UV) light (Stratalinker™ by Stratagene). Isolated insert DNA from all seven differentially displayed clones were radiolabeled using PrimeIt™ (Stratagene) and [α-^{32}P]CTP, and subsequently hybridized to the membranes for 16 h at 42°C. The blots were washed free of nonspecifically bound probe, and radioactivity was visualized and quantitated using a phosphoimager™ and ImageQuant™ Software from Molecular Dynamics (Eugene, OR).

In Situ Hybridization

The full-length big-h3 cDNA previously subcloned in pBluescript (2.7 kb) was linearized either with *Xba*I and transcribed using T7 RNA polymerase to make the antisense cRNA probe or with *Xho*I and T3 RNA polymerase for the sense cRNA. To produce DIG-labeled RNA, transcription was conducted in the presence of DIG-UTP, according to the manufacturer's instructions (DIG RNA Labelling kit, Boehringer-Mannheim). To increase the tissue penetration of the probes, controlled hydrolysis under alkaline conditions (SureSite II, Novagen) was performed, and the size of each probe was reduced to 300 bp in length, following the directions of the manufacturer.

Human developing bones were collected from 83-day-old embryos under NIH-IRB-approved protocols. After harvest, the tissue was decalcified and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded, and sectioned onto slides previously coated with 3-aminopropyltriethoxysilane. After deparaffinization, sections were treated to remove lipids using 0.2% Triton in PBS. Sections were treated with 0.2 N HCl/proteinase K (100 µg/ml) for 10 min at 37°C in TE buffer (pH 8.0) and the enzymatic digestion terminated by incubating the sections in 0.1 M glycine in PBS. Postfixation in 4% paraformaldehyde was performed for 5 min with subsequent wash in PBS. Nonspecific staining was further prevented by acetylation in a solution of 0.25% acetic acid/0.1 M triethanolamine (pH 8.0).

Before the hybridization step, sections were prehybridized for 15 min in hybridization solution (50% deionized formamide, 5× SSC, 10% dextran sulphate, 5× Denhardt's, 2% sodium dodecyl sulfate (SDS) and 100 µg/ml of sheared sperm DNA) without the probe at 50°C. Probes were denatured for 7 min at 70°C and placed on ice for 5 min. Sections covered with hybridization solution containing the probe were then incubated in a humidified chamber at 50°C for >18 h. After hybridization, the sections were washed 4 times, 5 min each, in 2× SSC/0.1% SDS at 25°C, followed by higher stringency washes at 50°C twice for 10 min each in 0.1× SSC/0.1% SDS. Incubation in 10 µg/ml RNase A for 15 min at 37°C was performed for further reduction of nonspecific binding.

To detect specifically hybridized probes sections were incubated for 2 h with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim) in buffer 1 (0.1 M Tris/0.1 M NaCl, 2 mM $MgCl_2$/3% bovine serum albumin [BSA], pH 7.5) at 25°C. After washing, alkaline phosphatase was detected by incubation in nitroblue tetrazolium containing 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrate in the dark for 3 h (Boehringer-Mannheim). Sections were then washed by soaking in distilled water for 10 min and were mounted for analysis.

RESULTS

Dexamethasone in combination with AscP stimulated hBMSC proliferation in primary human marrow stromal cell cultures (data not shown). The presence of DEX in passaged cultures notably altered hBMSC morphology, causing formation of more complex, multilayered cultures with abundant extracellular matrix (Fig. 1A). At lower densities, cells showed a polygonal shape, while cells cultured without DEX/AscP displayed a typical fibroblastic morphology, including elongated spindle-shaped cytoplasm and long processes (not shown).

Seven clones obtained by the differential display procedure (Fig. 1B) were found to encode the following human genes: TGF-β-induced gene product (βig-h3), calphobindin II, cytosolic thy-

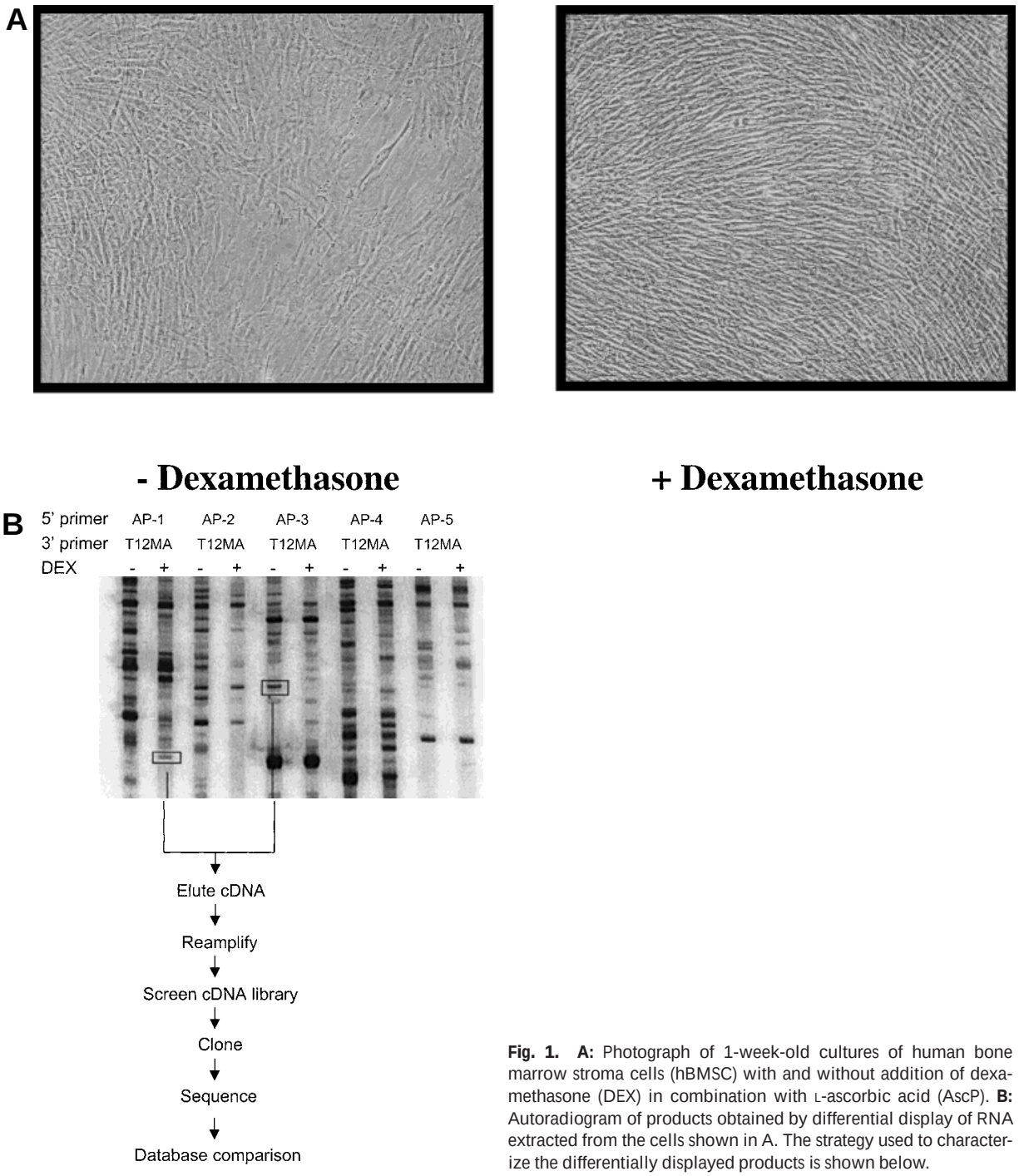

Fig. 1. **A:** Photograph of 1-week-old cultures of human bone marrow stroma cells (hBMSC) with and without addition of dexamethasone (DEX) in combination with L-ascorbic acid (AscP). **B:** Autoradiogram of products obtained by differential display of RNA extracted from the cells shown in A. The strategy used to characterize the differentially displayed products is shown below.

roid hormone-binding protein, 22-kDa smooth muscle protein (SM22), and the extracellular matrix proteins osteonectin/SPARC, type III collagen, and fibronectin. Table I provides the clone, gene accession number, and reference.

To confirm that these genes were regulated by DEX, hBMSC were treated continuously with this hormone for periods of 2–30 days; steady-state mRNA levels were measured by Northern blot analysis. Figure 2 depicts a representative Northern blot analysis of one of the genes under study. All genes showed some level of regulation by DEX (Fig. 3, Table II). The most profound regulation by DEX was observed in the βig-h3 gene, which was dramatically inhibited after 2 days of treatment (Figs. 2, 3), resulting in a 10-fold repression of βig-h3 mRNA. Also inhibited by DEX, although to a lesser extent,

TABLE I. Genes Found in Human Marrow Stromal Cells, Using DEX Treatment and Differential Display Analysis

Gene	Gene bank accession #	cDNA size (bp)	Reference
Human TGF-β-induced gene product (βig-h3), sequence 1 from patent US 5444164	I14045	2691	Skonier et al., 1992
Human calphobindin II	D00510	2391	Iwasaki et al., 1989
Human TCB gene encoding cytosolic thyroid hormone-binding protein	M26252	2306	Kato et al., 1989
Human osteonectin/SPARC	J03040	2133	Swaroop et al., 1988
Human 22-kDa smooth muscle protein (SM22)	M95787	1083	Thweatt et al., 1992
Human 3′ region for pro α1 (III) collagen	X06700	2520	Mankoo and Dalgleish, 1988
Human fibronectin gene extra type III repeat (EDII)	M18178	354	Gutman and Kornblihtt, 1987

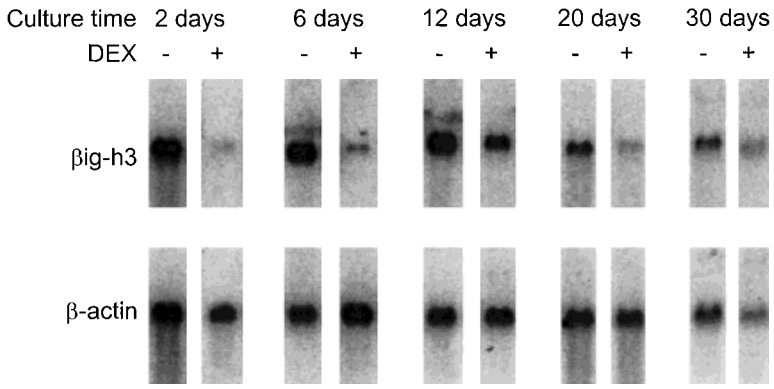

Fig. 2. Representative Northern blot analysis of mRNA expression in HMSC treated with (+) or without (−) dexamethasone (DEX) for 2, 6, 12, 20, and 30 days. **Top**, pattern of expression of TGF-β induced gene (βig-h3); **bottom**, β-actin on the same Northern blot as a reference for loading differences.

were genes encoding cytosolic thyroid-binding protein, SM22, osteonectin, and fibronectin. Genes encoding calphobindin II and type III collagen demonstrated some degree of stimulation by DEX, depending on the extent of culture time. When the mRNA levels of all the genes tested were examined in a parallel set of cultures treated, the presence of β-glycerol phosphate the relative expression patterns were identical to those described above and are summarized in Table II.

Since βig-h3 gene expression was dramatically regulated by DEX, we wanted to determine the specificity of this regulation in osteogenic cells. Therefore, we examined its expression in different populations of connective tissue cells. Various cell types, stromal and nonstromal in nature, were cultured for 7 days with or without DEX. None of the cell types under study (i.e., human thymus stromal fibroblasts, spleen stromal fibroblasts, and foreskin fibroblasts) was regulated by DEX, except for hBMSC (Fig. 4). In contrast to DEX, treatment of hBMSC with 5 ng/ml TGF-β showed upregulation of βig-h3 mRNA after 48 hs of treatment (Fig. 5).

To determine the localization of βig-h3 in developing bone, in situ hybridization was performed. When mRNA encoding βig-h3 was transcribed in both sense and antisense orientations, specific hybridization was observed in the primary spongiosa (Fig. 6A). Substantial amounts of specific hybridization were also observed in pre-osteoblasts in both the periosteum and perichondrium (Fig. 6B).

DISCUSSION

In this study, we used a differential display procedure to study changes in gene expression that underlie DEX-induced differentiation of hBMSC. The technique identified new genes expressed in substantial amounts by hBMSC, as judged by DNA sequencing and Northern blot analysis. Seven genes were subsequently found to be regulated in by DEX. The following discussion focuses on the nature of the identified genes and speculation about how they may be related to DEX-induced regulation of bone differentiation.

The most profound effect of DEX was observed in the βig-h3 gene. The effect appeared to be marrow stromal cell specific. βig-h3 is an

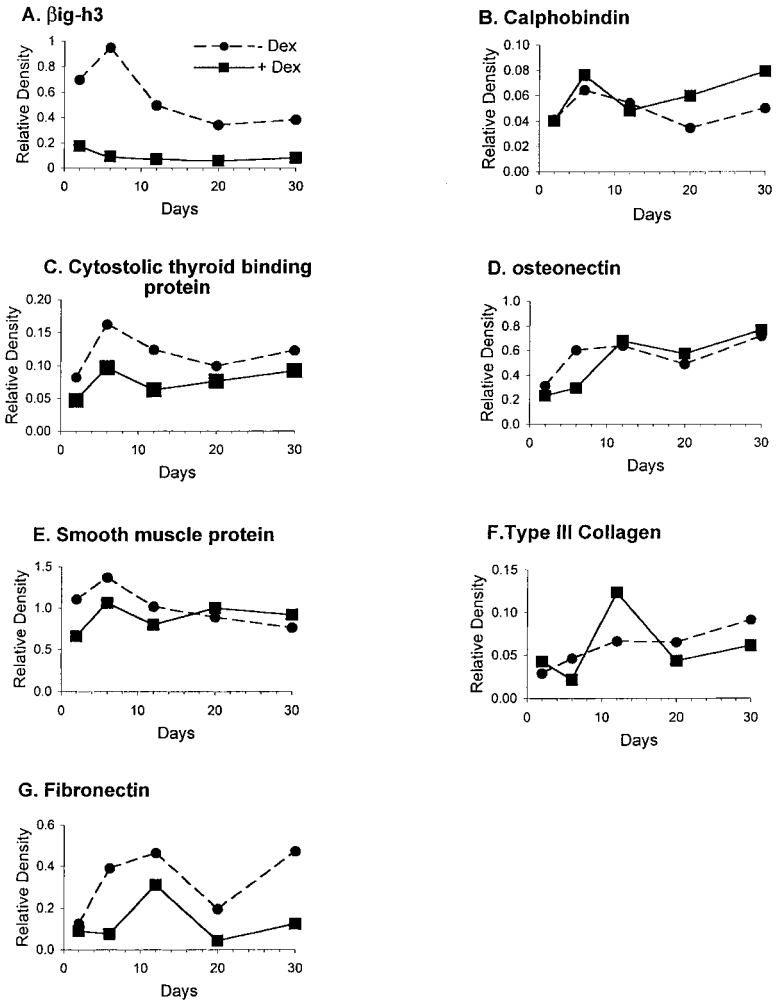

Fig. 3. Quantitation of relative abundance of mRNA of the differentially displayed genes isolated in this study. Relative levels were determined by counting the amount of radioactivity in each lane corresponding to a specific gene and then normalizing the value with β-actin. **A:** TGF-β-induced gene (βig-h3). **B:** Calphobindin II. **C:** Cytosolic thyroid hormone-binding protein. **D:** Osteonectin. **E:** Smooth muscle protein (SM22). **F:** Type III collagen. **G:** Fibronectin. The experiment was also performed in the presence of β-glycerolphosphate with identical relative patterns of gene expression.

extracellular matrix gene that can be induced by TGF-β1 in several cell types, including human melanoma cells, human mammary epithelial cells, human keratinocytes, and human lung fibroblasts [Skonier et al., 1994]. We show that TGF-β1 treatment increased βig-h3 mRNA in human bone marrow stromal cells as well. Other genes have recently been isolated by differential screening of TGF-β1-treated cells and include TGF-β1-regulated gene (TEIG) [Subramaniam et al., 1995] and TGF-β-induced factor 2 (TIF2) [Carey and Chang, 1998]. Both genes are quite distinct from βig-h3; the former is a zinc finger-containing transcription factor [Subramaniam et al., 1995] and the latter a membrane adhesion protein that confers resistance to cell killing by tumor necrosis factor (TNF)-α [Carey and Chang, 1998]. The opposing effects of DEX and TGF-β1 on βig-h3 is similar to what has previously been noted for collagen metabolism in treated human fibroblasts, namely, down-regulation by DEX and up-regulation by TGF-β1 [Slavin et al., 1994]. Further investigation will be required to determine whether the two agents share signaling mechanisms in controlling common matrix genes.

TGF-β1 belongs to a family of proteins that regulate growth and differentiation of many cells [Massague, 1990; Sporn and Roberts, 1990]; it has been shown to influence both the proliferation and differentiation of osteoprogenitor cells [Centrella et al., 1986; Faucheux et al., 1997; Potchinsky et al., 1996]. Both TGF-β1 and βig-h3 are highly conserved between species [Rawe et al., 1997]. Several studies suggest the involvement of βig-h3 expression in differentiation, wound healing, and morphogenesis. In rabbit corneal tissue, βig-h3 mRNA is present in fetal stromal cells and in endothelium- and stroma-derived cells in healing corneal wounds, but it is not detectable in normal adult endothelium and stroma [Rawe et al., 1997]. A

TABLE II. Summary of Relative mRNA Expression Patterns Judged by Northern Blot Analysis

	Regulation by DEX		
	Culture time		
Gene	Early (proliferation)	Middle (matrix-producing)	Late (Bone nodule formation)
TGF-β-induced gene product (βig-h3)	↓↓↓	↓↓	↓↓
Calphobindin II	→	→	↑
Cytosolic thyroid hormone-binding protein	↓↓	↓↓	↓
Osteonectin	↓	→	→
Smooth muscle protein (SM22)	↓	→	→
3′ region for pro-α1 (III) collagen	→	↑	→
Fibronectin	↓	↓↓	↓↓

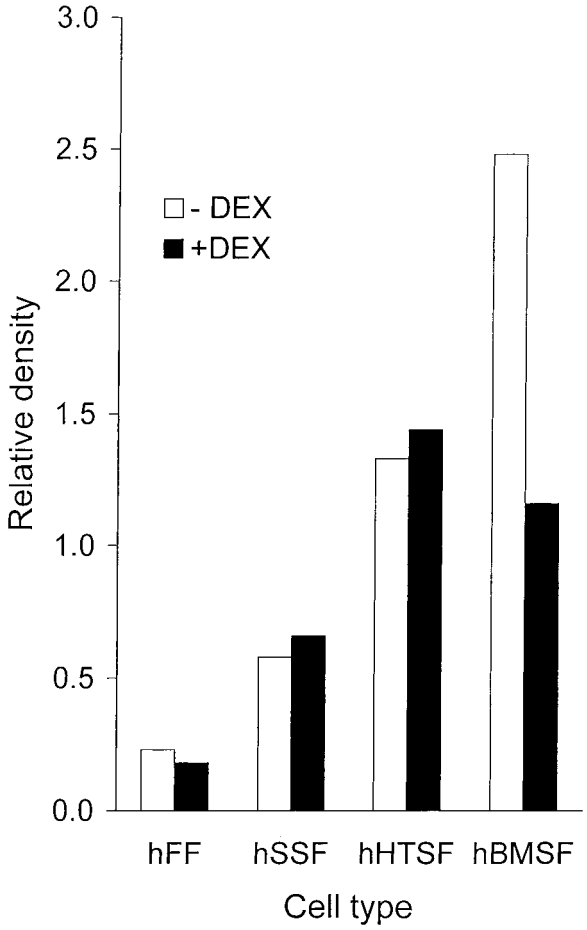

Fig. 4. Quantitation of relative abundance βig-h3 mRNA in different cell types treated with (+) or without (−) dexamethasone (DEX) for 7 days. Relative levels were determined by counting the amount of radioactivity in each lane and then normalizing the value with GAPDH. hFF, human foreskin fibroblasts; hSSF, human spleen stromal fibroblasts; hTSF, human thymus stromal fibroblasts; hBMSC, human bone marrow stromal cells.

role for disturbed morphogenesis has been assigned to βig-h3 missense mutations in families affected with human autosomal dominant corneal dystrophies [Munier et al., 1997]. Taken together, these findings may indicate that βig-h3 expression is involved in TGF-β1-induced differentiation in various species. Other studies imply a role for βig-h3 in cell attachment via cell-collagen interactions. For instance, βig-h3 co-localizes with type VI collagen in abnormal subepithelial matrix and at the stromal/Desçemet's membrane interface in dystrophic corneas implicating a cooperative role with collagen to anchor these tissues to adjacent stromal [Hirano et al., 1996]. RGD-CAP is a recently described protein that is identical or closely related to βig-h3 protein that was identified as a collagen fiber-associated protein within the fiber-rich fraction of pig cartilage [Hashimoto et al., 1997]. Other studies showed βig-h3 localized to regions of interface between collagen fibers and the outer margins of elastic fibers implicating a function "bridging" collagen fibrils to other matrix structures [Gibson et al., 1997]. Such functions may, in turn, be related to its transformation inhibiting activity [Skonier et al., 1994] noted in vitro and in vivo.

The dramatic decrease of βig-h3 expression by DEX suggests that βig-h3 is a negative regulator of hBMSC. In situ hybridization using βig-h3 in human fetal bone shows high levels of expression in newly recruited osteoblastic cells in the primary spongiosa and subperiosteal presteoblasts, further suggesting an involvement in the early stages of bone formation. In this regard, it is interesting to note that when 1,500 different cDNA were screened using mRNA from fibroblast cells cultured from a lesion in a patient with melorheostosis, the most prominently affected message was βig-h3 [Kim et al., in press]. Melorheostosis is a rare, benign connective tissue disorder characterized by cortical thickening of bone with irregular dense hyperostosis that appears to flow along the cortex [Rozencwaig et al., 1997]. It will be inter-

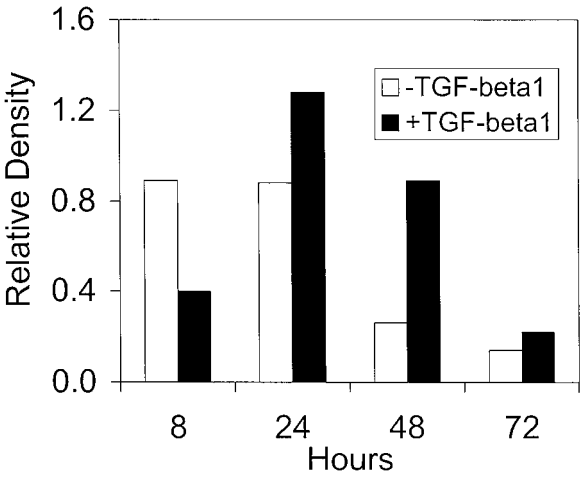

Fig. 5. Quantitation of relative abundance of βig-h3 mRNA in hBMSC treated with 5 ng/ml TGF-β1 for 0–72 h. Relative levels were determined by counting the amount of radioactivity in each lane and then normalizing the value with of β-actin. Ethidium bromide labeling of the RNA confirmed the equal loading of each sample on the gel (not shown).

esting to explore the relationship of βig-h3 to normal human bone development and to the etiology of this unusual human skeletal disease.

The other genes identified, although less strongly regulated, may be important to DEX-induced bone cell differentiation. Fibronectin [Gutman and Kornblihtt, 1987] is an RGD (gly-arg-asp) containing extracellular matrix protein that appears early during osteogenesis [Cowles et al., 1998; Yu et al., 1991]. Regulation of the gene may be complex, apparently depending on cell density and cell-cell contact [Perkinson et al., 1996]. Using a mineralizing organ culture system, fibronectin mRNA and protein were down-regulated by DEX [Gronowicz et al., 1991], implicating similar control mechanisms between rat and human osteogenesis. Like βig-h3, fibronectin plays a role in cell attachment and appears to be critical for osteoblast differentiation in vitro ([Moursi et al., 1996, 1997]. Deciphering the function of fibronectin in humans is further complicated by the fact that transcribed mRNA gives rise to numerous variants, in turn generating up to 20 polypeptides from a single gene [Gutman and Kornblihtt, 1987].

SPARC/osteonectin, an extracellular matrix gene identified in the present study [Swaroop et al., 1988], is implicated to have numerous functions, including anti-adhesion [Motamed and Sage, 1998], Ca^{2+} binding [Gehron Robey and Boskey, 1996; Gehron Robey, 1989; Young et al., 1993], and regulation of cell proliferation [Sage et al., 1995]. SPARC induces matrix metalloproteinase-2 (MMP-2) activation in breast cancer cells [Gilles et al., 1998] and MMP-9 and MMP-1 in treated human monocytes [Shankavaram et al., 1997]. By contrast, cleavage of SPARC by the metalloproteinases collagenase-3, gelatinases A and B, matrilysin, and stromelysin-1 [Sasaki et al., 1997] increased its affinity for collagen types I, IV, and V. These functions may be related to the proposed roles of SPARC in controlling tissue remodeling, cell movement, and proliferation and tumor invasion. Recent studies showed that mice deficient in SPARC have lower bone mass compared with their littermates; double fluorescent labeling experiments further demonstrated that the observed osteopenia was caused by a decrease in bone formation [Delany et al., 1998]. Taken together, it is tempting to speculate that SPARC has similar functions in humans, perhaps even functioning at the level of the bone cell precursors used in the present study.

Calphobindin II (annexin VI) [Iwasaki et al., 1989] is an anticoagulant considered to be closely related to the Ca^{2+}-dependent phospholipid binding proteins known as annexins [Ishikawa et al., 1998]. Interestingly, members of this protein family have previously been shown to be major components of mineralizing matrix vesicles [Genge et al., 1990; Wu et al., 1991]. The function of calphobindin in hBMSC is unclear but, considering the close proximity of bone marrow stromal cells to the blood vessels, it may be related to control of coagulation. The gene is also expressed in primary cultures of rat osteoblasts [Suarez et al., 1993], indicating an evolutionary conserved nature of expression in cells of osteoblast lineage.

The cytosolic thyroid hormone-binding protein gene (*TCB*) was discovered in an attempt to identify non-nuclear proteins that mediate thyroid hormone action [Kato et al., 1989]. Thy-

Fig. 6. In situ hybridization using DIG-labeled antisense (left) and sense (right) strands of βig-h3 RNA. **A:** Sections trough human developing bone showing trabeculae and specific mRNA expression in the preosteoblasts (arrows) within the primary spongiosa at lower (top) and higher magnifications (bottom). **B:** Sections through periosteum and perichondrium (bottom) of human developing bone showing expression of βig-h3 mRNA in the pre-osteoblasts (arrows) in areas of bone formation. Tb, trabeculae; pob, preosteoblasts; f, fibrous tissue; c, cartilage; pc, perichondrium; m, marrow.

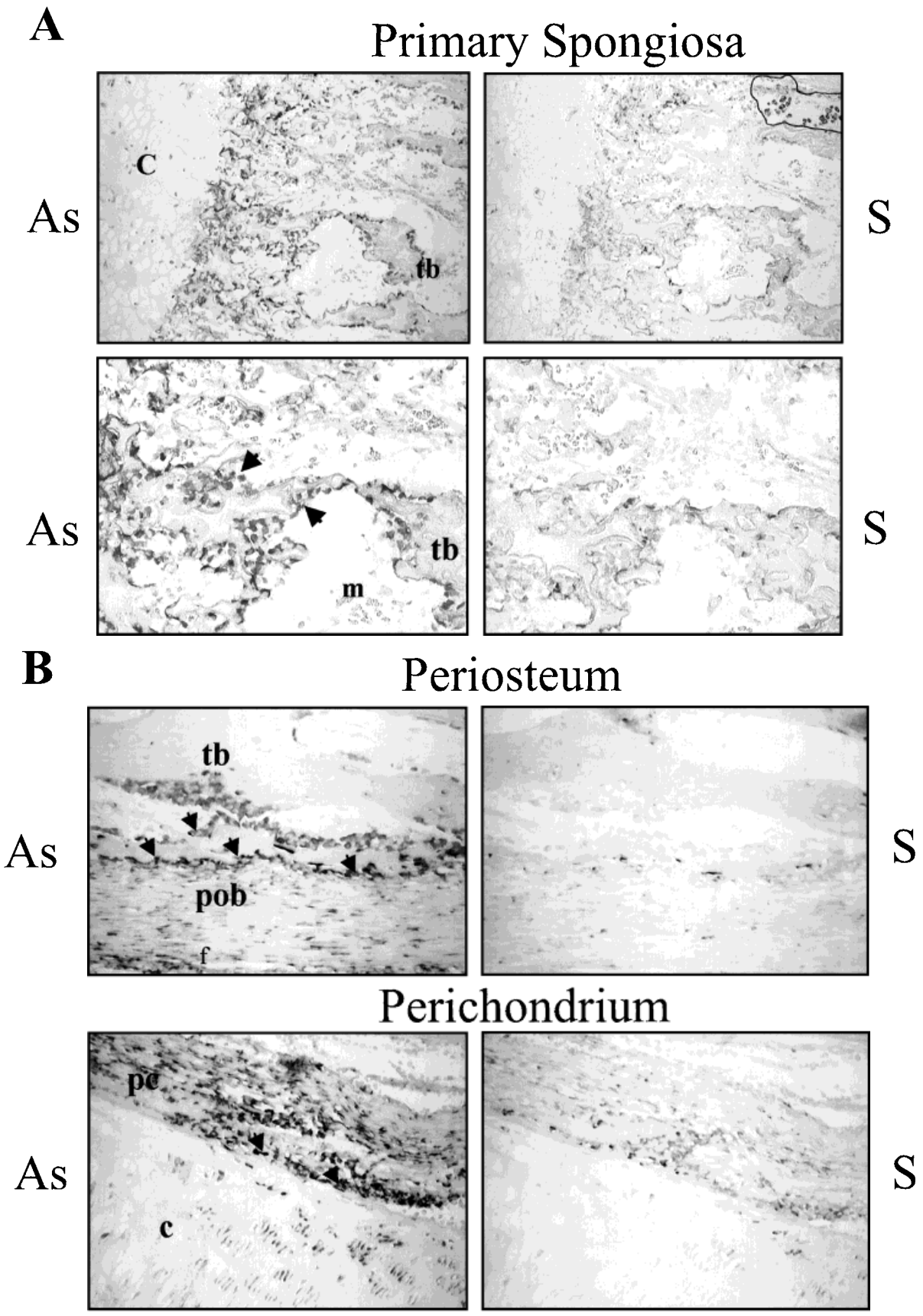
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Figure 6.

roid hormone has powerful effects on bone development and function [Ishikawa et al., 1998] and appears to act selectively at different skeletal sites [Suwanwalaikorn et al., 1997; Milne et al., 1998; Gouveia et al., 1997]. TCB is a cytosolic protein that binds to the thyroid hormone 3,3′,5-triiodo-L-thyronine (T3); it was subsequently cloned using monoclonal antibodies to the purified protein [Kato et al., 1989]. DNA sequence analysis showed that the gene encodes a monomer of pyruvate kinase. When converted to a tetrameric form, it leads to decreased pyruvate kinase activity. Such inhibitions are theorized to alter the rate of glycolysis and, ultimately, O_2 consumption, a role long attributed to thyroid hormone action. Further investigation will be required to determine whether similar enzymatic functions occur in hBMSC through the actions of TCB expression.

Human 22-kDa smooth muscle protein (SM22) is a novel protein widely distributed in smooth muscle cells [Lees-Miller et al., 1987] that has no known function. In an attempt to isolate genes that were overexpressed in senescent fibroblasts [Thweatt et al., 1992], a cDNA library was constructed from a patient with Werner syndrome of premature aging [Murano et al., 1991]. Patients afflicted with this syndrome have several features of aging, including osteoporosis, atherosclerosis, neoplasia, and premature death. Cultured cells from Werner subjects similarly undergo premature senesence, preceded by slow growth, cellular enlargement, and irregularity in cell shape. One gene clearly overexpressed in the cells was characterized and shown to be the human homologue of SM22, the same gene identified in our DEX studies. While regulation by DEX was marginal in hBMSC its Ca^{2+}-binding role implies a function in bone related to ion binding, or perhaps even cell senescence or aging.

Collagen has long been known to be critical for the structure and function of bone. The precise role of type III collagen [Mankoo and Dalgleish, 1988] in hBMSC is unclear, but recent studies implicate a role in marrow fibrosis. hBMSC are not differentiated and, as such, they produce a matrix that is not conducive to mineralization; that is, they produce type I/III/V collagens with low levels of bone extracellular matrix proteins. Marrow fibrosis is the result of the inability of hBMSC to differentiate into osteoblasts; subsequently, the extracellular matrix (ECM) is rich in type I/III/V collagen instead of being primarily type I. When levels of the N-terminus of the type III propeptide (PIII-INP) were examined in the serum of patients with multiple myeloma, a positive correlation was observed between the incidence of the disease and response to treatment. Bone marrow fibrosis is observed in 10–30% of cases of this disease. Type I collagen deposits were unaffected [Abildgaard et al., 1997]. Understanding the function of type III in the skeleton will likely unfold in the near future by isolating genetic mutants or by direct manipulation of the genes in vitro and in vivo, or both.

Glucocorticoids have long been known to have a key role in tissue development [Salomon and Pratt, 1979]. In vitro DEX appears to stimulate osteogenesis by influencing precursor cell proliferation and/or differentiation [Bellows et al., 1990; Fromigue et al., 1997; Peter et al., 1998]. Using cultured human bone marrow cells, we show that the expression of a newly identified bone matrix gene, βig-h3, is very high early in culture, gradually decreasing with time. The presence of DEX appears to accelerate the natural down-regulation of βig-h3 mRNA expression observed in our in vitro model of bone cell differentiation. Similarly, in vivo, βig-h3 is highly expressed in pre-osteoblasts within the primary spongiosa, the perichondrium, and the periosteum in regions characterized by early bone formation events. On the basis of these observations, we speculate that DEX accelerates the differentiation process by altering numerous genes, some of which are identified in the present study. While the underlying mechanism of DEX action in skeletal tissue is not completely clear, differential expression of cell- or stage-specific variants of the glucocorticoid receptor [Bamberger et al., 1996] may be involved. This may also explain the specificity of DEX action observed in the current study for stromal cells obtained from different sources.

In summary, differential display was an excellent method for the initial characterization of expression patterns in hBMC in response to DEX. In addition, by cDNA cloning and sequencing, gene products were identified that were either (1) not previously known to be expressed by HMSC or (2) known to be regulated by DEX in this cell type. One drawback to the method was the lengthy analysis required to identify the clones (i.e., prepare and screen hBMSC libraries to obtain additional 5′ DNA sequences). Another drawback to the method is that only

part of the differentially expressed genes could be analyzed. For example, other studies on DEX regulation showed clear down-regulation of the matrix genes bone sialoprotein and osteopontin [Cheng et al., 1996] and biglcyan [Kimoto et al., 1994], neither of which was detected in our investigation. In order to characterize and quantify large numbers of mRNA simultaneously, new methods must be explored, such as representational differences analysis [Hubank and Schatz, 1994; Lisitsyn and Wigler, 1993], SAGE [Velculescu et al., 1995], or cDNA microarray [Schena et al., 1995]. Other considerations in this analysis are the concomitant expression of the corresponding protein; recent studies showed considerable variation in the correlation between protein and mRNA abundance in yeast [Gygi et al., 1999]. Nevertheless, differential display of DEX-treated hBMSC has showed unique patterns of gene expression and has provided new information about transcriptional events that accompany and, potentially, regulate the osteogenic differentiation of hBMSC. This approach provides an important basis for future studies on the characterization of hBMSC during differentiation of the skeleton.

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