Journal of Cellular Biochemistry 75:382–392 (1999)

Human Bone Marrow Osteoprogenitors Express Estrogen Receptor-Alpha and Bone Morphogenetic Proteins 2 and 4 mRNA During Osteoblastic Differentiation

Richard O.C. Oreffo, Vesna Kusec, Silke Romberg, and James T. Triffitt*

MRC Bone Research Laboratory, Nuffield Department of Orthopaedic Surgery, University of Oxford, Oxford OX3 7LD, England

Abstract Understanding the mechanisms that control the proliferation and commitment of human stem cells into cells of the osteogenic lineage for the preservation of skeletal structure is of basic importance in bone physiology. This study examines some aspects of the differentiation in vitro of human bone marrow fibroblastic cells cultured in the absence (basal media) or presence of 1nM dexamethasone and 50 μg/ml ascorbate for 6, 10, 14, and 21 days. Northern blot analysis and in situ hybridisation with digoxygenin-labelled riboprobes for Type I collagen, osteocalcin, bone morphogenetic proteins 2 (BMP-2), and 4 (BMP-4) and the estrogen receptor alpha (ERα), together with immunocytochemical analysis of ERα expression and histochemical staining of alkaline phosphatase was performed. In basal media, alkaline phosphatase activity and collagen expressions were detected at day 6, ERα from day 10 and osteocalcin from day 10. In the presence of dexamethasone and ascorbate, cell proliferation and alkaline phosphatase were markedly stimulated over 10 to 14 days with a dramatic increase in the temporal expression of Type I collagen, ERα, and osteocalcin mRNAs in these cultures. Northern blot analysis showed cells cultured in basal media, expressed the highest levels of the mRNA for each marker protein at day 14, whereas in the presence of ascorbate and dexamethasone, the highest levels for alkaline phosphatase, ERα, osteocalcin, BMP-2, and BMP-4 were observed at day 21. ERα, BMP-2, and BMP-4 expression were found to correlate temporally with induction of the osteoblast phenotype as determined by alkaline phosphatase, collagen, and osteocalcin expression. These results give additional information on the development of the osteoblast phenotype from early fibroblastic stem cells and on the biological factors involved in this process. These studies suggest a role for estrogen and BMP-2 and -4 in the differentiation of osteoprogenitor cells. J. Cell. Biochem. 75:382–392, 1999.

Key words: estrogen receptor; bone morphogenetic proteins; osteoprogenitors; mesenchymal; osteogenesis; differentiation

According to current hypothesis, osteoblasts, the cells responsible for bone formation, arise from stem cells of the mesenchymal lineage. These cells are self-renewing and multipotential in nature and generate progenitors committed to fibroblastic, osteogenic, chondrogenic, adipogenic, myogenic, and reticular cell phenotypes [Friedenstein et al., 1987; Owen and Friedenstein, 1988; Beresford, 1989; Gimble et al., 1996; Triffitt, 1996; Triffitt and Oreffo, 1998]. The classical experiments of Friedenstein showed that such cells and certain of their progeny were capable of generating fibroblastic colonies in vitro, with each colony proven to be derived from a single cell, termed a colony forming unit–fibroblastic (CFU-F) [Friedenstein, 1973; Friedenstein et al., 1987]. In vivo studies in a number of animal systems indicate that a proportion of these CFU-F have high proliferative and differentiation capacity, and can give rise to osteogenic tissue within diffusion chambers [Ashton et al., 1980; Bab et al., 1984; Mardon et al., 1984; Friedenstein et al., 1987; Benayahu et al., 1989; Kamalia et al., 1992; Gundle et al., 1995; Bruder et al., 1997]. How-

Richard O.C. Oreffo's present address is University Orthopaedics, CF86, University of Southampton, Southampton General Hospital, Southampton S016 6YD, England.

*Correspondence to: Dr. J.T. Triffitt, MRC Bone Research Laboratory, Nuffield Department of Orthopaedic Surgery, University of Oxford, Oxford OX3 7LD, England. E-mail: james.triffitt@ndos.ox.ac.uk

Received 26 February 1999; Accepted 26 April 1999

ever, the identity and specific characteristics of the stem cell, the cell stage-dependent changes that occur during the generation of the progeny, and the biological controls that result in linear progression and human osteoblast differentiation remain unclear.

Over the last 10 years, knowledge on the development, differentiation, and maturation of the osteoblast phenotype has evolved from the use of primary cultures of fetal rodent calvarial osteoblasts [Bellows et al., 1986, 1987; Ecarot-Charier et al., 1988; Owen et al., 1990, 1991; Harris et al., 1994; Stein and Lian, 1996; Boden et al., 1997, 1998]. These cells in prolonged culture undergo a defined series of events from proliferation and the expression of early marker genes (c-fos, histone H4, TGFβ1) and type I collagen to maturation, with the expression of osteopontin, alkaline phosphatase, bone sialoprotein, and osteocalcin and, ultimately, mineral formation [reviewed in Stein and Lian, 1996]. Induction of the osteoblast phenotype by a variety of growth factors including glucocorticoids and ascorbate has been reported in murine and human bone cell cultures [Cheng, 1994; Beresford et al., 1994; Grigoriadis et al., 1988; Kasugai et al., 1991; Leboy et al., 1991; Owen et al., 1990; Bellows et al., 1994; Jaiswal et al., 1997; Oreffo et al., 1997].

As a major steroid hormone, it is well recognised that estrogen plays a major role in bone physiology. Estrogen deficiency observed at the menopause is associated with an increased rate of bone loss and greater risk of skeletal fracture [reviewed in Prince, 1994]. Other significant regulators of osteogenic differentiation include the bone morphogenetic proteins (BMPs). The BMPs, members of the transforming growth factor-beta superfamily, are key factors in the process of bone formation and act as morphogens during embryogenesis [reviewed in Rosen et al., 1996]. BMPs have been shown to induce differentiation of multipotential mesenchymal cells [Ahrens et al., 1993], enhance rat osteoblast proliferation, differentiation, matrix synthesis as well as the production of bone nodules in fetal rat calvarial cell cultures [Rickard et al., 1994; Hughes et al., 1995]. BMP-2 has been shown to induce osteoblast differentiation from pluripotent murine stem cell cultures and from rat bone marrow stromal cultures [Katagiri et al., 1990; Yamaguchi et al., 1991; Rickard et al., 1994; Wang et al., 1993], increasing alkaline phosphatase, collagen, and osteocalcin expression. However, there are species differences between rodent and human osteoblasts and, furthermore, many of the studies detailing the induction of the osteoblast phenotype have been performed using passaged rather than primary stromal cultures. Much is known about the agents required for the induction of the osteoblast phenotype and the factors controlling the differentiation between lineages, for example between the adipocyte and osteoblast phenotypes. However, the temporal expression of estrogen receptor alpha (ERα) and the bone morphogenetic proteins and their relationship to osteoblast differentiation remains unclear. The major aim of the present study was to determine the expression of these moieties during sequential differentiation of human bone marrow osteoprogenitors with time in in vitro culture using Northern blot analysis together with in situ hybridisation techniques. The current study details the development of the human osteoblast phenotype from early fibroblastic stem cells, which can be accelerated by treatment with dexamethasone and ascorbate. Furthermore, the present results clearly show the appearance of ERα, BMP-2, and BMP-4 mRNA expressions during osteogenic cell development and suggest a direct role for estrogen in the subsequent differentiation of the committed osteoprogenitors.

MATERIALS AND METHODS

Materials

Tissue culture reagents were obtained from Gibco/BRL (Paisley, Scotland). Dexamethasone, alkaline phosphatase kits, and other reagents were from Sigma Chemical Company (Poole, Dorset). The cDNA plasmid used for detection of estrogen receptor-alpha (ERα) was a generous gift from P. Chambon (IGBMC-LGME-U.184-ULP, Strasbourg, France). The Type I collagen probe was provided by E. Vuorio (University of Turku, Finland), the BMP probes by Genetics Institute (Cambridge, MA), and the osteocalcin probe was provided by M. Noda (Merck, Sharp and Dohme, West Point, PA). Reagents for RNA extraction and hybridisation were of molecular biology grade from Sigma Chemical Company. $1,25(OH)_2D_3$ was a generous gift from Roche Products Ltd. (Welwyn Garden City, Herts., England) and Leo Pharmaceuticals (Dr. L. Binderup, Leo Pharmaceuticals Ltd, Aarhuus, Denmark).

Cell Culture

Bone marrow samples were obtained from haematologically normal patients (19 patients, 29–81 years of age, 11 female and 8 male) undergoing routine total hip replacement surgery or corrective surgery. Only tissue which would have been discarded was used with the approval of the local hospital management committee. Primary cultures of bone marrow cells were established as previously described [Oreffo et al., 1997]. In brief, marrow cells were harvested using αMEM from trabecular bone marrow samples and pelleted by centrifugation at 500*g* for 5 min at 4°C. The cell pellet was resuspended in 10 ml αMEM and passaged through nylon mesh (90 micron pore size; Lockertex, Warrington, England). Samples of cell suspension were diluted with 0.5% ($^{w}/_{v}$) trypan blue in 0.16M ammonium chloride and the number and viability of nucleated cells determined. Cells were plated out on glass slides in square petri dishes at 2×10^7 nucleated cells/dish or at 10^7 nucleated cells / T-175cm^3 flask for Northern analysis. Cells were cultured in αMEM supplemented with 10%($^{v}/_{v}$) foetal calf serum alone (basal medium) or 10%($^{v}/_{v}$) foetal calf serum containing dexamethasone (10^{-8}M) and 50μg/ml ascorbate-2-phosphate. Cultures were fed after 6 days and thereafter every 3 days for up to 21 days. Cultures maintained in basal medium were analysed at day 6, 10, 14, and 21 and cultures maintained in dexamethasone and ascorbate at days 6, 10, and 14. At these time points cultures were analysed for mRNA expression of Type I collagen, estrogen receptor, and osteocalcin by in situ hybridisation and stained for alkaline phosphatase activity. Cultures were maintained at 37°C in a gassed incubator, 5% CO_2 in air. At the completion of cell culture, the medium was removed, the cell layer washed in phosphate-buffered saline (PBS), and cultures fixed in 95% ($^{v}/_{v}$) ethanol or 4% paraformaldehyde for 30 min prior to dehydration in graded alcohols (50%, 70%, 100% ethanol) and in situ hybridisation. For osteocalcin in situ hybridisation and Northern blot analysis, cells were cultured in 10% foetal calf serum containing $1,25(OH)_2D_3$ (10^{-8} M) for the final 48 h of the culture period. For Northern analysis, cells were washed twice in PBS and stored at −135°C until total RNA was isolated.

Histochemical Staining: Alkaline Phosphatase Activity

Cultures were rinsed three times in PBS and fixed in 95% ($^{v}/_{v}$) ethanol and stained using a Sigma alkaline phosphatase kit (no.85) according to the manufacturer's instructions. Histological examination of the human bone marrow cultures was performed using a Zeiss Axiophot photomicroscope (Carl Zeiss Ltd, Welwyn Garden City, Herts, UK), and recorded on Kodak tungsten film (Kodak, Hemel Hempstead, UK).

Alkaline Phosphatase Assay and DNA Content

Cell layers were washed with PBS and stored at −70°C until assayed for alkaline phosphatase activity. For assay, the cell layer from each well was scraped into 0.5 ml 0.1% ($^{v}/_{v}$) triton-X-100. Alkaline phosphatase activity was measured using p-nitrophenyl phosphate as substrate in 2-amino-2-methyl-1-propanol alkaline buffer solution (1.5 M, pH 10.3 at 25°C). DNA content was measured according to the method of West et al. [1985]. Alkaline phosphatase specific activity was expressed as nanomoles of p-nitrophenol/h/μg DNA.

In Situ Hybridisation: Preparation of RNA Probes

Digoxigenin-labelled single-strand RNA probes were prepared for hybridisation using a DIG RNA labelling kit (Boehringer Mannheim Biochemica, Lewes, Sussex) according to the manufacturer's instructions. Hybridisation was performed at 60°C for ERα, 55°C for Type I collagen and 45°C for osteocalcin for 16 h and the detection of the hybridised probe was performed using an alkaline phosphatase-coupled anti-digoxigenin antibody. Slides were assessed by an independent observer using a Zeiss Axiophot photomicroscope and a positive result was recorded when most of the cells within hybridised areas of the slide were found to contain the colour reaction product (bromo-chloro-indolyl phosphate/nitroblue tetrazolium). Intensity of the colour reaction product was not taken into account when evaluating results obtained. Control hybridisations were performed with sense probes.

Isolation of Total RNA and Northern Blot Analysis

Total RNA was isolated from T-175 cm^2 flasks using acid guanidine thiocyanate-phenol-chloroform as described by Chomczynski and Sacchi [1987]. Poly $(A)^+$ RNA was purified from 22

μg of total RNA using oligo (dT) coated magnetic microspheres (Dynabeads; Dynal UK Ltd., Wirral, England) according to the manufacturer's instructions and electrophoresced on 1% ($^{w}/_{v}$) agarose gel before blotting on Biodyne nylon membrane (Pall Biosupport, Portsmouth, UK) under alkaline conditions. The membrane was baked for 45 min at 80°C to immobilise the transferred RNA. Three samples for each treatment group were used.

The blot was hybridised with [^{32}P]-labelled riboprobes (Amersham International, England) for BMP-2 and 4, ERα, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcribed from linearised plasmids and random primed cDNA probes (Megaprime kit, Amersham International, England) for osteocalcin and alkaline phosphatase. Prehybridisation and hybridisation solution consisted of 50% ($^{v}/_{v}$) formamide, 1% ($^{v}/_{v}$) SDS, 4 × SSPE (0.18 M NaCl, 0.01 M NaH_2PO_4, 0.001 M Na_2EDTA, pH 7.7), 0.05% ($^{w}/_{v}$) non-fat dried milk and 300 μg/ml denatured salmon sperm DNA. Probes were used at ~1 × 10^6 cpm/ml for hybridisation at 68°C for BMP-2 and BMP-4, 60°C for estrogen receptor alpha, 42°C for alkaline phosphatase and osteocalcin, and, 65°C for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Post hybridisation washes were in 2 × SSC with 0.1% ($^{v}/_{v}$) SDS followed by 0.1 × SSC with 0.1% ($^{v}/_{v}$) SDS at the respective temperatures. Oligo(dT)15–18 were labelled using a 3′-end labelling kit (Promega Ltd., Southampton, England). Prehybridisation (2 to 3 h) and hybridisation (overnight) were carried out in a solution consisting of SSC (5×), SDS (0.5% [$^{w}/_{v}$]), Tris-HCL (10mM, pH7), and Denhardt's solution (1×; ficoll [0.02% ($^{w}/_{v}$)], polyvinylpyrrolidone (0.02% [$^{w}/_{v}$]), BSA (0.02% [$^{w}/_{v}$]). Hybridisation was performed at 37°C overnight after which the membranes were washed three times in SSC (2×) with SDS (0.1% [$^{w}/_{v}$]) at room temperature. The signal was obtained on autoradiographs by exposing the blot to pre-flashed Kodak X-OMAT film (Kodak Ltd., Hemel Hempstead, England) at −70°C. For quantitative measurement of the relative levels of the mRNAs, autoradiographs were digitised with a video camera and analysed using OPTIMAS image analysis software (DataCell Ltd, Maidenhead, Berks, England). mRNA expression was normalised to Oligo $(dT)_{15–18}$ levels and the results expressed as mRNA levels relative to control/untreated samples (mean ± SD from triplicate samples).

Statistics

All experiments were performed at least three times. Results of representative experiments are presented except where otherwise indicated. Values are expressed as mean ± SEM. Statistical analysis was performed by Kruskal-Wallis (non-parametric) one-way analysis of variance (ANOVA) with the Dunn multiple comparison test and by Student's *t*-test.

RESULTS

Alkaline Phosphatase Expression and Cell Proliferation

Alkaline phosphatase expression was detected at the earliest time point examined, day 6, in cultures grown on in αMEM alone (Table

TABLE I. Time Course of Alkaline Phosphatase Expression and DNA Content in Human Bone Marrow Cells Grown in the Presence and Absence of Ascorbate-2-Phosphate and Dexamethasone[a]

Time point (days)	Alkaline phosphatase specific activity (nM p-nitrophenol/ h/μg DNA		Total DNA content (μg)	
	Basal media	Osteogenic media	Basal media	Osteogenic media
6	11.45 ± 1.87	23.05 ± 5.59**	27.65 ± 2.91	42.35 ± 9.38*
10	13.35 ± 3.08	26.25 ± 10.06*	41.30 ± 12.77	46.46 ± 9.20
14	30.48 ± 2.64	38.92 ± 6.67*	52.41 ± 1.88	66.59 ± 10.47*
21	56.81 ± 2.74	91.14 ± 19.73**	59.41 ± 3.4	65.54 ± 7.77

[a]Basal media (αMEM + 10% FCS); Osteogenic media (αMEM + 10% FCS + ascorbate-2-phosphate + Dex). Cells plated at 2 × 10^6 cells/well in basal media or osteogenic media and harvested at the indicated time points. Data presented as mean ± SD, n = 4.

*$P \leq 0.05$.

**$P \leq 0.01$ vs. respective basal control.

I). At this early time point, small fibroblast colonies, of varying size, morphology, and number could be observed (not shown). Cell proliferation, as assessed by DNA content, and alkaline phosphatase expression were observed to increase to day 21 (Table I).

Culture of human bone marrow cells in dexamethasone and ascorbate resulted in accelerated cell proliferation and differentiation as assessed by DNA content and alkaline phosphatase expression (Table I). At day 6, numerous colonies, predominantly alkaline phosphatase positive, could be observed by microscopy (not shown). In all patients examined, irrespective of age or gender, significant induction of alkaline phosphatase activity compared to parallel cultures (in basal media) was observed (Table I). Extensive matrix deposition was observed in cultures maintained in dexamethasone and ascorbate but no von Kossa staining was observed. No mineralised areas or nodule formation was detected at day 14 in dexamethasone and ascorbate cultures, or in cultures grown for 21 days in αMEM alone, in any of the marrow samples cultured under these conditions (data not shown).

In Situ Hybridisation Studies: Profile of Type I Collagen, ERα, and Osteocalcin mRNA Expression

Type I collagen expression was detectable in only one of four marrow patient marrow cultures examined at the earliest time point (day 6) following culture in basal medium. The frequency of expression of type I collagen increased to 73% of samples at day 10 and peaked to approximately 90% of samples at day 21 (Figs. 1a, 2). In contrast, following culture in dexamethasone and ascorbate, expression of type I collagen increased to 62% of marrow samples examined at day 6 and peaked to 72% over 10 to 14 days (Figs. 1b, 2).

ERα expression was not detected in day 6 cultures grown in basal media, however by day 10, high levels of estrogen receptor expression could be detected and was maintained throughout the culture period with all samples at the 21-day time point showing strong expression (Fig. 1a). The increase in cell proliferation, as assessed by DNA content (Table I), and accelerated expression of collagen mRNA was paralleled by an increase in ERα mRNA expression (Figs.1a,b, 2). Expression of $1,25(OH)_2D_3$-stimulated osteocalcin was only detected in four out of 10 marrow samples cultured in basal medium at day 10. However, by day 21 expression of osteocalcin was detected in 66% marrow samples examined. Culture in dexamethasone and ascorbate resulted in earlier expression of osteocalcin mRNA although even under these osteogenic conditions, expression was still only observed in 50% of samples at day 14 (Figs. 1a,b, 2). No differences in the patterns of expression for alkaline phosphatase, ERα, and osteocalcin mRNA were observed between female (n = 11) and male (n = 8) patients, or, patients over 55 (n = 44) or under 55 (n = 5; data not

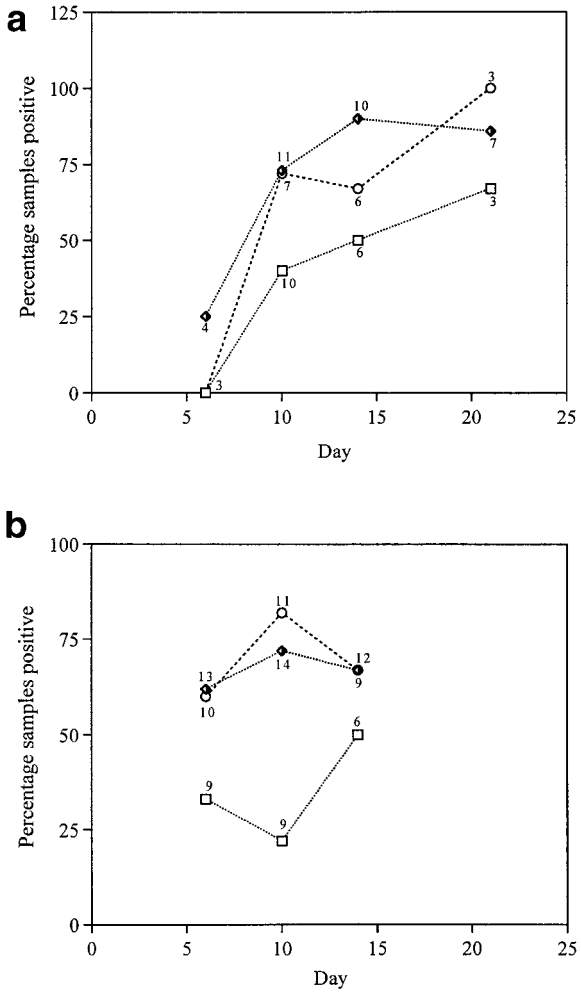

Fig. 1. Temporal expression of mRNA for Type I collagen (◇), ERα (○), and osteocalcin (□) in primary cultures of human osteoprogenitors. Cells were cultured in (**a**) basal medium (αMEM supplemented with 10% FCS) for 6, 10 14 and 21 days or (**b**) αMEM supplemented with 10% FCS, ascorbate-2-phosphate and dexamethasone for 6, 10, and 14 days prior to mRNA expression analysis. Number of patient samples assessed for each probe and at each time point is indicated.

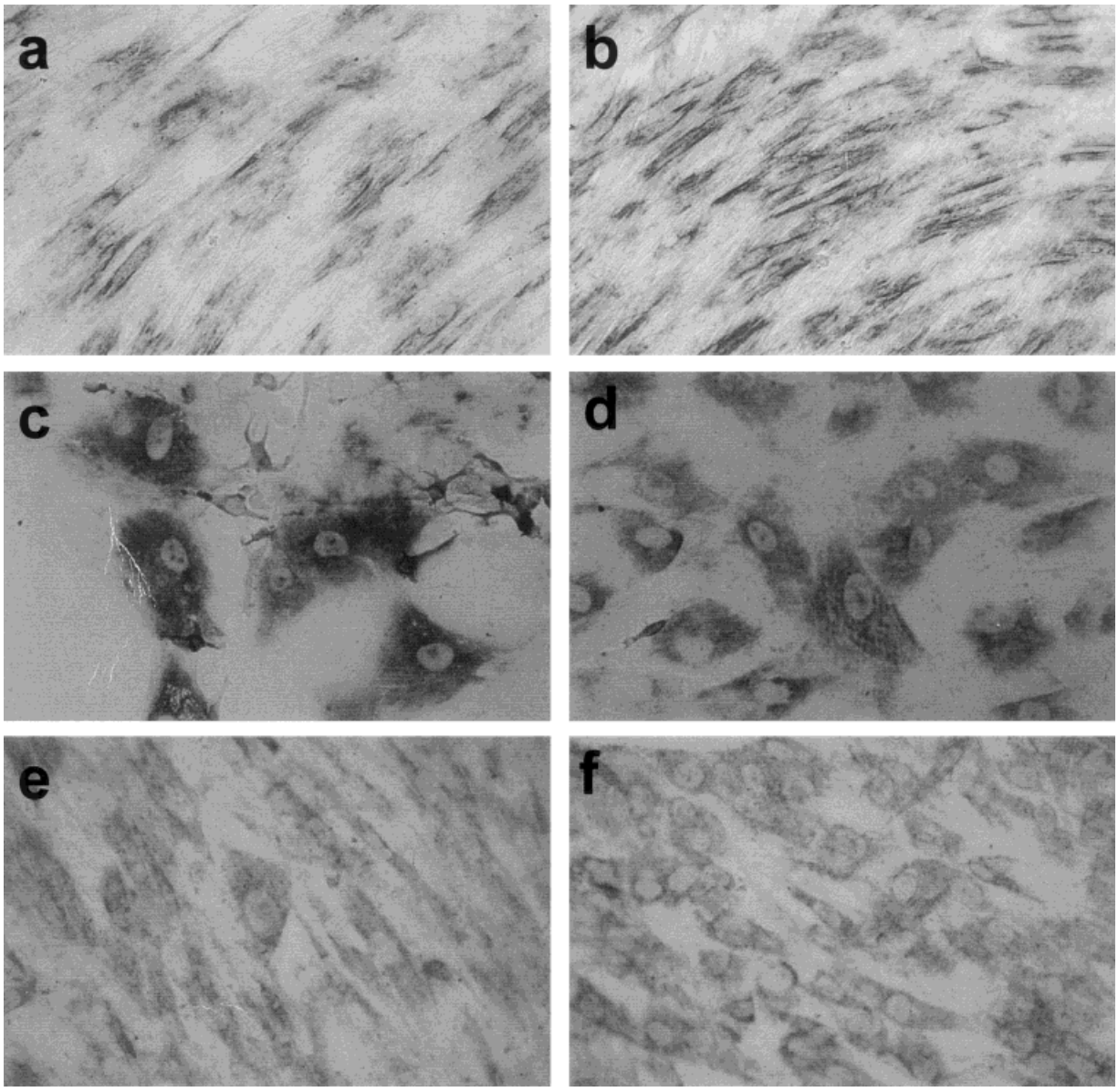

Fig. 2. Photomicrographs of human osteoprogenitors hybridised to the Type I collagen antisense probe (**a,b**), ERα antisense probe (**c,d**), osteocalcin antisense probe (**e,f**). Cells were cultured in basal media (a,c,e) or ascorbate-2-phosphate/dexamethasone (b,d,f) for 14 days. Original magnification ×40.

shown). All sense probes showed no specific staining in all samples (not shown).

Northern Blot Analysis

The changes observed in gene expression by in situ hybridisation were quantitated by northern blot analysis. mRNA expressions of alkaline phosphatase (2.5 kb), ERα (6.6 kb), osteocalcin (0.6 kb), BMP-2 (5.0 kb), and BMP-4 (2.3 kb) relative to total mRNA were examined in cultures maintained in basal media alone or in the presence of dexamethasone and ascorbate for up to 3 weeks. In cultures maintained in basal media alone, maximum levels of alkaline phosphatase, ERα, osteocalcin, BMP-2, and BMP-4 mRNA expressions were observed at day 14 (Figs. 3, 4). Under these conditions the expression of mRNAs for alkaline phosphatase, ERα, and BMP-2 were observed to increase significantly from day 10 and to fall from day 14 to day 21 as the cells reached confluency. Osteocalcin mRNA tended to follow this pattern but the changes were not statistically significant and

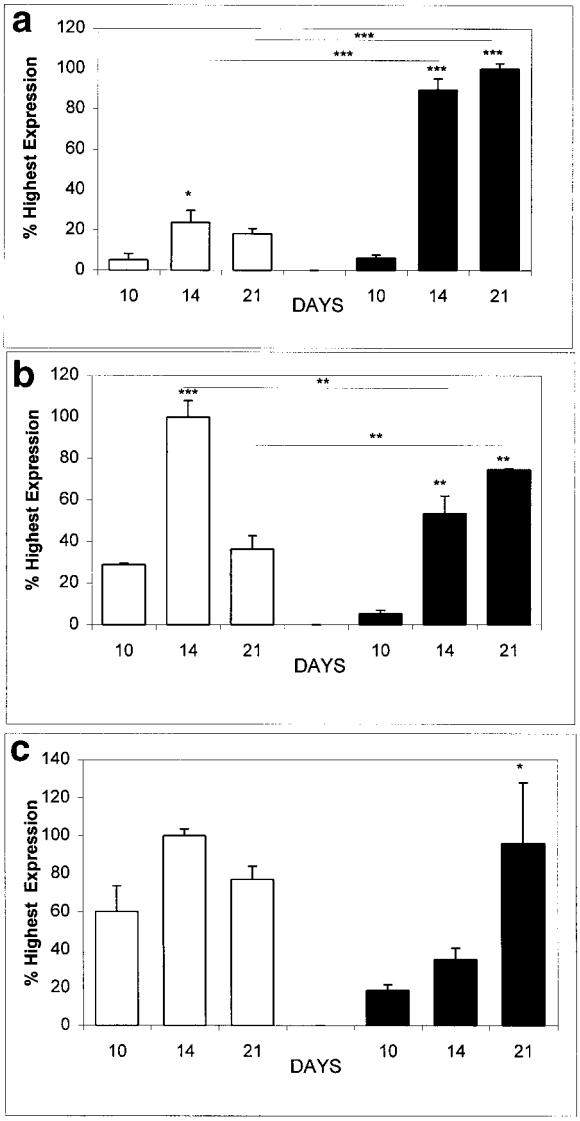

Fig. 3. Modulation of alkaline phosphatase (**a**), ERα (**b**), and osteocalcin (**c**) mRNA expression as assessed by Northern blot analysis in primary human bone marrow fibroblastic cells cultured in basal media (□) or ascorbate-2-phosphate and dexamethasone (■) for 10 to 21 days. Each point represents the mean ± SEM from triplicate samples. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ vs. respective control (□) αMEM/FCS day 10 group; (■) ascorbate-2-phosphate/dexamethasone day 10 group. Line indicates significant difference between groups ($**P \leq 0.01$, $***P \leq 0.001$).

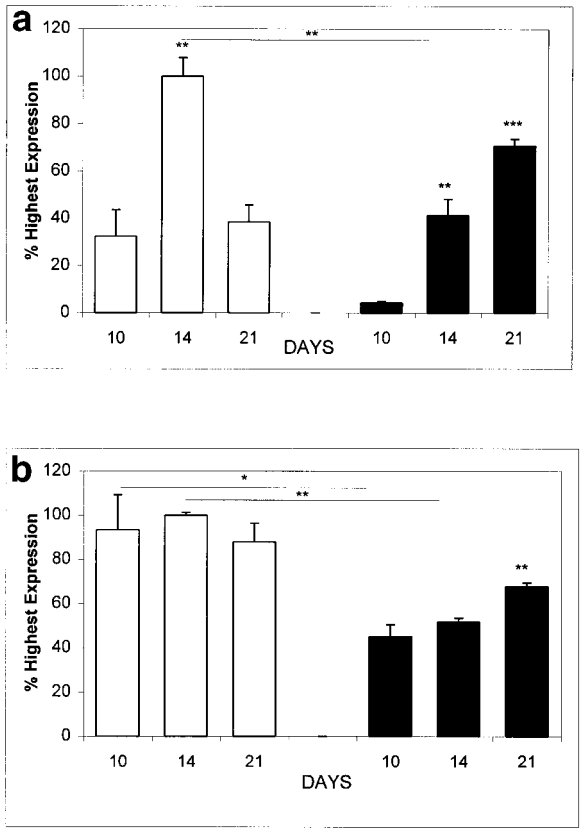

Fig. 4. Modulation of BMP-2 (**a**), and BMP-4 (**b**) mRNA expression as assessed by Northern blot analysis in primary human bone marrow fibroblastic cells cultured in basal media (□) or ascorbate-2-phosphate and dexamethasone (■) for 10 to 21 days. Each point represents the mean ± SEM from triplicate samples. $*P \leq 0.05$ vs. ascorbate-2-phosphate/dexamethasone day 10 group. Line indicates significant difference between groups ($*P \leq 0.05$, $**P \leq 0.01$).

BMP-4 showed no change with time in basal medium (Figs. 3, 4).

In the presence of dexamethasone and ascorbate, mRNA expression of alkaline phosphatase, osteocalcin, ERα, BMP-2 and BMP-4 increased dramatically and significantly from day 10 to day 21 (Figs. 3 and 4). Expression of alkaline phosphatase, ERα, and BMP-2 was most marked between day 10 and day 14, indicating increased expression of each of the selected markers with osteoblast differentiation. All of the examined markers showed elevated expression from day 14 through to day 21 with osteocalcin showing the greatest increase, providing further evidence of the expression of these markers with osteoblast differentiation. Comparison of mRNA expression under both culture conditions employed, showed the highest levels of relative expression of the mRNAs for alkaline phosphatase and osteocalcin in dexamethasone and ascorbate-treated cultures (Fig. 3). Osteocalcin expression was not observed in the absence of $1,25(OH)_2D_3$ (data not shown). However, treatment of the human bone marrow cells with dexamethasone and ascorbate did not induce maximum expression of all examined mRNAs. The highest relative levels of ERα, BMP-2, and BMP-4 were seen in cultures maintained in basal media (Fig. 3, 4).

DISCUSSION

The present study details the development and expression of osteoblast gene products in primary cultures of human bone marrow. In accordance with observations in rodent models, in situ hybridisation studies in combination with histochemical studies showed a temporal sequence of events with alkaline phosphatase and collagen expression preceding osteocalcin expression. The expression of type I collagen, was found to peak over 10 to 14 days. Osteocalcin, was undetectable until day 10 with only four of 10 marrow samples expressing osteocalcin mRNA at this time in cultures maintained in basal medium. Expression was increased upon cessation of cell growth and matrix production, as evidenced by light microscopy and DNA analysis. Parallel cultures in the presence of dexamethasone and ascorbate exhibited an accelerated pattern of proliferation, differentiation and expression of osteoblast genes. In contrast to marrow samples grown in basal conditions, in situ hybridisation showed collagen expression and, in three from nine patient samples, osteocalcin expression at the earliest time point examined day 6. ERα expression was found to correlate with the induction of the osteoblast phenotype, in particular, expression of Type I collagen. However, no correlation was observed in the expression of ERα mRNA, or any of the analysed parameters, as a function of age, or, with respect to sex. Northern analysis confirmed the temporal expression of mRNA for alkaline phosphatase, ERα, and osteocalcin as well as BMP-2 and BMP-4, with maximal expression of ERα, osteocalcin, and BMP-2 and BMP-4 observed at 14 days in cells cultured in basal media and 21 days in cells cultured in ascorbate and dexamethasone. The results presented reflect the proportions of mRNA relative to total mRNA and indicate the presence of the selected markers with osteoblast differentiation, although the relative levels of each of the selected mRNA may not be directly proportional to secreted protein.

The temporal expression of osteoblast gene products as a function of osteoblast differentiation has been described in a number of studies using primary cultures of rodent calvaria [Bellows et al., 1987; Owen et al., 1990; 1991; Harris et al., 1994; reviewed in Stein and Lian, 1996; Bodine et al., 1998]. In the rodent calvarial culture system a number of stages have been identified in the differentiation of the osteoblast, namely, proliferation of undifferentiated osteogenic precursors, maturation and matrix synthesis, and mineralisation. Accompanying these different stages are the expressions of collagen and alkaline phosphatase, which fall before mineralisation, and the late expression of osteocalcin and other matrix proteins with mineralisation. Recently, Bodine and coworkers [1998] have shown that ERα expression is developmentally regulated during osteoblast differentiation in cultures of rat calvarial-derived osteoblasts, with a biphasic increase in ERα expression that peaks in mature fetal rat calvarial-derived osteoblasts. In addition, examination of estradiol effects on mineralisation in these cultures, showed selective differences in regulation of type I procollagen, TGFβ1, osteocalcin, alkaline phosphatase, and ERα expression suggestive of a functional relationship between ERα expression and osteoblast differentiation. In the present study, using human bone marrow stromal fibroblasts, ERα, BMP-2, and BMP-4 were found to correlate with induction of the osteoblast phenotype. The presence of receptors in osteoblasts and in preosteoclasts and the observation that functional disruption of the estrogen receptor in a young adult male resulted in unfused epiphyses and reduced bone density suggests a direct role of estrogen on bone tissue [Eriksen et al., 1988; Komm et al., 1988; Korach, 1994; Smith et al., 1994; Hoyland et al., 1997; Kusec et al., 1998; Oreffo et al., 1999]. Migliaccio and coworkers [1995] have shown that injection of diethylstilbestrol to pregnant female mice can modulate the early phases of bone tissue development with permanent changes in skeletal mass in the offspring, further indicating estrogen may play a key role in osteoprogenitor differentiation.

In the present study, using cultures of human bone marrow fibroblasts, alkaline phosphatase, collagen, and estrogen receptor mRNA expressions were all observed to peak at day 14 in cultures maintained in basal media alone. This corresponded with confluency of the cell cultures. From day 14 to day 21, increased matrix synthesis and, in some cultures, multilayering of the cells was observed. In situ hybridisation showed a similar dramatic increase in each of these proteins to day 14 and both estrogen receptor alpha and osteocalcin mRNA were increased between days 14 and 21. In cultures grown in the presence of dexamethasone and ascorbate-2-phosphate, collagen and estrogen receptor alpha expression were detectable as

early as day 6 and by in situ hybridisation. It has been shown that dexamethasone, 1,25 dihydroxyvitamin D_3 (1,25$[OH]_2D_3$) and ascorbate induce alkaline phosphatase expression and increase colony number [Bellows et al., 1987; Ecarot-Charier 1988; Cheng et al., 1994; Beresford et al., 1994; Oreffo et al., 1997] and the addition of 1,25$(OH)_2D_3$ to dexamethasone-treated cultures increases expression of alkaline phosphatase, bone sialoprotein and osteocalcin, recognised markers of the osteoblast phenotype [Triffitt., 1996]. Furthermore, in contrast to the rodent systems, 1,25$(OH)_2D_3$ is required to observe osteocalcin production in the human osteoblast culture system, as previously reported [Beresford et al., 1994].

Northern blot analysis confirmed the high levels of expression of these marker proteins by day 14 and their continued expression to day 21. The differences in the expression of collagen, alkaline phosphatase and osteocalcin observed in this culture system compared to the fetal rat calvariae model are probably due to i) species differences between rat and human osteoblasts, ii) variations in phenotypic properties between calvarial derived and marrow derived osteoblast populations, and iii) the experimental conditions employed. In the human cell culture model detailed here, mineralisation is not observed at day 21 in the absence of exogenous phosphate whether the cells are cultured in the presence or in the absence of ascorbate and dexamethasone.

It has long been suspected that the primary target cell for BMP action is an early osteoblast progenitor or mesenchymal stem cell. BMPs have been shown to induce differentiation of multipotential mesenchymal cells [Ahrens et al., 1993; Wang et al., 1993] and to induce osteoblast differentiation in rat marrow stromal cell cultures and W-20–17 bone marrow stromal cells [Rickard et al., 1994; Thies et al., 1991]. In particular, expression of BMP-2 and BMP-4 in the murine mesenchymal C3H10T1/2 cell line induces differentiation into distinct mesenchymal cell lineages [Ahrens et al., 1993]. Furthermore, culture of fetal rat calvarial cells results in differentiation into both osteoblasts and chondroblasts. More recently, Rickard and coworkers have shown that estrogen selectively increases BMP-6 production in an estrogen responsive human immortalised osteoblast cell line indicating some of the effects of estrogen on bone and cartilage turnover may be mediated by BMP-6. In the present study, BMP-2 and BMP-4 expression was found to correlate with expression of the osteoblast phenotype in basal and osteogenic culture. Whether the expression of BMP-2 and BMP-4 modulates osteoblast differentiation (via autocrine mechanisms) from stem cells or the progenitor pool remains to be demonstrated, however, data from C3H10T1/2 does not preclude such a mechanism [Ahrens et al., 1993].

In summary, these results demonstrate the detailed development of the osteoblast phenotype from early human osteoprogenitors in primary culture, which can be accelerated by treatment with dexamethasone and ascorbate. Furthermore, these results suggest a role for estrogen and bone morphogenetic proteins in the subsequent differentiation of the committed osteoprogenitors.

ACKNOWLEDGMENTS

We thank A. Bennett and S. Bord for excellent technical assistance and Dr. A.S. Virdi for helpful discussions. We would like to thank and acknowledge the support of Prof. John Kenwright, the consultant orthopaedic surgeons and surgical staff at the Nuffield Orthopaedic Centre for provision of bone marrow samples. Support from the Medical Research Council is gratefully acknowledged.

REFERENCES

Ahrens M, Ankenbauer T, Schroder D, Hollnagel A, Gross G. 1993. Expression of human bone morphogenetic proteins -2 or -4 in murine mesenchymal C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. DNA Cell Biol 12:871–880.

Arts J, Kuiper GGJM, Janssen JMMF, Gustafson J-A, Löwik CWGM, Pols HAP, Van Leeuwen JPTM. 1997. Differential expression of estrogen receptors α and β mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 138:5067–5070.

Ashton BA, Allen TD, Howlett CR, Eaglesom CC, Hattori A, Owen M. 1980. Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. Clin Orthop Rel Res 151:294–307.

Bab I, Ashton BA, Syftestad GT, Owen M. 1984. Assessment of an in vivo diffusion chamber method as a quantitative assay for osteogenesis. Calcif Tissue Int 36:77–82.

Bellows CG, Aubin JE, Heersche JNM. 1987. Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated calvarial cells in vitro. Endocrinology 121:1985–1992.

Bellows CG, Wang Y-H, Heersche JNM, Aubin JE. 1994. 1,25 Dihydroxyvitamin D_3 stimulates adipocyte differentiation in cultures of fetal rat calvarial cells: Comparison with the effects of dexamethasone. Endocrinology 134: 2221–2229.

Benayahu D, Kletter Y, Zipori D, Weintroub S. 1989. Bone marrow-derived stromal cell line expressing osteoblastic phenotype in vitro and osteogenic capacity in vivo. J Cell Sci 140:1–7.

Bennett JH, Joyner CJ, Triffitt JT, Owen ME. 1991. Adipocytic cells cultured from marrow have osteogenic potential. J Cell Sci 99:131–139.

Beresford JN, Joyner CJ, Devlin C, Triffitt JT. 1994. The effects of dexamethasone and 1,25- dihydroxyvitamin D_3 on osteogenic differentiation of human marrow stromal cells in vitro. Arch Oral Biol 39:941–947.

Beresford JN. 1989. Osteogenic stem cells and the stromal system of bone and marrow. Clin Orthop Rel Res 240:270–280.

Boden SD, Hair G, Titus, L, Racine M, McCuaig K, Wozney JM, Nanes MS. 1997. Glucocorticoid-induced differentiation of fetal rat calvarial osteoblasts is mediated by bone morphogenetic protein-6. Endocrinology 138:2820–2828.

Bodine PVN, Henderson RA, Green J, Aronow M, Owen T, Stein GS, Lian JB, Komm BS. 1998. Estrogen receptor-α is developmentally regulated during osteoblast differentiation and contributes to selective responsiveness of gene expression. Endocrinology 139:2048–2057.

Bruder SP, Fink DJ, Caplan AI. 1994. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. J Cell Biochem 56:283–294.

Bruder SP, Jaiswal N, Haynesworth SE. 1997. Growth kinetics, self renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and cryopreservation. J Cell Biochem 64:278–294.

Cheng S, Yang JW, Rifas L, Zhang S, Avioli LV. 1994. Differentiation of human bone marrow osteogenic stromal cells in-vitro: induction of the osteoblast phenotype by dexamethasone. Endocrinology 134:277–286.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Ecarot-Charrier B, Shepard N, Charette G, Grynpas M, Glorieux FH. 1988. Mineralization in osteoblast cultures: a light and electron microscopic study. Bone 9:147–154.

Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC, Riggs BL. 1988. Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241: 84–86.

Friedenstein AJ. 1976. Precursor cells of mechanocytes. Int Rec Cytol 47:327–355.

Friedenstein AJ, Chailakhyan RK, Gerasimov UV. 1987. Bone marrow osteogenic cells: in vitro cultivation and transplantation in diffusion chambers. Cell Tiss Kinet 20:263–272.

Gimble JM, Robinson CE, Wu X, Kelly KA. 1996. The function of adipocytes in the bone marrow stroma: an update. Bone 19:421–428.

Grigoriadis A E, Heersche JNM, Aubin JE. 1988. Differentiation of muscle, fat, cartilage and bone from progenitor cells present in bone-derived clonal cell populations: effect of dexamethasone. J Cell Biol 106:2139–2151.

Gundle R, Joyner CJ, Triffitt JT. 1995. Human bone tissue formation in diffusion chamber culture in vivo by bone-derived cells and marrow stromal fibroblastic cells. Bone 16:597–601.

Harris SE, Bonewald LF, Harris MA, Sabatini M, Dallas S, Fenq JQ, Ghosh-choudhury N, Wozney JM, Mundy GR. 1994. Effects of transforming growth factor β on bone nodule formation and expression of bone morphogenetic protein 2, osteocalcin, osteopontin, alkaline phosphatase, and type I collagen mRNA in long-term cultures of fetal rat clavarial osteoblasts. J Bone Miner Res 9:855–863.

Hoyland JA, Mee AP, Baird P, Braidman IP, Mawer EB, Freemont AJ. 1997. Demonstration of estrogen receptor mRNA in bone using in situ reverse-transcriptase polymerase chain reaction. Bone 20:87–92.

Hughes FJ, Collyer J, Stanfield M, Goodman SA. 1995. The effects of BMP-2, -4 and -6 on differentiation of rat osteoblasts in vitro. Endocrinology 136:2671–2677.

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. 1997. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 64:295–312.

Kassem M, Harris SA, Spelsberg TC, Riggs BL. 1996. Estrogen inhibits interleukin-6 production and gene expression in a human osteoblastic cell line with high levels of estrogen receptors. J Bone Miner Res 11:193–199.

Katagiri T, Yamaguchi A, Ikeda T, Yoshiki S, Wozney JM, Rosen V, Wang EA, Tanaka H, Suda T. 1990. The non-osteogenic mouse pluripotent cell line C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Comm 172:295–299.

Kasugai S, Todescan R, Nagata T, Yao K, Butler W T, Sodek J. 1991. Expression of bone matrix proteins associated with mineralized tissue formation by adult rat bone marrow cells in vitro: inductive effects of dexamethasone on the osteoblastic phenotype. J Cell Physiol 147:111–120.

Komm BS, Terpening CM, Benz DJ, Graeme KA, Gallegas A, Kore M, Greene GL, O'Malley BW, Haussler MR. 1988. Estrogen binding receptor mRNA and biologic response in osteoblast-like osteosarcoma cells. Science 241:81–84.

Korach KS. 1994. Insights from the study of animals lacking functional estrogen receptor. Science 266:1524–1527.

Kusec V, Virdi AS, Prince R, Triffitt JT. 1998. Localisation of estrogen receptor α in human and rabbit skeletal tissues. J Clin Endocr Metab 83:2421–2428.

Leboy PS, Bereford JN, Devlin C, Owen ME. 1991. Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol 146:370–378.

Mardon HJ, Bee J, von der Mark K, Owen ME. 1987. Development of osteogenic tissue in diffusion chambers from early precursor cells in bone marrow of adult rats. Cell Tiss Res 250:157–165.

Migliaccio S, Dacis VL, Gibson MK, Gray TK, Korach KS. 1992. Estrogens modulate the responsiveness of osteoblast-like cells (ROS 17/2.8) stably transfected with estrogen receptor. Endocrinology 130:2617–2624.

Onoe Y, Miyaura C, Ohta H, Nozawa S, Suda T. 1997. Expression of estrogen receptor β in rat bone. Endocrinology 138:4509–4512.

Oreffo ROC, Virdi AS, Triffitt JT. 1997. Modulation of osteogenesis and adipogenesis by human serum in human bone marrow cultures. Eur J Cell Biol 74:251–261.

Oreffo ROC, Kusec V, Virdi AS, Flanagan AM, Grano M, Zambonin-Zallone A, Triffitt JT. In press. Expression of estrogen receptor-alpha in cells of the osteoclastic lineage. Histochem Cell Biol.

Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS. 1990. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143:420–430.

Owen TA, Aronow MS, Barone LM, Bettencourt B, Stein GS, Lian JB. 1991. Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. Endocrinology 128:1496–1504.

Owen ME, Friedenstein AJ. 1988. Stromal stem cells: marrow derived osteogenic precursors. In: Evered D, Harnett S, editors. Cell and molecular biology of vertebrate hard tissues. Chichester Ciba Foundation Symposium vol 136, pp 42–60.

Parfitt AM. 1979. Quantum concept of bone remodeling and turnover: implications for the pathogenesis of osteoporosis. Calcif Tissue Int 28:1–16.

Prince RL. 1994. Counterpoint: estrogen effects on calcitropic hormones and calcium homeostasis. Endocr Rev 15:301–309.

Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I. 1994. Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev Biol 161:218–228.

Rickard DJ, Hofbauer LC, Bonde SK, Gori F, Spelsberg TC, Riggs BL. 1998. Bone morphogenetic protein-6 production in human osteoblastic cell lines—selective regulation by estrogen. J Clin Invest 101:413–422.

Robinson JA, Harris SA, Riggs BL, Spelsberg TC. 1997. Estrogen regulation of human osteoblastic cell proliferation and differentiation. Endocrinology 138:2919–2927.

Rosen V, Cox K, Hattersley G. 1996. Bone Morphogenetic Proteins. In: Bilizekian J, Raisz L, Rodan G, editors. Principles of bone biology. San Diego: Academic Press Inc. pp 661–671.

Shalhoub V, Conlon D, Tassinari M, Quinn C, Partridge N, Stein GS, Lian JB. 1992. Glucocorticoids promote development of the osteoblast phenotype by selectively modulating expression of cell growth and differentiation associated genes. J Cell Biochem 50: 425–440.

Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS. 1994. Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Eng J Med 331:1056–1061.

Stein GS, Lian JB, Stein JL, Van-Wijnen AJ, Montecino M. 1996. Transcriptional control of osteoblast growth and differentiation. Physiol Rev 76:593–629.

Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM, Rosen V. 1991. Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20–17 stromal cells. Endocrinology 130:1318–1324.

Triffitt JT, Oreffo ROC. 1999. The osteoblast lineage. In: Zaidi M, editor. Molecular and cellular biology of bone. Advances in Organ Biology Series. Connecticut: JAI Press Inc. vol 5B:pp 429–451.

Triffitt JT. 1996. The stem cell of the osteoblast. In: Bilizekian J, Raisz L, Rodan G, editors. Principles of bone biology. San Diego: Academic Press Inc. pp 39–50.

Wang EA, Israel DI, Kelly S, Luxenberg DP. 1993. Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. Growth Factors 9:57–71.

West DC, Sattar A, Kumar S. 1985. A simplified in situ solubilization procedure for the determination of DNA and cell number in tissue cultured mammalian cells. Analyt Biochem 147:289–295.

Yamaguchi A, Kahn AJ. 1991. Clonal osteogenic cell lines express myogenic and adipocytic developmental potential. Calcif Tiss Int 49:221–225.