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ARTICLES

Characterisation of the Temporal Sequence of Osteoblast Gene Expression During Estrogen-Induced Osteogenesis in Female Mice

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Abstract Osteoblast differentiation under in vitro conditions is associated with increased expression of non-collagenous bone proteins including osteocalcin, osteopontin, and osteonectin, the exact function of which remain poorly understood. To determine whether these proteins play an important role in the formation of mineralised bone matrix by osteoblasts in vivo, we analysed the time-course of their expression during estrogen-induced osteogenesis in female mice, and compared this with the formation of new cancellous bone. Female mice were sacrificed prior to or following treatment with 17β-estradiol for up to 32 days (500 μg/animal/week). Total RNA was extracted from femurs, and changes in expression of genes for a range of osteoblast-derived proteins assessed by Northern blot analysis. In parallel experiments, the time course of cancellous bone formation was determined by measuring bone mineral density (BMD) of the distal femur. Estrogen led to a rapid increase in BMD, which reached significance by Day 16. This was preceded by three-fold increases in expression of alkaline phosphatase (ALP) and type I collagen (COL I) at Days 8 and 12 respectively. In contrast, osteocalcin, osteopontin, and osteonectin expression showed no change during this initial period, although modest increases were observed at later times (i.e., Days 20 and 24). Our results suggest that osteocalcin, osteopontin, and osteonectin are not involved in the initial phase of the osteogenic response to estrogen, suggesting that these non-collagenous bone proteins do not play a direct role in the formation of mineralised bone matrix by osteoblasts in vivo. J. Cell. Biochem. 82: 683–691, 2001.

Key words: Northern blot analysis; bone mineral density; bone formation

A decline in osteoblast function is thought to contribute to the remodelling imbalance which underlies many forms of osteoporosis [Eastell, 1999]. To improve understanding of the pathogenesis of this condition, the mechanisms involved in osteoblast development have been extensively investigated. Osteoblasts are thought to arise from multipotential precursors within the bone marrow stroma [Friedenstein et al., 1987; Bianco and Robey, 2000]. In vitro studies utilising primary bone cell cultures and osteoblastic cell lines suggest that osteoblast differentiation can be divided into distinct phases based on which osteoblast genes are expressed. For example, maximal expression of alkaline phosphatase (ALP) and type I collagen (COL I) is observed at the stage where proliferation ceases and osteoblast differentiation commences [Owen et al., 1990; Stein et al., 1990; Quarles et al., 1992; Malaval et al., 1994], whereas, late genes such as osteocalcin are expressed by relatively mature osteoblasts at the onset of mineralisation [Stein et al., 1989; Owen et al., 1990; Quarles et al., 1992; Yao et al., 1994].

ALP and COL I both play an important role in the formation of mineralised bone matrix of which the skeleton is constituted. For example, ALP deficiency in hypophosphatasia is associated with a significant impairment in bone matrix mineralisation [Whyte, 1999], while COL I is the major protein component of bone matrix. In contrast, the exact function of non-collagenous proteins produced by mature osteoblasts, such as osteocalcin, is poorly understood. For example, there is no known disease state in humans associated with deficiency of osteocalcin or related non-collagenous proteins. Analysis of the skeletal phenotype of mice lacking

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osteocalcin, osteonectin, and osteopontin suggest that, while these may play a role in regulating bone remodelling, they do not directly influence the ability of osteoblasts to produce mineralised bone matrix [Ducy et al., 1996; Rittling et al., 1998; Delany et al., 2000].

Our recent studies suggest that estrogen-treated mice represent a useful model for exploring the mechanisms involved in bone formation in vivo. During this response, new cancellous bone can be detected within long bone metaphysis 12 days after commencing estrogen, which extends to fill the diaphysis by 24 days [Samuels et al., 1999]. Further observations suggest that estrogen-induced bone formation involves the differentiation of new osteoblasts from early precursors in bone marrow [Perry et al., 2000]. In the present study, we used this model to investigate whether the non-collagenous bone proteins osteocalcin, osteonectin, and osteopontin are involved in the production of mineralised bone matrix by osteoblasts in vivo, by comparing the time-course of expression of these proteins after estrogen with that of the appearance of new cancellous bone.

MATERIALS AND METHODS

Animals

Eight-week old female CBA-1 mice from the University of Bristol breeding colony were divided into weight-matched groups (mean pre-treatment weight 19.3 g) and administered 500 μg/animal/week 17β-estradiol (Sigma, Poole, Dorset, UK) in corn oil by subcutaneous injection. Animals were sacrificed by cervical dislocation 1, 2, 4, 8, 12, 16, 20, 24, or 32 days after the initial injection. A further group did not receive estrogen and were sacrificed at Day 0 (untreated baseline controls). Throughout the experimental period all animals received standard diet (rat and mouse standard diet; B&K Ltd., Humberside, UK) and were kept on a 12-h light/dark cycle. All experimental procedures complied with the guiding principles in the "Care and Use of Animals".

Measurement of Bone Mass at the Distal Femur

Femurs were removed, cleaned, and fixed in 70% ethanol at 4°C for two weeks. Bone mineral density (BMD) were measured by dual-energy x-ray absorbtiometry (DXA) using a PIXImus scanner (Lunar, Maddison, WI) with small animal software. Femurs were placed on a plastic attenuator and scanned using software version 1.44. To enhance detection of estrogen-induced bone formation, which commences at the metaphysis of long bones [Samuels et al., 1999], BMD was measured at the distal femur at a site approximating to the distal metaphysis. This region of interest (ROI) consisted of a 1.2×1.8 mm rectangle positioned over the longitudinal axis of the distal femur at the site associated with maximal BMD. Coefficient of variation for BMD measurements based on this ROI, obtained after scanning four mouse femurs six times each, with re-positioning, was 3.9%.

Histological Analysis

Following analysis by DXA, whole femurs were dehydrated through graded alcohols (80%, 90% and three changes of 100% ethanol for 24 h each), cleared in chloroform for 24 h, placed in 100% ethanol for 24 h and embedded undecalcified in LR White Hard Grade resin (London Resin Company, Reading, UK). Longitudinal sections (7 μm) were cut on a Reichert-Jung 2050 microtome (Heidelberg, Germany) with a D profile tungsten carbide knife. Sections were stained with 0.1% toluidine blue in 0.01 M citrate phosphate buffer and observed under light microscopy (Leica). Images were acquired using Neotech Image Grabber PC1 version 2.01 software.

Northern Blot Analysis

Three independent experiments were performed to assess changes in gene expression in mouse femurs at varying time following commencement of estrogen administration. In experiment one, female mice were sacrificed immediately prior to and following 1, 2, 4, 8, 12, and 16 days of estrogen treatment as described above, and whole femoral RNA subsequently extracted. The protocol for experiment 2 was identifical to that for experiment 1, except for the inclusion of two additional groups following 20 and 24 days of treatment. In experiment three, mice were sacrificed after treatment with estrogen as in experiment one, with the exception that marrow was immediately flushed from femurs using 0.5 ml 1X phosphate buffered saline (PBS) pH 7.4, and RNA subsequently extracted separately from marrow and the remaining bone. In all cases,

four animals were used per time-point, and RNA pooled from eight femurs prior to subsequent analysis.

Femurs were removed immediately following sacrifice, freed from soft tissue, snap-frozen in liquid nitrogen, and stored at −80°C for not more than two weeks. Bones (whole femurs and flushed femurs) were ground into a fine powder with a mortar and pestle (Fisher Scientific, Loughborough, Leics, UK) under liquid nitrogen. The resulting tissue was dissolved in 11 ml TRIzol reagent (Life Technologies, Paisley, UK) in polypropylene centrifuge tubes (Falcon, Becton Dickinson, Cowley, Oxon, UK) and incubated at room temperature for 10 min to allow for dissociation of nucleoprotein complexes and sedimentation of tissue fragments.

Phase separation was performed by addition of chloroform (BDH Laboratory Supplies, Poole, Dorset, UK; 1/5 volume of TRIzol) and incubation at room temperature for a further 10 min followed by centrifugation at 8,000 rpm for 20 min at 4°C in a Sorvall RC5-B centrifuge. The aqueous phase was collected and the RNA precipitated by addition of ice-cold isopropanol (BDH; 1/2 original volume of TRIzol used) followed by incubation on ice for at least 40 min and centrifugation as previously described for 30 min. The resulting RNA pellet was washed twice in 3 ml cold 70% ethanol (BDH; diluted in diethylpyrocarbonate (Sigma) treated water (DEPC-H_2O)) by incubation on ice for 15 min followed by centrifugation at 7,000 rpm for 15 min at 4°C. The RNA was air dried in a laminar flow hood, dissolved in at least 50 μl DEPC-H_2O, and stored at −70°C. The RNA yield was determined by measuring the absorbance at 260 nm (A_{260}) using a Gene Quant II RNA/DNA calculator (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK).

RNA transcripts were subsequently separated by electrophoresis on 1.2% Agarose (Life Technologies) denaturing gels containing 2.2 M formaldehyde (BDH) in 1X MOPS (3-[N-Morpholino]propanesulphonic acid) running buffer (20 mM MOPS, 5 mM sodium acetate, 10 mM EDTA; Sigma). After denaturation at 80°C for 10 min, 5–30 μg RNA were loaded per lane in buffer containing 40% w/v sucrose, 20 mM EDTA, 20 mM MOPS, 5 mM sodium acetate, 40% formamide, 2.2 M formaldehyde, 30 μg/ml ethidium bromide, 0.1% saturated bromophenol blue alongside 10 μg of 0.24–9.5 Kb RNA ladder (Life Technologies). Gels were washed twice in 1X MOPS to remove formaldehyde and the RNA transferred to Gene Screen nylon membrane (NEN Life Science Products, Hounslow, Essex, UK) overnight in 10X salt sodium citrate (SSC, pH 7.0; 0.3 M trisodium citrate, 3 M NaCl; BDH) with a 3 mm chromatography paper wick (Whatman International Ltd., Maidstone, Kent, UK). The RNA was covalently crosslinked to the filter by UV irradiation and stored at room temperature in sealed polythene until hybridization.

Northern blots were then hybridized with the following cDNA probes for proteins produced by osteoblasts (obtained from Dr. J. Lean, St Georges Hospital Medical School, London, UK): rat COL I ($p\alpha_1R1$) [Genovese et al., 1984] subcloned into the Pst1 site of pUC18; rat ALP (pRAP54) [Thiede et al., 1988]; rat osteocalcin (pR2211) [Celeste et al., 1986]; mouse osteonectin [Mason et al., 1986]; rat osteopontin [Yoon et al., 1987]. To provide a loading control, mouse β-actin [Leader et al., 1986] was obtained (Sigma) and a 988 bpr *Pst I*/*Bgl II* fragment cloned into the *Pst I* and *Bam HI* sites of pBluescript KS^+.

Hybridizations were performed at 65°C in a modification of Church and Gilbert (C&G) buffer [Church and Gilbert, 1984] containing 1% BSA, 0.125 M Na_2HPO_4, 0.14 M H_3PO_4, 0.25 M NaCl, 7% SDS. Filters were prehybridized for at least 6 h at 65°C in C&G. cDNA probes were labelled with α-^{32}P-dCTP (Amersham Pharmacia Biotech) using the random primer method (Life Technologies), added to the appropriate hybridization tube and incubated at 65°C overnight. Filters were washed twice in 4X SSC/0.1% SDS, once in 0.5X SSC/0.1% SDS and once in 0.2X SSC/0.1% SDS for 30 min at 65°C for all probes except β-actin where the final wash was performed in 0.1X SSC/0.1% SDS. The filters were exposed to Phosphor Screens (Version 4 Molecular Dynamics, Amersham Pharmacia Biotech) and the resulting signal quantified using ImageQuaNT version 4.2a software (Molecular Dynamics). All blots were also exposed to Kodak Biomax photographic film. Blots were then stripped by washing twice in 0.1% SDS at ~90°C for 20 min before probing for β-actin as a loading control, as described above.

Statistical Analysis

Results were analysed statistically by one-way ANOVA with duration of estrogen treat-

ment as the variable. Where this was found to have a significant effect overall, between-group differences at specific time points were further analysed by Fisher's least significant difference test. Statistical significance was taken as $P < 0.05$.

RESULTS

Weekly administration of high-dose estrogen resulted in a significant osteogenic response within the distal femur by Day 16, as assessed by BMD and histology (Figs. 1 and 2). Two independent experiments revealed that by this time, ALP expression had increased by approximately three-fold, as assessed by Northern blot analysis performed on pooled femoral RNA samples (Fig. 3). The increase in ALP expression after estrogen was evident by Day 4, reached statistical significance by Day 8, and peaked at Day 20, when a five-fold increase was observed.

The cDNA probe for COL I cross-hybridised with unknown marrow species, making it impossible to detect a specific signal (data not shown). Therefore, COL I expression was assessed in femurs from a further experiment, where marrow had previously been removed. Both transcripts of COL I showed increased expression compared to baseline at 12 and 16 days after commencing estrogen (Fig. 4). Interestingly, the 5.7 Kb transcript increased to a greater extent than the 4.7 Kb transcript (approximately six-fold and three-fold increases respectively).

Little change in expression of osteocalcin, osteopontin, and osteonectin was seen over the first 16 days after estrogen administration, as assessed in two independent experiments (Figs. 5–7). In contrast, all three non-collagenous proteins demonstrated statistically significant increases in expression at Day 20. In the case of osteopontin, increased expression was also observed at Day 24.

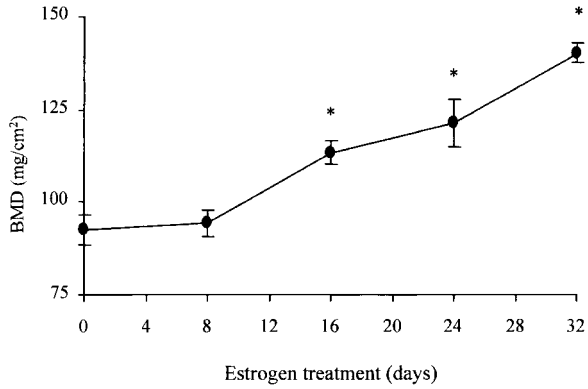

Fig. 1. Effect of estrogen treatment on BMD of the distal femoral metaphysis. Results show mean±SEM prior to and 8, 12, 16, 24, and 32 days following commencement of weekly injections of 17β-estradiol in female mice (six or seven animals per time-point). One way ANOVA revealed a significant ($P < 0.0005$) effect of treatment group for both parameters. *$P < 0.05$ vs. untreated controls.

DISCUSSION

We analysed the role of the non-collagenous bone proteins, osteocalcin, osteopontin, and osteonectin, in the formation of new cancellous bone in response to estrogen in vivo. Estrogen was administered at the same dose, to animals of identical age and strain, to that used in our recent study where estrogen increased cancellous bone formation within 12 days of commencing treatment, as assessed by histo-

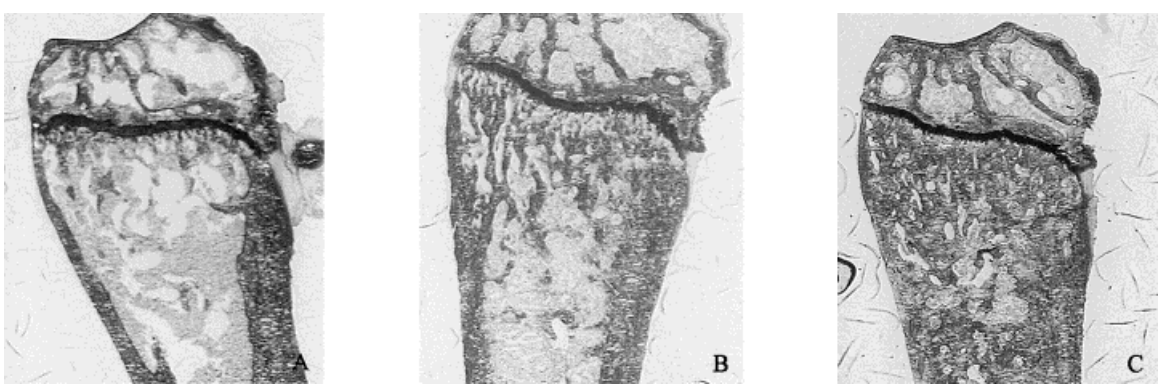

Fig. 2. Longitudinal sections of the distal femoral metaphysis of female mice (**a**) prior to, (**b**) 16, and (**c**) 32 days following commencement of weekly injections of 17β-estradiol in female mice. Sections are stained with toluidine blue. Magnification 20×.

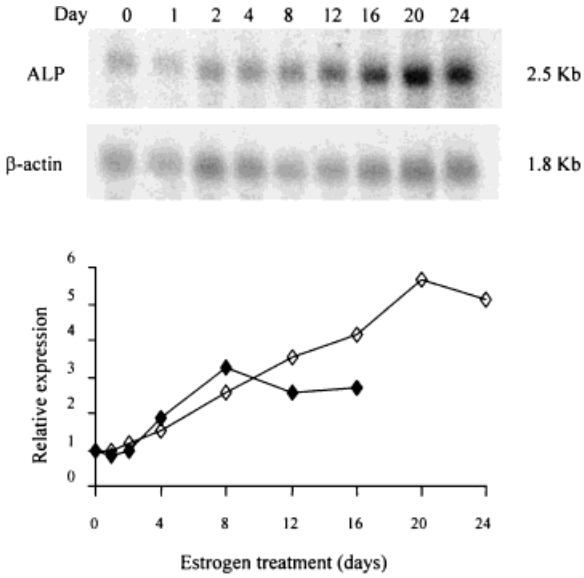

Fig. 3. Effect of estrogen treatment on ALP expression in mouse femurs. **Upper panel:** Northern blots hybridised with cDNA probes for ALP and β-actin; whole femurs were obtained from female mice (four animals per time-point) prior and 1, 2, 4, 8, 12, 16, 20, and 24 days following commencement of weekly injections of 17β-estradiol. **Lower panel:** ratio of ALP and β-actin expression as quantified by densitometry for experiment shown above, and a separate independent experiment where mice were treated with 17β-estradiol for 1, 2, 4, 8, 12, or 16 days. ALP expression was significantly increased compared to baseline at Day 8 and later time-points ($P < 0.05$) (ANOVA performed on combined data).

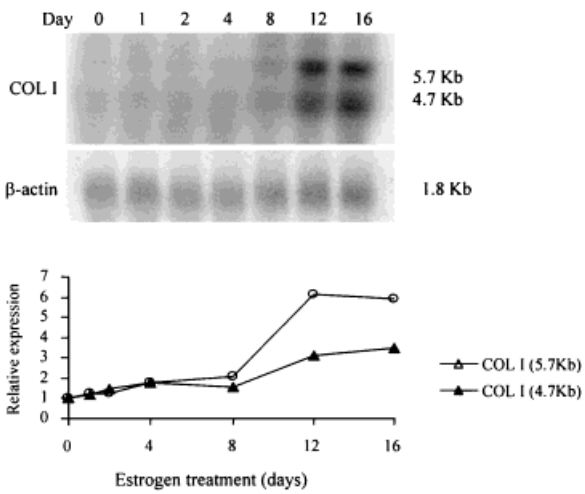

Fig. 4. Effect of estrogen treatment on COL I expression in mouse femurs. **Upper panel:** Northern blots hybridised with cDNA probes for COL I and β-actin, showing 4.7 and 5.7 Kb COL I transcripts and 1.8 Kb β-actin transcript (bottom panel); whole femurs were obtained from female mice (four animals per time-point) prior and 1, 2, 4, 8, 12, and 16 days following commencement of weekly injections of 17β-estradiol (marrow was removed prior to RNA extraction). **Lower panel:** ratio of COL I and β-actin expression as quantified by densitometry.

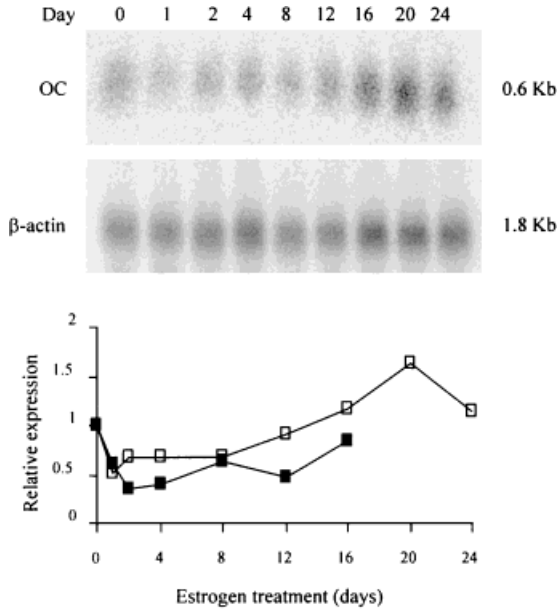

Fig. 5. Effect of estrogen treatment on osteocalcin (OC) expression in mouse femurs. **Upper panel:** Northern blots hybridised with cDNA probes for osteocalcin and β-actin; whole femurs were obtained from female mice (four animals per time-point) prior and 1, 2, 4, 8, 12, 16, 20, and 24 days following commencement of weekly injections of 17β-estradiol. **Lower panel:** ratio of osteocalcin and β-actin expression as quantified by densitometry for experiment shown above, and a further experiment where mice were treated with 17β-estradiol for 1, 2, 4, 8, 12, or 16 days. Osteocalcin expression was significantly increased at Day 20 compared to baseline ($P < 0.05$) (ANOVA performed on combined data).

morphometry performed on tibial longitudinal sections [Samuels et al., 1999]. In the present study, analysis of femoral BMD and histology from animals 16 days after commencing estrogen suggested that an equivalent response had occurred at the femur. This rapid formation of cancellous bone was preceded by marked increases in femoral expression of COL I and ALP, which are known to play important roles in bone matrix synthesis and mineralisation respectively. In contrast, little change was observed in expression of mRNA for osteocalcin, osteopontin, and ostenoectin within mouse femurs over the first 16 days, suggesting these are not involved in the initial osteogenic response to estrogen.

In a previous investigation of developing neonatal rat bone, expression of osteocalcin and osteopontin was found to be limited to mature osteoblasts immediately adjacent to bone surfaces, consistent with our findings that these proteins are not expressed until after bone formation has commenced [Weinreb et al.,

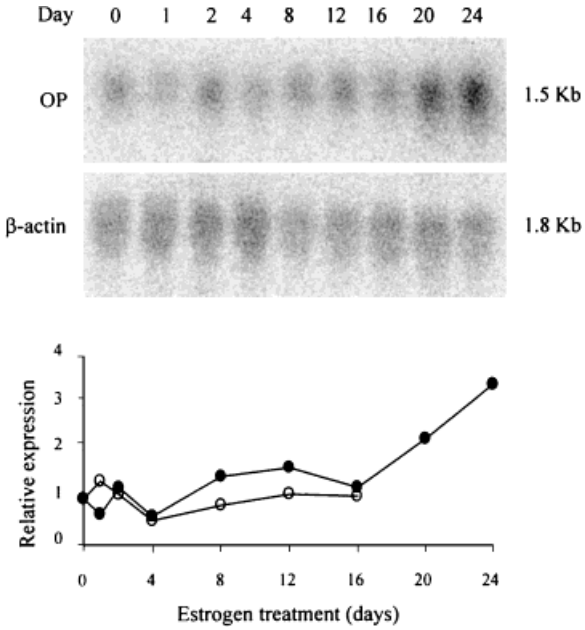

Fig. 6. Effect of estrogen treatment on osteopontin (OP) expression in mouse femurs. **Upper panel:** Northern blots hybridised with cDNA probes for osteopontin and β-actin; whole femurs were obtained from female mice (four animals per time-point) prior and 1, 2, 4, 8, 12, 16, 20, and 24 days following commencement of weekly injections of 17β-estradiol. **Lower panel:** ratio of osteopontin and β-actin expression as quantified by densitometry for experiment shown above, and a further experiment where mice were treated with 17β-estradiol for 1, 2, 4, 8, 12, or 16 days. Osteopontin expression was significantly increased at Days 20 and 24 compared to baseline ($P < 0.05$) (ANOVA performed on combined data).

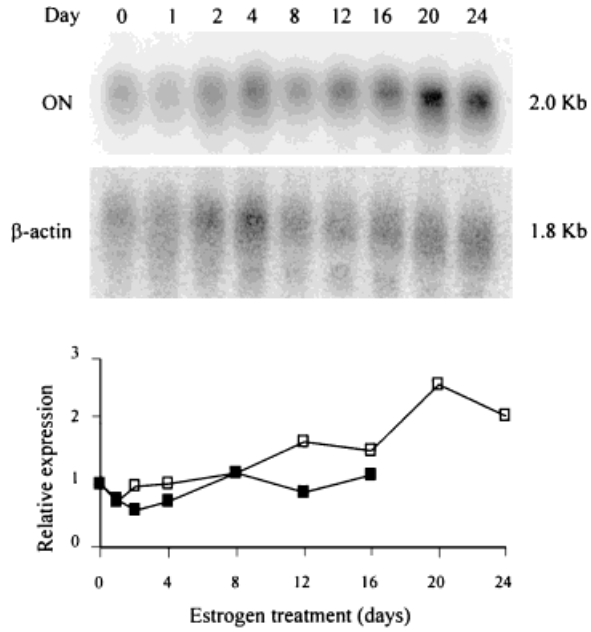

Fig. 7. Effect of estrogen treatment on osteonectin (ON) expression in mouse femurs. **Upper panel:** Northern blots hybridised with cDNA probes for osteonectin and β-actin; whole femurs were obtained from female mice (four animals per time-point) prior and 1, 2, 4, 8, 12, 16, 20, and 24 days following commencement of weekly injections of 17β-estradiol. **Lower panel:** ratio of osteonectin and β-actin expression as quantified by densitometry for experiment shown above, and a further experiment where mice were treated with 17β-estradiol for 1, 2, 4, 8, 12, or 16 days. Osteonectin expression was significantly increased at Day 20 compared to baseline ($P < 0.05$) (ANOVA performed on combined data).

1990]. Few previous studies have related changes in non-collagenous bone protein expression to bone formation in adult animals as in the present investigation. However, following marrow ablation in rats, increased osteopontin expression was reported to precede the subsequent osteogenic response, in contrast to osteocalcin expression which showed relatively little change [Suva et al., 1993]. A possible explanation for these contrasting findings is that, in the tibial ablation model, new cancellous bone formation is preceded by formation of a blood clot within the marrow cavity, which may induce a specific injury response associated with a distinct pattern of gene expression.

Important differences exist between osteoblast differentiation under in vitro and in vivo conditions. For example, many in vitro studies have been performed on transformed cell lines which may have lost certain characteristics of the normal osteoblast phenotype [Gerstenfeld et al., 1996]. Under in vitro conditions, osteoblast differentiation requires the addition of specific osteo-inducers, such as dexamethasone and ascorbic acid [Maniatopoulos et al., 1988; Aronow et al., 1990; Leboy et al., 1991], which may be unrelated to physiological regulators of bone formation in vivo. As well as producing growth factors required for stimulating osteoblast differentiation, the network of non-osteoblastic cells in bone marrow, which is either disrupted or absent under in vitro conditions, is also thought to play an important role in osteoblast differentiation by providing specific cell–cell contact [Schmitz et al., 1995]. However, despite these differences, the sequence of osteoblast-related gene expression which we observed is similar to that found in in vitro studies. For example, ALP and COL I have been found to be expressed during the initial phase of osteoblast differentiation, before the appearance of a mineralised matrix [Stein et al., 1990;

Malaval et al., 1994; Yao et al., 1994]. In contrast, osteocalcin, osteonectin, and osteopontin are expressed by relatively mature osteoblasts during the terminal phase of osteoblast differentiation, after matrix mineralisation has commenced [Stein et al., 1989, 1990; Owen et al., 1990; Strauss et al., 1990; Kasugai et al., 1991; Yao et al., 1994].

Though estrogen-induced osteogenesis is associated with a similar pattern of gene expression to that observed during osteoblast differentiation in vitro, certain differences were found. For example, the suggestion from our results that stimulation of osteoblast differentiation in vivo is associated with preferential use of the 5.7 Kb COL I transcript has not previously been reported during in vitro studies of osteoblast differentiation. In addition, ALP expression did not decrease at later time-points, in contrast to observations from in vitro studies that ALP expression falls in association with mineralisation [Malaval et al., 1994]. A possible explanation for the latter discrepancy is that, unlike in vitro systems where changes are assessed in individual osteogenic colonies containing cells at a single differentiation stage, our data represents changes occurring within whole bones which have greater cellular heterogeneity. Cells within the femur may also mature at different times in distinct spatial compartments as the osteogenic response extends proximally through the marrow cavity. This possibility could be addressed in further studies in which temporal changes in ALP expression following estrogen are analysed at distinct locations by in situ hybridisation.

The suggestion from our results that osteocalcin, osteopontin, and osteonectin are not directly involved in the formation of mineralised bone matrix is consistent with previous reports that the ability of osteoblasts to form bone matrix is relatively unaffected in mice in which these genes have previously been deleted [Ducy et al., 1996; Rittling et al., 1998; Delany et al., 2000]. Nevertheless, these non-collagenous bone proteins showed increased levels of expression at later time-points after estrogen, suggesting these are deposited within the mineralised matrix as part of a later maturation process. Although the precise role of these proteins remains unclear, evidence from knockout animals suggest that they play an important role in regulation of subsequent bone remodelling [Ducy et al., 1996; Rittling et al., 1998; Delany et al., 2000], possibly by directing responses to immune and inflammatory processes [Denhardt and Noda, 1998].

It has previously been suggested that estrogen's tendency to stimulate bone formation in mice, which is well recognised [Urist et al., 1950; Bain et al., 1993], represents a unique response with no counterpart in other mammals [Turner, 1999]. Although estrogen is recognised to increase bone mass in postmenopausal women [Stevenson et al., 1990], this action has been attributed to suppression of bone resorption rather than stimulation of bone formation [Christiansen et al., 1982]. However, reports that estrogen enhances osteoblast function in postmenopausal women [Vedi et al., 1999; Khastgir et al., 2001] and female rats [Takano-Yamamoto and Rodan, 1990; Chow et al., 1992] suggest that estrogen stimulates bone formation in other species apart from the mouse. Nevertheless, there may be important differences between effects of estrogen on bone formation in mice as compared to other species. For example, while estrogen induces the formation of new sites of cancellous bone in mouse long bones [Samuels et al., 1999], in other species, this hormone may predominantly act to suppress osteoblast apoptosis [Tomkinson et al., 1997, 1998].

Despite these reservations, cancellous bone formed in response to high-dose estrogen in female mice has a normal morphological appearance, and consists of lamellar bone as assessed by polarised light microscopy (our unpublished observations). Hence, this response is likely to prove an accurate model for studying the changes in gene expression which accompany bone formation in long bones in vivo. Since these studies were performed in growing animals, and as estrogen is also known to suppress bone resorption [Wronski et al., 1988], part of the gain in cancellous bone which occurred may also have been due to suppression of resorption of newly-formed primary spongiosa. Nevertheless, our previous histomorphometric analysis demonstrated that formation of new cancellous bone accounts for the great majority of the gain in bone observed under these experimental conditions [Samuels et al., 1999].

In summary, we characterised the sequence of expression of genes for a range of osteoblast-derived proteins, during the rapid formation of cancellous bone within mouse femurs following

estrogen administration. DXA and histological analysis suggested that new cancellous bone is formed within 16 days of commencing estrogen. This response was preceded by increased expression of ALP and COL I, which had risen three-fold by Days 8 and 12 respectively. In contrast, the non-collagenous bone proteins osteocalcin, osteopontin, and osteonectin showed little change over the first 16 days after commencing estrogen, although modest changes at subsequent time-points were observed. The lack of change in expression of these proteins by Day 16, despite evidence that estrogen had already induced new bone formation by this time, supports previous studies which indicate that osteocalcin, osteopontin, and osteonectin are not directly involved in mineralised matrix formation by osteoblasts.cc

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