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Estrogen Stimulates Differentiation of Megakaryocytes and Modulates Their Expression of Estrogen Receptors α and β

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Abstract Estrogen has multifunctional effects influencing growth, differentiation, and function in many tissues. High-dose estrogen has been shown to produce anabolic skeletal effects in the skeleton of postmenopausal women with increased megakaryocyte (MK) population in the bone marrow, suggesting a possible role for these cells in bone remodelling. To investigate if estrogen stimulates megakaryocytopoiesis and affects on estrogen receptor (ER) expression, CD34(+) cells were cultured for 6, 9, and 14 days plus or minus low-dose or high-dose 17β estradiol (E). Cells were immunolocalised for CD61, CD41, ERα and β. ER mRNA expression was assessed by RT-PCR. Cells formed more CD61 positive MK colonies with low- and high-dose E treatment ($P < 0.001$) at 6 and 9 days. CD41 expression was increased dose-dependently in MK (3- and 5-fold $P < 0.001$) at 9 days. E-stimulated ERα expression at 6 days ($P < 0.001$) whilst ERβ was dose-dependently increased only at 9 days ($P < 0.01$). ERα mRNA was increased at 6 days but not at 14 days whilst ERβ mRNA expression was only increased at 14 days with E treatment. These results demonstrate that E stimulates the colony forming potential of CD34(+) cells to a more megakaryocytic phenotype in vitro. This finding together with the stimulation of ER protein and mRNA expression adds to the increasing evidence for a role for MKs in estrogen-induced bone formation. J. Cell. Biochem. 92: 249–257, 2004.

Key words: megakaryocytopoiesis; immunocytochemistry; ERα; ERβ; bone remodelling; estradiol; CD34(+); CD61; CD41

Estrogen has multifunctional effects influencing growth, differentiation, and function in many tissues [Turner et al., 1994; Tsai et al., 1998]. It is essential in skeletal development and the maintenance of bone health in both men and women [Harris et al., 1996]. Estrogens diffuse in and out of cells, but are retained in target cell nuclei by the estrogen receptor (ER) protein. Once bound by estrogens, the ER undergoes conformational change, allowing the receptor to interact with chromatin and to modulate the expression of target genes [Brzozowski et al., 1997].

Within the bone marrow compartment, stem cells differentiate along mesenchymal and haematopoietic lineages, regulated by local and systemic factors. Megakaryocyte (MK) progenitors produce numerous colony-forming units (CFU-MK) that differentiate and undergo endomitosis with the generation of large cells with lobulated nuclei. During the final stages of maturation, a complex demarcation system is developed, culminating in the release of platelets into the circulation [Hoffman et al., 1987; Ellis et al., 1995]. Recent evidence shows that in addition to platelet release, MKs express a number of factors, some of which are involved in the regulation of bone remodelling [Kelm et al., 1992; Thiede et al., 1994; House et al., 1997; Genever et al., 1999]. We have recently reported an increase in the MK population in the bone marrow of postmenopausal women

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treated with estrogen [Bord et al., 2000]. In addition, we have shown increased expression of TGFβ and its receptors in these MKs [Bord et al., 2001a] leading to the hypothesis that MKs may play a role in mediating the E-induced effects seen in bone. Thus, the aim of this investigation was to determine the effect of estrogen on MK differentiation and the expression of ER protein and mRNA by these cells.

MATERIALS AND METHODS

Cell Culture

CD34(+) cells were isolated from human cord blood by magnetic bead technology (MACS). Umbilical cord blood was obtained from natural and caesarean deliveries from 0 to 19 h prior to CD34(+) cell selection. Cord blood was anticoagulated with sodium heparin in PBS, centrifuged at 800*g* (10 min), the plasma removed and discarded, and red cells removed by lysis in 5–10 volumes of ammonium chloride red cell lysis buffer (incubated 10 min at room temperature). Cells were centrifuged as previously, and washed once in PBS. The remaining cells were selected for CD34(+) using the MACS system (Miltenyi Biotec, Bisley, Surrey, UK) according to the manufacturer's instruction. Briefly, cells were counted, suspended in MAC buffer, incubated in MAC blocking reagent and MAC CD34 antibody (clone QBEND/10, isotype mouse IgG, 15 min at 4°C). Following washing and centrifugation cells were resuspended in MACS buffer and microbeads conjugated to an anti-hapten antibody and passed through a positive selection column surrounded by a magnetic field. Finally, CD34(+) cells were collected by eluting from the column and cultured for protein and mRNA determinations.

Immunocytochemistry

To determine the effects of estrogen on MK differentiation and their expression of ERs, cells were cultured for 6, 9 and 14 days in Lab-Tek chamber slides (Nalge Nunc International Rochester, NY) containing a serum-free collagen-based system supplemented with thrombopoietin, interleukin-3 and inteleukin-6 (MegaCult, StemCell Techology, Vancouver, BC). Cultures were treated with a physiological dose 10^{-10} M (low-dose) or a supra-physiological dose 10^{-8} M (high-dose) 17β estradiol (E) (Sigma, Poole, Dorset, UK) or carrier (cyclodextrin, Sigma) as untreated controls. At the end of the incubation period, chamber surrounds were removed and the collagen films dehydrated using nylon membranes and filters (StemCell Technologies). The collagen films/cells were fixed in acetone/methanol (3:1) for 30 min prior to air-drying. After hydrating the films in PBS for 10 min they were immunostained using an indirect immunoperoxidase method as described previously [Bord et al., 2001a]. Briefly, following blocking steps and washes primary antibodies to CD41 (1 in 150) and CD61 (1 in 500) (monoclonal antibodies, Dako, Ely, Cambs, UK) ERα (1.0 μg/ml, rabbit polyclonal HC-20 mapping to the carboxy-terminus of ERα and shown not to cross-react with ERβ), and ERβ (1.0 μg/ml, rabbit polyclonal H-150 raised against a recombinant protein corresponding to amino acids 1–150 mapping at the amino-terminus of human ERβ and shown to be non-cross-reactive with ERα; ER antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA) were applied and incubated overnight at 4°C. A biotinylated secondary antibody (horse anti-rabbit anti-mouse, Vector Laboratories, Burlingame, CA, at 3.5 μg/ml) was added and sites of antigenicity amplified by avidin–biotin complex (ABC) Elite substrate (Vector Laboratories). Signal was detected using DAB (Vector Laboratories). Cells were lightly counterstained with Gills Haematoxylin (1:50, 45 sec, Sigma) and air-dried. Specificity of antibody reaction was confirmed by substituting the primary antibody with non-immune serum at the same IgG concentration, and omission of primary and secondary antibodies. To standardise staining and measurements, all slides in each experiment were immunolocalised at the same time at precisely timed intervals. Image analysis was used to quantify protein expression. All slides for each antibody were measured at the same time with the same threshold parameters. Thus, for each antibody, it was possible to detect protein expression changes induced by E.

Some cultures were maintained for 14 days and stained using GpIIb/IIIa (CD41) by the immuno-alkaline phosphatase procedure (APAAP) with a standard staining kit (StemCell Technologies) according to the manufacturer's instructions to detect mature MKs.

Quantitation of Immunolocalisation

Cells were examined by light microscopy with a Nikon E-800 fitted with a Basler digital

camera. Intensity and extent of staining were measured using Lucia G image analysis. To quantify protein expression for each antibody used, thresholds on the image analysis programme were set to detect and measure only positive staining. Five fields of view were examined for each antibody for each experiment. Experiments were repeated three times. Results are shown as the mean of the experiments (±SD).

RNA Isolation, Determination, and Relative Quantification of Gene Expression

To determine ER mRNA expression by both early and mature MKs in response to E, isolated CD34(+) cells (as above) were cultured in IMDM liquid (Gibco BRL, Invitrogen, Paisley, UK) supplemented with 5% human serum (Labtech International, Lewes, East Sussex, UK) and thrombopoietin (10 ng/ml) plus or minus high-dose E for 6 and 14 days. Poly(A) RNA was extracted from cultured cells using the MicroPoly(A)Pure^{TM} (Ambion, Huntingdon, UK) according to the manufacturer's instructions.

PCR products for ERα, ERβ, and GAPDH were made by RT-PCR with primer sequences shown in Table I. PCR products were cloned into the T-tailed vector PCR ll-TOPO (Invitrogen) which has opposing SP6 and T7 RNA polymerase promotor sites. BamHl restriction sites were added to forward primers so that the orientation of each insert could be determined by cutting the plasmids with BamHl. Plasmids containing inserts suitable for transcription with SP6 RNA polymerase were selected and cut with Not1. RNA transcripts were purified using Microspin S-300 columns (Amersham Biosciences UK, Little Chalfont) after treatment with DNase to remove plasmid DNA.

Levels of mRNA expression were determined using one-step RT-PCR reagents (Applied Biosystems, Foster City, CA) in a Gene Amp 5700 SDS with Primer Express^{TM} software used to design primers and probes. Primer pairs were chosen to include introns in the gene sequence and probes to span intron-exon boundaries (Table II). A standard curve was included in each assay so that the overall efficiency of the assay could be calculated. mRNA levels were quantified using the comparative threshold-cycle (C_T) method [Fink et al., 1998] which assumes that the efficiencies of the PCR reactions are close to 1, as described previously [Ireland et al., 2002].

Four replicates were performed for each experimental point and experiments repeated three times. The results in each group are expressed as the mean relative change in the E-treated cells compared to untreated cells (±SD).

Stastistical Analysis

Stastistical analysis for protein and mRNA data was performed using the approximate test for unequal variance based on the *t*-distribution [Armitage and Berry, 1994].

RESULTS

Cord blood was used to isolate CD34(+) cells, with the positive magnetic bead sorting resulting in a 60–70% purity of CD34(+) cells.

The three-dimensional semi-solid collagen matrix allowed the proliferation of single haematopoietic progenitors to form distinct colonies containing recognisable progeny. Within some colonies large MKs with lobulated nuclei could be detected. Immunolocalisation demonstrated that the CD34(+) cells formed distinct colonies of the MK lineage which were increased in cultures treated with E.

Protein Expression

To determine the effect of E on early megakaryocytopoiesis cultures were immunolocalised for CD61, an early marker of MKs and CD41, a specific MK antigen, at 6 and 9 days using an immunoperoxidase method. Cells

TABLE I. Sequences of Cloning Primers

	Cloning primers	
Gene	Forward primer	Reverse primer
GAPDH	TGAAGGTCGGAGTCAACGGATTTG	GTTGGTGGTGCAGGAGGCATTGCT
ERα	AATTCAGATAATCGACGCCAG	GTGTTTCAACATTCTCCCTCCTC
ERβ	TAGTGGTCCATCGCCAGTTAT	GGGAGCCACACTTCACCAT

TABLE II. GeneAmp 5700 SDS Primers and Probes

Gene	Forward primer	Reverse primer	Probe
GAPDH	TTTTAACTCTGGTAAAGTGGATATTGTTG	TGACGGTGCCATGGAATTT	ATTGACCTCAACTACATGGTTTACATGTTCCAATATG
ERα	TGCTTCAGGCTACCATTATGGA	GTTTTTATCAATGGTGCACTCGTA	ACACATATAGTCGTTATGTCCTTGAATACTTCTCTTGAAGAA
ERβ	TCAAAAGAGTCCCTGGTGTGAAG	CTCTTTGAACCTGGACCAGTAACAG	CGGTTCCCACTAACCTTCCTTTTCAGTGTCTCT

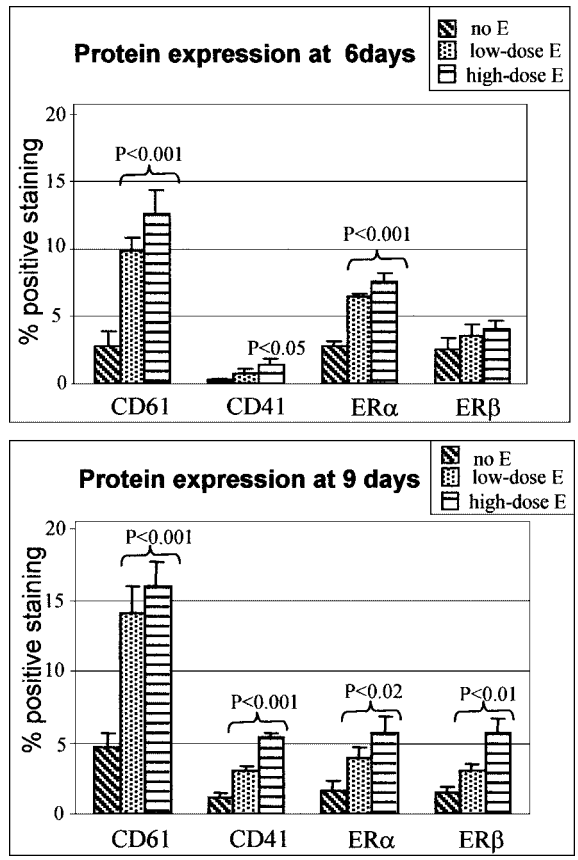

Fig. 1. Protein expression: cells were cultured in the presence of vehicle, low-, and high-dose E. After 6 and 9 days culture cells were immunolocalised for CD61, CD41, and ERs. Positive protein expression was measured by image analysis and shown as a percentage of the field of view with positive staining. Results show mean of three experiments ± SD. *P* values are given for E-treated cultures compared to untreated cultures.

cultured in the presence of both low- and high-dose E formed more MKs than untreated cells. At 6 days, MKs were highly CD61 positive with a 3.04- and 3.48-fold ($P < 0.001$) increase in expression in low- and high-dose E, respectively compared to controls (Fig. 1). Greater increases were seen at 9 days with 3.5- and 4.5-fold induction in low- and high-dose E-treated cells ($P < 0.001$) compared to untreated cultures (Figs. 1 and 2a,b). At 6 days, very few untreated cells expressed CD41. E induced a small increase with high-dose E treatment ($P < 0.05$) (Fig. 1). After 9 days culture there was a dose-dependent increase in CD41 expression with E treatment ($P < 0.01$) (Fig. 1).

At both 6 and 9 days E-stimulated ER expression. ERα expression was mainly confined to MK colonies with very little staining

seen in other cells. The greatest effect of E on ERα expression was seen at 6 days with a highly significant 2.29- and 2.72-fold increase in expression with low- and high-dose, respectively ($P < 0.001$). At 9 days, no further increase was seen ($P < 0.02$) (Figs. 1 and 2c,d). Low-level ERβ expression was seen in many CD34(+) cells. This was increased with E treatment with intense nuclear staining seen in the MKs. In contrast to ERα expression, the only significant increase in ERβ was seen at 9 days with a dose-dependent increase in expression (2.2- and 4.0-fold induction with low- and high-dose E, $P < 0.01$). At the earlier time point there was no significant changes from basal levels (Figs. 1 and 2e,f). All negative controls showed absence of staining (Fig. 2g,h).

To determine the long-term effect of E on MK colony formation, some cultures were maintained for 14 days and immunolocalised using an APAAP method. The number of positive staining MK colonies increased in the presence of high-dose E compared to untreated controls (Fig. 2i,j). There was considerable variability in MK colony number between cord blood collections (1.16, 12.26, 2.24% area occupied by MK colonies in untreated cultures to 3.88, 19.78, 6.26% area, respectively occupied by MK colonies in E-treated cultures) possibly due to the variations in waiting time between cord blood collection and CD34(+) selection. However, the percentage increase in all cases was similar, with E eliciting a 2.44-fold (mean ±0.45, $P < 0.02$) induction of MKs. The MK colonies were larger and stained more intensely in the E-treated groups. All cultures showed some negative stained colonies denoting their non-MK lineage.

The developing MK colonies, as demonstrated by increasing CD61 expression, showed

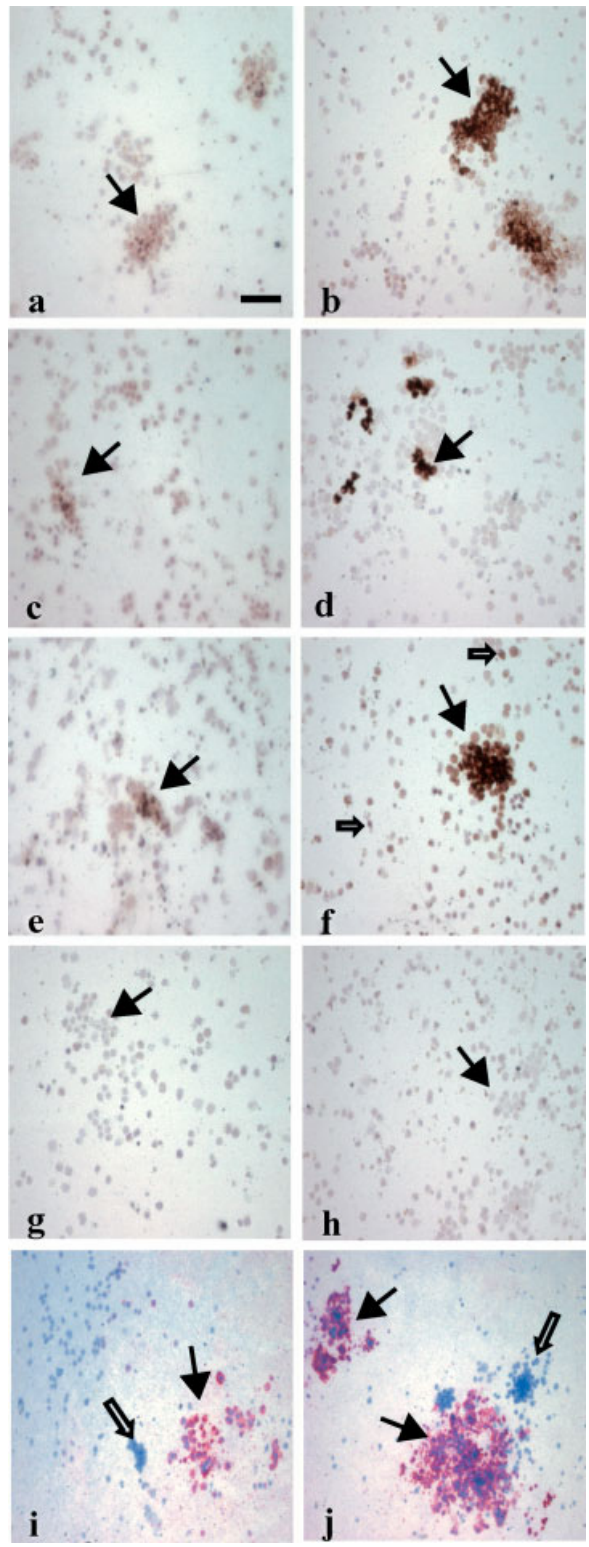

Fig. 2. Megakaryocytes (MKs) generated from CD34(+) cells derived from human cord blood and cultured for 9 days in serum-free collagen based system ± high-dose E. **a**–**h**: These were immunolocalised in an indirect peroxidase system with positive expression indicated by the brown colour reaction of DAB. a,c,e,g show cells cultured with no E. b,d,f,g, show cells cultured with high-dose E. Bar = 200 μm. a, b: localisation of CD61 shows increased positive staining of MKs colonies (arrows) in E-treated cultures. c, d: localisation of ERα with increased expression in MK colonies (arrows) in the E-treated cultures. e, f: localisation of ERβ shows increased expression in MK colonies (arrows) and some surrounding cells (open arrows) in the E-treated cultures. g, h: Rabbit IgG negative control shows absence of staining in all cells including CFUs (arrows). **i**, **j**: Cells were cultured for 14 days and immunolocalised for CD41 to detect positive staining MK colonies (arrows). Increased colonies were seen in the E-treated cultures. Colonies of non-MK lineage are denoted by the blue staining (open arrows).

increased ERα and ERβ expression. To test that these MK colonies were responsible for the ERα and ERβ expression some cultures were co-localised with CD61 and ERα or CD61 and ERβ. The majority of CD61(+) staining CFUs in the 9 day E-treated cultures co-localised with ERα (78 ± 7%) and ERβ (83 ± 6%).

mRNA Expression

ER mRNA expression was quantified after 6 and 14 days in liquid culture ± high-dose E to determine the effects on immature and mature MKs. Generally, in the untreated cultures ERβ was more highly expressed than ERα at both 6 and 14 days (Ct values: ERβ 32.5 at 6 day and 32.8 at 14 day compared to ERα 35.8 and 34.1 at 6 and 14 day, respectively). In the E-treated cultures ERα was more highly expressed at 6 day than 14 day (Ct values, 33.4 and 34.8), whilst ERβ levels increased between day 6 and 14 (Ct values, 33.3 and 31.9). Results were normalised to GAPDH. Untreated controls were given a value of 1 and the E-treated cultures expressed as a relative ratio. At 6 days, there was a trend towards suppression of ERβ gene expression in the E-treated cultures compared to controls, although this failed to reach statistical significance. ERα mRNA showed an increase in all E cultures at 6 days but, there was considerable variability between samples, and these results only just reached statistical significance ($P < 0.05$) (Fig. 3). At 14 day, there was no difference in ERα expression between treated and untreated cells. In contrast, at 14 day ERβ expression was significantly increased ($P < 0.01$) by E (Fig. 3).

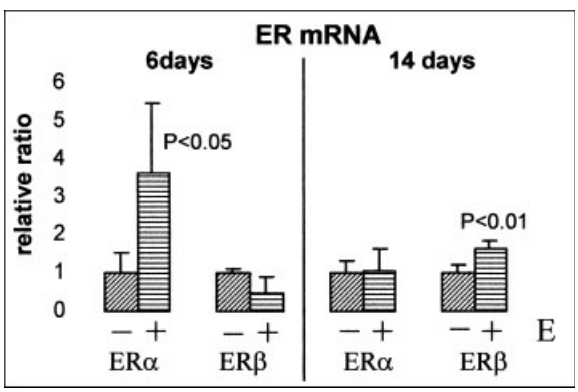

Fig. 3. CD34(+) cells were cultured ± E for 6 and 14 days. mRNA levels of ERs were determined by RT-PCR. Results were normalised to GAPDH. Controls have been given a value of 1 and the E-treated cultures a relative ratio. Results show the mean of three experiments ± SD.

In addition, CD34(+) cells were cultured in liquid medium for 6 days ± high-dose E, after which time MKs were isolated by CD61 positivity by magnetic bead sorting. RNA was extracted from the CD61(+) MKs and analysed for ERα and ERβ mRNA expression. E was shown to induce a 3.2 (±1.05, $P < 0.02$)-fold increase in ERα and a 1.8 (±0.9, ns)-fold increase in ERβ mRNA expression.

DISCUSSION

This in vitro study, demonstrates that E promotes proliferation, differentiation, and maturation of MKs and elicits changes in their cellular protein and mRNA expression. MKs, which are among the rarest of haematopoietic cells, serve the essential function of producing platelets. However, evidence is accumulating that MKs may also have other roles. It has been reported that MKs express calcium-sensing receptors [House et al., 1997], *N*-methyl-D-aspartate (NMDA)-type glutamate receptors [Genever et al., 1999] and synthesise the bone matrix proteins osteonectin [Kelm et al., 1992] and osteocalcin [Thiede et al., 1994]. Thrombopoietin, the major regulator of megakaryocytopoiesis [Thiede et al., 1996; Yan et al., 1996], has been shown to inhibit osteoclastogenesis [Wakikawa et al., 1997] and it seems possible that this reported inverse relationship between MK and osteoclast number may be mediated through alterations in the inter-medullary synthesis of MK-derived growth factors and cytokines. Normal human CD34(+) cells, MK progenitors, and MKs synthesise and secrete numerous regulatory molecules that form the basis of intercellular cross-talk networks and regulate, in an autocrine and/or paracrine manner, the various stages of normal human haematopoiesis [Majka et al., 2001]. These factors also have effects on stromal cells derived from mesenchymal stem cells (MSCs) or MSCs themselves. Evidence suggests that human MSCs may affect MK development by their production of thrombopoietin, a primary regulator of megakaryocytopoiesis, and their proximity to MKs in the bone marrow microenvironment [Cheng et al., 2000].

We have previously reported an increase in MK number in the bone marrow of postmenopausal women treated with 2 years conventional HRT compared with pretreatment values. A further increase was shown in a group

of women treated with high doses of estradiol [Bord et al., 2000]. As there was also evidence of anabolic skeletal effects [Vedi et al., 1999] we hypothesise that MKs may play a role in regulating these E-induced effects. Thus, this study was designed to identify the effects of E on MK differentiation and to investigate if their ER expression would be up-regulated by E and, thereby support our hypothesis. Studies in mice by Samuels et al. [1998, 1999] demonstrated that high-dose E-induced de novo medullary bone formation, with a rapid increase in cancellous bone volume and new bone formation in the medullary cavity. They also showed an increase in MK number after 4 days of high doses of E. Another study by the same group [Perry et al., 2000] reported a transient increase in MK number in E-treated mice, with an initial increase of 50% at 2 days followed by a gradual decline of 75% by day 12. The first two of these reports are consistent with our findings that E stimulates MK formation, differentiation, and maturation. However, results of Perry et al., differ from those of our study and also to a previous in vivo study in which MK number was increased in women treated with E for many years [Bord et al., 2000]. The reasons for this disparity are not known but may relate to species differences or different time points at which numbers of MK were evaluated. In addition, very high doses of E used in the mouse studies may be relevant.

Although in this particular study, we did not have information on the circulating platelet numbers a similar cohort of postmenopausal women treated with long-term high-dose E all had platelet counts within the normal range. This is consistent with other studies in which there were no differences in platelet counts between pre and postmenopausal women [Carter et al., 1991] nor between postmenopausal HRT treated and untreated women [Ranganath et al., 1996]. This suggests that there may be feedback mechanisms that control platelet levels or, alternatively, that different types of MKs may have different functions.

We have previously demonstrated the in vivo protein expression of TGFβs and TGFβRs by MKs and the up-regulation of TGβ2, TFβRl, and TGFβRll by high-dose E [Bord et al., 2002]. As TGFβ is a known potent mitogen of osteoblasts [Zheng et al., 1992] and inhibits bone resorption by mediating the induction of osteoclast apoptosis [Hughes and Boyce, 1998], the E-induced up-regulation of MK-derived TGFβ may contribute to the skeletal effects E.

Tarantino et al. [1994] first presented evidence of the presence ERs in MK in 1994. At that time only the α subtype had been identified. Subsequently both ERα and β have been demonstrated in MKs: we have reported expression of both ERα and ERβ protein in MKs in samples of human adult iliac crest biopsies and neonatal rib bone specimens [Braidman et al., 2001; Bord et al., 2001b]. Braidman et al. [2001] demonstrated ERβ protein expression in MKs in normal and fracture callus bone. Our findings of changes in ER protein and gene expression with E treatment indicates that these skeletal effects could be mediated, at least in part, by the ERs. It has been previously shown in osteoblasts that an increase in ERβ/ERα ratios prevents ligand-dependent down-regulation of ERα transcription, resulting in reduced osteoblast proliferation and increased maturation [Ireland et al., 2002]. Our results demonstrate that E induced an early increase in MK ERα protein expression. This is consistent with reports in other cell types of increasing ERα during proliferation [Ireland et al., 2002]. CD61, an early MK marker, was dose-dependently increased in the E-treated cells at 6 days. After 9 days culture CD61 expression levels in both E-treated groups were similar whilst CD41, a late MK marker, showed a dose-dependent increase. Also at 9 days ERβ was more highly expressed than ERα, consistent with a more differentiated cell type. By 14 days the CD34(+) cells had generated many mature MK colonies, which increased more than 2-fold with E treatment.Then, 6 and 9 day time-points were chosen to establish the effects of E on ER mRNA expression levels in differentiating and mature MKs. ERα mRNA was increased at 6 day but not at 14, whilst ERβ was suppressed at 6 day and significantly increased at 14 day. This is consistent with the protein data, again indicating a switch in ERα/ERβ ratios with differentiation. A recent study showed only the presence of ERβ mRNA in mature MK and platelets, ERα not being detected [Khetawat et al., 2000] supporting our findings that ERα is the dominant form in proliferating cells whilst ERβ is more highly expressed in mature cells.

In summary, we have shown that E stimulates differentiation and maturation of MK progenitor cells and modulates their ER protein

and mRNA expression. These results support our original in vivo findings and support our hypothesis that MK may play a role in bone remodelling.

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