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Estrogen-Induced Osteogenesis in Mice Is Associated With the Appearance of Cbfa1-Expressing Bone Marrow Cells

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Abstract Cbfa1 is a transcription factor recognised as being involved in early osteoblast differentiation during embryonic skeletogenesis. To determine whether Cbfa1 plays a similar role in bone formation in the adult, we analysed whether its expression is altered during estrogen-induced osteogenesis, following our recent studies which suggest that this response involves the generation of early osteoblast precursors within bone marrow. To facilitate identification of Cbfa1-expressing cells, these studies were performed in mice heterozygous for a cbfa1 gene deletion (cbfa1$^{+/-}$) using β-galactosidase (lacZ) as a genetic marker. Cbfa1-expressing cells were identified by lacZ staining of longitudinal sections of the proximal tibial metaphysis. Treatment of cbfa1$^{+/-}$ mice with 17β-estradiol 0.5 mg/week for 24 days led to the appearance of new cancellous bone surfaces. This response was associated with a marked increase in number of Cbfa1-expressing cells within the metaphysis, consisting not only of osteoblasts on bone surfaces but also of cells within the adjacent bone marrow. We subsequently enumerated Cbfa1-expressing cells at earlier time-points following estrogen, in sections co-stained for ALP activity. After 4 days of estrogen treatment, a population of cells appeared within the marrow cavity which expressed Cbfa1, but were negative for ALP. At later time-points, large numbers of Cbfa1 + bone marrow cells were still present, but the majority of these were close to new trabecular bone surfaces at sites which showed high levels of ALP activity. An equivalent distribution of Cbfa1-expressing cells was observed in further studies where Cbfa1 expression was analysed in wild-type mice by immunohistochemistry. We conclude that estrogen-induced osteogenesis is associated with the appearance of a population of Cbfa1-expressing cells within bone marrow, which we hypothesize to represent the osteoblast precursor population responsible for subsequent new bone formation. J. Cell. Biochem. 84: 285–294, 2002.

Key words: estrogen; mice; Cbfa1; osteogenesis; bone

An adequate supply of osteoblasts is required for several processes on which skeletal integrity depends, including embryonic skeletogenesis, prepubescent bone growth and maintenance of bone mass in adulthood. Several factors are thought to play important roles in osteoblast differentiation [Yamaguchi et al., 2000], during which multipotent bone marrow stromal stem cells form mature osteoblasts via as yet poorly defined intermediary stages [Bianco and Robey, 2000]. The transcription factor Cbfa1 is essential for osteoblast differentiation during embryonic skeletogenesis, based on observations that mice which are homozygous for a cbfa1 gene deletion do not form osteoblasts in utero [Komori et al., 1997; Otto et al., 1997]. Cbfa1, which belongs to the *Drosophila* runt domain gene family [Ogawa et al., 1993], induces a range of genes associated with osteoblast maturation [Yamaguchi et al., 2000], and is itself induced by osteogenic growth factors such as bone morphogenetic proteins [Yamaguchi et al., 2000].

The observation that over-expression of a dominant negative Cbfa1 DNA-binding construct suppressed bone formation in adult mice, while having no effect on osteoblast number [Ducy et al., 1999], suggests that Cbfa1 also controls the function of mature osteoblasts. Whether Cbfa1 regulates osteoblast differentiation in the adult is less clear. In a recent study of

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stromal cell lines generated from bone marrow of adult mice, osteogenic capacity and Cbfa1 expression were found to be poorly correlated [Satomura et al., 2000]. Based on these observations, it was suggested that Cbfa1 expression by marrow stromal cells in the adult reflects osteogenic imprinting as a consequence of their embryological origin, rather than a regulatory step in the generation of new osteoblasts.

It is well-recognised that high-dose estrogen induces a marked osteogenic response in long bones of female mice [Urist et al., 1950; Bain et al., 1993]. In a recent time-course study, we found that this action involves the generation of new sites of cancellous bone formation within the marrow cavity [Samuels et al., 1999]. This response also coincides with a marked increase in number of early osteoblast precursors within the marrow cavity, as assessed by counting alkaline phosphatase (ALP) positive fibroblastic colony forming units (CFU-f) in ex-vivo adherent bone marrow cultures [Perry et al., 2000]. Taken together, these findings suggest that estrogen induces osteogenesis in female mice at least in part by stimulating the generation of osteoblast precursors from marrow stromal stem cells.

Based on our observations which suggest that estrogen-induced osteogenesis provides a useful in vivo model for studying the mechanisms which regulate early stages of osteoblast commitment and differentiation in the adult, we used this approach to study the role of Cbfa1 in this process. In particular, we wished to determine whether estrogen-induced osteogenesis is associated with up-regulation of Cbfa1 expression in bone, and if so, whether this simply reflects increased numbers of osteoblasts, or whether there is also evidence that Cbfa1-expressing osteoblast precursors are generated within the marrow cavity as part of this response. To facilitate analysis of Cbfa1 expression, these studies utilised mice which were heterozygous for a cbfa1 gene deletion ($cbfa1^{+/-}$) in which β-galactosidase (lacZ) has been used as a genetic marker [Otto et al., 1997].

MATERIALS AND METHODS

Animals

A breeding colony of $cbfa1^{+/-}$ mice bred into a C57 Black/6 background (C57BL/6) was established in Bristol, heterozygotes being distinguished from wild-types at 6 weeks of age by the presence of lacZ activity in tail vertebrae. The latter were fixed in 0.2% glutaraldehyde, and then incubated in a lacZ substrate (X-Gal; Sigma, Poole, Dorset, UK). In subsequent experiments, 10-week-old female $Cbfa1^{+/-}$ or wild-type mice were administered 17β-estradiol 500 μg/animal (E_2; Sigma) or vehicle (Sigma) by weekly sc injection for up to 24 days. Throughout, 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 Laboratory Animals."

Histomorphometry

Histomorphometry was performed on longitudinal tibial sections obtained from $cbfa1^{+/-}$ mice, 24 days after commencing weekly estrogen and compared with results from untreated and wild-type controls. Tibiae were freed from soft tissue, ethanol fixed, dehydrated through a series of graded alcohols, and embedded undecalcified in LR White Hard Grade (London Resin Company, Reading, UK). Seven micrometer longitudinal sections of the proximal tibial metaphysis were then cut on a Reichert-Jung 2050 microtome with a 'd' profile tungsten carbide knife, stained in 1% toluidine blue, and mounted in DPX (BDH). Histomorphometric analysis of the proximal tibial metaphysis was subsequently performed using transmitted and epifluorescent microscopy linked to a computer assisted image analyser (Osteomeasure, Osteometrics, Atlanta, Georgia). All sections were examined blind. The sampling site consisted of a standard area of 0.36 mm^2, the proximal border of which was situated 0.3 mm below the growth plate to exclude primary spongiosa [Samuels et al., 1999]. Cancellous bone parameters were measured at 200 × magnification. Cancellous bone volume was assessed on two non-consecutive sections stained with toluidine blue for each animal, and expressed as a percentage of total tissue volume (BV/TV).

Localisation of β-Galactosidase Activity in Tissue Sections

To analyse Cbfa1 expression in $cbfa1^{+/-}$ mice, cells were identified which express the lacZ marker in longitudinal tibial sections, indicating activation of the cbfa1 gene promotor. Immediately following sacrifice, hindlimbs were removed with surrounding soft tissue

intact. The limb was coated in Tissue-tek (Sakura Finetek Europe, Netherlands), frozen by immersion in isopentane cooled in dry ice, and 5 μm sagittal sections subsequently cut in a Bright OF/AS cryostat at −20°C using a 'd' profile tungsten steel knife. Air-dried sections were then glutaraldehyde-fixed, and incubated overnight with X-gal prepared as above. Slides were subsequently washed, counterstained with Nuclear Fast Red (Vector Laboratories), dehydrated through graded alcohols, cleared in xylene, and mounted in DPX.

Co-Localisation of β-Galactosidase and Alkaline Phosphatase Activity

We then analysed whether Cbfa1-expressing cells identified in this way co-express the osteoblast phenotypic marker, ALP, based on previous studies which indicate that the latter is an early marker of the osteoblast phenotype [Stein et al., 1990; Malaval et al., 1994], and is up-regulated in mouse bone marrow in response to estrogen [Plant and Tobias, 2001]. Experimental methods which combined immuno-labelling for ALP with lacZ staining or immunolabelling for Cbfa1 (see below) were unsuccessful. As an alternative approach, combined lacZ and ALP staining was performed using a leukocyte ALP detection kit (Sigma) to assess ALP activity of tibial sections from $cbfa1^{+/-}$ mice incubated with X-gal substrate as above. ALP staining of bone marrow is not specific for osteoblasts, since ALP is also produced by haemopoietic cells. However, osteoblasts are associated with relatively high ALP activity, and in preliminary studies, we found that estrogen-induced osteogenesis is associated with a marked increase in marrow ALP activity as assessed using this method (data not shown). $Cbfa1^{+/-}$ mice were sacrificed before and 4, 8, and 12 days after E_2 treatment, and fresh frozen longitudinal tibial sections subsequently prepared. Cbfa1-expressing cells within the proximal tibial metaphysis were subsequently counted blind, using a sub-region corresponding to that used for histomorphometry as described above. Results were recorded separately according to whether cells co-expressed ALP.

Cbfa1 Immunohistochemistry

To confirm that estrogen-induced osteogenesis is associated with increased numbers of bone marrow cells which express Cbfa1, we analysed expression of Cbfa1 protein. Wild-type female mice were sacrificed prior to or following treatment with E_2 for 4, 8, or 12 days. Formaldehyde-fixed tibiae were decalcified and paraffin-embedded, after which 6 μm longitudinal sections were cut on a Reichert-Jung 2050 microtome, dewaxed with xylene, rehydrated through graded alcohols, permeabilised in 0.2% triton-X-100 (PBS/TX), and incubated in 1% hydrogen peroxide to quench endogenous peroxidase. Following incubation in blocking solution, sections were incubated in polyclonal rabbit anti-mouse Cbfa1 primary antibody (at 1/500 dilution) kindly provided by G Karsenty [Xiao et al., 1998], followed by peroxidase-conjugated goat anti-rabbit secondary antibody (at 1/200), and biotinylated tyramide amplification reagent with streptavidin-peroxidase (NEN Life Science Products, Hounslow, Essex, UK). Cbfa1 immunoreactivity was detected by incubating with 3,3′-diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories), producing a black precipitate.

Northern and Western Blot Analysis

Female wild-type mice (ten per treatment group) were sacrificed immediately prior to and following 1, 2, 4, 8, 12, and 16 days of E_2 treatment as described above. Femurs were removed immediately following sacrifice, freed from soft tissue, snap-frozen in liquid nitrogen, and subsequently ground into a fine powder. In a parallel experiment, mice (ten per group) were treated for 4 days with E_2 or vehicle and bone marrow aspirated from both femurs using 0.5 ml phosphate buffered saline (PBS). For Northern blot analysis, femurs/marrow were pooled within the same treatment group, transferred to TRIzol (Life Technologies, Paisley, UK), and total RNA subsequently extracted by phase separation. $PolyA^+$ RNA was then isolated using the PolyATtract kit (Promega, Southampton, UK), loaded onto a denaturing agarose gel, and transcripts separated by electrophoresis. Following transfer to a nylon membrane, RNA was hybridised with a Cbfa1 cDNA probe [Ogawa et al., 1993], kindly provided by G. Stein, and the resultant signal visualised by phosphoimager. To provide a loading control, blots were stripped and re-probed for β-actin [Leader et al., 1986]. For Western blot analysis, protein was extracted from pulverised bone tissue, separated by gel electrophoresis, transferred to nitrocellulose membrane, and blocked

overnight with 5% non-fat milk powder. Blots were then incubated with the rabbit anti-mouse Cbfa1 antibody used for immunohistochemistry studies above, or goat anti-human AML3 polyclonal antibody obtained from Calbiochem (Cambridge, MA, USA), diluted to 1/500 in blocking solution. The primary antibody was detected using a Vectastain Elite ABC detection kit.

RESULTS

Results for cancellous bone volume (BV/TV) revealed that estrogen induced a similar osteogenic response in cbfa1$^{+/-}$ mice and C57BL/6 wild-type controls (Fig. 1). The magnitude of this response was similar to that previously observed in CBA-1 mice (10), suggesting this is not influenced by genetic background of animals. The distribution of Cbfa1 expression within the proximal tibial metaphysis was then assessed by examining activity of the lacZ marker gene in sections from un-treated cbfa1$^{+/-}$ mice to identify cells in which the cbfa1 promotor is activated. Despite the relatively poor cellular morphology due to the use of frozen sections, lacZ positive osteoblasts and chondrocytes could be clearly identified by the presence of characteristic blue staining (Fig. 2A–C). A nuclear pattern of staining was observed, which is expected since the lacZ construct used in the generation of the transgene contained a nuclear localisation signal. No nuclear staining was present in sections from wild-type controls (Fig. 2D), however occasional cells with punctate cytoplasmic (rather than nuclear) staining could be seen in both groups demonstrating endogenous lacZ activity (not shown).

Cbfa1$^{+/-}$ mice were subsequently used to analyse the effect of estrogen treatment on Cbfa1 expression within the proximal tibial metaphysis. Estrogen-induced osteogenesis was associated with a marked increase in number of Cbfa1-expressing cells, as assessed by analysing lacZ-stained sections from cbfa1$^{+/-}$ mice 24 days after commencing E2 (Fig. 3A–C). Cbfa1 was expressed by osteoblasts lining existing bone surfaces and forming bridges between trabeculae. In addition, Cbfa1 + cells lacking in specific morphological characteristics were observed within the marrow cavity close to, but distinct, from bone surfaces. We subsequently related temporal changes in Cbfa1 expression following estrogen administration

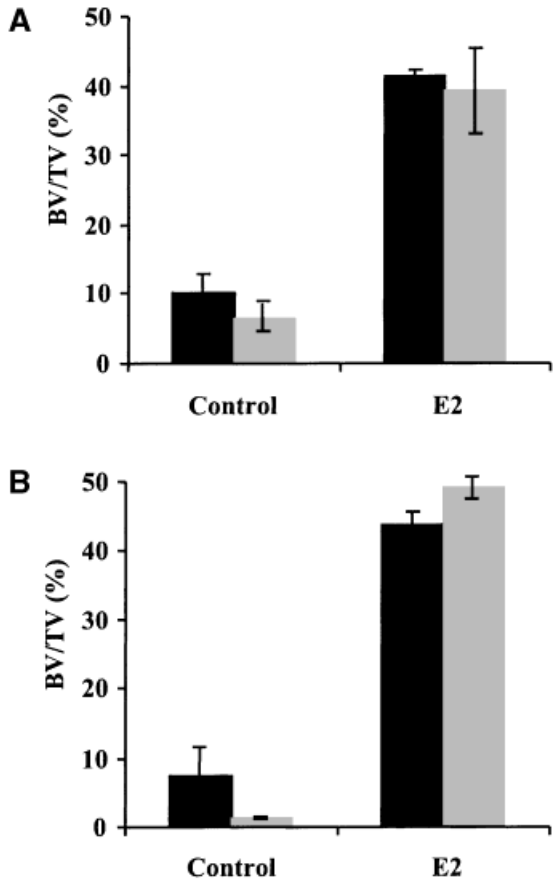

Fig. 1. Effect of treatment with 17β-estradiol on cancellous bone volume (BV/TV) in cbfa1$^{+/-}$ and wild type C57BL/6 mice. Results (mean ± SEM) are shown for wild type (black) and cbfa1$^{+/-}$ (grey) female controls, and age-matched animals treated with 17β-estradiol 0.5 mg/week for 24 days (E_2). BV/TV was analysed at the proximal tibial metaphysis. No significant differences were observed between the two mouse genotypes.

with those in ALP activity. In untreated animals, Cbfa1 expression was confined to osteoblasts adjacent to bone surfaces at sites associated with high levels of ALP expression (Fig. 3D). Analysis of sections from animals 4 days after estrogen administration revealed a marked increase in number of Cbfa1 + cells within the proximal tibial metaphysis (Fig. 3E). These largely consisted of ALP − cells within the marrow cavity located some distance from bone surfaces, lacking in specific morphological characteristics. At later time-points, less Cbfa1 + ALP − cells were observed within the marrow cavity, while in contrast, large numbers of Cbfa1 + ALP + cells were present at ALP-rich sites at cancellous bone surfaces and adjacent regions of bone marrow (Fig. 3F). These

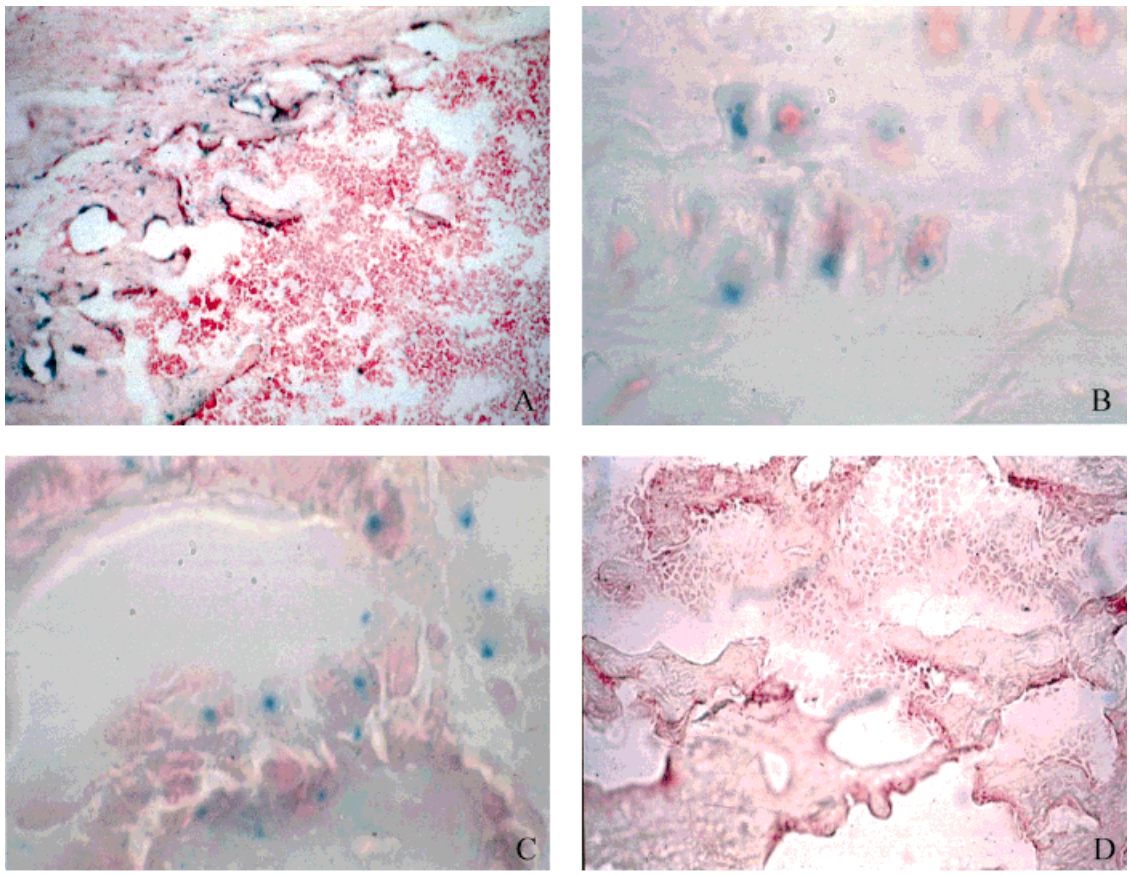

Fig. 2. Distribution of Cbfa1 positive cells in the proximal tibial metaphysis of $cbfa^{+/-}$ adult mice. Cbfa1 positive cells were subsequently identified by blue lacZ staining on frozen longitudinal sections. **A**: Cbfa1 expression is largely restricted to cancellous bone surfaces as assessed under low power (100 ×); (**B**) Cbfa1 expression in hypertrophic chondrocytes (400 ×); (**C**) Cbfa1 expression by osteoblasts (400 ×); (**D**) absence of lacZ activity in a wild-type animal (100 ×).

observations were supported by Cbfa1 + cell counts (Fig. 4).

Having identified cells with an activated Cbfa1 promotor based on analysis of lacZ reporter activity, we studied whether a similar cellular distribution is observed when assessing Cbfa1 protein expression. Immunohistochemistry was performed on decalcified sections of the proximal tibial metaphysis from wild-type mice, using an anti-Cbfa1 polyclonal antibody. In untreated animals, Cbfa1 expression was restricted to chondrocytes and osteoblasts as observed above (Fig. 5A–C). By 4 days after estrogen, a population of Cbfa1 + cells could be seen within the marrow cavity, whose distribution and morphological features were equivalent to the Cbfa1-expressing bone marrow population identified at this time-point by lacZ staining (Fig. 5D). At subsequent time-points, clusters of Cbfa1-expressing bone marrow cells could be observed adjacent to new bone formation surfaces (Fig. 5E). Control sections from estrogen-treated animals where the primary antibody was omitted showed no immunoreactivity (Fig. 5F).

We also assessed skeletal Cbfa1 expression at the level of whole bone, by performing Western and Northern blot analysis on protein and RNA extracts pooled from mouse femurs. We were unable to detect Cbfa1 protein expression using either anti-Cbfa1 antibody (data not shown). This may have been due to relatively low levels of Cbfa1 protein expression, which is consistent with the requirement of amplification to detect Cbfa1 protein by immunocytochemistry. Two distinct Cbfa1 mRNA transcripts of ~6.4 and ~7.5 Kb were identified, the expression of which was unaltered after estrogen (data not shown). We also compared mRNA levels in bone marrow aspirated from femurs before and after

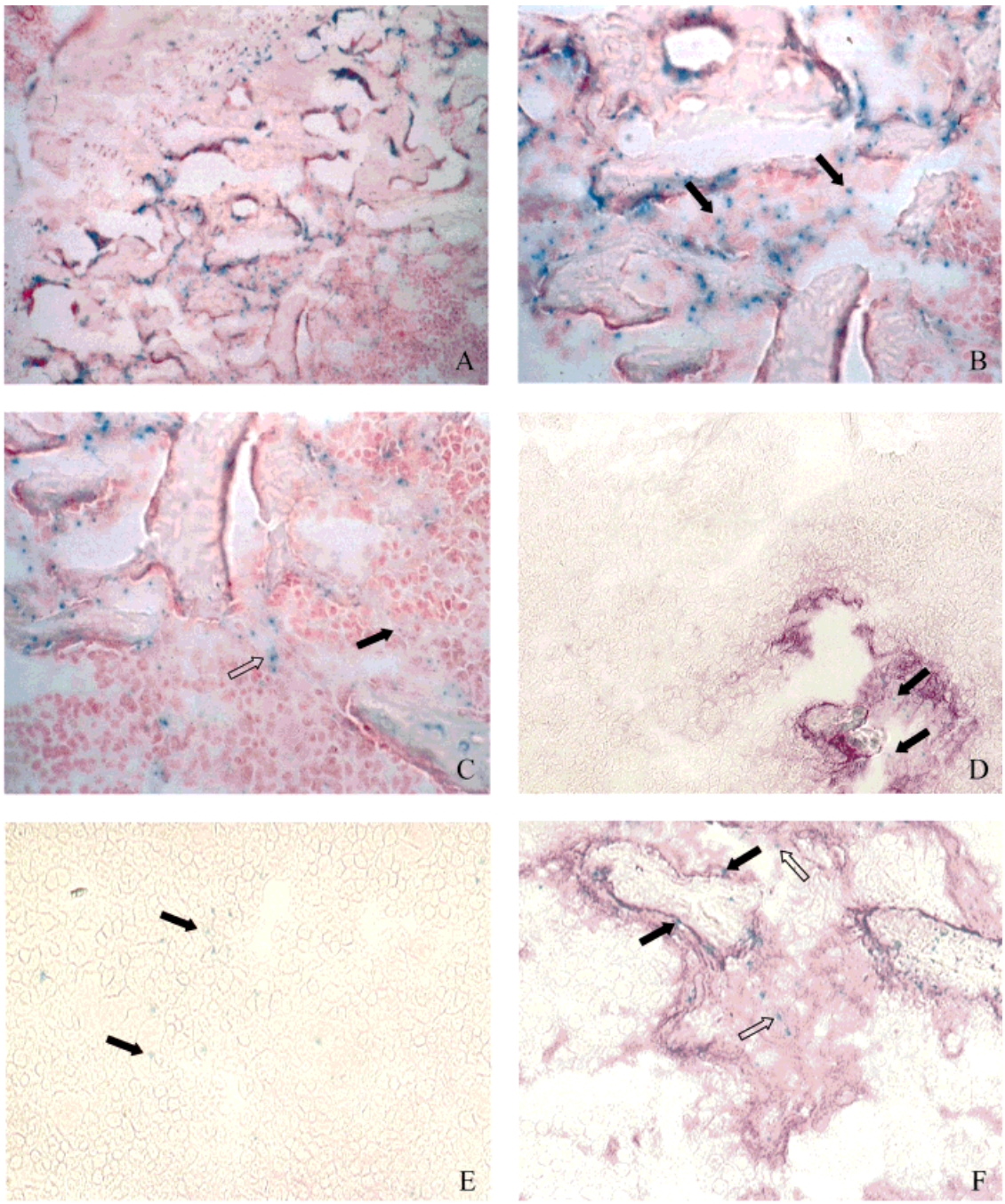

Fig. 3. Effect of treatment with 17β-estradiol (E_2) 0.5 mg/week on Cbfa1 expression in the proximal tibial metaphysis of cbfa1$^{+/-}$ adult mice. Animals were treated with E_2 for 24 days, and Cbfa1 positive cells subsequently identified by lacZ staining on frozen longitudinal sections; (**A**) increased Cbfa1 expression as compared with untreated animals in Figure 2A (100 ×); (**B**) Cbfa1 is expressed both by osteoblasts lining bone surfaces, and cells within bone marrow (arrows) (200 ×); (**C**) Cbfa1 is expressed by osteoblasts forming trabecular bridges (open arrows) and bone marrow cells (arrows) (200 ×). Longitudinal tibial sections were also analysed after staining for lacZ and ALP activity; (**D**) section from an untreated animal, in which Cbfa1 is confined to osteoblasts co-expressing ALP (arrows) (200 ×); (**E**) section obtained 4 days after commencing E_2, showing a population of Cbfa1-expressing cells within bone marrow which do not co-express ALP (arrows) (200 ×); (**F**) section obtained 8 days after commencing E_2, showing Cbfa1-expressing cells which co-express ALP, both on bone surfaces (arrows) and within the adjacent bone marrow (open arrows) (300 ×).

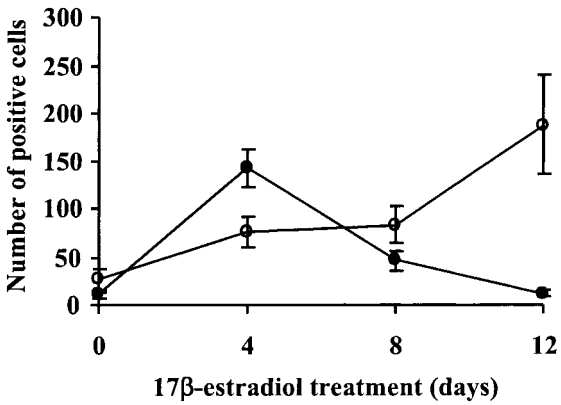

Fig. 4. Effect of estrogen treatment on number of Cbfa1 positive cells in the proximal tibial metaphysis of cbfa1$^{+/-}$ mice, according to ALP co-expression. Animals were sacrificed before or after treatment with 17β-estradiol 0.5 mg/week (E_2) for varying durations, and longitudinal tibial sections were stained for lacZ and ALP activity. Results show mean ± SEM for four animals per time-point. Two-way analysis of variance revealed significant effects of time ($P = 0.002$) and staining pattern ($P = 0.02$), and a significant interaction term ($P = 0.0002$).

treatment with estrogen for 4 days. Femoral bone marrow from untreated mice expressed relatively high levels of each Cbfa1 mRNA transcript as compared to MCF-7 cells, with no further change observed after estrogen treatment (data not shown).

DISCUSSION

We investigated the role of Cbfa1 in early osteoblast differentiation in adult mice by analysing changes in Cbfa1 expression during estrogen-induced bone formation based on our results which suggest that this response involves the generation of early osteoblast precursors from bone marrow [Perry et al., 2000]. Estrogen-induced osteogenesis was associated with a marked increase in the number of cells which express Cbfa1 within the tibial metaphysis, as assessed both by lacZ staining of sections from cbfa1$^{+/-}$ mice and immunocytochemistry performed on sections from wild-type animals. Part of this increase reflected the emergence of Cbfa1 + osteoblasts lining new cancellous bone surfaces. However, the majority of Cbfa1 + cells which appeared after estrogen were not immediately adjacent to bone surfaces and lacked characteristic morphological appearances of osteoblasts. We speculate that this latter population represent precursors which form in bone marrow in response to estrogen and give rise to osteoblasts which appear during later phases of the osteogenic response.

The possibility that Cbfa1 + bone marrow cells represent osteoblast precursors was supported by subsequent studies in which we examined co-expression of ALP, which was employed as an early osteoblast differentiation marker [Stein et al., 1990; Malaval et al., 1994]. We found that Cbfa1 + cells which initially appeared were evenly distributed throughout the marrow cavity and failed to express ALP. At subsequent time-points, this population was replaced by a population of Cbfa1 + ALP + cells close to, but distinct from, trabecular bone surfaces. Taken together, these findings suggest that Cbfa1 + cells are initially present as ALP − cells and subsequently migrate towards bone surfaces coincident with co-expression of ALP. This pattern of cellular migration and ALP expression is very suggestive of progressive differentiation within an early osteoblast precursor population.

Several lines of evidence support this suggestion that Cbfa1 + bone marrow cells represent osteoblast precursors. For example, the appearance of large numbers of Cbfa1 + bone marrow cells as early as 4 days after commencing estrogen preceded the subsequent osteogenic response by several days; in previous time course studies using equivalent experimental conditions, cancellous bone formation surfaces and type I collagen mRNA expression showed no increase until 12 days after commencing estrogen [Samuels et al., 1999; Plant and Tobias, 2001]. In addition, the time-course over which Cbfa1 + bone marrow cells appeared was similar to that over which estrogen was found to expand the bone marrow osteoblast precursor population as assessed by ex-vivo CFUf assay [Perry et al., 2000]. Finally, previous studies indicate that Cbfa1 acts to induce a range of genes associated with osteoblast differentiation including ALP [Ducy et al., 1997; Xiao et al., 1998; Harada et al., 1999; Kern et al., 2001].

In view of the recent suggestion that stromal cells may express Cbfa1 without necessarily being osteogenic [Satomura et al., 2000], it is possible that Cbfa1 + ALP − bone marrow cells induced by estrogen represent an expanded stromal population rather than committed osteoblast precursors. Further studies are planned to address this question, by investigating whether Cbfa1 + ALP − bone marrow cells identified in this study are restricted to the

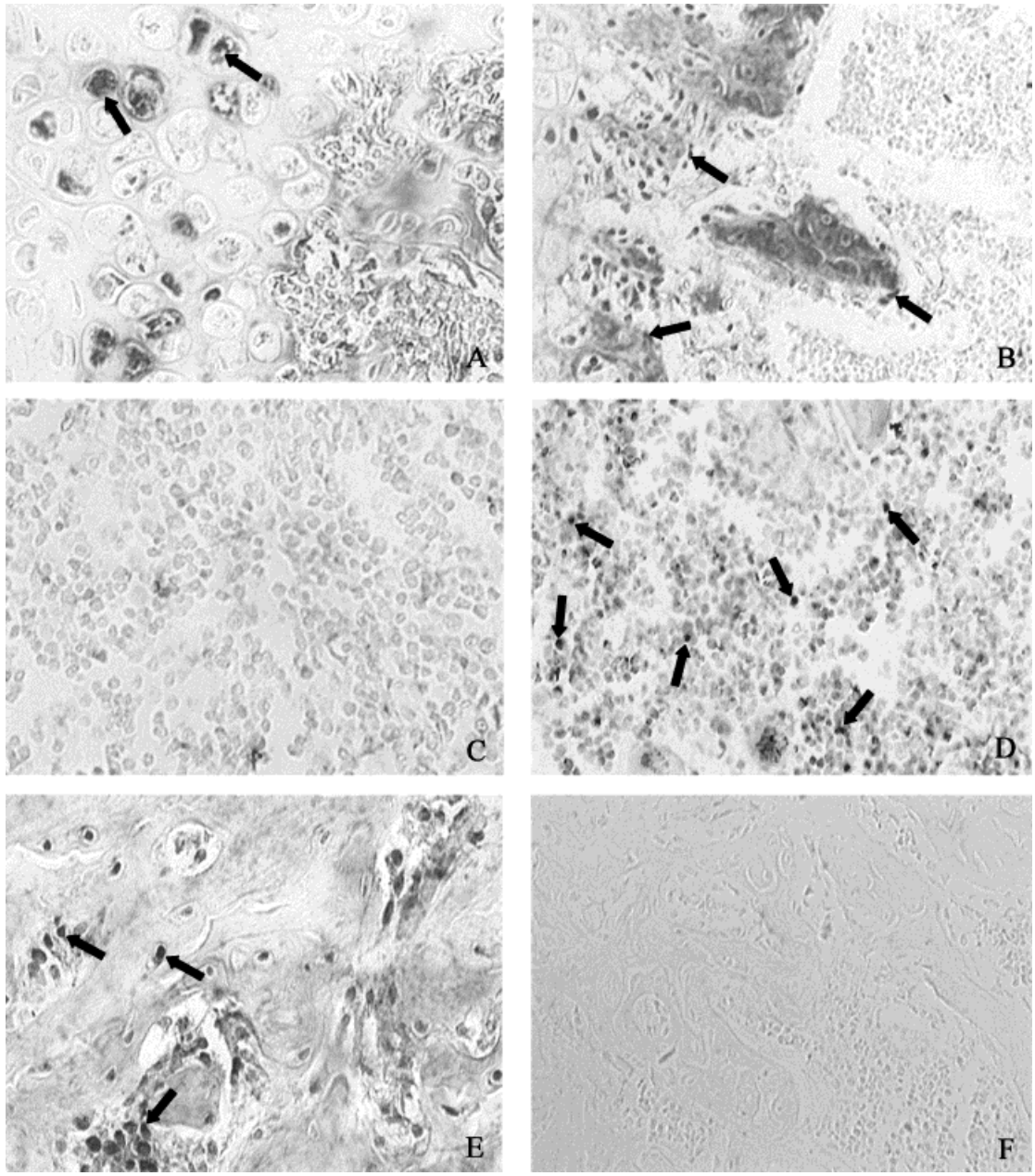

Fig. 5. Effect of estrogen treatment on Cbfa1 expression in the proximal tibial metaphysis of wild-type mice. Animals were sacrificed before or after treatment with 17β-estradiol 0.5 mg/week (E_2) for varying durations, and Cbfa1 immunoreactivity subsequently analysed in longitudinal tibial sections. **A**: Growth plate from an untreated mouse showing Cbfa1 expression by hypertrophic chondrocytes (arrows) (200 ×); (**B**) primary and secondary spongiosa from an untreated animal showing Cbfa1 expression in osteoblasts (arrows) (200 ×); (**C**) bone marrow from an untreated animal showing very few Cbfa1-expressing cells (200 ×); (**D**) bone marrow section obtained 4 days after commencing E_2 in which approximately 25% of cells show nuclear immunoreactivity for Cbfa1 (arrows) (200 ×); (**E**) section obtained 12 days after commencing E_2, showing cluster of Cbfa1-expressing bone marrow cells adjacent to new sites of bone formation (arrows) (200 ×); (**F**) bone marrow section obtained 4 days after commencing E_2, in which the primary antibody has been omitted, showing lack of Cbfa1 expression (200 ×).

osteogenic lineage, unlike other stromal populations which are generally pluripotent [Bianco and Robey, 2000]. If these Cbfa1 + ALP − bone marrow cells are found to represent committed early osteoblast precursors, this would be consistent with previous embryological studies which suggest Cbfa1 plays an important role in early osteoblast differentiation [Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997]. Although Cbfa1 has previously been found to

influence the function of mature osteoblasts in adult mice [Ducy et al., 1999], to our knowledge, this is the first study to investigate the role of Cbfa1 in early osteoblast differentiation in the adult.

To study the role of Cbfa1 in early osteoblast differentiation in adult mice, we analysed changes in Cbfa1 expression during estrogen-induced osteogenesis in cbfa1$^{+/-}$ mice, in which Cbfa1-expressing cells were readily identified by detection of activity of the lacZ marker gene. Cbfa1 haplo-insufficiency is known to affect skeletogenesis in these animals, in which minor skeletal abnormalities equivalent to cleidocranial dysplasia occur, as in humans with the same gene defect [Otto et al., 1997]. However, our finding that estrogen induces bone formation in cbfa1$^{+/-}$ mice to a similar extent to wild-type controls suggests that osteoblast function in adult animals is unaffected by haplo-insufficiency of cbfa1, and that studies of the role of Cbfa1 in osteoblast differentiation using cbfa1$^{+/-}$ mice are applicable to wild-type animals. The similarity of findings from immunohistochemistry studies performed in wild-type mice support this conclusion.

Whether our observations reflect specific regulation of Cbfa1 expression by estrogen is unclear, given that estrogen does not stimulate Cbfa1 expression in cultured osteoblast-like cells in vitro [Banerjee et al., 1998]. However, estrogen-induced Cbfa1 expression may be mediated by other local regulatory factors implicated in estrogen-induced osteogenesis [Samuels et al., 1999, 2001; Plant and Tobias, 2000], of which bone morphogenetic proteins have previously been found to induce Cbfa1 [Ducy et al., 1997; Yamaguchi et al., 2000]. Though estrogen has an exaggerated tendency to stimulate new bone deposition in mice [Turner, 1999], recent findings suggest that estrogen also stimulates osteoblast function in postmenopausal women [Vedi et al., 1999; Khastgir et al., 2001] and elderly men [Falahati-Nini et al., 2000]. Hence, further study of the mechanisms by which estrogen regulates Cbfa1 expression in vivo may yield insights into the pathogenesis and treatment of osteoporosis.

Unlike the marked increase in Cbfa1 expression observed histologically, no change in Cbfa1 mRNA expression was seen during estrogen-induced osteogenesis. Furthermore, bone marrow extracts from untreated animals contained relatively high levels of Cbfa1 mRNA compared to whole bone, which differed from results from histological analysis where very few Cbfa1-expressing cells were found in bone marrow of untreated mice. These findings were unexpected, since results from analysis of lacZ marker expression reflect level of cbfa1 promotor activity which would be expected to correlate with findings from quantification of mRNA levels. One explanation for this apparent discrepancy is that other cell lineages within bone marrow express Cbfa1 at low levels which contribute to overall mRNA levels as assessed by Northern blot analysis, but are not detected by histological methods utilised here. Consistent with this possibility, related factors such as Cbfa2 are thought to play an important role in haematopoiesis [Okuda et al., 1996]. Though non-osseous tissues are not generally found to express Cbfa1 [Ducy et al., 1997], observations that Cbfa1 is expressed by T cells demonstrate that Cbfa1 is not entirely osteoblast specific [Ogawa et al., 1993], and to what extent Cbfa1 is expressed by haematopoietic cells has not, to our knowledge, been addressed previously.

In summary, we have found that estrogen-induced osteogenesis is associated with the appearance of a population of Cbfa1-expressing cells within long bone marrow of adult female mice. The time-course over which this population appears, its tissue distribution, and preliminary phenotypic analysis suggest that this represents a discrete osteoblast precursor population within bone marrow which has not been recognised hitherto. Further studies are planned to determine whether Cbfa1-expressing bone marrow cells identified in this study represent a committed osteoblast precursor population distinct from bone marrow stromal cells.

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