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# The temporal expression of estrogen receptor alpha-36 and runx2 in human bone marrow derived stromal cells during osteogenesis ☆

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ABSTRACT

During bone maintenance *in vivo*, estrogen signals through estrogen receptor (ER)-α. The objectives of this study were to investigate the temporal expression of ERα36 and ascertain its functional relevance during osteogenesis in human bone marrow derived stromal cells (BMSC). This was assessed in relation to runt-related transcription factor-2 (runx2), a main modulatory protein involved in bone formation. ERα36 and runx2 subcellular localisation was assessed using immunocytochemistry, and their mRNA expression levels by real time PCR throughout the process of osteogenesis. The osteogenically induced BMSCs demonstrated a rise in ERα36 mRNA during proliferation followed by a decline in expression at day 10, which represents a change in dynamics within the culture between the proliferative stage and the differentiative stage. The mRNA expression profile of runx2 mirrored that of ERα36 and showed a degree subcellular co-localisation with ERα36. This study suggests that ERα36 is involved in the process of osteogenesis in BMSCs, which has implications in estrogen deficient environments.



## 1. Introduction

Bone marrow derived stromal cells (BMSC) contain a population of stem/progenitor cells that differentiate into a variety of mesenchymal lineages depending on their environment [1]. Runt-related transcription factor 2 (runx2) plays a vital role in their commitment to the osteoblast lineage [2] and positively regulates osteoblast proliferation and differentiation through the upregulation of bone-related genes (e.g. type I collagen, osteopontin and osteocalcin). The ablation of runx2 in mice results in a complete lack of osteoblast differentiation and retardation of chondrocyte differentiation [3].

Estrogen is essential for skeletal development and in the maintenance of bone health by maintaining a focal balance between bone formation and bone resorption. It has antiapoptotic effects on osteoblasts (responsible for bone formation) and proapoptotic effects on osteoclasts (responsible for bone resorption) preventing high bone turnover [4]. Loss of estrogen, for example, at menopause, can lead to a reduction in bone density and an increased risk of osteoporotic fractures [5].

Estrogen signals through estrogen receptors (ER), which belong to the nuclear hormone receptor family, the two main forms of which are ERα and ERβ. ERα is the predominant form in osteoblasts [6]. Previous studies have shown that knocking out ERα in mice significantly reduces osteoblast activity in cancellous bone but retains osteoclast activity suggesting ERα in vital in maintaining bone mass and strength [7].

ERα consists of a N-terminal ligand-independent activation function 1 (AF-1), a DNA binding domain and a C-terminal ligand-binding domain that also harbours an activation function 2 (AF-2) [8]. Three ERα isoforms have been identified: the full length ERα66; ERα46, which lacks AF-1 as a result of skipping exon 1, and ERα36, which lacks both transcriptional activation domains AF-1 and AF-2 (Fig. 1). ERα36 mRNA is generated from an alternative promoter located in the first intron of the ERα66 gene and despite lacking the AF-1 and AF-2 domains, the 36 kDa isoform

*Abbreviations:* BMSC, bone marrow stromal cells; ERα, estrogen receptor alpha; Runx2, Runt-related transcription factor 2.

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(ERα36) retains the DNA-binding domain and partial dimerisation and ligand-binding sites [9]. It also has a unique 27 amino acid domain at the ligand binding C-terminal region, which results in a broader ligand-binding spectrum [6]. This significantly influences the response of ERα36 to estrogens and antiestrogens compared to ERα66 [10]. Whilst ERα66 and ERα46 are often expressed in the nucleus and mediate genomic estrogen signalling, ERα36 is predominantly expressed on the cell surface and mediates membrane-initiated or non-genomic estrogen signalling [8].

There are no studies investigating the temporal expression of ERα, in particular ERα36, and runx2 in human primary bone marrow mesenchymal stromal cells during osteogenesis. This investigation studies the mRNA expression profiles and subcellular localisation of the different ERα isoforms in BMSCs during osteogenic commitment and differentiation, correlated with that of runx2. ERα36 mRNA expression rapidly rose during differentiation. The mRNA expression profile of ERα36 not only reflected that of runx2 but ERα36 protein also showed a degree of subcellular co-localisation with runx2.

## 2. Materials and methods

### 2.1. Cell isolation and culture

Ethical approval was obtained from the Local Research Ethics Committee (LREC) and all patients signed informed consent prior to participation. Inclusion criteria included patients aged 16–65 who underwent surgery to the pelvic ring or acetabulum. Bone marrow aspirates were harvested from the iliac crest of these patients during surgery using a bone marrow aspiration needle (Mana-Tech Ltd.). Exclusion criteria included pre-existing conditions (e.g. connective tissue disease, diabetes, malignancy), or medication (e.g. steroids/cytotoxic agents). The mononuclear cells (MNC) were isolated from four bone marrow aspirates (3 males aged 22, 34 & 58, & 1 female aged 55) using histopaque-1077 (Sigma). Up to $4 \times 10^7$ MNC were placed into 75 $cm^2$ tissue culture flasks in 10 ml commercial Alpha-MEM (Gibco) with 10% FCS and antibiotics, and incubated for 7 days in a humidified 37 °C incubator under 5% $CO_2$. During this time, a fraction of the MNC population (bone marrow derived stromal cells: BMSC) adhered to the plastic and proliferated. Subsequently cultures were washed in PBS and fed every 3–4 days until the cell population reached 60–70% confluence at which time they were sub-cultured or cryopreserved in liquid nitrogen.

### 2.2. Real time PCR analysis

ERα36 mRNA, and key markers of osteogenesis (e.g. runx2 and alkaline phosphatase) were assessed by real time PCR. GAPDH was used as the house keeping (HK) gene. RNA was isolated from cell cultures at 0, 7, 10, 14 and 17 days differentiation using RNeasy plus mini kit (Qiagen, UK) and 1 μg mRNA was reverse transcribed into cDNA in a total volume of 20 μl as per manufacturer's instructions (high capacity cDNA reverse transcription kit, Applied Biosystems). The PCR reactions were run at 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s using Platinum SYBR Green qPCR Supermix UDG (Invitrogen) and the iCycler MyIQ Real-time PCR detection system (BioRad Laboratories). This was followed by a melt curve analysis. The cycle threshold (CT) of amplification was computed according to the manufacturer's instructions and the relative gene expression level determined by 2^(HK CT-Test CT). The sequences of primer oligonucleotides used are as follows: GAPDH (forward: 5′TCA TTG ACC TCA ACT ACA TGG T-3′ and reverse: 5′TCT CGC TCC TGG AAG ATG GTG-3′), ERα36 (forward: 5′CCA AGA ATG TTC AAC CAC AAC CT-3′ and reverse: 5′GCA CGG TTC ATT AAC ATC TTT CTG-3′), runx2 (forward: 5′CCT AGG CGC ATT TCA GGT GCT T-3′ and reverse: 5′CTG AGG TGA CTG GCG GGG TGT-3′) and alkaline phosphatase (forward: 5′GAC CCT TGA CCC CCA CAA T-3′ and reverse: 5′GCT CGT ACT GCA TGT CCC CT-3′).

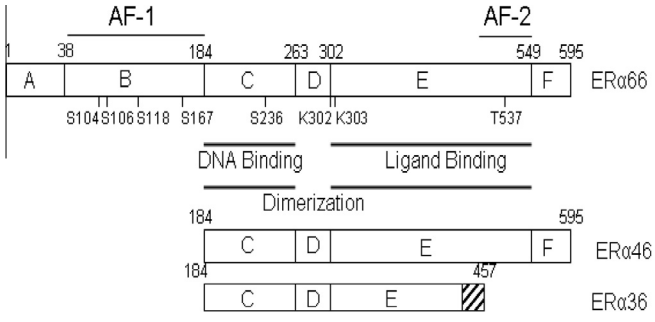


**Fig. 1.** Domain structure and function representation of human estrogen receptor-α isoforms (Wang et al. [9]). The last 27 amino acids of ERα36 are indicated by the filled box.

### 2.3. Immunocytochemistry

The BMSCs, differentiated for 0, 7, 10, 14 and 17 days in the 96 well sensoplates, were rinsed in PBS and fixed in 10% buffered formalin at room temperature for 1 h. After the BMSCs were permeabilized for 10 min in PBS containing 0.1% Triton-X (PBS-T) and non-specific binding sites blocked with 0.5% BSA in PBS-T (blocking buffer) for 30 min, the BMSCs were incubated with primary antibody diluted in 0.2% BSA in PBS-T overnight at 4 °C. The primary antibodies used were mouse anti-human runx2 (3 μg/ml), mouse anti-human collagen I (1 in 1000 dilution) (Sigma–Aldrich), and rabbit anti-human ERα36 (1 in 200 dilution) (kindly donated by Dr. Zhao-Yi Wang, Creighton University, California Plaza, USA). Unbound primary antibody was removed by washing 3 times with PBS-T and incubated with FITC-conjugated anti-mouse (collagen I), Cy5-conjugated anti mouse (runx2) or FITC-conjugated anti rabbit (ERα36) (1 in 200 dilution in 0.2% BSA in PBS-T) were added for 1 h at room temperature. ERα36 was stained independently and in combination with runx2. Unbound secondary antibody was removed by washing 3 times with PBS-T and BMSCs were stained with DAPI (1 in 1000 dilution in PBS) for 1 min. Cells were immersed in PBS for analysis by confocal microscopy.

### 2.4. Statistics

The data is presented as mean ± SE and all non-parametric statistical testing was performed using Statistica version 6.

## 3. Results

### 3.1. Temporal expression of estrogen receptor α and runx2 during osteogenesis

ERα36 mRNA expression was assessed in BMSC throughout the process of osteogenesis by real time PCR and immunohistochemistry. ERα36 mRNA was observed to be low in BMSC cultures at ~70% confluence before the addition of osteogenic media, but rose following the addition of osteogenic media to day 7 (Fig. 2). Expression then declined by day 10 ($p = 0.006$) before increasing during the later stages of osteogenesis. This pattern of expression was closely mimicked by runx2 mRNA expression (Fig. 2). Both ERα36 and runx2 mRNA expression remained low within the untreated control group throughout the process of osteogenesis.

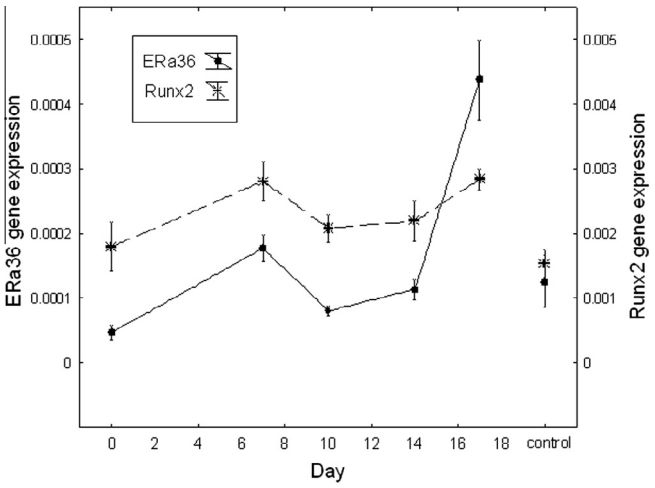


**Fig. 2.** ERα36 and runx2 mRNA expression throughout the process of BMSC osteogenesis ($n = 4$). Gene expression relative to GAPDH. ERα36 significantly increased from day 0 to day 7 ($p = 0.014$), day 14 ($p = 0.027$) and day 17 ($p = 0.014$). In the latter stages of differentiation ERα36 significantly increased to day 17 ($p = 0.021$) from day 14. Runx2 increased from day 0 to day 7 ($p = 0.047$) and day 17 ($p = 0.009$). The undifferentiated control cultures retained low ERα36 and runx2 mRNA expression throughout the 17 days.

### 3.2. Osteogenic commitment

BMSC cultures showed clear commitment to the osteogenic lineage with positive extracellular matrix production and mineralisation demonstrated by collagen I production and Alizarin red S staining respectively (Fig. 3C and A). Runx2 and ALP protein expression were increased following osteogenic differentiation as assessed by immunocytochemistry (Fig. 3B and D respectively). Runx2 and alkaline phosphatase (ALP) mRNA were significantly upregulated by the addition of osteogenic medium as quantified by real time PCR.

### 3.3. Co-expression of ERα and runx2

The protein expression and sub-cellular localisation of ERα36 and runx2 was assessed by immunocytochemistry throughout the process of osteogenesis (Fig. 4). ERα36 was expressed both within the nucleus and cytosol of the BMSCs, whereas runx2 was largely expressed within the nucleus. They showed some degree of co-localisation within the nucleus of the cells.

## 4. Discussion

This study aimed to investigate the temporal expression of ERα36 and runx2 during osteogenesis in human stromal cells derived from bone marrow. Their similar pattern of expression and colocalisation within the nucleus, in the absence of exogenous estrogen, indicates an interplay between the two proteins during osteogenesis. The co-localisation at distinct domains within the nucleus has also been shown previously [5]. No co-localisation was shown within undifferentiated control cultures that had poor runx2 production.

A previous study with rat bone marrow derived stroma showed a similar temporal expression of ERα mRNA during osteogenesis, but they did not specifically investigate the expression of isomer ERα36 at the mRNA level or equate ERα expression with that of runx2 [6]. The pattern of expression, with a reduction in mRNA levels by day 10 followed by an increase in the expression towards the latter stages of osteogenesis may represent a dynamic change within the culture between the proliferative/maturation and differentiative phases. Cultures were osteogenically stimulated at approximately 70% confluence (3–4 days) so were still within their proliferative phase when the supplements were added.

Estrogen signalling is vital during bone formation and ERα36 is the predominant ER isoform involved in bone regulation. Being expressed on the cell surface, ERα36 is able to respond to much lower estrogen concentrations than nuclear based isomers. Higher concentrations of ERα36 are found on the osteoblasts of normal postmenopausal women compared to osteoporotic

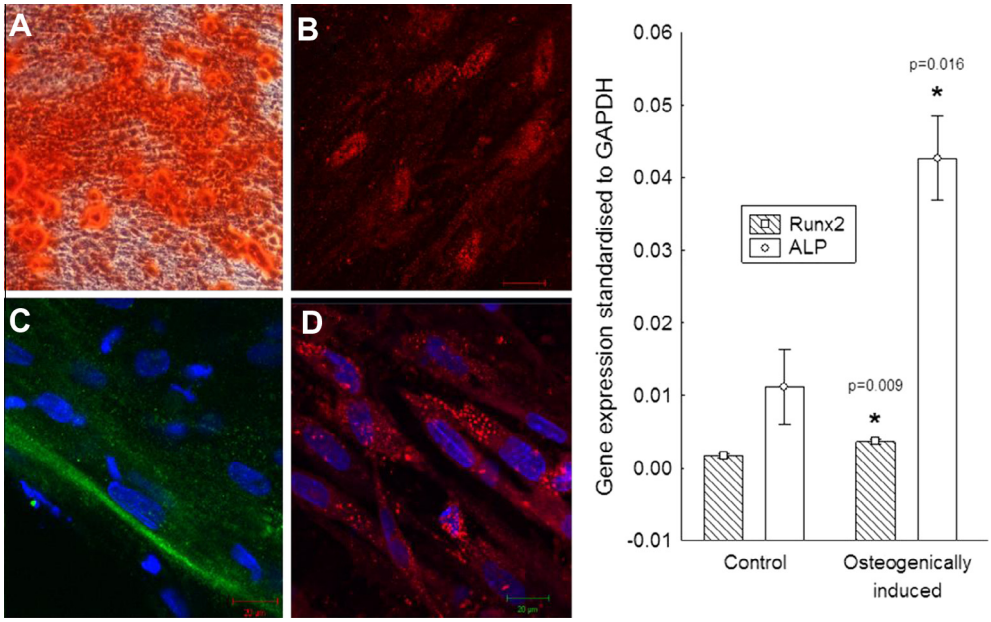


**Fig. 3.** Markers of osteogenesis at day 17 differentiation. Differentiated BMSC cultures demonstrated mineralisation with alizarin red s staining (A); stained positively for runx2 (B, red) & collagen I (C, green) by immunohistochemistry using confocal imaging, and were alkaline phosphatase positive (D, red) using a Vector Laboratories kit. Differentiated BMSC cultures had significantly more runx2 mRNA ($p = 0.009$) and alkaline phosphatase mRNA ($p = 0.016$) at day 17 differentiation than the undifferentiated control cultures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

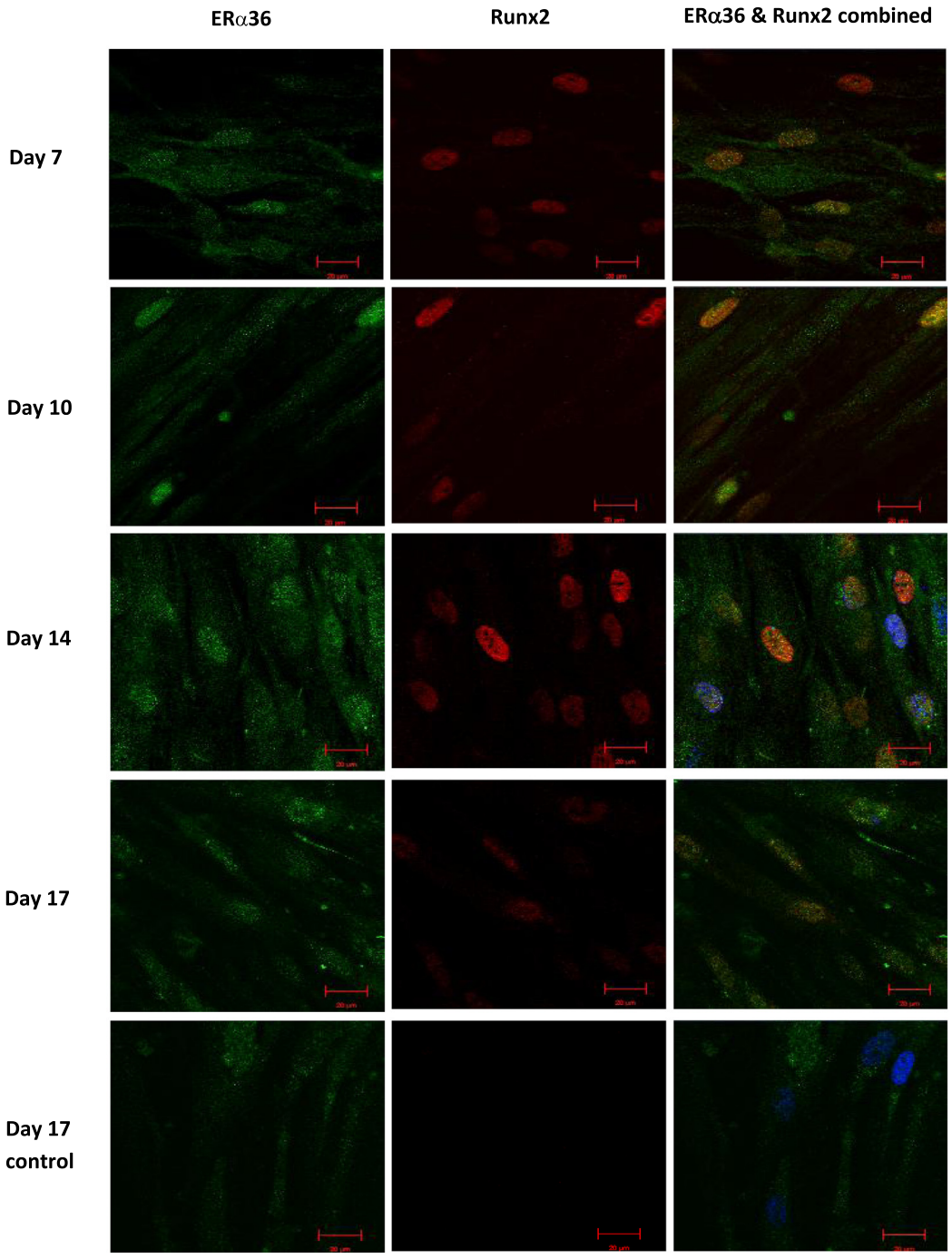


**Fig. 4.** Protein expression of ERα36 (green, 1st column), runx2 (red, 2nd column) and combined (merged colours, 3rd column) in BMSCs osteogenically differentiated over 17 days. The nuclear stain DAPI (blue) has been considerably reduced for clarity purposes to show the co-localisation of ERα36 and Runx2. Only in the day 17 control is the DAPI available to show nuclear expression in the absence of clear runx2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

postmenopausal women, which is consistent with normal postmenopausal women ability to respond to estrogen at much lower concentrations to maintain bone turnover [4].

The immunocytochemical staining of ERα36 and runx2 showed the majority of BMSCs are ERα36 and runx2 positive following osteogenic stimulation. Runx2 was predominantly within the nucleus, whereas ERα36 was found in the nucleus, membrane and cytosol. Forced expression of runx2 in osteoblastic cells has been shown to increase estrogen production [11] by directly interacting with the aromatase gene promoter to stimulate aromatase expression, which ultimately converts androgens to estrogen. Estrogen acts upon ERα36 at the cell surface of mesenchymal stromal cells and osteoblasts to promote bone formation. *In vitro* studies have shown that ERα directly interacts with, and inhibits runx2 in the presence of 17β-estrogen [3,5]. These positive and negative interactions are likely to be vital in estrogen-deficient environments [3,11]. More work needs to be done to characterise the interaction between ERα36 and Runx2 and its functional role in osteogenic differentiation.

The passage 2 human BMSCs used for this investigation observed the traditional MSC phenotype [12] with positive expression for CD73, CD90 and CD105, and negative expression for the haemopoietic markers CD14 (macrophage), CD19 (lymphocyte), CD34 (haemopoietic progenitor) and CD45 (leukocyte marker) (data not shown). They differentiated efficiently down the osteogenic lineage, significantly up-regulating their gene and protein expression of alkaline phosphatase and runx2 leading to the development of a mineralised extracellular matrix. These cells have also been shown to differentiate down the adipogenic and chondrogenic lineages (results not shown), which is characteristic of BMSCs.

In conclusion ERα36 is the predominant isoform of ERα involved in bone regulation in human BMSCs. ERα36 expression is significantly upregulated during the process of osteogenesis, indicating that it may play a vital role alongside runx2 in the regulation of bone formation. Further work needs to be done to confirm co-localisation and investigate the functional relationship between ERα36 and runx2.

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