Estrogen stimulates leptin receptor expression in ATDC5 cells via the estrogen receptor and extracellular signal-regulated kinase pathways

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Abstract
Regulation of the physiological processes of endochondral bone formation during long bone growth is controlled by various factors including the hormones estrogen and leptin. The effects of estrogen are mediated not only through the direct activity of estrogen receptors (ERs) but also through cross talk with other signaling systems implicated in chondrogenesis. The receptors of both estrogen and leptin (OBR (LEPR)) are detectable in growth plate chondrocytes of all zones. In this study, the expression of mRNA and protein of OBR in chondrogenic ATDC5 cells and the effect of 17β-estradiol (E2) stimulation were assessed using quantitative PCR and western blotting. We have found that the mRNA of Obr was dynamically expressed during the differentiation of ATDC5 cells over 21 days. Application of E2 (10^{-7} M) at day 14 for 48 h significantly upregulated OBR mRNA and protein levels (P<0.05). The upregulation of Obr mRNA by E2 was shown to take place in a concentration-dependent manner, with a concentration of 10^{-7} M E2 having the greatest effect. Furthermore, we have confirmed that E2 affected the phosphorylation of ERK1/2 (MAPK1/MAPK3) in a time-dependent manner where a maximal fourfold change was observed at 10 min following application of E2. Finally, pretreatment of the cells with either U0126 (ERK1/2 inhibitor) or ICI 182 780 (ER antagonist) blocked the upregulation of OBR by E2 and prevented the E2-induced phosphorylation of ERK. These data demonstrate, for the first time, the existence of cross talk between estrogen and OBR in the regulation of bone growth whereby estrogen regulates the expression of Obr in growth plate chondrocytes via ERs and the activation of ERK1/2 signaling pathways.

Introduction
The development of bones is a result of endochondral bone formation that occurs at the epiphyseal growth plate through a process whereby cartilage is formed and then remodeled into bone tissue. This complex process is regulated by several growth factors and hormones. One of the key regulators of endochondral bone development is estrogen (Perry et al. 2008). Estrogen exerts its effect via traditional genomic interactions with two nuclear receptors, namely estrogen receptor α (ERα (ESR1)) and β (ERβ (ESR2)) as well as by rapid nongenomic mechanisms. Studies have demonstrated that ERα and ERβ are expressed in all zones of the growth plate (van der Eerden et al. 2002, Nilsson et al. 2002, 2003). Our recent study (Li et al. 2012) observed gender- and region-specific differences in ERα and ERβ gene expression in the limb and spine growth plate during development in both male and female rats. The direct binding of estrogen to ERα and ERβ results in the initiation of signal transduction. In addition, the nongenomic mechanism has been suggested to be of importance for the regulation of bone growth (Sylvia et al. 2001, Ekstein et al. 2005, McMillan et al. 2006, Windahl et al. 2007).

Another hormone that also plays an important role in the regulation of endochondral bone formation is leptin, which modulates several stages of the process of growth plate chondrocyte differentiation and leptin and estrogen in hypothalamic neurons and cancer cells (Maeso Fortuny et al. 2006, Ray et al. 2007, Gao & Horvath 2008, Fusco et al. 2010). In addition, 17β-estradiol (E2), acting through ERα, can induce the expression of leptin in placental trophoblastic cells through genomic and nongenomic actions including the MAPK and PI3K pathways (Gambino et al. 2010). In rodents, ovariectomy reduces Obr expression in the hypothalamus but increases Obr
expression in subcutaneous adipose tissue, and such effects can be reversed by estrogen treatment (Mayes & Watson 2004, Meli et al. 2004, D’Eon et al. 2005).

The fact that the expressions of ERs and OBR coexist in the growth plate and that both estrogen and leptin play a role in the regulation of chondrocyte differentiation suggests that the two hormones may interact during longitudinal bone growth. However, the direct effect of estrogen on OBR in the growth plate has not been fully elucidated. The purpose of this study was to examine how estrogen modulates the expression of OBR in chondrocytes. The study used mouse chondrogenic ATDC5 cells, which are an excellent in vitro model for studying the regulation of endochondral bone growth plate and that both estrogen and leptin play a role in

Our results also indicated that leptin regulates ERs via the ERK1/2 (2012) also suggest that ATDC5 cells are an appropriate model for studying the cross talk between leptin and estrogen. In our previous study (Wang et al. 2011), we found that both ERα and ERβ were dynamically expressed during the ATDC5 cell differentiation from 4 to 21 days. Our results also indicated that leptin regulates ERs via the ERK1/2 (MAPK1/MAPK3) signaling pathway in ATDC5 cells, suggesting a cross talk between leptin and ERs in the regulation of bone formation. In this study, we hypothesized that estrogen could induce an upregulation of the expression of Obs during chondrocyte differentiation and that this upregulation was dependent on ERs and the ERK1/2 signaling pathways.

Real-time PCR analysis

Following the differentiation protocol, total RNA was extracted from ATDC5 cells using a 1 ml TRIzol reagent kit (Invitrogen). The total RNA was then dissolved in 0.1% diethylpyrocarbonate water and quantified by spectrophotometry using a wavelength of 260 nm. After quantification, 2 μg total RNA was reverse transcribed into cDNA using a TaKaRa RNA PCR Kit version 2.1 (TaKaRa Biotechnology Dalian Co. Ltd., Dalian, China) according to the manufacturer’s protocol. The sequences for primers (TaKaRa Biotechnology Dalian Co. Ltd.) used to amplify the mRNA were as follows: Obr: 5’-GACAGAACACGGCGACACTGTGTA-3’, 5’-AAGCAGACTGACTCCACAGCA-3’; Col2 (Col2a1): 5’-GTCCTGAAGGTGCTCAAGGTTCTC-3’, 5’-AGGAATACCATCAGTCCCTGG- GTTA-3’; Col110 (Col10a1): 5’-AGAACGCCGACCCCTAGGATACCTTCTCATGCAAC-3’, 5’-ATGGAGCCCGATCCACA-3’. A LightCycler rapid thermal cycler system (Roche Diagnostics) was then used to perform quantitative real-time PCR using 1 μg cDNA and SYBR Green (Bio-Rad) in 96-well plates according to the manufacturer’s instructions. Melting curve analysis was performed using RotorGene 6.0 Analysis Software (Corbett Research, Sydney, Australia) to identify, analyze, and quantify the PCR products. The abundance of each gene product was normalized to β-actin and the 2^–DDCT (cycle threshold) method was used to calculate relative gene expression levels as described previously (Maymo et al. 2010). The samples analyzed were generated from triplicate experiments and the results are expressed as fold change in gene expression normalized to a housekeeping gene (β-actin) and relative to control conditions (untreated cultures).

Materials and Methods

Cell culture and treatments

ATDC5 cells, obtained by courtesy of Prof. Lin Chen (Third Military Medical University, Chongqing, China), were cultured and maintained in a 1:1 mixture of DMEM/F12 medium (Invitrogen) containing 5% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin (Invitrogen) and incubated at 37 °C in 5% CO₂/95% humidity. The cells were initially plated in six-well plates (Corning, New York, NY, USA) at a density of 6 × 10⁴ cells/well. After reaching 70–80% confluency, the cells were cultured with a differentiation medium, which was similar to the maintenance medium but with the addition of 1% insulin/transferrin/sodium selenite (ITS; Sigma). The day of addition of the differentiation medium was designated as day 0 and the differentiation medium was replaced every other day from days 2–14. After 14 days, differentiation medium was removed and replaced with an ITS-free medium containing 0.5% FBS. The cells were incubated in the ITS-free medium for 24 h and then stimulated with E₂ (Sigma) for another 24 or 48 h.

ATDC5 cells were washed with PBS (10 mM, pH 7.4) and total protein was extracted using a Western & IP Cell Lysis Kit (Beyotime, Shanghai, China). The concentration of protein in the cell lysate was determined using a Bio-Rad assay kit (Invitrogen). The total RNA was then dissolved in 0.1% diethylpyrocarbonate water and quantified by spectrophotometry using a wavelength of 260 nm. After quantification, 2 μg total RNA was reverse transcribed into cDNA using a TaKaRa RNA PCR Kit version 2.1 (TaKaRa Biotechnology Dalian Co. Ltd., Dalian, China) according to the manufacturer’s protocol. The sequences for primers (TaKaRa Biotechnology Dalian Co. Ltd.) used to amplify the mRNA were as follows: Obr: 5’-GACAGAACACGGCGACACTGTGTA-3’, 5’-AAGCAGACTGACTCCACAGCA-3’; Col2 (Col2a1): 5’-GTCCTGAAGGTGCTCAAGGTTCTC-3’, 5’-AGGAATACCATCAGTCCCTGG-GTTA-3’; Col110 (Col10a1): 5’-AGAACGCCGACCCCTAGGATACCTTCTCATGCAAC-3’, 5’-ATGGAGCCCGATCCACA-3’. A LightCycler rapid thermal cycler system (Roche Diagnostics) was then used to perform quantitative real-time PCR using 1 μg cDNA and SYBR Green (Bio-Rad) in 96-well plates according to the manufacturer’s instructions. Melting curve analysis was performed using RotorGene 6.0 Analysis Software (Corbett Research, Sydney, Australia) to identify, analyze, and quantify the PCR products. The abundance of each gene product was normalized to β-actin and the 2^–DDCT (cycle threshold) method was used to calculate relative gene expression levels as described previously (Maymo et al. 2010). The samples analyzed were generated from triplicate experiments and the results are expressed as fold change in gene expression normalized to a housekeeping gene (β-actin) and relative to control conditions (untreated cultures).

Western blot analysis

ATDC5 cells were washed with PBS (10 mM, pH 7.4) and total protein was extracted using a Western & IP Cell Lysis Kit (Beyotime, Shanghai, China). The concentration of protein in the cell lysate was determined using a Bio-Rad assay according to the manufacturer’s protocol (Bio-Rad). For each sample, whole cell extracts equivalent to 40 μg total protein were loaded into a 8% SDS–polyacrylamide gel, separated by electrophoresis, and the proteins electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Nonspecific binding was blocked by incubating the PVDF membrane with 10 μM TBS with 1% Tween 20 and 1% dehydrated skimmed milk. Following the blocking procedure, the membranes were incubated overnight with antibodies against OBR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-phospho ERK1/2 (Cell Signaling Technology, Beverly, MA, USA) at 4 °C. The performance of immunoblotting on the same membranes using antibodies against β-actin (Beyotime) or total-ERK1/2 (Cell Signaling Technology) was used as a loading control to assay the relative
amounts of protein loaded into each gel. Following incubation with a primary antibody, the membranes were washed with TBST and incubated with alkaline phosphatase-linked secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA). The membranes were then washed once more and the immunoreactive bands visualized using NBT/BCIP as a substrate. Densitometric analysis was done using the NIH ImageJ Software to quantify the protein present in the detected bands. β-actin content was assayed as standardization of sample loading. Quantitative densitometric values of each protein of interest were normalized to β-actin or to the nonphosphorylated form of the protein.

Statistical analysis

All data were presented as mean ± S.E.M. for at least three independent experiments. Statistical analyses were performed using the SPSS 11.5 Statistical Software program (SPSS Inc, Chicago, IL, USA). Comparative studies of means between groups were determined by the one-way ANOVA followed by the least significant difference test or by the unpaired Student’s t-test with a statistically significance at P values <0.05.

Results

Differentiation of ATDC5 cells into chondrocytes

We have evaluated ATDC5 cell proliferation and differentiation in vitro by light microscopy and typical chondrogenic differentiation markers (Fig. 1). As shown in Fig. 1A, from days 4 to 21, ATDC5 cells proliferated gradually to form the typical cartilage nodules, which increased in size and number with time in culture. The expression of extracellular matrix genes, including those for type II and type X collagens, was used to characterize the chondrogenic differentiation of ATDC5 cells. Changes in the relative expression of these two chondrogenic differentiation markers were assayed using real-time PCR (Fig. 1B). The differentiation of mesenchymal cells into chondrocytes was indicated by an increase in Col2 expression in ATDC5 cells following a single day in the differentiation medium. The expression of Col2 continued to increase from days 4 to 14, indicating the early stages of chondrocyte differentiation, and then decreased to near-baseline levels from days 14 to 21. The level of Col10 mRNA also increased but the effect was most noticeable starting on day 7, and the high level of Col10 expression was maintained during days 14 and 21, indicating the later stages of chondrocyte differentiation. By day 21, the expression of type II collagen in ATDC5 cells had been replaced by the expression of type X collagen. The expression pattern of chondrocyte differentiation markers indicates that undifferentiated ATDC5 cells can differentiate into proliferative chondrocytes and then to hypertrophic chondrocytes under certain conditions. This result also validates the use of this cell line as an in vitro model that can be used to study chondrocyte differentiation.
Expression of the leptin receptor in ATDC5 cells

We further examined changes in the expression of Obr during the differentiation of ATDC5 cells over 21 days. The results of real-time PCR confirmed that Obr mRNA was dynamically expressed in ATDC5 cells during all the differentiation phases (Fig. 1C). The levels of Obr mRNA increased sharply with the progression of chondrogenic differentiation (days 4–14) and then continued to increase but at a slower rate after day 14 during the hypertrophy stages, which was indicated by the change of predominant collagen expression from type II to type X (days 14–21).

Effect of E2 on leptin receptor in ATDC5 cells

The role of E2 in regulating the expression of Obr in chondrocytes was determined by measuring the amount of Obr mRNA present in ATDC5 cells following treatment with E2 (10^{-7} M) on day 14. The cells were treated with E2 (10^{-7} M) for 24 or 48 h and the expression of the Obr gene was analyzed by real-time PCR (Fig. 2A). When compared with control cells, no significant difference in the level of Obr mRNA was observed in ATDC5 cells after 24 h of exposure to E2. However, after 48 h, the level of Obr mRNA was significantly increased in those cells stimulated with E2 compared with the control (P<0.05).

We then examined whether the changes in Obr mRNA expression were followed by a corresponding increase in protein level using western blotting on cells treated with E2 (10^{-7} M) for 24 and 48 h (Fig. 2B and C). Similar to the mRNA expression level, no significant change in OBR protein expression was noted at 24 h after E2 stimulation. However, the presence of E2 significantly increased the level of OBR protein after 48 h of treatment compared with control cells (P<0.05). The results also showed that the level of OBR protein expression was significantly enhanced at 48 h after E2 treatment in comparison to the level of protein expression before treatment (0 h; P<0.05). The level of protein expression for the housekeeping protein, β-actin, was unchanged at all the time points examined. These data suggest that E2 stimulation regulates the expression of Obr mRNA and consequently the changes of protein level.

To confirm whether the regulatory effect of E2 on the expression of Obr mRNA occurred in a concentration-dependent manner, we treated ATDC5 cells with various concentrations of E2 for 48 h. Analysis of the results indicated that the concentration of E2 required to significantly increase the expression of Obr mRNA was in the range of 10^{-8} to 10^{-7} M, with a concentration of 10^{-7} M being the most effective and 10^{-8} M only slightly less effective (Fig. 3). Both concentrations produced an ~150% increase in Obr mRNA expression. In addition, the application of 10^{-9} and 10^{-6} M E2 produced increases in Obr mRNA expression that were not significant but were greater than control, indicating that the effect of E2 was concentration dependent.

Figure 2 Effect of E2 on OBR mRNA and protein. ATDC5 cells were cultured in DMEM/F12 containing 5% FBS and 1% ITS in six-well plates at a density of 6×10^4/well for 14 days. Cultured cells were then treated without and with E2 at 10^{-7} M for 24 and 48 h. Obr gene expressions (A) were analyzed by real-time PCR, normalized against β-actin, and compared with the control group. OBR protein levels were analyzed by western blot using specific antibodies as indicated. Bands (B) show representative western blots, whereas graphs (C) show normalized data. Data represent mean ± S.E.M. from triplicate samples in three independent experiments. *P<0.05 vs control, #P<0.05 vs 0 h.

To verify whether the gene regulation is through ERα or ERβ, cells were treated with the ERα-selective agonist PPT (4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triy) trisphenol; 10^{-7} M) and the ERβ-selective agonist DPN
(2,3-bis(4-hydroxyphenyl)-propionitrile; 10^{-7} M). PPT and DPN were obtained from Tocris Bioscience (Ellisville, MO, USA). OBR mRNA and protein levels were significantly upregulated at 48 h after PPT stimulation ($P<0.05$) at day 14. However, no significant difference was found after DPN treatment (Fig. 4A and B).

As type X collagen is expressed exclusively by mature chondrocytes, and its expression is a differentiation marker of the ATDC5 cell model, we treated the cells with E2, PPT, and DPN at 10^{-7} M for 24 h at day 14; a significant increase in Col10 mRNA levels was observed after E2 or PPT stimulation (Fig. 5).

**Activation of ERK signaling by E2 in ATDC5 cells**

Using western blotting in ATDC5 cells, we next performed a time-course experiment to identify whether E2 can activate the phosphorylation of ERK1/2 in a time-dependent manner on day 14. After treatment of ATDC5 cells with 10^{-7} M E2, a rapid and transient phosphorylation of ERK1/2 was detected from 5 min and peaked at the 10 min time point and then gradually declined to basal levels within 60 min (Fig. 6A and B). A maximum fourfold stimulation was observed at 10 min. The protein levels of the internal controls, total ERK1/2 and $\beta$-actin, remained unchanged between samples.

**Role of ERs and ERK signaling in E2-induced expression of leptin receptors**

Our earlier research suggested that the ERK pathway should have an important role in the chondrocyte differentiation process and indicated that this pathway should be activated by ER stimulation. In order to analyze the involvement of ERK1/2 in E2-induced expression of Obr, we used an inhibitor of ERK1/2 (U0126; Cell Signaling Technology) and an antagonist of ERs (ICI 182 780; Tocris Bioscience)

Figure 3  Effect of E2 on Obr mRNA expression in a dose-dependent manner. ATDC5 cells were cultured in DMEM/F12 containing 5% FBS and 1% ITS in six-well plates at a density of 6\times10^4/well for 14 days. Cultured cells were then treated without and with E2 at 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M for 48 h. Obr gene expressions were analyzed by real-time PCR, normalized against $\beta$-actin, and compared with the control group. Data represent mean±S.E.M. from triplicate samples in three independent experiments. *$P<0.05$ vs control.

Figure 4  Effect of PPT and DPN on OBR mRNA and protein. ATDC5 cells were cultured in DMEM/F12 containing 5% FBS and 1% ITS in six-well plates at a density of 6\times10^4/well for 14 days. Cultured cells were then treated without and with PPT or DPN at 10^{-7} M for 48 h. Obr gene expressions (A) were analyzed by real-time PCR, normalized against $\beta$-actin, and compared with the control group. OBR protein levels were analyzed by western blot using specific antibodies as indicated. Bands (B) show representative western blots, whereas graphs (C) show normalized data. Data represent mean±S.E.M. from triplicate samples in three independent experiments. *$P<0.05$ vs control.
to examine their effects on E2-induced phosphorylation of ERK1/2 by western blotting. Cells were pretreated with U0126 (5 or 20 μM) and ICI 182 780 (1 μM) for 90 min and then incubated with or without E2 (10^{-7} M) for 10 min (Fig. 4C and D). The application of U0126 suppressed the effect of E2 on ERK1/2 phosphorylation in ATDC5 cells in a concentration-dependent manner. Even the lowest concentration of U0126 used (5 μM) significantly decreased the phosphorylation of ERK1/2 by 80% compared with the no inhibitor-treated cells (P<0.05). Similarly, treatment with ICI 182 780 prevented E2-induced increases in the amount of p-ERK1/2 protein. However, neither U0126 nor ICI 182 780 changed the total amounts of ERK1 and ERK2 proteins. These results confirmed that ERK phosphorylation occurs as a result of ER-mediated activation of ERK signaling.

In order to demonstrate that the E2-induced expression of the Obr gene is dependent on the increase in ERK phosphorylation resulting from ER activation, ATDC5 cells were pretreated with U0126 (20 μM) or ICI 182 780 (1 μM) for 90 min and then incubated with E2 (10^{-7}) for 48 h. The alterations in Obr expression produced by this treatment were quantified using real-time PCR and western blotting. Analysis of the results indicated that the upregulation of OBR mRNA and protein expression mediated by E2 was significantly blocked by U0126 or ICI 182 780 (Fig. 7A and B; P<0.05), in agreement with the effect of E2-mediated phosphorylation of ERK1/2. Treatment with ICI 182 780 or U0126 alone did not alter the steady-state level of OBR mRNA or protein. Taken together, these results strongly suggest that estrogen exerts its effect through the action of ERs on Obr expression and reinforce the notion that E2 has a role in regulating OBR mRNA and protein expression via the ERK1/2 pathway.

**Discussion**

The activation of OBR in growth plate chondrocytes modulates several events associated with the differentiation and maturation of chondrocytes (Nakajima et al. 2003, Kishida et al. 2005). In this study, experiments in the chondrogenic ATDC5 cell line have elucidated for the first time some of the mechanisms whereby estrogen was able to regulate the OBR. The results of the study showed that OBR was dynamically expressed during chondrocyte differentiation in increasing amounts and that the application of E2 for 48 h on day 14 could upregulate both OBR mRNA and protein during the middle stages of differentiation. This effect was mediated through the activation of ERs and induction of the ERK1/2 signaling pathway.

The ATDC5 cell line is an excellent growth plate chondrocyte model. The use of this model was validated in our study with the cells expressing chondrogenic markers in a relevant progression over a 21-day period. In particular, cells initially experienced an increase in the expression of mRNA of type II collagen, which is the predominant extracellular matrix in the proliferating stage and coincides with the differentiation phase. The increase in type II collagen was then reversed, whereas the expression of collagen was dominated by type X, a unique marker in the hypertrophic stage of growth plate chondrocytes. This pattern of collagen expression in ATDC5 cells is in agreement with previous reported results (Nakajima et al. 2009, Challa et al. 2010, Snelling et al. 2010). Therefore, the change in collagen type II and X expressions clearly define two critical events that occur during cartilage formation: the early differentiation of committed stem cells into chondrocytes and the terminal differentiation of proliferating to hypertrophic chondrocytes. Thus, we investigated the role of E2 in Obr expression during differentiation at day 14, a time point where the maximum amounts of both types of collagen were expressed, which occurs during the middle stage between the generation of proliferative and prehypertrophic chondrocytes.

In parallel with the results of collagen expression analysis, we also confirmed that ATDC5 cells express OBR starting from the first day of culture and steadily increasing over the next 21 days as the cells move through the various differentiation phases. This observation is consistent with results from a previous study in which ATDC5 cells were cultured for 6 weeks (Kishida et al. 2005). The finding that the expression of Obr increases over the differentiation stages suggests that OBR may mediate different functions. This study also provided evidence that Obr expression could be regulated by E2 (Fig. 2). The concentration of E2 used in the study was selected based on the study by Kato et al. (2010) who used 10^{-7} M E2 to demonstrate a cross talk between ERα and the P38/MAPK pathway in ATDC5 cells. Rodd et al. (2004) also indicated that only 10^{-7} M E2 was capable of inducing the maturation of prehypertrophic chondrocytes into the hypertrophic phenotype, which was associated with
the appearance of type X collagen in bovine fetal epiphyseal growth plate chondrocytes. Here we have reconfirmed those results as our application of increased concentrations of E2 from 10^{-7} to 10^{-6} M resulted in a concentration-dependent change in Obr mRNA, with 10^{-7} M being the most effective (Fig. 3). Interestingly, both the lower and higher concentrations of E2 had a similar effect producing a bell-shaped effect curve whereby the Obr gene expression profiles suggested that the optimal effects of E2 occur within a narrow range.

One hypothesis for the narrow concentration window for E2 activity in relation to Obr expression is that the effects of estrogen are mediated through several opposite pathways. During longitudinal bone growth, ERα stimulates bone growth while ERβ inhibits bone growth (Chagin et al. 2004, Perry et al. 2008, Borjesson et al. 2010). In addition, ERβ can antagonize ERα activity in some control studies, evidence that supports a ‘yin yang’ relationship between ERβ and ERα (Perry et al. 2008). For example, at low concentrations of estrogen, ERβ is able to inhibit ERα-activated transcription from estrogen response elements. In contrast, at high concentrations of a ligand, ERβ does not inhibit ERα action; moreover, it induces its own transcription (Hall & McDonnell 1999). Thus, at different concentrations of estrogen, ERβ might have the capacity to promote different effects. Using mouse mammary epithelial cells, Helguero et al. (2005) showed that activation of ERβ opposes ERα-induced proliferation and increases apoptosis. Other studies have focused on the ratio of the ERα to ERβ expression (Shin et al. 2007, Yi et al. 2008) and showed that the ratio of ERα to ERβ expression in adipocytes is an important potential regulatory factor in leptin expression. As discussed above, the reported ability of ERβ to antagonize ERα activity adds complexity to the interpretation of this experiment; therefore, the functions of the two subtypes in ATDC5 cell were to be defined in this study. We used PPT and DPN to examine the selective role of ERα and ERβ in regulation of OBR in ATDC5 cells and found that PPT was more effective than DNP on Obr expression.
expression. It could thus be speculated that the effect of E2 on OBR in ATDC5 cells was predominantly through the actions on ERα.

In addition to the fact that E2 only has an effect within a narrow concentration range, the application of E2 for 24 or 48 h to ATDC5 cells shows that 48 h stimulation is required to produce a significant change in mRNA and consequent protein levels. Different mechanisms may be involved during the first 24 h of E2 exposure vs the latter half of the 48 h cell culture. From our observation that 24 h exposure to E2 did not induce significant change in OBR mRNA and protein, we speculated that E2 may indirectly stimulate the level of OBR mRNA and protein. This delay in the positive regulation of OBR by E2 during middle-stage chondrogenesis could be related to transcription levels and stability of Obr mRNA. The late response of protein suggested that the regulation may take place through an intermediate gene product, but the precise molecular mechanisms by which this effect is only seen following exposure for 48 h are not clear. Another possible explanation may be due to intracellular changes in the ATDC5 cells. Albrecht et al. (2009) found that in vitro culture conditions changed dramatically with time, and this change would influence the expression profile of the hormones and their receptors that are involved during the growth and differentiation of growth plate chondrocytes. In this study, we replaced the culture medium every other day (48 h) to lessen its influence; hence, we did not analyze receptor activation at additional time points such as 72 and 96 h.

Studies indicated that the MAPK pathway involving ERK (ERK1 and ERK2) played a critical role in chondrocyte differentiation as inhibition of ERK1/2 signaling with U0126 blocked insulin-induced chondrogenesis (Nakajima et al. 2004, Phornphutkul et al. 2006). It is also well known that ERs can initiate various phosphorylation-related signal transduction cascades (Acconcia & Kumar 2006). Thus, we hypothesized that the upregulation of OBR by E2 was due to an increase in ERK1/2 caused by E2 acting at ERs. Our results showed that E2 strongly activated the ERK1/2 pathway in ATDC5 cells and that the resultant ERK1/2 phosphorylation could be blocked in a dose-dependent manner by U0126. These findings were consistent with a previous report demonstrating that E2 caused a rapid phosphorylation of ERK1/2 at 9 min in rat growth plate chondrocytes (McMillan et al. 2006). Similarly, the application of the ER inhibitor ICI 182 780 blocked the E2-mediated upregulation of OBR. These observations indicated that activation of ERK1/2 and functional ERs is essential for E2-induced Obr expression during chondrogenic differentiation of ATDC5 cells. However, the molecular mechanisms about how ERK mediates the effects of E2 on Obr expression are unknown. ERK1/2 should not be the unique pathway activated in the ATDC5 cells, we believe, and multiple signaling pathways may be involved potentially in the regulation of chondrocyte differentiation.

In conclusion, this study indicated that Obr expression in chondrocytes increases over the duration of the cell differentiation process, and this expression could be upregulated by E2 in a leptin-independent manner requiring the presence of ERs. Furthermore, our findings suggested that this upregulation by E2 was mediated through ERK signaling.

Figure 7 Effect of U0126 and ICI 182 780 on E2-induced Obr expression. ATDC5 cells were cultured in DMEM/F12 containing 5% FBS and 1% ITS in six-well plates at a density of 6×10⁴well for 14 days. Cultured cells were pretreated with U0126 (20 µM) and ICI 182 780 (1 µM) for 90 min followed by treatment with 10⁻⁷ M E2 for 48 h. (A) Obr gene expressions were analyzed by real-time PCR, normalized against β-actin, and compared with the control group. (B) OBR protein levels were analyzed by western blot using specific antibodies as indicated. Bands show representative western blots, whereas graphs show normalized data. Data represent mean±S.E.M. from triplicate samples in three independent experiments.

*P<0.05 vs control, *P<0.05 vs noninhibited control.
pathways. The results of our research may have significant implications in understanding the mechanisms of longitudinal bone growth, but further studies are needed to confirm our findings and define the other possible mechanisms involved.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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