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Leptin Regulates Estrogen Receptor Gene Expression in ATDC5 Cells Through the Extracellular Signal Regulated Kinase Signaling Pathway

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ABSTRACT

Both estrogen and leptin play an important role in the regulation of physiological processes of endochondral bone formation in linear growth. Estrogen receptors (ERα and ERβ) are known as members of the superfamily of nuclear steroid hormone receptors and are detected in all zones of growth plate chondrocytes. They can be regulated in a ligand-independent manner. Whether leptin regulates ERs in the growth plate is still not clear. To explore this issue, chondrogenic ATDC5 cells were used in the present study. Messenger RNA and protein analyses were performed by quantitative PCR and Western blotting. We found that both ERα and ERβ were dynamically expressed during the ATDC5 cell differentiation for 21 days. Leptin (50ng/ml) significantly upregulated ERα and ERβ mRNA and protein levels 48 h after leptin stimulation ($P < 0.05$) at day 14. The up-regulation of ERα and ERβ mRNA by leptin was shown in a dose-dependent manner, but the most effective dose of leptin was different (100 and 1,000 ng/ml, respectively). Furthermore, we confirmed that leptin augmented the phosphorylation of ERK1/2 in a time-dependent manner. A maximum eightfold change was observed at 15 min. Finally, a specific ERK1/2 inhibitor, UO126, blocked leptin-induced ERs regulation in ATDC5 cells, indicating that ERK1/2 mediates, partly, the effects of leptin on ERs. These data demonstrate, for the first time, that leptin regulates the expression of ERs in growth plate chondrocytes via ERK signaling pathway, thereby suggesting a crosstalk between leptin and estrogen receptors in the regulation of bone formation. J. Cell. Biochem. 113: 1323–1332, 2012.

KEY WORDS: LEPTIN; ESTROGEN RECEPTOR; ATDC5; GROWTH PLATE; CHONDROCYTE

HIGHLIGHTS: Both ERα and ERβ were dynamically expressed during the ATDC5 cell differentiation. Leptin significantly upregulated ERα and ERβ mRNA and protein levels. Leptin augmented the phosphorylation of ERK1/2 in a time-dependent manner. The phosphorylation of ERK1/2 was involved in the effects of leptin on estrogen receptors.

Longitudinal bone development occurs at the epiphyseal growth plate by a complex process called endochondral ossification in which cartilage is first formed and then remodeled into bone tissue. The growth plate consists of three principal layers: the resting zone, proliferative zone, and hypertrophic zone. The proliferation, hypertrophy, and extracellular matrix secretion of chondrocytes result in chondrogenesis. The newly formed cartilage is invaded by blood vessels and bone cell precursors, and finally replaced by bone. This process is controlled by various endocrine, paracrine, and autocrine agents including growth factors, glucocorticoid, thyroid hormone, sex hormones, and leptin [Nilsson et al., 2005; Savendahl, 2005]. Any disturbances of the epiphyseal development and physiology will result in various skeletal abnormalities known as dysplasia [Burdan et al., 2009].

Estrogen plays a crucial role in the regulation of longitudinal bone growth. In humans, estrogen is responsible for the initiation of the pubertal growth spurt and closure of the growth plate in both sexes [Karperien et al., 2005; Perry et al., 2008]. The direct effects of estrogen are mediated through two known nuclear receptors, namely estrogen receptor-α (ERα) and -β (ERβ). Many studies have demonstrated the expression of ERα and ERβ in all zones of growth plate chondrocytes in various species including humans, rabbits, and rats [Nilsson et al., 2002, 2003]. ERα and ERβ exert opposite effects in the regulation of longitudinal bone growth: the former

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accelerates bone growth and contributes to growth spurt in puberty, while the latter inhibits growth [Chagin et al., 2004; Nilsson et al., 2005; Perry et al., 2008; Borjesson et al., 2010]. It has been reported that ERα is the main functional estrogen receptor and that ERβ can antagonize ERα activity in some species and tissues, supporting a "yin yang" relationship between ERβ and ERα [Hertrampf et al., 2008; Perry et al., 2008]. The expressions of ERs in the growth plate are regulated by hormones that is involved in longitudinal growth [van der Eerden et al., 2002; Yao et al., 2006].

Leptin, a 16-kDa protein encoded by the obese (ob) gene, is a circulating hormone secreted primarily from adipocytes. Leptin, like estrogen, also plays an important role in the regulation of physiological processes of endochondral bone formation in linear growth, modulating several events associated with differentiation, proliferation, and mineralization of growth plate chondrocytes [Kishida et al., 2005; Bertoni et al., 2009]. The effect of leptin on bone development is a combined result of a direct local endocrine/paracrine mechanism and an indirect mechanism via the central nervous system. The direct peripheral actions of leptin act through ubiquitously leptin receptors (Ob-Rs), which at least have six alternatively spliced forms [Bjorbaek and Kahn, 2004] and are present in growth plate chondrocytes [Nakajima et al., 2003; Kishida et al., 2005]. Upon binding to its receptor, leptin can activates many signaling pathways involving the Janus kinase/signal transducer and activator of transcription (JAK/STAT), as well as phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) which play a key role in regulating chondrocyte differentiation [Ben-Eliezer et al., 2007; Otero et al., 2007].

Colocalization of ERs and Ob-R in the growth plate and overlapping function of estrogen and leptin in the regulation of chondrocyte differentiation may suggest some interactions in long-bone growth. In recent years, several studies have found a cross talk between leptin and estrogen receptors in normal hypothalamus neurons and breast cancer cells [Maeso Fortuny et al., 2006; Gao and Horvath, 2008; Fusco et al., 2010]. Leptin directly regulates the activation and expression of ERα in the absence of its ligand and potentiates the effects of estradiol induced ERα activation [Catalano et al., 2004; Maeso Fortuny et al., 2006]. Binai et al. [2009] verified that ERα strongly increased leptin-induced STAT3 transactivation and target gene expression in breast cancer cells and down-regualtion of ERα abolished leptin-induced STAT3 phosphorylation. 17β-estradiol (E2) can induces leptin expression in placental trophoblastic cells through genomic and nongenomic actions involving ERα and MAPK and PI3K signal transduction pathways [Gambino et al., 2010]. While leptin can induce functional activation of ERα via extracellular signal-regulated kinase 1/2 (ERK1/ERK2, one of the MAPK cascades) signal pathways in breast cancer cells [Catalano et al., 2004]. Leptin has also been shown to interfere with the antiestrogen effect on ERα degradation produced by ICI 182,780 in breast cancer cells [Garofalo et al., 2004]. Yi et al. [2008] indicated that ERα exerted stimulatory effects on leptin production in adipocytes, whereas ERβ produced inhibitory effects. Taken together, these findings suggest a close connection between leptin and estrogen.

However, the direct effect of leptin on estrogen receptors in growth plate chondrocytes has not been fully elucidated. The purpose of the present study was to examine how leptin modulates the estrogen receptors. In our experimental system, we used the mouse chondrogenic ATDC5 cells, which can mimic the growth plate chondrocyte differentiation processes from chondroprogenitors to fully differentiated hypertrophic chondrocytes in the presence of insulin and thereby provide a excellent model to study the mechanisms underlying the regulation of endochondral bone growth in vitro [Challa et al., 2010; Snelling et al., 2010]. Both ERs and Ob-R were detected in ATDC5 cells [Kishida et al., 2005; Galal et al., 2008]. In this study, we observed that leptin induces an upregulation of ERα and ERβ in the middle stage of chondrocyte differentiation. Furthermore, we demonstrated, for the first time, that these actions are mediated through the ERK1/2 signaling pathway.

MATERIALS AND METHODS

REAGENTS

Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12; Invitrogen), fetal bovine serum (FBS; Gibco), insulin/transferrin/sodium selenite (ITS; Sigma), penicillin–streptomycin (Invitrogen). Alcian blue 8GX (Sigma). Recombinant mouse leptin (ProSpec bio). Rabbit polyclonal antibody to ERα (Santa Cruz) and ERβ (Abcam, Hongkong), mouse monoclonal antibody to β-actin (Beyotime, China). Monoclonal anti-phospho-ERK1/2 (Thr202/Thr204) and polyclonal antitotal-ERK1/2 antibody (Cell Signaling Technology). Monoclonal anti-Phospho-p38 MAPK (Thr180/Tyr182) and antitotal-p38 MAPK antibody (Cell Signaling Technology). MEK1/2 inhibitor U0126 (Cell Signaling Technology).

CELL CULTURE AND TREATMENTS

ATDC5 cells were cultured in a maintenance medium consisting of a 1:1 mixture of DMEM/F12 medium containing 5% FBS and 1% penicillin–streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air.

ATDC5 cells were plated at a density of 6×10^4/well in 6-multiwell plastic plates (Corning, New York, NY). Cells were subcultured at 70–80% confluency using differentiation medium, which is identical to maintenance media with the addition of 1% ITS. The differentiation medium was changed every other day from day 2 to 14. After differentiation for 14 days, cells were incubated in ITS-free medium containing 0.5% FBS for 24 h and then stimulated with leptin for another 24 or 48 h.

ALCIAN BLUE STAINING

ATDC5 cells were cultured for 4, 7, 14, and 21 days. Cells were rinsed with phosphate buffered saline (PBS) twice, fixed with 4% paraformaldehyde for 15 min, and then stained with 1% Alcian blue 8GX (pH 2.5) for 30 min. The stained cells were washed with PBS three times, and then photographed. Experiments were performed in triplicates.

REAL-TIME PCR ANALYSIS

Total RNA isolated from ATDC5 cells using 1 ml TRIzol reagent kit (Invitrogen) was dissolved in 0.1% diethylpyrocarbonate (DEPC) water and quantified by spectrophotometry at 260 nm (absorbance). cDNA was synthesized from 2 μg total RNA through reverse transcription using a TaKaRa RNA PCR Kit Ver. 2.1 (TaKaRa Bio)

according to manufacturer's protocol. The sequences for primers (TaKaRa Biotechnology Co. Ltd) used to amplify mRNA were as follows: ERα: 5′-TGGGCTTATTGACCAACCTAGCA-3′, 5′-AGAATCTCCAGCCAGGCACAC-3′; ERβ: 5′-GACTGTAGAACGGTGTGGTCATCAA-3′, 5′-CCTGTGAGGTAGGAATGCGAAAC-3′; Col II: 5′-GTCCTGAAGGTGCTCAAGGTTCTC-3′, 5′-AGGAATACCATCAGTCCCTGGGTTA-3′; Col X: 5′-AGAACGGCACGCCTACGAT-3′, 5′-CTGTGAGCTCCATGATTGCA-3′; β-actin: 5′-CATCCGTAAAGACCTCTATGCCAAC-3′, 5′-ATGGAGCCACCGATCCACA-3′. Quantitative Real-time PCR was carried out using 1 μg of cDNA and SYBR Green (Bio-Bad) in 96-well plates in a LightCycler rapid thermal cycler system (Roche Diagnostics) according to the manufacturer's instructions. PCR products were subjected to melting curve analysis, with the data analyzed and quantified using RotorGene 6.0 analysis software. Relative quantification of each gene was normalized to β-actin. We used the $2^{-\Delta\Delta Ct}$ (cycle threshold) method to calculate relative gene expression levels as previously described [Maymo et al., 2010]. For the treated samples, results are presented as fold changes in gene expression normalized to β-actin, and relative to control conditions (untreated cultures). Analysis of the results is based on triplicate (or more) samples.

WESTERN BLOT ANALYSIS

Protein extracts were assayed using a Western & IP Cell lysis Kit (Beyotime, China). The protein of cell lysate was determined by Bio-Rad assay using manufacturer's protocol (Bio-Rad). Forty-micrograms of total protein from each sample was separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). After the PVDF membrane had been incubated with 10 mM TBS with 1.0% Tween 20 and 10% dehydrated skim milk to block nonspecific protein binding, the membranes were incubated at 4°C overnight with specific antibody against ERα, ERβ, or anti-phospho ERK1/2 antibody or anti-phospho p38 antibody. Loading controls were performed by immunoblotting the same membranes with anti-β-actin or anti total-ERK 1/2 antibody or anti total-p38 antibody. The membranes were washed with TBST and then incubated with alkaline phosphatase-linked secondary antibodies (Jackson Immunoresearch, PA). After being washed with TBST, immunoreactive bands were visualized using NBT/BCIP as substrate. Protein expression was quantified by densitometry analysis using the NIH image J software. Quantitative densitometric values of each protein were normalized to β-actin or to the nonphosphorylated form of the protein.

STATISTICAL ANALYSIS

All data were presented as mean ± standard error of the mean (SEM) for at least three independent experiments, each with at least three or more independent observations. Statistical analyses were performed by the one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test or the unpaired Student's *t* test using the SPSS 11.5 statistical software program. A P values <0.05 was considered significant.

RESULTS

DIFFERENTIATION IN ATDC5 CELLS

Chondrogenic differentiation of ATDC5 cells was characterized by expression of extracellular matrix genes such as type II and type X collagens. We evaluated the expression of chondrogenic differentiation markers by using real-time PCR. As Figure 1A shows, Col II expression began to increase at day 1 in culture, which indicates the differentiation of mesenchymal cells into chondrocytes, markedly increased between days 7 and 14, which indicates early-stage differentiation of chondrocytes. The level of Col X mRNA gradually increased from day 7 onwards and maintained high levels between days 14 and 21, indicating late-stage differentiation of chondrocytes. On Day 21, ATDC5 cells expressed type X collagen which

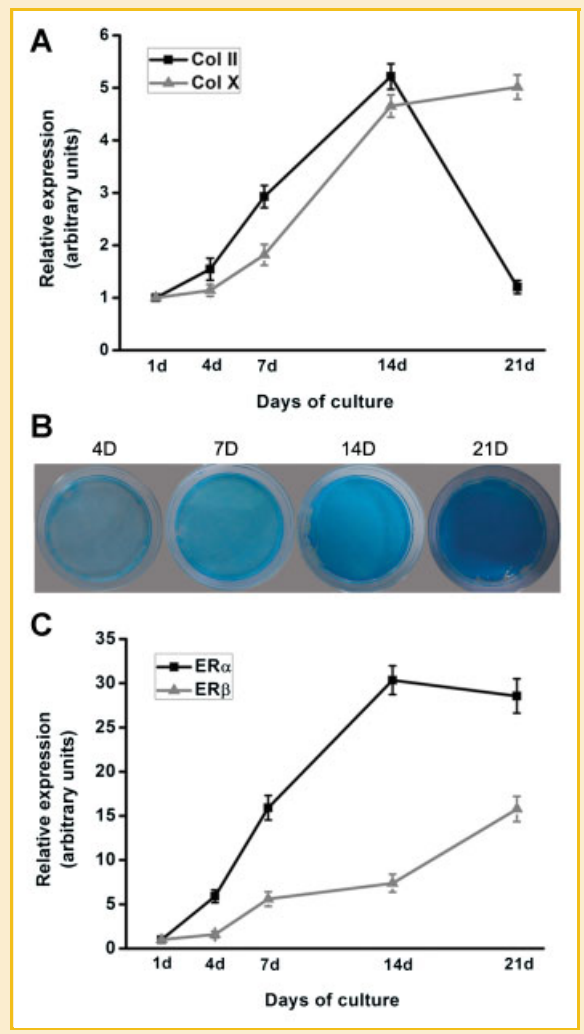

Fig. 1. ATDC5 cell differentiation in culture. ATDC5 cells were cultured in 6-well plates at a density of 6×10^4/well in DMEM/F12 medium containing 5% FBS and ITS for 1, 4, 7, 14, and 21 days. Relative expression of collagen types II and X (A) and ERα and ERβ (C) was determined by real-time PCR. Data represent mean ± SEM from triplicate samples in three independent experiments normalized against β-actin. B: Alcian blue staining of cells for 4, 7, 14, and 21 days. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

substituted for type II collagen. Matrix proteoglycan synthesis was verified by stained nodules with Alcian blue dye, as shown in Figure 1B, staining intensities noted in ATDC5 cells cultured with insulin was gradually increased in a time-dependent manner from 4 to 21 days.

These results showed that undifferentiated ATDC5 cells differentiate in culture into proliferative chondrocytes then to hypertrophic chondrocytes, validating the use of this cell system as an in vitro model to study chondrocyte differentiation.

EXPRESSION OF ESTROGEN RECEPTORS IN ATDC5 CELLS

We further examined the expression changes of estrogen receptors during ATDC5 cell differentiation in culture. The results of real-time PCR confirmed that both ERα and ERβ were dynamically expressed in ATDC5 cells during all of the differentiation phases (Fig. 1C).

The ERα mRNA levels were sharp increased with the progression of chondrogenic differentiation (days 4–14), and slightly decreased after days 14. The ERβ levels were increased slowly before days 14 and then increased greatly during the hypertrophy stages as characterized by the induction of type II and type X collagen transcripts (days 14–21).

EFFECT OF LEPTIN ON ESTROGEN RECEPTORS IN ATDC5 CELLS

To determine whether leptin plays a role in regulating the expression of ERs in chondrocytes, we first tested the effect of leptin on the mRNA of ERα and ERβ on day 14 in ATDC5 cells. ATDC5 cells were treated with 50 ng/ml leptin for up to 48 h. Then ERα and ERβ expression was analyzed by real-time PCR.

We first compared the gene expression of ERs in the ATDC5 cells in response to leptin treatment for 24 and 48 h, respectively, with the control. No significant difference in ERα gene levels was observed except for a decreasing tendency between the leptin treated cells and the control at 24 h while ERβ was significantly increased. After 48 h, both the mRNA of ERα and ERβ was significantly increased as stimulated by leptin compared with the control (Fig. 2A,B). The results also show that ERα and ERβ mRNA expression was significantly enhanced by leptin treatment for 48 h in comparison to that for 0 h.

To confirm the regulation effect on the expression of ERs by leptin, we treated ATDC5 cells with increasing concentrations of leptin for 48 h. Consequently, increasing concentrations of leptin resulted in further upregulation of ERα mRNA, with the concentration of 100 ng/ml being the most effective, whereas for the upregulation of ERβ mRNA, the dose of 1,000 ng/ml was the most effective (Fig. 2C). Thus, the results were shown in a dose-dependent manner.

We then examined whether the changes in the ER mRNA were followed by a corresponding change in the protein. After ATDC5 cells were treated with 50 ng/ml leptin for 24 and 48 h, ER protein level was analyzed by Western blot. The cell lines expressed higher levels of ERα and ERβ protein. No significant change in ER protein level was noted at 24 h after leptin stimulation. The levels of ERα and ERβ protein significantly increased in the presence of leptin after 48 h of treatment compared to the control (Fig. 3). The results also showed that ERα and ERβ protein levels were significantly enhanced at 48 h after leptin treatment comparison with that at 0 h. No change in protein level of housekeeping protein, actin, was noted in immunoblots at any time points examined. These data suggest that the regulation of ER gene expression by leptin correlates with changes at the protein level.

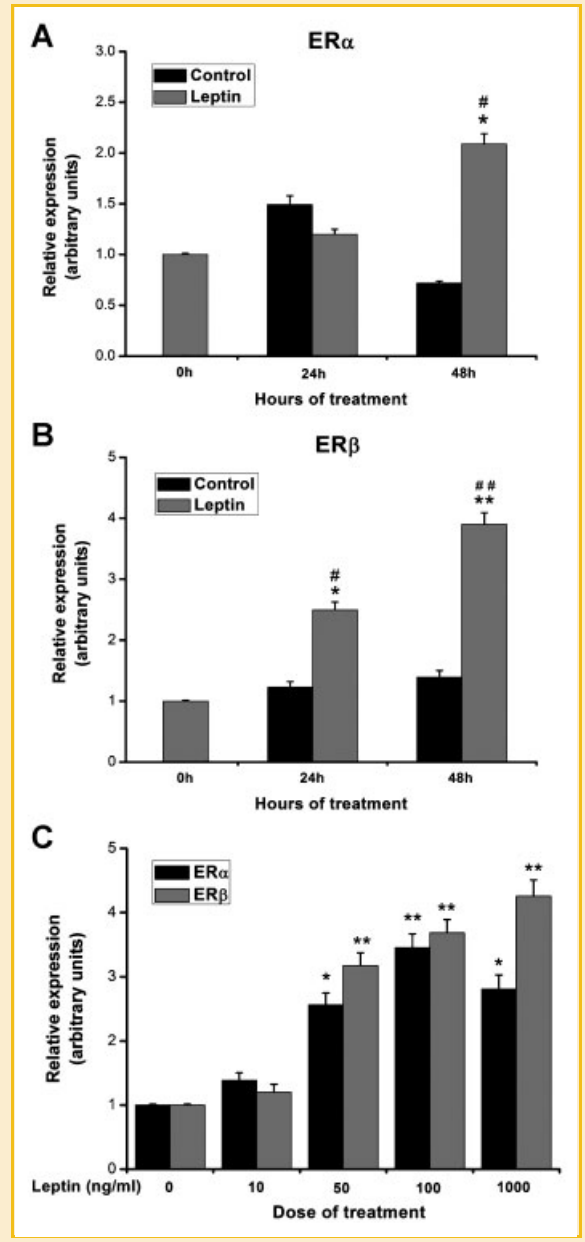

Fig. 2. Effect of leptin on ERα and ERβ mRNA expression. ATDC5 cells were cultured in DMEM/F12 containing 5% FBS and ITS in 6-well plates at a density of 6×10^4/well for 14 days. Cultured cells were then treated without and with leptin at 50 ng/ml for 24 and 48 h. ERα (A) and ERβ (B) gene expressions were analyzed by real-time PCR, normalized against β-actin and compared to the control group. C: Cultured cells were treated without and with leptin at 10, 50, 100, and 1,000 ng/ml for 48 h. ERα and ERβ gene expressions were analyzed by real-time PCR, normalized against β-actin and compared to the control group (without leptin). Data represent mean ± SEM from triplicate samples in three independent experiments. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ versus 0 h, $^{\#\#}P < 0.01$ versus 0 h.

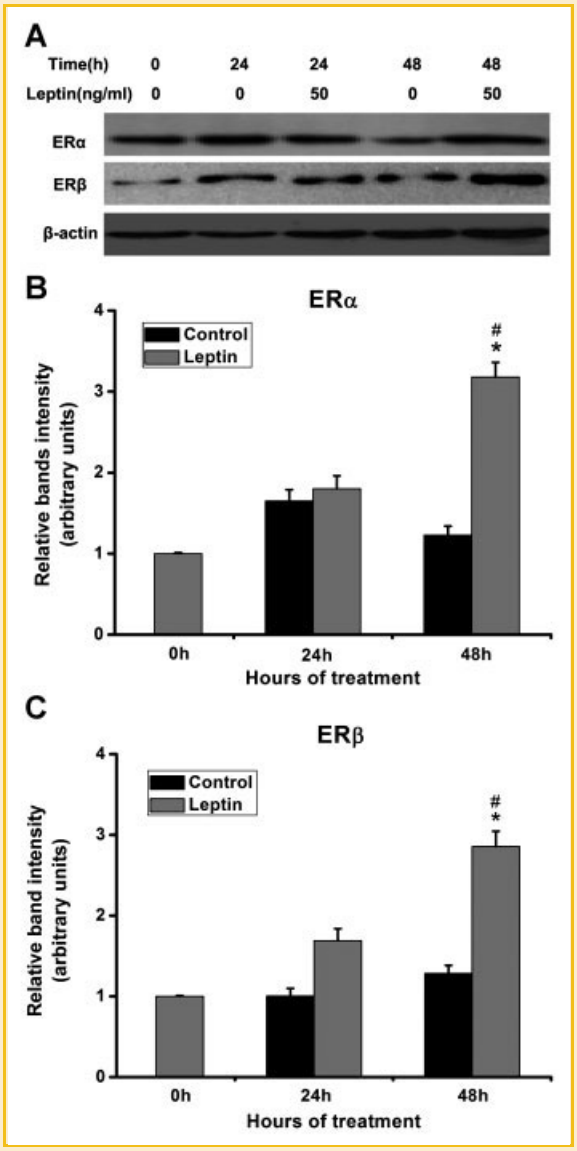

Fig. 3. Effect of leptin on ERα and ERβ protein levels. ATDC5 cells were cultured in DMEM/F12 containing 5% FBS and ITS in 6-well plates at a density of 6×10^4/well for 14 days. Cultured cells were treated without and with leptin at 50 ng/ml for 24 and 48 h. ERα and ERβ protein levels were analyzed by Western blot using specific antibodies as indicated. Bands (A) show representative Western blots, whereas graphs (B,C) show normalized data. Data represent mean ± SEM from triplicate samples in three independent experiments. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ versus 0 h, $^{\#\#}P < 0.01$ versus 0 h.

ACTIVATION OF ERK SIGNALING BY LEPTIN IN ATDC5 CELLS

To identify whether leptin can activate the phosphorylation of ERK1/2 in a time-dependent manner on day 14, we next performed a time-course experiment using Western blotting.

Treatment of ATDC5 cells with 50 ng/ml leptin induced rapid and transient phosphorylation of ERK1/2 which was detected from 5 min after treatment and peaked at the 15 min time point and then gradually declined to basal levels within 60 min. (Fig. 4A,B). A maximum eightfold stimulation was observed at 15 min. As internal controls, total protein levels of ERK1/2 and actin remained unchanged between samples.

ROLE OF ERK SIGNALING IN LEPTIN-INDUCED ESTROGEN RECEPTORS

As noted above, the ERK pathway has an important role in the chondrocyte differentiation process. To assess the involvement of ERK1/2 in leptin induced expression of estrogen receptors, we first investigated the inhibitory effects of ERK1/2 inhibitors (U0126) on leptin-induced phosphorylation of ERK1/2 by Western blotting. Our data indicate that U0126 dose-dependently suppressed the effect of leptin on ERK1/2 phosphorylation in ATDC5 cells (Fig. 4C,D). Even low concentrations (5 μM) significantly decreased phosphorylation levels by 80% compared to the leptin-induced, noninhibited culture. The results confirmed that MEK was involved in the ERK phosphorylation induced by leptin.

To further demonstrate whether leptin-induced expression of the ER gene is mediated through ERK pathway, ATDC5 were pretreated with ERK1/2 antagonist U0126 (5 or 20 μM) for 1 h and then incubated with 50 ng/ml leptin for 48 h. Using RT-PCR and Western blot analyses, we found that the effect of leptin on upregulation of ERα and ERβ mRNA (Fig. 5C) and protein (Fig. 5A,B) were blocked by U0126. Total inhibition was observed already with the lowest concentration of U0126 used (5 μM), in agreement with the effect on phosphorylation. Taken together, these results strongly suggest that the regulation of ER mRNA and protein by leptin is mediated by ERK1/2 phosphorylation.

EFFECT OF LEPTIN ON p38 MAPK IN ATDC5 CELLS

We further performed additional experiments to examine the effect of leptin (50 ng/ml) on p38 phosphorylation with anti-phospho-p38 and anti-p38 antibodies. As shown in Figure 6, leptin-induced p38 phosphorylation was not shown in a time-dependent manner in the ATDC5 cells at days 14. Phosphorylation was maximal after 5 min and decreased thereafter. Assessment of phosphorylated-p38 levels relative to total-p38 showed that incubation with leptin could activate p38 MAPK by up to 1.8-fold after 5 min. However, unlike ERK1/2, we failed to find significant difference of leptin on p38 phosphorylation, suggesting that p38 is not a major MAPK pathway on day 14.

DISCUSSION

Estrogen receptors (ERα and ERβ), which plays an essential role in longitudinal bone growth, can be transcriptionally activated in a ligand-independent manner. Considering that unliganded ER is an effector of MAPK signal and that leptin is able to activate the MAPK pathway [Machinal-Quelin et al., 2002; Catalano et al., 2004], in the present study we utilized the ATDC5 chondrogenic cell line to investigate the ability of leptin to regulate ERs. We found that both ERα and ERβ were dynamically expressed during chondrocyte differentiation. Moreover, pretreatment of leptin for 48 h can upregulate both ERα and ERβ expression in the middle stage of differentiation, and that such regulation was through leptin induced ERK1/2 signaling pathway. To the best of our knowledge, this

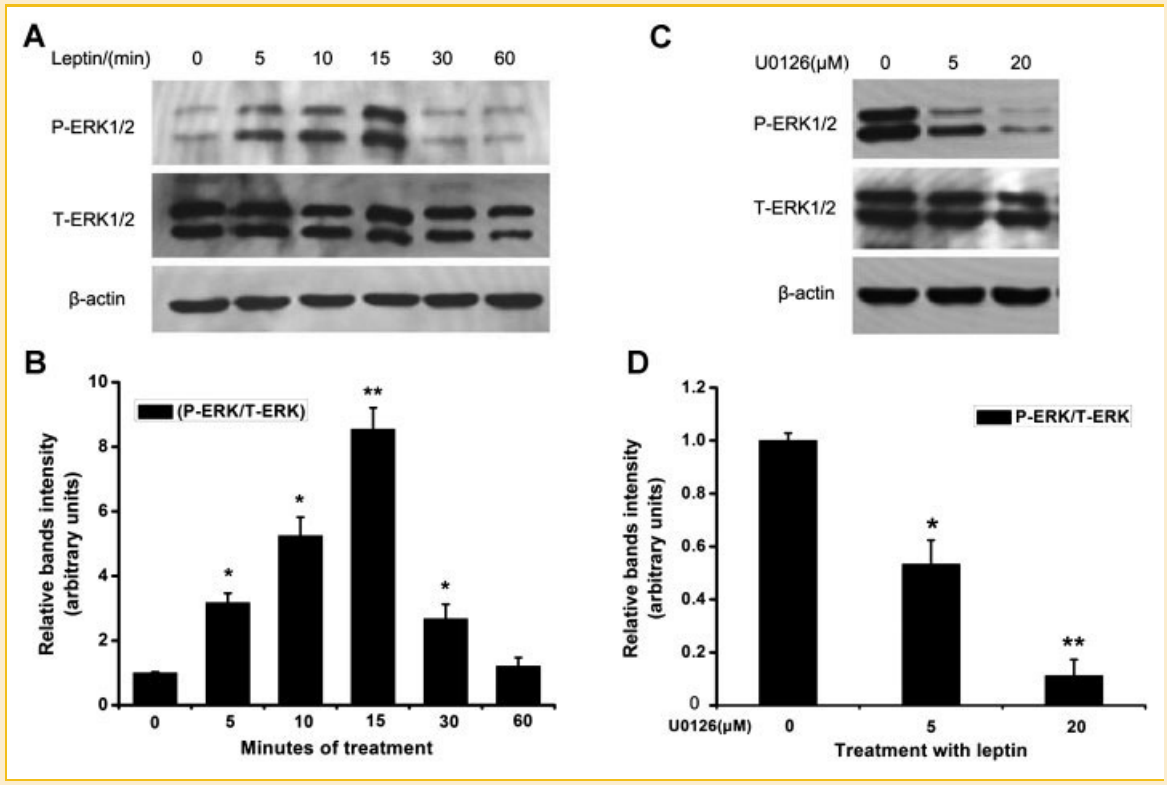

Fig. 4. Effect of leptin on ERK1/2 phosphorylation levels. ATDC5 cells were cultured in DMEM/F12 containing 5% FBS and ITS in 6-well plates at a density of 6×10^4/well for 14 days. A,B: Cultured cells were treated with 50 ng/ml leptin for indicated times. P-ERK1/2 and ERK1/2 protein levels were analyzed by Western blot. Bands (A) show representative Western blots, whereas graphs (B) show normalized data. C,D: Cultured cells were treated with U0126 (5 and 20 μM) for 60 min and then treated with 50 ng/ml leptin for 15 min. P-ERK1/2 and ERK1/2 protein levels were analyzed by Western blot. Bands (A) show representative Western blots, whereas graphs (B) show normalized data. Data represent mean ± SEM from triplicate samples in three independent experiments. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control.

research is the first to shed light on the effects of leptin on estrogen receptors in ATDC5 cells.

ATDC5 cell line is an excellent growth plate model and less phenotypically diverse than primary cultures. The cells were validated in this study and were shown to express chondrogenic genes in a relevant progression over a 21-day period. With regard to the differentiation, cells initially experienced a increase and then a decrease in the mRNA of type II collagen, which is the predominant extracellular matrix in the proliferating stage, and at last the mRNA of type II collagen was replaced by type X, which was a unique marker in the hypertrophic stage of growth-plate chondrocytes. These observations were in agreement with previously shown results [Challa et al., 2010; Snelling et al., 2010]. Therefore, we can clearly define two critical events during cartilage formation: the early differentiation of committed stem cells into chondrocytes and the terminal differentiation of proliferating to hypertrophic chondrocytes. In the present study, leptin was added to the culture medium from day 14, a time when ATDC5 cells expressed a large amount of type II and type X collagen mRNA, during the middle stage between proliferative and prehypertrophic chondrocytes.

We confirmed that ATDC5 cells expressed two estrogen receptor isoforms, starting from the first day of culture and throughout its duration. Both ERα and ERβ were dynamically changed during the differentiation phase. In the human growth plate, both ERα and ERβ expression was seen in all zones, with high levels in proliferative and prehypertrophic chondrocytes and lower levels in the late hypertrophic zone [Bord et al., 2001; Nilsson et al., 2002]. Yao et al. [2006] detected that ERα was preferentially localized in the cytoplasm, whereas ERβ showed a mainly nuclear stain in the mouse growth plate chondrocytes.

The finding that dynamic expression of ERs correlated with the differentiation stages in the current study may suggest that ERα and ERβ mediate different functions. The pattern of receptor that dominates the regulation of skeletal growth depends on the sex, species, and even the dose of estrogen [van der Eerden et al., 2002; Chagin et al., 2004; Takano et al., 2007]. In humans, higher doses of estrogen during puberty can lead to growth plate fusion, which are primarily mediated through ERα [Nilsson and Baron, 2005]. At low concentrations of estrogen, ERβ had the capacity to repress ERα-activated transcription from estrogen response elements. In contrast, at high concentrations of ligand, ERβ did not inhibit ERα action but induced its own transcription [Hall and McDonnell, 1999; Pettersson et al., 2000]. Thus, ERβ might have the capacity to promote different effects depending on the concentrations of estrogen. Recent study [Chagin et al., 2006] has shown that endogenously produced estrogen had the capacity to promote chondrocyte proliferation and protect them from programmed cell death.

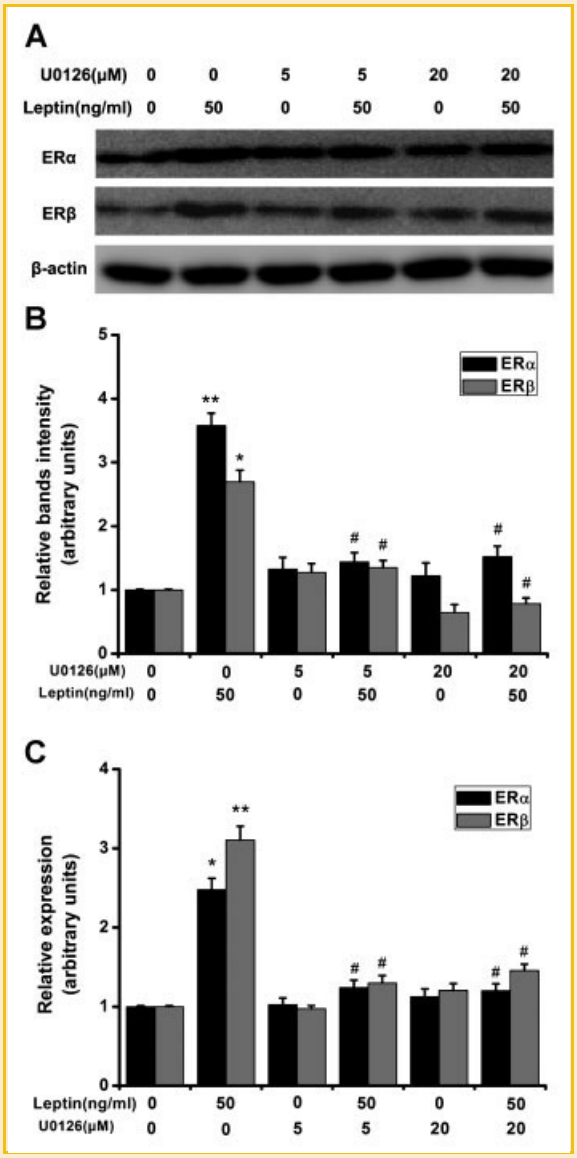

Fig. 5. Effect of U0126 on leptin-induced ERα and ERβ expression. ATDC5 cells were cultured in DMEM/F12 containing 5% FBS and 1% ITS in 6-well plates at a density of 6×10^4/well for 14 days. ATDC5 cells were then pretreated with U0126 (5 and 20 μM) for 60 min followed by treatment with 50 ng/ml leptin for 48 h. A,B: ERα and ERβ protein levels were analyzed by Western blot using specific antibodies as indicated. Bands (A) show representative Western blots, whereas graphs (B) show normalized data. C: ERα (A) and ERβ (B) gene expressions were analyzed by real-time PCR, normalized against β-actin and compared to the control group. Data represent mean ± SEM from triplicate samples in three independent experiments. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ versus noninhibited control.

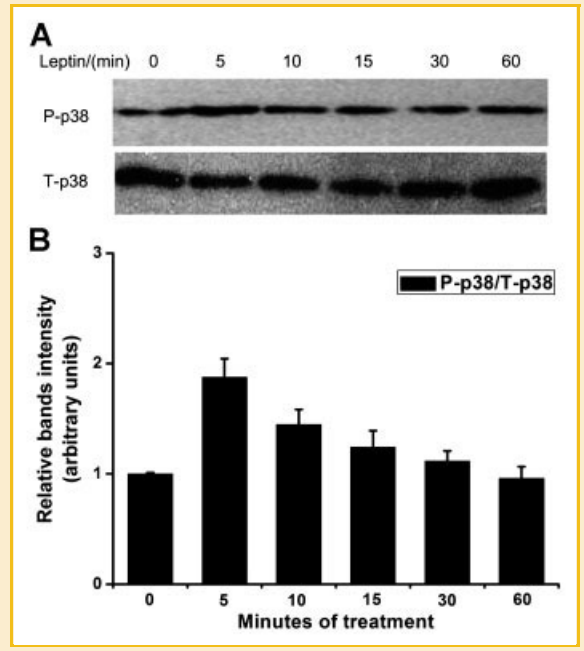

Fig. 6. Effect of leptin on p38 phosphorylation levels. ATDC5 cells were cultured in DMEM/F12 containing 5% FBS and ITS in 6-well plates at a density of 6×10^4/well for 14 days. A,B: Cultured cells were treated with 50 ng/ml leptin for indicated times. P-p38 and p38 protein levels were analyzed by Western blot. Bands (A) show representative Western blots, whereas graphs (B) show normalized data. Data represent mean ± SEM from triplicate samples in three independent experiments.

Experiments with estrogen receptor knockout mice have demonstrated the different role of ERα and ERβ in the regulation of longitudinal bone growth [Vidal et al., 2000; Chagin et al., 2004]. With the ERβ knockdown, the inhibition of appendicular- and axial-skeletal growth is noted in young adult female mice [Chagin et al., 2004]. ERβ also inhibits chondrocyte proliferation and reduces the ratio of proliferative to hypertrophic chondrocytes in the growth plate [Chagin et al., 2004]. Using the ERα knockdown mice, ERα is the main functional ER in the mouse growth plate and ERβ slightly modulates longitudinal bone growth in female but not in male mice [Windahl et al., 1999; Vidal et al., 2000]. The loss of ERα in mice is associated with shortening of the long bones, suggesting a growth-plate effect, while female ERβ deficient mice have increased trabecular bone density, suggesting that ERβ played a role in bone matrix synthesis [Lindberg et al., 2001; Cao et al., 2003]. However, Borjesson et al. [2010] found that ERα was not required for skeletal growth during early sexual maturation.

The present study has provided evidence that leptin can regulate ER expression. The dose of leptin we selected based on a previous study [Ben-Eliezer et al., 2007] in which 50 ng/ml leptin was used to demonstrate pathways in the differentiation of ATDC5 cells. Kishida et al. [2005] clearly indicated that 1–10 ng/ml of leptin, equivalent to normal circulating level, strongly inhibited matrix mineralization, decreased the rate of apoptosis and altered the type X collagen expression pattern. However, in the current study, no significant change was found in the mRNA levels of ERs after 10 ng/ml leptin treatment. Leptin with concentrations increasing from 10 to 1,000 ng/ml has induced a dose-dependent change in the mRNA levels of ERs. Interestingly, the most effective concentration of leptin that affected ERα was different from ERβ (100 vs. 1,000 ng/ml).

Here, we report that leptin act as a positive regulator of ERα and ERβ at the middle stage chondrogenesis. The regulation could be related to transcription levels and mRNA stability of ERα and ERβ.

The 24 and 48 h experiment in ATDC5 cells revealed a significant change in mRNA level till 48 h after treatment. This late response suggested the regulation may take place through an intermediate gene product. Furthermore, we showed that the change in ER expression at the mRNA level was followed by an enhanced production of the corresponding protein. Catalano et al. [2004] have shown that leptin can down-regulate mRNA and total protein of ERα in MCF-7 cells and that leptin had the ability to induce ERα nuclear localization. On the contrary, Garofalo et al. [2004] showed that leptin alone had no significant effects on ERα expression in the cytoplasmic and nuclear compartments in MCF-7 cells. Feuermann et al. [2009] found that leptin upregulated the expression of ERα in the mammary epithelial cells in a dose-dependent manner. These findings supported our hypothesis that leptin regulated the expression of ERs in ATDC5 cells. Whether ERs are upregulated or downregulated may depend on many factors, such as cell type, culture conditions, leptin concentrations, and time.

Ob-Rs are localized in growth plate chondrocytes. Leptin modulates several events associated with differentiation and matrix maturation of chondrocytes in growth plate [Nakajima et al., 2003; Kishida et al., 2005]. Kume et al. [2002] suggested that leptin exert its influence on endochondral ossification by regulating angiogenesis. Other authors revealed the molecule mechanism that leptin may affect chondrocyte proliferation and differentiation by activating the PTHTP-Ihh and Wnt/β-catenin signaling pathway in the growth plate [Gat-Yablonski et al., 2007; Ohba et al., 2010]. From these findings, we conclude that leptin has the potential to modify chondrogenesis and that its effects may indirectly influence the expression of ERs by affecting chondrocyte proliferation and differentiation.

The mitogen-activated protein kinase pathway involving ERK (ERK1 and ERK2) kinases was crucially involved in chondrocyte differentiation as inhibition of ERK signaling with U0126 blocked insulin-induced chondrogenesis [Phornphutkul et al., 2006]. In the present study, we found that the specific inhibitor of ERK, U0126, inhibited the expression of ER at both mRNA and protein levels 48 h after leptin stimulation, indicating that activation of ERK1/2 is essential for leptin-induced ER expression in chondrogenic differentiation of ATDC5 cells. The findings described here were consistent with previous results reported by Ben-Eliezer et al. [2007]. They also found that leptin can induce the translocation of ERK1/2 into the nucleus and that the effects of leptin on type X collagen expression were specifically mediated through ERK1/2.

In MCF 7 cells, leptin induced a strong phosphorylation of the ERK1/2 pathways with an increased cell viability and proliferation associated with an increased expression of ERα receptor. The effects induced by leptin were inhibited when ObR was neutralized using either a monoclonal inhibitory antibody to ObR or ObR gene silencing siRNA [Fusco et al., 2010]. These data suggest that there is a bidirectional communication between ObR and ERα. Further observation strengthened that leptin-induced proliferation of breast cancer cells correlates with ER expression levels [Ray et al., 2007]. Binai et al. [2009] demonstrated that ERα stimulated the leptin-mediated STAT3 in the cytoplasm in breast cancer cells independent of ERα ligands, underlying a crosstalk between leptin stimulation and ERα expression.

Interestingly, we observed that ERα mRNA 24 and 48 h after leptin stimulation had different tendency. The precise molecular mechanisms by which the regulation tendency was opposite between 24 and 48 h are not clear. We speculate that different dominant mechanisms may be involved for 24 or 48 h cell culture. One of the possible mechanisms may be the intracellular changes in ATDC5 cells. Albrecht et al. [2009] found that in vitro culture conditions were dramatically changed with time which influence the expression profile of the hormones and their receptors that are involved during the growth and differentiation process. We compared ERs at 24 and 48 h with at 0 h, they indeed had some changes.

However, the reported ability of ERβ to antagonize ERα activity adds complexity to the interpretation of these experiments. In mouse mammary epithelial cells, the activation of ERβ opposed ERα-induced proliferation and increased apoptosis [Helguero et al., 2005]. It could thus be speculated that the observed decrease in ERβ after leptin treatment could cause an enhanced ERα-mediated signaling in the ATDC5 cells. Some studies focused on the ratio of the ERα to ERβ expression [Shin et al., 2007; Yi et al., 2008] and found that the ratio of the ERα to ERβ expression in adipocytes was an important potential regulatory factor in leptin expression. The effect of leptin on ERs may be also related to the relative ratio of ERα to ERβ expression during ATDC5 differentiation. These results may explain the reasons why leptin regulates both ERα and ERβ expression dynamically.

We found that the regulation of ER expression by leptin was prevented by specific blockade of the ERK1/2 signaling pathway. However, the molecular mechanisms by which ERK mediates the effects of leptin on ER expression are unknown. As many signaling pathways involved in the regulation of chondrocyte differentiation, it seems reasonable for us to speculate that ERK1/2 is not the exclusive pathway activated by leptin. Although JAK/STAT3 and PI-3K have already been suggested to mediate the effects of leptin on gene expression in ATDC5 cells [Ben-Eliezer et al., 2007; Otero et al., 2007], it remains to be determined whether these pathways play a role in leptin-induced expression of ERs in chondrocytes.

Based on our data, leptin exerts its activity not only through leptin receptor, but also through crosstalk with other signaling systems implicated in chondrogenesis. In this study, we focused our attention on the relationship between the leptin axis and the estrogen receptors. We have for the first time demonstrated estrogen-independent ER activation mechanisms caused by leptin in growth plate chondrocytes. Furthermore, our findings provide a possible explanation for the observed results that leptin regulate ERs through ERK signal pathways.

The results obtained from these studies may have significant implications in understanding the mechanisms involved in the endochondral ossification. Studies are needed to confirm our findings and further define the other mechanisms involved.

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