RESEARCH

Leptin stimulates aromatase in the growth plate: limiting catch-up growth efficiency

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Abstract

Catch-up growth (CUG) in childhood is defined as periods of growth acceleration, after the resolution of growth attenuation causes, bringing the children back to their original growth trajectory. Sometimes, however, CUG is incomplete, leading to permanent growth deficit and short stature. The aim of this study was to investigate the mechanisms that limit nutritional-CUG. Specifically, we focused on the crosstalk between leptin, increased by re-feeding, and sex hormones, which increase with age. *In vivo* studies were performed in young male Sprague Dawley rats fed *ad libitum* or subjected to 10/36 days of 40% food restriction followed by 90–120 days of re-feeding. *In vitro* studies were performed on ATDC5 cells. Analyses of mRNA and protein levels were done using qPCR and Western blot, respectively. CUG was complete in body weight and humerus length in animals that were food-restricted for 10 days but not for those food-restricted for 36 days. *In vitro* studies showed that leptin significantly increased aromatase gene expression and protein level as well as the expression of estrogen and leptin receptors in a dose- and time-dependent manner. The effect of leptin on aromatase was direct and was mediated through the MAPK/Erk, STAT3 and PI3K pathways. The crosstalk between leptin and aromatase in the growth plate suggests that re-feeding during puberty may lead to increased estrogen level and activity, and consequently, irreversible premature epiphyseal growth plate closure. These results may have important implications for the development of novel treatment strategies for short stature in children.

Key Words

- catch-up growth (CUG)
- leptin
- aromatase
- growth plate
- ATDC5

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Introduction

Temporary growth attenuation in children may be due to endocrinological, nutritional, medical or emotional disorders. Catch-up growth (CUG) is a phase of accelerated growth that follows the correction of these disorders, making it possible for the children to resume their pre-illness growth curve (Prader *et al.* 1963, Boersma *et al.* 2002). Occasionally, however, recovery is incomplete, leading to a permanent growth deficit and short stature. Therefore, means to increase the efficiency of this process is required.

The association between nutrition and linear growth in children is well accepted. The growth of the human skeleton requires many different nutritional factors for building materials and participation in regulatory

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processes (Gat-Yablonski & De Luca 2017). To resume normal growth or initiate CUG, undernourished children must regain up to 85% of their weight for height (Kay's & Hindmarsh 2006).

Long bones are formed through endochondral ossification wherein a cartilage template is formed by condensed mesenchymal cells and later replaced by bone tissue. The process of endochondral ossification starts with the axial proliferation of resting early chondrocytes located at the most epiphyseal end of the growth plate (GP), near the end of the long bones. The chondrocytes then align in columns (proliferation zone) parallel to the long axis of the bone and ultimately mature into hypertrophic chondrocytes (hypertrophic zone). The hypertrophic cells cease dividing, increase in volume by five- to tenfold and promote the deposition of components of the extracellular matrix and the secretion of matrix vesicles that contain matrix-processing enzymes and serve as centers of mineralization. Thereafter, the chondrocytes undergo either programmed cell death with calcification of the extracellular matrix or trans-differentiation to endochondral osteoblasts, switching from the synthesis of cartilage collagens (mostly type II and X) to the synthesis of type I collagen (Yang *et al*. 2014, Zhou *et al*. 2014). Blood vessels invade the tissue, and initiate the formation of bone, supplanting the cartilage. GP fusion occurs when the GP chondrocytes exhaust their proliferative potential in a process of senescence during puberty that is regulated by sex hormones. Senescence was shown to be accelerated by estrogen (Weise *et al*. 2001, Chagin *et al*. 2007, Nilsson *et al*. 2014).

There are multiple factors, both systemic and local, that coordinate and couple chondrocyte proliferation and differentiation at the GP. Many of them are affected by nutritional status, including growth hormone (GH), insulin-like growth factor (IGF)-I, thyroid hormone, leptin, growth and differentiation factor 5 (Shtaif *et al*. 2015), corticosterone and sex hormones (Stevens & Williams 1999, Chrysis *et al*. 2003).

Estrogen is the principal regulator of GP fusion in both males and females, as shown in studies of males with mutations in the genes encoding estrogen receptor α (*ESRα*) (Smith *et al*. 1994) or aromatase (*CYP19A1*) (Carani *et al*. 1997), in addition to animal experiments (Chagin *et al*. 2007). In males, most estrogen is synthesized by peripheral tissues through local aromatization of circulating androgens. Aromatase catalyzes the rate-limiting step in the conversion of C19 androgens (androstenedione and testosterone) to C18 estrogenic steroids (estrone and estradiol). It is found in many tissues, including adipose tissue, brain, skin and bone (Sasano *et al*. 1997), and in GP chondrocytes (Oz *et al*. 2001, Eshet *et al*. 2004).

The aim of this study was to investigate the crosstalk between leptin, which is increased by re-feeding after food restriction (Pando *et al*. 2014), and sex hormones, which increase with age, in order to gain insight into the mechanism that limits nutritional-induced CUG. Experiments were performed *in vivo*, using a male Sprague Dawley rat model of food-restriction-induced growth attenuation followed by re-feeding, and *in vitro*, using chondrogenic ATDC5 cells.

Materials and methods

In vivo studies

All *in vivo* experiments were performed on pre-pubertal 24-day-old male Sprague–Dawley rats with an average weight of 50 g (Envigo, Ltd., Jerusalem, Israel), maintained under the same experimental conditions (25 ± 1°C, humidity 50 ± 2%, 12 h light/dark cycle; lights off at 18:00 h) and fed the same commercial rat chow (Teklad Rodent Diet, 2018SC; WI, USA). The rats were housed separately in single cages to allow for monitoring of food intake. All experiments were approved by the Tel Aviv University Institutional Animal Care and Use Committee (committee protocol approval number: M-12096).

Dietary manipulation

At the age of 24 days, after three days of acclimatization to the solitary cages, the rats were randomly divided into two groups: a control group which had unlimited access to regular rat chow (AL group) and a restricted group fed 60% of the normal daily intake of the same chow for 10 days (Even-Zohar *et al*. 2008) or 36 days (Masarwi *et al*. 2016), depending on the specific experiment. The restricted group was further divided into two subgroups: continued restriction (RES group) or unrestricted re-feeding for various periods (CU group). Animals that were food-restricted for 10 days were followed for up to 120 days; those food-restricted for 36 days were followed for up to 90 days.

Serum analysis

At the end of the experiments, blood was collected by cardiac puncture, serum-separated and stored at −70°C. Serum levels of leptin and testosterone were determined using commercial kits: Mouse/Rat Leptin Quantikine ELISA Kit (cat. no. MOB00, R&D Systems); Mouse/Rat

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Testosterone ELISA kit (cat. no. IB79174, Immuno-Biological Laboratories (IBL-America), Minneapolis, MN, USA).

Histological staining and measurement of growth plate height

The tibiae and humeri of each animal were carefully removed, cleaned and measured for length with a digital caliper. The tibiae were fixed in 4% neutral buffered formalin, decalcified with EDTA and HCl (Calci-Clear Rapid, cat. no. HS-105, National Diagnostics, Atlanta, GE, USA), dehydrated with a graded ethanol series (70, 95, 100%) and stabilized by two sequential changes of chloroform for paraffin embedding. Histological studies and GP height measurements were performed on paraffin sections of 6 µm, photographed and analyzed using Image-Pro software (version 4.5.1.22, Media Cybernetics, Rockville, MD, USA).

For immunohistochemistry, paraffin-embedded tissue sections were de-paraffinized in xylene, dehydrated in a graded series of ethanol and washed with PBS. Endogenous peroxidase was quenched using 3% hydrogen peroxide in methanol for 30 min. Nonspecific background was blocked by incubation with 10% goat non-immune serum (cat. no. 95-6143B; Zymed). Sections were incubated with primary antibodies: 3 different anti-aromatase antibodies (cat. no. MCA2077s, Serotec, Oxford, UK; cat. no. ab18995, Abcam; cat. no. 3599-100, Biovision, Milpitas, CA, USA) and anti-leptin receptor (Ob-Rb; cat. no. ab177469, Abcam), followed by a biotinylated second antibody and streptavidin-peroxidase conjugated with aminoetyl carbazole substrate (Histostain-SP Kit, Zymed Laboratories, South San Francisco, CA, USA). An IgG isotype was used as a negative control.

In vitro studies

The chondrogenic ATDC5 cell line (ATCC) (Atsumi *et al.* 1990) was cultured in Dulbecco's Modified Eagle Medium/Ham's-F12 (1:1 mixture, Gibco/Life Technologies, Thermo-Fisher Scientific) containing 5% heat-inactivated fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin (from Biological Industries, Beit HaEmek, Israel) at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were seeded at an initial cell density of 12×10^3 cells/cm^2 in a 6-multiwell plate (Corning) as previously reported (Pando *et al.* 2014). The next day, the cells were induced to differentiate by the addition of insulin, transferrin and sodium selenite (ITS 0.2%) (Sigma-Aldrich) for 4, 7, 14 or 21 days to obtain cells at different stages of differentiation.

The cells were stimulated with recombinant murine leptin (Protein Laboratories Rehovot, Rehovot, Israel) in ITS-free medium (phenol-red-free DMEM/F-12; Gibco/Life Technologies, Thermo-Fisher Scientific) containing 0.5% charcoal stripped fetal bovine serum (Biological Industries) for 24 h. Leptin stimulation was performed for 3, 6, 12 and 24 h.

Western blot analysis

ATDC5 cells were homogenized in a radio-immuno-precipitation assay buffer (50 µL/well of 20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 1% NP-40, 0.25% Na-deoxycholate) supplemented with a protease inhibitor cocktail (Roche) in a 1:12 ratio and with a phosphatase inhibitor cocktail (Roche) in a 1:10 ratio. The mixture was incubated on ice (20 min) followed by centrifugation at 21,000 ***g*** for 5 min. For each sample, 50 µg of proteins was analyzed on 7.5% SDS-PAGE and transferred to a nitrocellulose blotting membrane (cat. no. 10600008, GE Healthcare). The nitrocellulose membranes were then incubated in TBS-T (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) with 5% skim milk for 1 h to block nonspecific binding. The membranes were incubated with primary antibodies against aromatase (cat. no. MCA2077s, Serotec), and leptin receptor (Ob-Rb) (cat. no. ab177469, Abcam), overnight at 4°C, washed with TBS-T, incubated with a secondary fluorescent antibody (LI-COR Biosciences, Lincoln, NE, USA), decorated with IRDye for 1 hour and washed again. β-Actin served as the reference. Quantification was performed using the Odyssey Classic Imaging System with Odyssey Application Software (version 2.1.) (LI-COR Biosciences).

Cell-signaling studies

For the signal transduction studies, ATDC5 cells were incubated with the same ITS-free medium described earlier for 24 h and then treated for 1 h with specific inhibitors. Leptin was then added to the cells (100 ng/mL), the effect on phosphorylation was assayed after 1, 5, 15, 30 and 60 min (depending on the target) and the effect on aromatase expression was assayed after 6 h. The following inhibitors were used (all from Abcam): mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK1/2) (MEK1/2) inhibitor U0126 (cat. no. ab120241-5-B; 5 µM or 20 µM); phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (cat. no. ab120243-5-B, 5 µM or 10 µM) or Wortmannin (10 µM); and signal transducer and activator of transcript (STAT)-3 inhibitor Stattic (cat no. 120952-10-B; 2.5 µM or 5 µM). To follow the effect on phosphorylation, the following antibodies were used

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(all from Cell Signaling): anti-phospho ERK1/2 (cat. no. 4376) and rabbit monoclonal anti-total ERK1/2 (cat. no. 9102); anti-phospho Akt antibody (Ser473, cat. no. 4060) and rabbit monoclonal anti-total-Akt antibody (cat. no. 4691); anti-phospho STAT3 (anti-pTyr705, cat. no. 9131 and anti-pSer727, cat no. 9134); and rabbit monoclonal anti-total STAT3 (cat. no. 12640).

RNA isolation

Total RNA was extracted from ATDC5 cells using 1 mL TRIzol Reagent (Ambion/Life Technologies, Thermo-Fisher Scientific), and stored at −70°C. First-strand cDNA was synthesized using 2 µg total RNA (ratio of 260/280 ≥ 1.9) as a template with the PrimeScript RT reagent Kit (Perfect Real Time, cat. No. RR037A, Clontech Laboratories, TaKaRa Bio) and the high capacity RNA to cDNA kit (cat. no. AB-4387406, Applied Biosystems).

Real-time qPCR

Quantitative real-time polymerase chain reaction (qPCR) was performed using TaqMan technology and commercially available FAM-labeled assays (all reagents were from Applied Biosystems): aromatase (Cyp19a1, Mm00484049_m1), Ob-Rb (Lepr, Mm01265583_m1), ER-α (Mm00433149_m1), collagen II (Mm01309565_m1) and collagen X (Mm00487041_m1); TATA-box binding protein (TBP, Mm00446973_m1) served as the endogenous control. TaqMan Fast Advanced Master Mix was used at a final reaction volume of 10 µL/well (20 µL/well for aromatase and Ob-Rb) in 96-well plates. Reactions were performed in triplicate on cDNA using the StepOne Real-Time PCR system (version 2.2.2, Applied Biosystems). Cycling parameters were: 50°C * 2 min, 95°C * 10 min, then 50 cycles of 95°C * 15 s, 60°C * 1 min.

The relative quantity of mRNA was first normalized to the endogenous reference TBP by subtracting the *Ct* of TBP from the *Ct* of the gene of interest to obtain Δ*Ct*. The Δ*Ct* of the leptin-treated samples was compared with that of the control samples, and the difference was designated as ΔΔ*Ct*. The fold change between the treated and control samples was then calculated as $2^{-\Delta\Delta Ct}$.

Luciferase assay

Transient transfection experiments were done using the pGL3 vector expressing the human aromatase promoter II and I.3 sequence ligated to a firefly luciferase reporter gene. (The PGL3PII/I.3 plasmid was provided by Dr Kristy A Brown, Hudson Institute of Medical Research, Clayton, Australia.) The pRL-CMV-Renilla luciferase expression vector (Promega) served as a control. At 24 h after transfection, cells were serum-starved for 24 h, followed by the addition of leptin for another 6 h (a total of 54 h) and then harvested. The luciferase activity assay was performed using the Dual-Luciferase Reporter Assay system (Promega). Relative luciferase activity was calculated as the ratio of the Firefly luciferase activity to the Renilla luciferase activity and compared to cells transfected with the same vectors but not treated with leptin.

Statistical analysis

Data are presented as mean ± S.D. The significance of differences between experimental groups was determined with one-way analysis of variance (ANOVA) with Tukey's *post hoc* test (multiple groups). Differences in weight were analyzed with ANOVA with repeated measures. Data were generated and analyzed with SPSS version 21 (IBM) software. Differences were considered statistically significant at $P<0.05$.

Results

In vivo studies

Effect of food restriction on CUG

Male Sprague Dawley rats (24 days) were subjected to 10 days of 40% food restriction followed by unrestricted re-feeding for 1, 2, 7 (Even-Zohar *et al.* 2008), 26, 60 or 120 days (CU group); complete CUG (bone length and body weight) was noted after 60 days of re-feeding (Supplementary Fig. 1, see section on supplementary data given at the end of this article). The control group of the 26-day study that was food-restricted for a total of 36 days showed increased accumulation of fat in the bone marrow (Supplementary Fig. 2).

We speculated that fat accumulation in the bones may indicate that the effect of malnutrition on bones was already irreversible; therefore, in the next experiments, 24-day-old rats were subjected to 36 days of 40% food restriction followed by unrestricted re-feeding for 24 days or 90 days (CU group). Their average body weights are presented in Fig. 1A and C. The body weight of the re-fed animals increased, but remained significantly lower than that of control rats fed *ad libitum* for the whole experiment (AL group) (Fig. 1C, $P<0.0001$). Re-feeding also led to an increase in the weight of the internal organs, but values remained significantly lower than those of the controls at all time points checked. The growth rate was different for each organ (Fig. 1B). After 90 days of re-feeding, the

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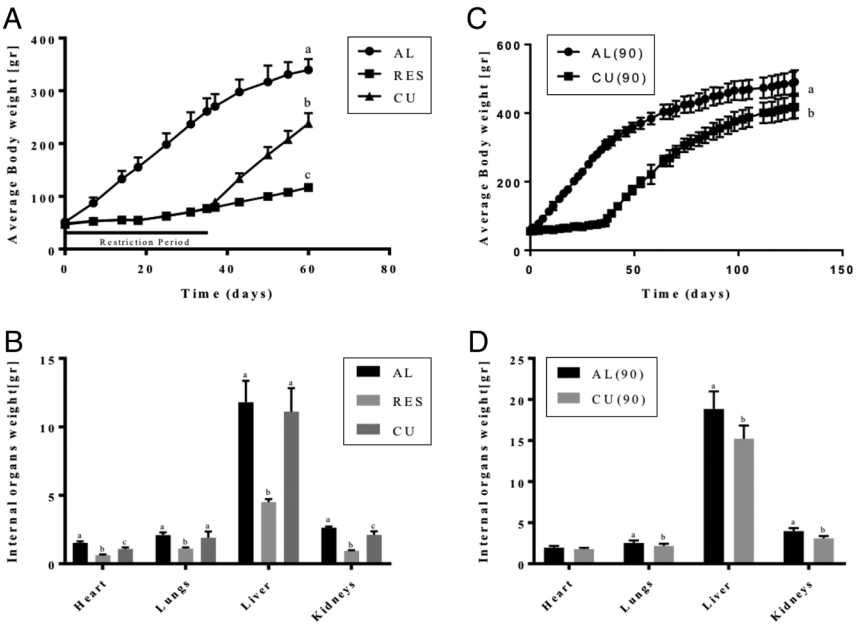

Figure 1
Effect of food restriction and re-feeding on body and internal organ weight. Body weight was monitored throughout the experiment. (A) Body weight and (B) internal organ weight of rats subjected to 36 days of food restriction followed by 24 days of re-feeding. Data were analyzed by one-way ANOVA. (C) Body weight and (D) internal organ weight of rats subjected to 36 days of food restriction followed by 90 days of re-feeding. Data were analyzed by *t*-test. Superscripts within the graphs designate significant differences at $P<0.05$; curves/columns marked with the same letter did not differ significantly. AL – rats fed *ad libitum* (control group); RES – food-restricted group; CU – catch-up group.

lungs, liver and kidneys weighed significantly less in the CU than the AL group (Fig. 1D), but the heart weighed only slightly less.

The length of the humerus was significantly lower in the CU than in the AL group after both 24 days and 90 days of re-feeding (Fig. 2A and C). The height of the GP was significantly greater in the CU than in the AL group after 24 days (Fig. 2B, $P<0.0001$), but at 90 days, there was no significant between-group difference (Fig. 2D, $P=0.1$), probably reflecting the age-related shrinkage of the GP.

Effect of sex hormones on CUG

The inhibitory effect of estrogen on GP growth is well known (Chagin *et al.* 2007, Nilsson *et al.* 2014). As the animals in the AL and CU groups were of the same age, we were able to investigate whether the nutritional manipulation stimulated estrogen production, thereby leading to premature cessation of growth. Our earlier studies in similar models showed that leptin is significantly affected by nutritional manipulation: its level was significantly reduced in food-restricted rats and increased rapidly already after 1 day of re-feeding (Pando *et al.* 2014). In the present experiments, leptin and testosterone showed a similar pattern of secretion. Mean leptin levels were 3.1±1.44 ng/mL in the AL group, 0.47±0.28 ng/mL in the RES group (food-restricted for 36 days) and 2.3±0.83 ng/mL in the CU group (re-fed for 1 day). Corresponding testosterone levels were 1.81±0.51, 0.42±0.1 and 1.47±0.96 ng/mL. For both factors, there was a significant difference between the RES and AL groups ($P<0.05$) but not between the CU and AL groups.

Detection of leptin receptor and aromatase in the growth plate

Immunohistochemistry staining (Fig. 3) showed the presence of both aromatase (Fig. 3A, B and C), and the long form of leptin receptor (Ob-Rb, Fig. 3D, E and F) in the GP of all groups of rats. Aromatase was detected mostly in the resting zone (Fig. 3A, B and C), and Ob-Rb

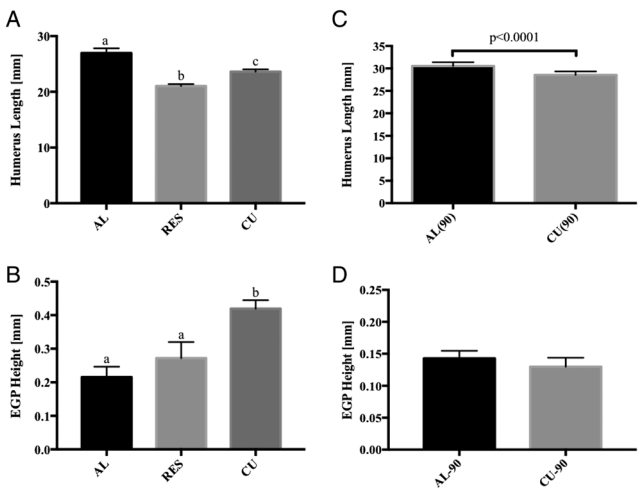

Figure 2
Effect of re-feeding on humerus length and growth plate height. (A) Humerus length and (B) growth plate (EGP) height in rats subjected to food restriction for 36 days followed by 24 days of re-feeding. Data were analyzed by one-way ANOVA. (C) Humerus length and (D) EGP height in rats subjected to food restriction for 36 days followed by 90 days of re-feeding. Data were analyzed by *t*-test. Superscripts within the graphs designate significant differences at $P<0.05$; columns marked with the same letter did not differ significantly. AL – rats fed *ad libitum* (control group); RES – food-restricted group; CU – catch-up group.

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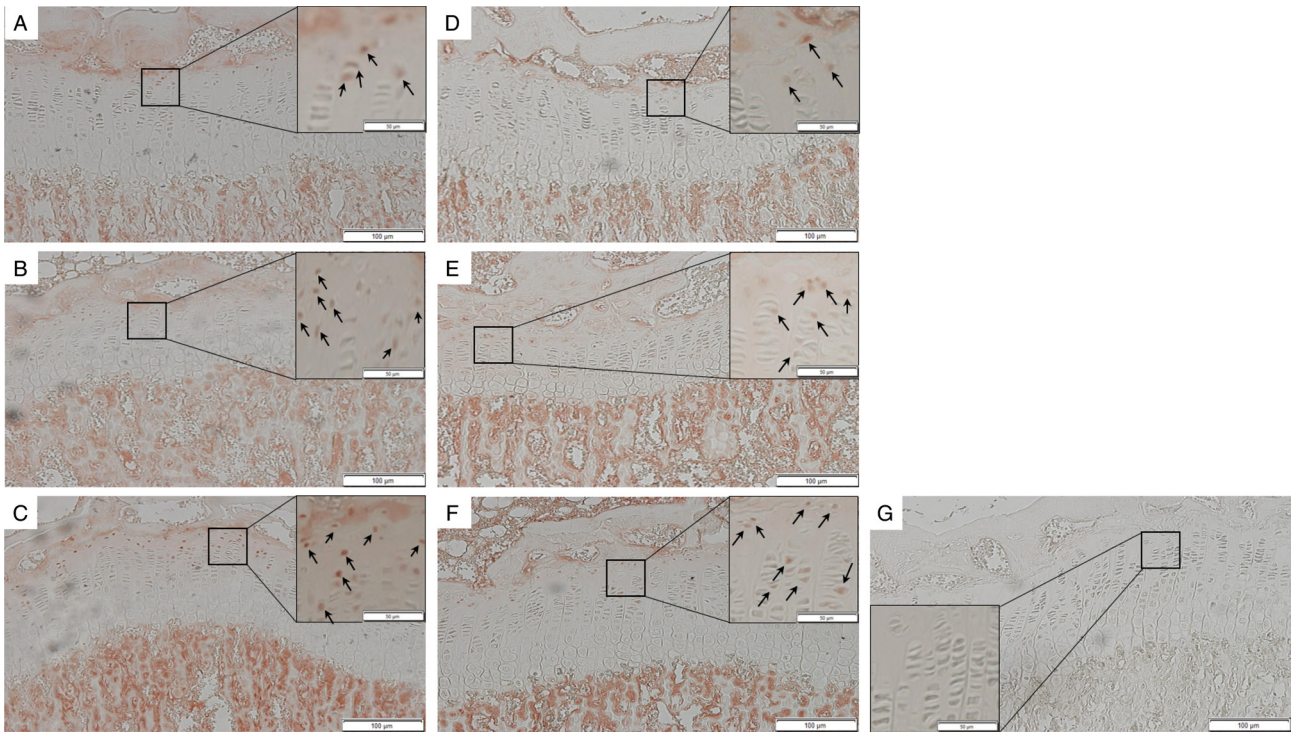

Figure 3
(Revised version): immunohistochemistry of aromatase enzyme and the long form of leptin receptor (Ob-Rb) in the humeral growth plate. Animals were fed *ad libitum* (AL) or food-restricted (RES), or food-restricted and then re-fed for 1 day (CU). All animals were killed at the same day. Aromatase-(A) rats fed *ad libitum*, (B) rats food-restricted throughout the experiment, (C) rats food-restricted followed by re-feeding stained with anti-aromatase antibody. Staining is located mainly in the resting zone in the AL and RES groups, whereas in the CU group, resting and proliferating chondrocytes were stained (arrows). Ob-Rb-(D) rats fed *ad libitum*, (E) rats food-restricted throughout the experiment, (F) rats food-restricted followed by re-feeding stained with anti-Ob-Rb antibody. (G) Negative control. In all groups, staining is located mainly in the resting and proliferating zones (arrows). Insets in all figures are of the same sections photographed with higher magnification (×20). A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0028.

(Fig. 3D, E and F) was detected in both the resting and proliferative zones. In sections taken from RES animals, we noted a significant reduction in the level of Ob-Rb during food restriction, followed by a rapid increase at 1 day of re-feeding mainly in the proliferative zone. The level of aromatase was very low in the sections of the control groups, with a slight increase in sections of the RES group and a marked increase in sections taken form the CU groups, in both the resting and proliferative zones. Similar results for aromatase were obtained when real-time PCR was performed on total RNA extracted from the GP (data not shown).

In vitro studies

Effect of leptin on aromatase gene expression

Although leptin was found to significantly increase aromatase gene expression and protein level in other cell types (Catalano *et al.* 2003), its effect on aromatase in chondrocytes is unknown. As the expression of both Ob-Rb and aromatase was mostly confined to the resting and proliferative zones of the GP, we performed our studies on ATDC5 cells at their early differentiation stages.

Cells were grown for 4 or 14 days, serum-depleted for 24 h, and then treated with leptin (100 ng/mL) for different time periods (Fig. 4A). The results showed that leptin stimulated the expression of aromatase mRNA in a time-dependent manner. A tendency to increase in aromatase mRNA was detected after 3 h of leptin treatment ($P=0.06$), and a significant peak was noted at 6 h ($P<0.01$). Accordingly, for the dose–response study, cells grown for 4 days were similarly treated with leptin at different concentrations for 6 h. As shown in Fig. 4B, the expression of aromatase followed a bell-shaped curve that peaked at 100 ng/mL leptin ($P=0.015$).

Effect on leptin receptor and estrogen receptor alpha expression

We next studied the effect of leptin on the expression of Ob-Rb and estrogen receptor alpha (Er-α). Leptin (100 ng/mL) increased Ob-Rb gene expression after 3 h ($P=0.043$) and 6 h ($P=0.007$) of incubation (Fig. 5A),

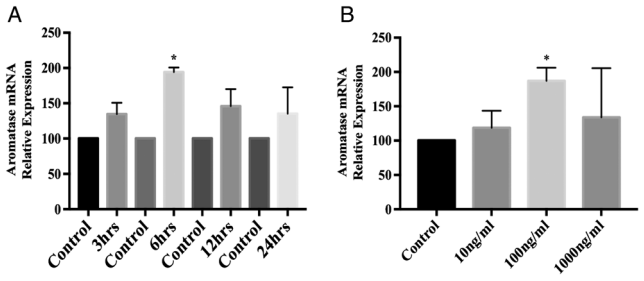

Figure 4
Effect of leptin on aromatase mRNA expression. ATDC5 cells were cultured in DMEM/F12 containing 5% FCS and ITS for 4 days. (A) Cells incubated with or without (control) 100 ng/mL leptin for 3, 6, 12 and 24 h. (B) Cells treated with leptin at 0, 10, 100 and 1000 ng/mL for 6 h. Aromatase gene expression was analyzed by qPCR, normalized against TBP and compared to the control group. Data represent the mean (±S.D.) value of triplicate samples in three independent experiments.

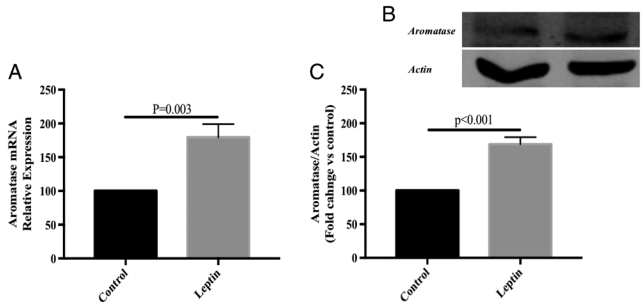

Figure 6
qPCR analysis and Western blot of aromatase expression in ATDC5 cells. Cells were cultured in DMEM/F12 containing 5% FCS and ITS for 14 days, treated overnight with ITS-free medium containing 0.5% FCS, and incubated with 100 ng/mL leptin for 48 h. (A) Aromatase mRNA expression. Gene expression was analyzed by qPCR, normalized against TBP and compared to the control group. (B) Representative Western blot of aromatase. (C) Western-blot-normalized data. Data represent the mean (±S.D.) value of triplicate samples in three independent experiments.

whereas the expression of ER-α significantly increased after 6 h of leptin incubation (Fig. 5B; $P<0.001$).

Effect of leptin on aromatase gene expression and protein levels at 14 days of ATDC5 differentiation

Western blot analysis of aromatase was unsuccessful when performed after 4 days of differentiation owing to its very low level of expression. However, at 14 days of differentiation, the protein level could be reliably detected. In cells serum-starved for 24 h, 48 h of treatment with 100 ng/mL leptin significantly increased both aromatase gene expression (Fig. 6A) and protein levels (Fig. 6B and C).

Effect of leptin on the activity of the human aromatase promoter PII/PI.3

We next investigated the activity of the aromatase promoter PII/PI.3 in ATDC5 cells treated with 100 ng/mL leptin. Analysis with the luciferase reporter assay showed that leptin significantly increased aromatase promoter activity after 6 h of incubation (Fig. 7).

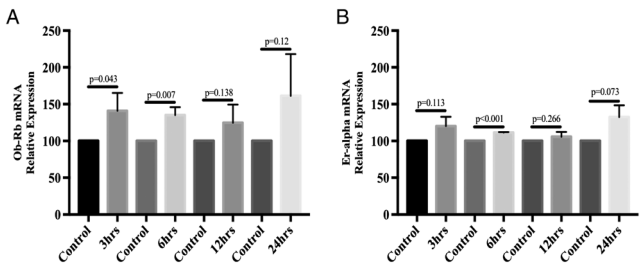

Figure 5
Effect of leptin on gene expression of the long form of leptin receptor (Ob-Rb) and estrogen receptor alpha (Er-α). ATDC5 cells were treated with 100 ng/mL leptin for 3, 6, 12 and 24 h. (A) Expression of Ob-Rb. (B) Expression of Er-α. Gene expression was analyzed by qPCR, normalized against TBP and compared to the control group. Data represent the mean (±S.D.) value of triplicate samples in three independent experiments.

Mechanism of leptin stimulation of aromatase gene expression

We have previously shown that leptin exerts its biological action by binding to and activating Ob-Rb, in both GP chondrocytes and ATDC5 cell (Gat-Yablonski *et al.* 2004, Ben-Eliezer *et al.* 2007). Ob-Rb activates several classical signal transduction pathways: MAPK (MEK1/2) cascade via ERK1/2; STAT-3 and PI3K/Akt pathways. To assess which of these is activated by leptin in ATDC5 cells and therefore important for leptin-induced aromatase expression, we systematically assessed their phosphorylation status. As shown in Figs 8, 9 and 10, leptin enhanced the phosphorylation of ERK1/2, STAT3 and Akt in a time-dependent manner. Time-course experiments revealed that the phosphorylation of ERK1/2, STAT3 (Tyr705 and Ser727) and Akt was maximal after 1–5 min of leptin treatment and declined thereafter.

Next, we used specific inhibitors to delineate the role of each pathway in leptin-induced aromatase expression. U0126, the inhibitor of MEK1/2, suppressed the effect of leptin on ERK1/2 phosphorylation, as well as the effect of leptin on aromatase expression, in a dose-dependent manner (Fig. 8B). Leptin stimulated STAT3 (Fig. 9A) and Akt phosphorylation (Fig. 10A); pretreatment with Stattic and LY294002 profoundly reduced STAT3 (Tyr705 only) (Fig. 9B) and Akt (Fig. 10B) phosphorylation, respectively. Both STAT3 and PI3K inhibitors significantly reduced aromatase mRNA expression (Figs 9B and 10B, respectively).

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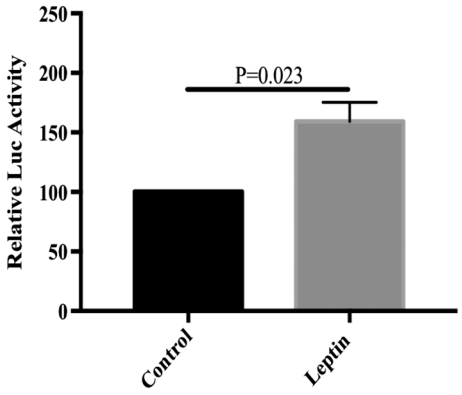

Figure 7
Effect of leptin on aromatase promoters PII/PI.3. ATDC5 cells were cultured in DMEM/F12 containing 5% FCS and ITS for 4 days. The cells were transiently transfected with pGL3 vector expressing the human aromatase promoter II and I.3 sequence ligated to a firefly luciferase reporter gene, together with pRL-CMV-Renilla luciferase expression vector. At 24 h after transfection, the cells were serum-starved for 24 h, and leptin was added for an additional 6 h. The relative luciferase activity was calculated as the ratio of the firefly luciferase activity to the Renilla luciferase activity and compared to cells transfected with the same vectors but not treated with leptin. Data represent the mean (±S.D.) values of triplicate samples in three independent experiments.

Discussion

Growth inhibition is common during childhood and may occur repeatedly, and each episode is usually followed by CUG. However, when the growth arrest is prolonged, and when close to completion of puberty, CUG may be incomplete, leading to a permanent growth deficit. The clinical toolbox for treating children with short stature is very limited, and identifying the mechanisms limiting CUG may have important implications for the development of novel means to improve height.

In this study, we specifically examined the crosstalk between leptin and sex hormones; we showed that leptin, increased with re-feeding, stimulates the activity of aromatase and together with the increase in testosterone by re-feeding and age, may be responsible for premature GP shrinkage and incomplete CUG. We have shown *in vitro* in ATDC5 cells that leptin induction of aromatase gene expression and protein levels occurred in a time- and dose-dependent manner via activation of the canonical signal transduction pathways, MAPK, STAT3 and PI3K.

The finding that leptin stimulates aromatase expression is not new (studies were done in endometrial fibroblasts (Liu *et al.* 2013), luteinized granulosa cells (Kitawaki *et al.* 1999) and MCF-7 breast cancer cells (Catalano *et al.* 2003)). Aromatase expression was reported in human mesenchymal stem cells of bone marrow (Heim *et al.* 2004) which can differentiate into osteoblasts, adipocytes and chondrocytes (Pittenger *et al.* 1999). Leptin plus vitamin D 1,25$(OH)_2D_3$ stimulated aromatase activity in the osteogenic lineage (Pino *et al.* 2006). However, to the best of our knowledge, it has never before been shown in chondrocytes.

Estrogen is responsible for both the growth spurt and GP fusion in both sexes (Cutler 1997, Stevens & Williams 1999). The process of GP fusion is associated with senescence, manifested by a decline in the proliferative capacity of the resting chondrocytes with age (Schrier *et al.* 2006) and culminating in growth cessation. Studies in juvenile ovariectomized female rabbits showed that

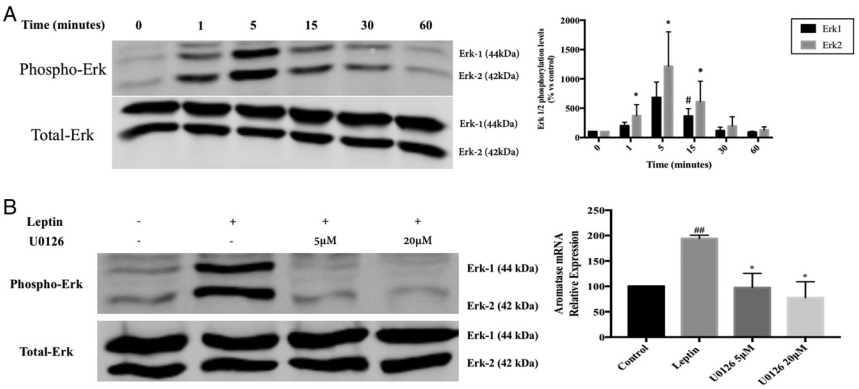

Figure 8
Leptin stimulation of aromatase expression-role of MAPK signaling pathway (ERK1/2). ATDC5 cells were cultured in DMEM/F12 containing 5% FBS and ITS for 4 days. Cells were treated overnight with ITS-free medium containing 0.5% FCS. (A) Cells treated with 100 ng/mL leptin for the indicated times. The left panel shows a representative Western blot; the right panel shows the normalized data. (B) Cells treated with the MEK1/2 inhibitor U0126 (5 μM and 20 μM) for 60 min before the addition of 100 ng/mL leptin for 5 min. P-ERK1/2 and total ERK1/2 protein levels were analyzed by Western blot. The left panel shows a representative Western blot; the right panel shows the effect on aromatase expression. Data represent the mean (±S.D.) values of triplicate samples in three independent experiments. Columns marked with octothorpe (#, ERK1) and asterisk (*, ERK2) are significantly different compared to baseline.

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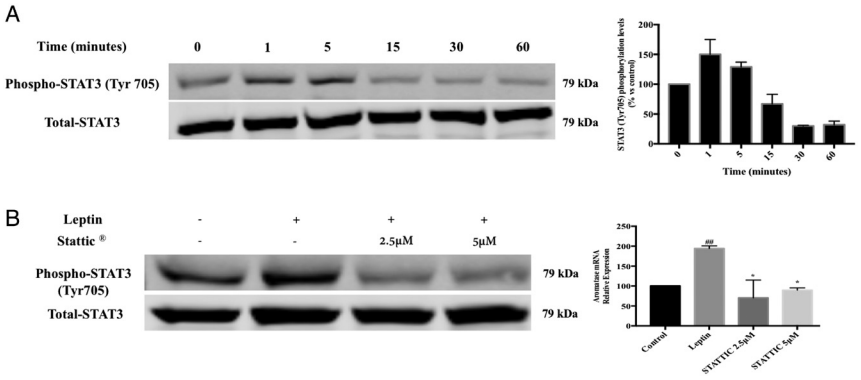

Figure 9
Leptin stimulation of aromatase expression-role of the STAT3 signaling pathways (Tyr 705). ATDC5 cells were cultured in DMEM/F12 containing 5% FCS and ITS for 4 days. Cells were treated overnight with ITS-free medium containing 0.5% FCS. (A) Cells were treated with 100 ng/mL of leptin for the indicated times. The left panel shows a representative Western blot of STAT3 Tyr 705; the right panel shows the normalized data. (B) Cells were treated with the STAT3 inhibitor Stattic (2.5 μM and 5 μM) for 60 min and then with 100 ng/mL leptin for 1 min. The left panel shows a representative Western blot; the right panel shows the effect on aromatase expression. Data represent the mean (±S.D.) values of triplicate samples in three independent experiments.

changes in estrogen level were associated with tibial chondrocyte proliferation rate, GP height, number of proliferative chondrocytes, number and size of hypertrophic chondrocytes and column density (Weise *et al.* 2001, Nilsson *et al.* 2014).

Sexually mature men with tall stature and unfused epiphyses have been reported in the literature. One such case had autosomal recessive inherited mutation in *ER-alpha* (Smith *et al.* 1994), and several others had mutations in *CYP19A1* encoding aromatase (Belgorosky *et al.* 2009). These findings emphasized the importance of estrogen and estrogen signals in GP maturation and fusion in human males (Attie *et al.* 1990) and has led the way to the treatment of short stature using aromatase inhibitors (AI). Accordingly, we previously reported that applying the AI Letrozole to young male mice led to a significant increase in body weight, tail and tibial length in association with a marked increase in GP height (Eshet *et al.* 2004). Aromatase was expressed mainly in the hypertrophic and pre-hypertrophic chondrocytes of the GP, and slightly in the proliferative and resting chondrocytes. By contrast, in the present study, we found that aromatase was expressed mainly in the resting and proliferating GP chondrocytes. This discrepancy in localization is due to the different ages of the animals in the two studies (Supplementary Fig. 3).

Treatment of young boys with growth failure using AI, alone (Hero *et al.* 2005) or in combination with testosterone (Wickman *et al.* 2001), or GH (in GH-deficient patients) delayed GP closure, thereby increasing height potential. In a study of Finnish adolescent boys with constitutional growth and puberty delay, AI were used to increase adult height (Wickman *et al.* 2001). Later, other studies confirmed that the treatment of GH-deficient male adolescents with AI increased height (by 6.7 cm after 36 months) compared to treatment with GH alone (Mauras *et al.* 2008), with a significant delay in the tempo of bone age acceleration and lower estrogen levels in treated boys. Nevertheless, the clinical use of AI is still limited due to

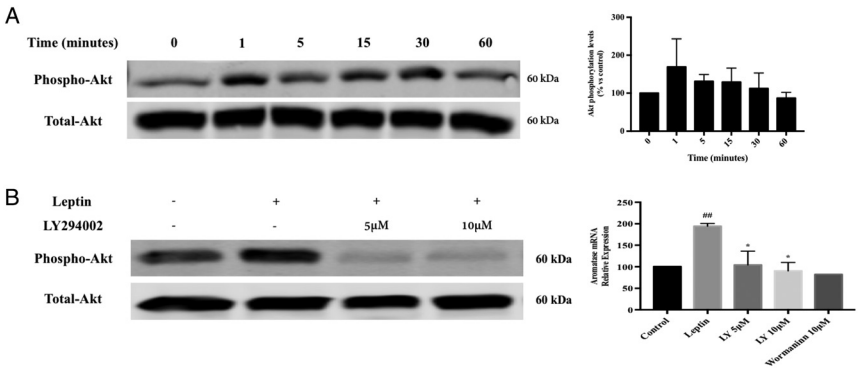

Figure 10
Leptin stimulation of aromatase expression-role of the PI3K signaling pathway. ATDC5 cells were cultured in DMEM/F12 containing 5% FCS and ITS for 4 days. Cells were treated overnight with ITS-free medium containing 0.5% FCS. (A) Cells were treated with 100 ng/mL of leptin for the indicated times. The left panel shows a representative Western blot; the right panel shows the normalized data. (B) Cells were treated with the PI3K inhibitors LY294002 (5 μM and 10 μM) and Wortmannin (10 μM) for 60 min and then 100 ng/mL leptin for 1 min. P-Akt and total Akt protein levels were analyzed by Western blot. The left panel shows a representative Western blot; the right panel shows the normalized data. Data represent the mean (±S.D.) value of triplicate samples in three independent experiments.

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concerns about an increased risk of vertebral deformities, decreased levels of high-density lipoprotein cholesterol and increased erythrocytosis (Wit *et al.* 2011).

Our group and others have extensively studied the role of leptin in regulating growth (Gat-Yablonski & Phillip 2008, Turner *et al.* 2013). The present study shows that leptin can also regulate growth by stimulating aromatase in the presence of testosterone. Leptin, encoded by the obese (*Ob*) gene, is produced and secreted mostly by adipose tissue, but also by other tissues such as fetal bone and cartilage (Kishida *et al.* 2005), and primary cultures of human osteoblasts (Reseland *et al.* 2001). Studies in humans have shown that leptin deficiency leads to developmental abnormalities of the skeleton and advanced bone age (Farooqi *et al.* 1999). We have previously shown that cultured mice mandibular condyles express Ob-R, and adding leptin to the culture increased mandibular condyle proliferation and differentiation (Maor *et al.* 2002). In a later study, leptin was found to significantly increase tibial length in normal mice compared to pair-fed control mice, probably acting through the parathyroid-hormone-related protein (PTHrP)/Indian hedgehog (Ihh) loop (Gat-Yablonski *et al.* 2007). Furthermore, the addition of leptin to ATDC5 cells increased their differentiation, as manifested by an increased expression of type X collagen, a unique marker of hypertrophic chondrocytes (Kishida *et al.* 2005, Ben-Eliezer *et al.* 2007). Leptin also enhanced chondrocyte proliferation and differentiation in the early stage in cultured chondrocytes derived from rabbit ribs (Nakajima *et al.* 2003).

Our finding that Ob-R is expressed mainly in resting and proliferating GP chondrocytes contrasts with the report of Kishida *et al.* (2005) of Ob-R expression in the terminal hypertrophic chondrocytes. This discrepancy may be due to differences in the animal model used (rats vs mice) or the different ages of the animals (61 days in our study vs 28 days in the earlier study). The immunohistochemistry staining revealed that like Ob-Rb, aromatase was expressed in resting and proliferating chondrocytes of the GP. This finding indicates that local interactions between aromatase and leptin in the resting and proliferation zones are indeed feasible.

Leptin binding to the Ob-Rb receptor activates the MAPK, STAT3 and PI3K signaling pathways (Hegyi *et al.* 2004, Ben-Eliezer *et al.* 2007, Yang & Barouch 2007). MAPK, present in all eukaryotic cells, coordinately regulates cellular growth and differentiation as well as chondrocyte differentiation (Phornphutkul *et al.* 2006). In the present study, leptin was found to stimulate the phosphorylation of ERK1/2, and this effect, along with the effect of leptin on aromatase, was abrogated with pretreatment with a MEK-specific inhibitor (U0126). These results are consistent with those of a previous study showing that leptin induced aromatase expression and activity in MCF7 cells through ERK1/2 signaling (Catalano *et al.* 2003).

STATs play an important role in mediating leptin-regulated gene transcription (Yang & Barouch 2007). In chondrocytes, previous studies showed that leptin regulates the expression of matrix metalloproteinases through STAT3 and STAT5 signals (Hui *et al.* 2012), with STAT3 increasing the expression of multiple genes in chondrocyte cells, such as type X collagen and Frizzled receptors (Ben-Eliezer *et al.* 2007, Ohba *et al.* 2010). STAT3 phosphorylation is important on both Tyr705 and on Ser727 (Aggarwal *et al.* 2009, Sakaguchi *et al.* 2012), as Ser727 phosphorylation regulates STAT3 nuclear translocation and cell survival (Sakaguchi *et al.* 2012). In the present study, leptin increased STAT3 phosphorylation on both tyrosine 705 (Fig. 9A) and serine 727 (data not shown). Blocking STAT3 signaling using the selective STAT3 inhibitor Stattic significantly reduced both the phosphorylation of STAT3, and aromatase mRNA expression, pointing to the biological significance of STAT3 signaling in regulating aromatase expression (Stattic is specific to Tyr 705; it was shown to inhibit STAT3 dimerization and translocation to the nucleus (Schust *et al.*)).

PI3K is a family of enzymes involved in cellular functions, such as cell growth, proliferation, differentiation and survival and intracellular trafficking. PI3K/Akt is considered the canonical PI3K signaling pathway (Carracedo & Pandolfi 2008) and plays a role in anabolic and catabolic processes in cartilage in response to IGF-I (Starkman *et al.* 2005), leptin (Hui *et al.* 2012) and other cytokines (Wegiel *et al.* 2008). PI3K/Akt induced the expansion of chondrocyte proliferation and blocked hypertrophic chondrocyte differentiation, perhaps as a consequence of the suppression of Runx2 expression (Kita *et al.* 2008). Consistent with other studies, we showed that leptin increased PI3K phosphorylation in a time-dependent manner. Adding LY294002 or Wortmannin to ATDC5 cells significantly reduced aromatase expression, and LY294002 significantly reduced Akt phosphorylation.

ADTC5 cells treated with leptin showed an increase in the gene expressions of leptin receptor, ER-α and aromatase. Similar results were previously shown by Wang *et al.* (2012*b*) who showed in ATDC5 cells that leptin increased estrogen receptor mRNA expression and protein levels in a dose-dependent manner by activating

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ERK1/2, and that estrogen significantly up-regulated Ob-R protein and mRNA levels (Wang *et al.* 2012*a*).

Our results are consistent with previous data showing that aromatase expression is regulated through MAPK (Erk1/2, P38 and JNK) and PI3K/Akt signaling in adipose tissue (Tan *et al.* 2015) and through STAT3, MAPK and PI3k/Akt in breast cancer cells (Catalano *et al.* 2003, Phuong *et al.* 2014). The organization of the aromatase first exon is complex and leads to 9 alternative tissue specific versions, while the coding region and the protein expressed are always the same (Harada *et al.* 1993). Aromatase promoters I.3, PII, I.4 and I.6 are expressed in bone and chondrocyte cells (Enjuanes *et al.* 2005, Jeong *et al.* 2010). The regulatory elements contain binding sites for c-Fos and c-Jun (AP-1) that can be activated by MAPK signaling, a binding site for STAT3 and others. In the present study, treatment of ATDC5 cells with leptin (100 ng/mL) significantly increased aromatase mRNA through MAPK Erk1/2 (Fig. 8), P38 and JNK (data not shown), STAT3 (Fig. 9) and PI3K/Akt (Fig. 10). Inhibiting every pathway alone was sufficient to significantly reduce aromatase expression.

Obesity is associated with high serum leptin levels due to the increase in adipose tissue concomitant with central resistance to circulating leptin. The differential sensitivity to the central effect of leptin (at the hypothalamus) (Jung & Kim 2013), and the peripheral effect of leptin (at the GP) (Maor *et al.* 2002, Kishida *et al.* 2005) may explain the accelerated growth of pre-pubertal obese children. Pre-pubertal obese children are taller than their peers, often start puberty earlier and show earlier maturation of the GP, which may be also associated with loss of the pubertal growth spurt (Shalitin & Kiess 2017) and final short stature.

To conclude, this study clearly shows that sex hormones limit the efficacy of CUG in pubertal male rats. For the first time, we describe a crosstalk between leptin and aromatase in chondrocytes. Re-feeding was associated with an increase in leptin level, augmented aromatase mRNA expression and protein content, and augmented leptin and estrogen receptors gene expression. Testosterone itself was also increased by re-feeding, as the animals were already old enough. The increase in aromatase expression enhanced the aromatization process of testosterone to estrogen, increased locally produced estrogen then bound to its receptors (also increased), enhancing the senescent decline in the GP chondrocyte proliferation rate, reducing GP height and leading to incomplete CUG. Our results are also supported by a recent study showing a significant higher longitudinal growth rate in animals treated with leptin + estrogen receptor inhibitor compared to leptin-treated group, indicating that the concurrent increase in estrogen levels during leptin treatment antagonizes the growth-promoting actions of leptin (Turner 2017).

Identifying factors that limit CUG may open new areas for research and pave the way to improved and new treatment modalities.

Limitations of the study

The limitations of the study include the fact that we could not follow the changes in the bones in each animal during the experiment, as we do not have access to *in vivo* CT scan. Additional limitations are the lack of data on aromatase activity and on serum and local estrogen levels. The commercially available estradiol ELISA kit was not sensitive enough to identify the low levels of estradiol in a reliable manner. In addition, owing to the very low level of expression, we could not detect changes in aromatase protein levels in ATDC5 cells after 4 days of differentiation. Other limitations include the small sample size in each group as well as the use of an animal model instead of children, due to ethical considerations.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0028.

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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Author contribution statement
All authors contributed to study design and have approved the final version of manuscript. M.M. contributed to conduct the study. M M and G-G Y contributed to data collection, data analysis and manuscript drafting and revision. M M, G-G Y, M P and R S contributed to data interpretation.

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