Bone 122 (2019) 166–175

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

The effect of a high-calorie diet on bone growth is mediated by the insulin receptor

Shufang Wu[a,b], Ying Zhang[b], Francesco De Luca[a,*]

[a] Section of Endocrinology and Diabetes, St. Christopher's Hospital for Children, Drexel University College of Medicine, Philadelphia, PA, United States of America
[b] Center for Translational Medicine, the First Affiliated Hospital of Xi'an Jiaotong University School of Medicine, 277 West Yanta Road, Xi'an, Shaanxi 710061. PR China

ARTICLE INFO

Keywords:
Insulin
Insulin receptor
High-calorie diet
Growth plate
Bone
Bone growth
Chondrocyte

ABSTRACT

Obese children grow faster than their normal-weight peers. Insulin resistance and hyperinsulinemia have been associated with obesity-related growth acceleration. To determine whether obesity-associated hyperinsulinemia promotes bone growth by activating the insulin receptor in the growth plate, we generated $^{TamCart}IR^{flox/flox}$ mice. The injection of 4 doses of tamoxifen in these mice (beginning at postnatal day 5th with 2 days interval between injections) resulted in the Insulin Receptor (*IR*) gene excision exclusively in the cartilage. $^{TamCart}IR^{flox/flox}$ tamoxifen-treated mice (KO mice) and their $IR^{flox/flox}$ control littermates (C mice) at 3 weeks of age were exposed to a standard or hypercaloric (high-fat) diet for 4 weeks. At the end of study, C and KO mice fed with a high-fat diet exhibited greater weight gain than the respective strains fed with a standard diet. Body and tibial growth and growth plate height of C mice fed with high-fat diet were greater than those of standard-diet-fed C mice; however, no difference was observed between KO mice fed with standard or high-fat diet with respect to body and tibial growth and growth plate height. Circulating levels of insulin, IGF-1 and leptin were significantly higher in C and KO mice exposed to high-fat diet compared to those in the same strain exposed to standard diet. Increased phosphorylation of Akt (one of the intracellular mediators of insulin action in bone) in the growth plate of C mice on high-fat diet (compared to those on standard diet) suggests that high-fat-mediated increased circulating insulin levels may directly affect growth plate function and bone growth. High-fat diet was not associated with any change of Akt phosphorylation in KO mice. In addition, in vitro studies in cultured primary chondrocytes revealed that Akt mediates the stimulatory effects of insulin on chondrocyte proliferation and differentiation. In conclusion, the activation of the insulin receptor in the growth plate of mice fed with a hypercaloric diet stimulates skeletal growth and growth plate chondrogenesis.

1. Introduction

Statural growth and adult height in humans depend on the interplay of multiple genetic variants and the environment. Among the environmental factors known to regulate growth, nutrition has a very significant role. It is well known that undernutrition/malnutrition is a major cause of growth failure, primarily (but not only) in less developed countries of the world. On the other hand, ample evidence indicates that obesity in children is associated with acceleration of statural growth and skeletal maturation [1–13]. Because of their advanced skeletal maturation, however, obese children reach their final height sooner than their non-obese peers; thus, despite their growth acceleration they do not typically experience any gain in adult height relative to their genetic potential [12]. The underlying mechanisms of the obesity-related growth acceleration remain poorly defined. The effects of a number of growth-promoting hormones have been implicated [14–20], including those of insulin [21–24].

Since obese children are often hyperinsulinemic resistant, it has been postulated that the elevated serum insulin levels typically detected in obese subjects may promote statural growth. Previously, we have shown that, in mice fed with a high-fat, hypercaloric diet, hyperinsulinemia may stimulate growth plate chondrogenesis and longitudinal bone growth [25]. However, our findings have not unequivocally demonstrated an insulin-mediated stimulation of the insulin receptor expressed in the rodent growth plate as the underlying molecular mechanism of accelerated bone growth.

To determine whether insulin can directly affect growth plate chondrogenesis and longitudinal bone growth, we used a transgenic

* Corresponding author at: Children's Mercy Kansas City, Division of Endocrinology and Diabetes, 3101 Broadway Boulevard, Suite 900, Kansas City, MO 64111, United States of America.
E-mail address: fdeluca@cmh.edu (F. De Luca).

https://doi.org/10.1016/j.bone.2019.02.021
Received 30 November 2018; Received in revised form 15 February 2019; Accepted 20 February 2019
Available online 21 February 2019
8756-3282/

mouse in which the Insulin Receptor (IR) gene was conditionally ablated postnatally by Cre-lox recombination only in chondrocytes. We then administered a high-fat diet to control and transgenic mice and monitored its effects on weight gain, body and tibial growth, and growth plate morphology.

2. Materials and methods

2.1. Generation of mice with cartilage-specific IR disruption

Briefly, the inducible $^{TamCart}IR^{flox/flox}$ mice were generated by breeding $IR^{flox/flox}$ mice (These mice *loxP*sequences flanking exon 4 of the IR gene) [26]. They were purchased from Jackson Laboratories, stock number, #006955-B6.129S4) with mice expressing a *CreERCart* transgene, which encodes a fusion protein of the Cre recombinase and a mutated estrogen-responsive element to confer sensitivity to tamoxifen [27,28] and is regulated by the α1(II) promoter for cartilage-specific expression [27] (these mice were kindly donated by Dr. Susan Mackem). The resulting $^{TamCart}IR^{flox/flox}$ mice were viable and fertile in the absence of tamoxifen. To produce *IR* gene knockout in chondrocytes of $^{TamCart}IR^{-/-}$ mice during postnatal growth, $^{TamCart}IR^{flox/flox}$ mice were injected with four doses of tamoxifen (0.2 mg/mouse) at 2-day intervals beginning at postnatal day 5 (Fig. 1A). To control for any non-specific effect of tamoxifen, $IR^{flox/flox}$ mice were also injected at the same time points. From now on, we will refer to the $^{TamCart}IR^{-/-}$ mice as KO mice and to $IR^{flox/flox}$ littermates as C mice.

2.2. Genotyping and determination of tissue-specific deletion of the IR gene

Genotyping was performed after extracting genomic DNA from tail snips and other tissues (heart, liver, spleen, lung, kidney, and the tibial growth plate) using DNeasy Blood and Tissue Kit (QIAGEN Inc., Valencia CA). Polymerase chain reaction (PCR) analyses of the DNA were performed to detect Cre and floxed-IR alleles using corresponding primer sets with standard conditions (30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C for 35 cycles). PCR was performed with a mixture of three primers (two forward primers, 5′-GAT GTG CAC CCC ATG TCT G-3′ and 5′- ACG CCT ACA CAT CAC ATG C-3′, and a reverse primer, 5′-CTG AAT AGC TGA GAC CAC AG-3′) to detect a 205 bp smaller product from the excised gene alleles, the wild-type allele generates a 280-bp product and the floxed allele generates 300-bp product, respectively [29]. The cre transgene was detected by PCR using the primers 5′-GAA AAT GCT TCT GTC CGT TTGC -3′ (forward) and 5′-ATT GCT GTC ACT TGG TCG TGGC -3′ (reverse) to amplify a 207-bp DNA product.

2.3. Animal care

Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals [DHEW Publication (NIH) 85-23, revised 1988]. All procedures were approved by the Institutional Animal Care and Use Committee of Drexel University College of Medicine.

Male and female mice were maintained in a temperature-(22 °C), humidity-, and light-(12 h of light, 12 h of darkness)-controlled environment. Three-week old KO and C mice were housed separately and allowed ad libitum access to chow and water. At the beginning of the 4-weeks study period (at P21, Fig. 1A), mice were assigned to 4 groups: two "Standard-Diet" groups (control, 3 males and 3 females. KO, 3 males and 3 females) and two "High-Fat-Diet" groups (control, 6 males and 5 females. KO, 3 males and 4 females). The Standard Diet consisted of standard chow (4% of energy as fat, 3.85 kcal/g), while the High-Fat-Diet had 60% of energy as fat, 5.24 kcal/g. Both diets were provided ad lib.

Weight and whole body length (from nose to anus) were measured weekly during the 4-week study period after the animals were sedated with an injection of ketamine hydrochloride (0.02 mg/100 g body weight) (Phoenix Pharmaceutical, Inc.) and acepromazine (0.1 mg/100 g body weight) (Roche Applied Science). Serial radiographs of the tibiae were obtained weekly, with each animal placed prone on an x-ray

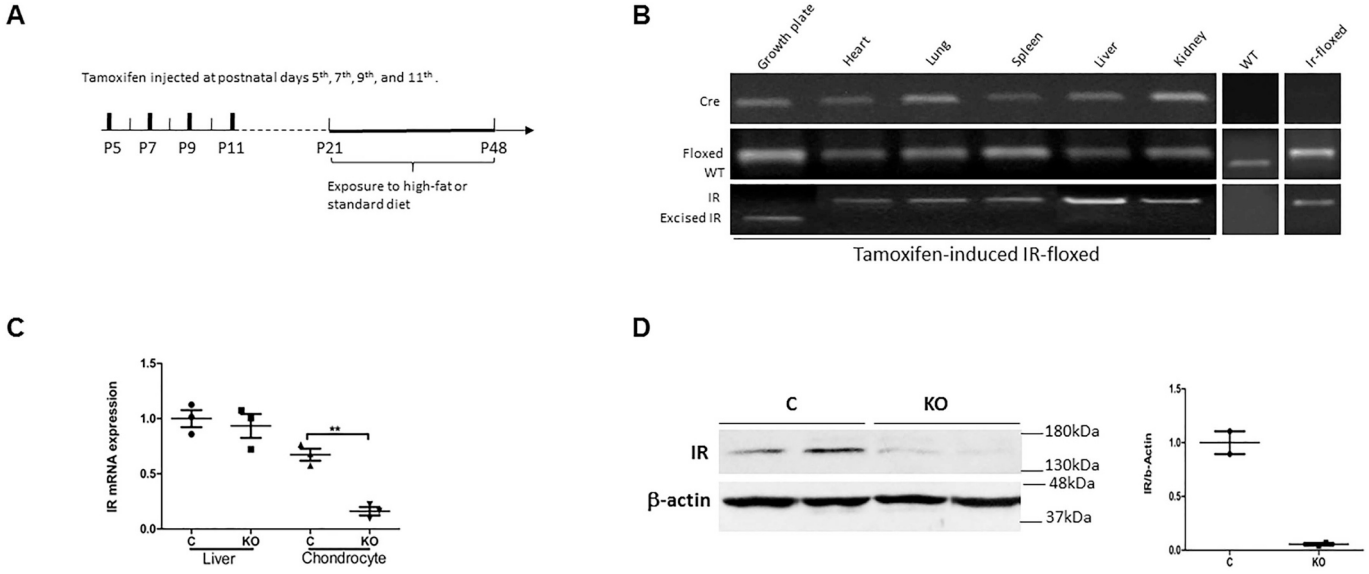

Fig. 1. Ablation of the *IR* in $^{TamCart}IR^{-/-}$(KO) mice.
A: Timeline of the experimental design: mice were injected with 4 doses of tamoxifen (0.2 mg/mouse) intraperitoneally at 2-day intervals beginning on day 5 after birth. Both control mice ($IR^{flox/flox}$) and KO mice ($^{TamCart}IR^{-/-}$) received tamoxifen. At postnatal day 21 (P21), mice were fed with high-fat diet or standard diet for 4 weeks. B: PCR analyses of genomic DNA extracted from different tissues of the $^{TamCart}IR^{-/-}$(KO) and of the $IR^{flox/flox}$ littermates (C) with primer sets for the Cre transgene, floxed-*IR* allele, and sequences after gene excision (*IR*) as described in "Materials and Methods." The labels "WT and IR-floxed" refer to liver samples. The bands labeled "Floxed" are obtained from F2 (IR-Floxed, col2a-cre) pups before tamoxifen injection. The bands labeled "IR/Excised IR" are obtained from F2 (IR-Floxed, col2a-cre) pups after 4 doses of tamoxifen injection. C: At the end of 4-week study period, total RNA was extracted from the liver and growth plate of KO and C mice (n = 6–11/group) and expression of *IR* mRNA was detected by real-time PCR. The mRNA level was normalized by β-actin in the same samples. D: At the end of the study period, protein extracted from the tibial growth plates of C and KO mice was electrophoresed and immunoblotted with an antibodies directed to IR and β-actin. A representative blot from three independent experiments is presented. **P < 0.01.

cassette. The lengths of both tibiae were measured on the radiograph, and the average value was calculated.

At the end of the 4-week study period, blood was collected by ventricular puncture from each animal before euthanasia. Serum samples were obtained and stored at −80 °C for insulin, leptin, IGF-1, and β-hydroxybutyrate analysis. After euthanasia, tibial growth plates were removed; half of them were stored at −80 °C for subsequent RNA extraction and half were fixed with 4% paraformaldehyde overnight, decalcified with Decalcifier II (Surgipath, Richmond, IL) for 1 h, and paraffin-embedded.

2.4. Measurement of serum hormones and metabolites

After collection, serum was stored at −80 °C before analysis. Insulin levels were determined by an ultrasensitive mouse-specific ELISA (ALPCO, Salem, NH) with intra- and inter-assay C.Vs of 3.9–6.6% and 5.7–5.83% respectively. IGF-1 levels were determined in duplicate by a mouse- specific Quantikine ELISA including a 500-fold dilution of all samples (R&D Systems, Inc. Minneapolis, MN) with intra- and inter-assay C·V. of 4.1 to 5.6% and 4.3 to 9.0% respectively. Leptin levels were determined in duplicate by a mouse-specific ELISA (Crystal Chem, Downers Grove, IL) both intra- and inter-assay C.V. were < 10%. β-hydroxybutyrate (β-HB) levels were determined in duplicate using an enzyme colorimetric assay (BioVision, Mountain View, CA).

2.5. Quantitative histology

At the end of the 4-week study period, tibiae (4–7 tibiae for each treatment group) were removed and fixed with 4% paraformaldehyde overnight, decalcified with Decalcifier II (Surgipath, Richmond, IL) for 1 h, and paraffin-embedded. Three 5–7-μm thick longitudinal sections from each bone were obtained and stained with hematoxylin/phloxine/saffron. Tibial growth plate epiphyseal, proliferative, hypertrophic zone heights (μm) as well as the height of whole growth plate were measured. All measurements were performed by a single observer blinded to the treatment regimen. The representative pictures shown in the figures were obtained at 20× magnification. The longitudinal sections were then scanned by Leica SCN400 and the size of hypertrophic chondrocytes was measured using a Leica Microsystems software. Using 5 mice in each group, we chose three grids per section with 10 cells included in each grid, and the chondrocyte size was measured and averaged in each grid. All measurements were made by the same observer blinded to the treatment and genotype categories.

2.6. RNA extraction and real time PCR

Total RNA was extracted from the tibial growth plate using the QIAGEN RNeasy Mini kit (QIAGEN Inc., Valencia CA). The recovered RNA was further processed using 1st Strand cDNA Synthesis kit for RT-PCR (AMV)(Roche Diagnostics Corp. Indianapolis, IN) to produce cDNA. 1 μg of total RNA and 1.6 μg of oligo-p$(dT)_{15}$ primer were incubated for 10 min at 25 °C, followed by incubation for 60 min at 42 °C in the presence of 20 Units AMV Reverse transcriptase and 50 Units of RNase inhibitor in a total 20 μl reaction. The cDNA products were directly used for PCR or stored at −80 °C for later analysis. Real-time quantitative PCR was carried out using StepOne Real time PCR System (Applied Biosystems, Foster City, CA) in a final volume of 25 μl containing 1 μl cDNA, 12.5 μl 2 x SYBR Green master mix (Applied Biosystems), 0.1 μM primers (Applied Biosystems) in DNAse-free water. Primers used were: mouse β-actin (forward 5′-TGT GAT GGT GGG AAT GGG TCA GAA-3′, reverse 5′-TGT GGT GCC AGA TCT TCT CCA TGT-3′); mouse IR (forward 5′-GTT CTT TCC TGC GTG CAT TTC CCA-3′, reverse 5′-ATC AGG GTG GCC AGT GTG TCT TTA-3′), mouse IGF-1R (forward 5′- GGA GGA GTT CGA GAC AGA GTA-3′; reverse, 5′-CGA TGC GGT ACA GAG TGA AA-3′). The PCR conditions were: 50 °C for 2 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Values were quantified using the comparative cycle threshold (CT) method [30], and samples were normalized to β-actin.

2.7. siRNA transfection

Primary cultured chondrocytes isolated from mouse fetal metatarsal bones (dpc 20) were transfected with pools of siRNAs targeted for Akt (sc-29,196; Santa Cruz Biotechnology) and then cultured in the absence or in the presence of insulin (100 nM) for 72 h. A pool of siRNAs consisting of scrambled sequences was similarly transfected as control siRNA (Santa Cruz Biotechnology) using LipofectAMINE 2000 (Invitrogen), according to the procedure recommended by the manufacturer. One day before transfection, cells were plated in 500 μl of growth medium without antibiotics, such that they were 30%–50% confluent at the time of transfection. The transfected cells were cultured in DMEM containing 10% fetal calf serum for 72 h after transfection.

2.8. TUNEL assay

Apoptotic cells in the growth plate were identified by an In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany; Cat.No.12156792910), according to the manufacturer's instructions. Nuclei were counterstained with DAPI. TUNEL-positive cells were stained with red fluorescence. The percentage of TUNEL-positive cells was calculated as the number of TUNEL-labeled cells per grid divided by the total number of cells per grid. For each sample, the fraction of labeled cells in three distinct grid locations was calculated and averaged. All determinations were made by the same observer blinded to the treatment category.

2.9. BrdU incorporation

Primary chondrocytes were exposed to BrdU (1:50) the last 4 h of culture. The BrdU-labeled cells were visualized utilizing a BrdU labeling Kit (Invitrogen; Cat. No.93–3943) according to the manufacturer's instructions. The percentage of BrdU-positive cells was calculated as the number of BrdU-labeled cells per grid divided by the total number of cells per grid. For each sample, the fraction of labeled cells in three distinct grid locations was calculated and averaged. All determinations were made by the same observer blinded to the treatment category.

2.10. Western blot

To evaluate the IR and phosphorylated–IR protein expression, at the end of the in vivo study protein were extracted from the tibial growth plates, solubilized with 1% SDS sample buffer, and electrophoresed on a 4–15% SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane and probed with specific monoclonal or polyclonal antibodies. The following antibodies were used: monoclonal mouse antibody directed to IR (Cat. # SC-57342, Santa Cruz); goat polyclonal antibody directed to phosphorylated-IR (Tyr 1162/1163) (Cat. # SC-25103, Santa Cruz); antibody directed to Akt (Cat# 4691, Cell Signaling Technologies, Danvers, MA); antibody directed to phosphorylated-Akt (Tyr 308, Cat# 13038, Cell Signaling Technologies); antibody directed to PCNA (Cat.# bs-0754R, Shanghai Universal Biotech Co., Ltd.) and rabbit polyclonal antibody directed to β-actin (Cat# A-2066, Sigma). The blots were developed using a horseradish-peroxidase-conjugated polyclonal IgG and enhanced chemiluminescence system (GE healthcare). Western blot bands were quantitated by Image J and data were expressed as mean ± S.E from three independent experiments.

2.11. Immunohistochemistry

To determine the localization of Insulin Receptor (IR) in the growth plate of long bones, three 5–7-μm thick longitudinal sections obtained from mouse tibiae were deparaffined in xylene and rehydrated in

graded ethanol. Sections were incubated in 1% H2O2 for 10 min followed by three rinses with PBS. For digestion, 0.1% trypsin for 12 min was used at room temperature (RT) followed by a triple wash in PBS. After pre-incubation with 1.5% blocking serum for 30 min at RT, sections were incubated for 30 min at RT with monoclonal antibody raised against mouse IR (1:150) (Cat. # SC-57342, Santa Cruz Biotechnology Inc., Santa Cruz, CA). The secondary antibody was an anti-mouse antibody conjugated with biotin, applied for 30 min at a dilution of 1:200. This step was followed by an incubation for 30 min with avidin and biotinylated horseradish peroxidase. The sections were then visualized with peroxide substrate for 5 min and mounted with Permount medium. Control experiments were performed using normal rabbit serum instead of the primary antibody.

2.12. Statistics

All data are expressed as the mean ± SE. Data were analyzed using SPSS version 16.0 (SPSS Inc., Chicago, IL) and statistical significance was determined by two-way ANOVA followed by Tukey-Kramer's multiple comparison post hoc test. P values < 0.05 were considered statistically significant.

3. Results

3.1. Tissue-specific deletion of the IR gene

We confirmed the specificity of gene excision in the KO mice by PCR analyses of genomic DNA from different tissues. As shown in Fig. 1B, gene excision occurred in the cartilage of the KO mice but not in other tissues from the same animals or in the growth plate of C mice. Ablation of IR mRNA expression in the growth plate was confirmed by qPCR (Fig. 1C) and of protein expression by Western (Fig. 1D) and by immunohistochemistry (Supplemental Fig. 1A). In our study, the injection of tamoxifen in $IR^{flox/flox}$ (C mice) did not induce any detectable adverse effect on growth plate morphology.

3.2. Effects of standard diet and high-fat diet on weight gain, body growth, and tibial growth of control (C) and KO (knock-out) mice

At the beginning of the 4-week in vivo study, the mean body weight, body length, and tibial length of the four study groups were equivalent (Supplemental Table 1). At the end of the study, both C and KO mice exposed to a high-fat-diet experienced greater weight gain compared to the respective groups exposed to standard diet (Fig. 2A, Supplemental Table 2). The high-fat-diet–related weight gain was quantitatively similar in C and KO mice. In C mice, the body length increase and tibial length increase after 4 weeks of high-fat-diet were significantly greater than those of C mice fed with a standard diet (Fig. 2B–C, Supplemental Tables 2 and 5). In contrast, KO mice exposed to standard diet or high-fat diet experienced similar body length increase and tibial length increase (Fig. 2B–C, Supplemental Tables 2 and 5). The body length increase and tibial length increase of KO mice fed with a high-fat-diet was significantly smaller than those of C mice fed with a high-fat-diet (Fig. 2B–C, Supplemental Tables 2 and 5). We then made the same comparisons after separating the groups according to the animal gender. The body length increase of male high-fat-fed KO mice was significantly smaller than that of male high-fat-fed C mice, while the increase of body length in female high-fat-fed KO mice was comparable with that of female high-fat-fed C mice. The tibial length increase of both male and female high-fat-fed KO mice was significantly smaller than that of male and female high-fat-fed C mice. (Supplemental Tables 3 and 5).

3.3. Effects of standard diet and high-fat diet on the growth plate morphology of C and KO mice

Whole growth plate, the epiphyseal, proliferative, and hypertrophic zone heights of high-fat-fed C mice were all greater than standard-diet-fed C mice; no difference in zone height was found between high-fat-fed and standard-diet-fed KO mice (Fig. 3; Supplemental Table 4, Supplemental Fig. 1B). Measurements of the tibial whole growth plate, and of the epiphyseal, proliferative, and hypertrophic zone height revealed that all these parameters were significantly greater in high-fat-fed C mice compared to high-fat-fed KO (Fig. 3, Supplemental Table 4, Supplemental Fig. 1B). No significant difference in zone heights was found after comparing standard-diet-fed KO and C mice (Fig. 3, Supplemental Table 4, Supplemental Fig. 1B). Since the hypertrophic zone height primarily depends on the hypertrophic chondrocyte size, we measured and compared this parameter in all 4 groups of mice. KO mice fed with either the standard diet or the high-fat diet exhibited significantly smaller hypertrophic chondrocyte size compared to C mice fed with the same diet regimen (Fig. 3E).

To study the effects of standard and high-fat diet on growth plate chondrogenesis, we analyzed PCNA (a marker of chondrocyte proliferation) protein expression and collagen X (a marker of chondrocyte differentiation) mRNA expression in the growth plate of C and KO mice. The expression of both markers was significantly greater in high-fat-fed C mice compared to standard-diet-fed C mice; we found no difference after comparing high-fat-fed KO mice and standard-diet-fed KO mice. Both PCNA and collagen X expression in the growth plate was greater in high-fat-fed C mice than in high-fat-fed KO mice (Fig. 4A–B). Lastly, the number of apoptotic chondrocytes (as assessed by TUNEL) was significantly greater in KO mice exposed to high-fat diet when compared to C mice fed with high-fat diet and all mice fed with standard diet. We found no difference between C mice and KO mice fed with standard diet (Fig. 4C, Supplemental Fig. 2A).

3.4. Effects of standard diet and high-fat diet on serum hormones and metabolites of control and IR KO mice

At the end of the study, we found no difference between C and KO mice fed with either the standard or the high-fat diet with respect to serum insulin, leptin, IGF-1 or β-hydroxybutyrate levels (Fig. 5). Both C and KO mice fed with high-fat diet exhibited greater serum insulin and leptin levels, and lower β-hydroxybutyrate compared to the same-genotype groups fed with standard diet. High-fat diet was associated with higher serum IGF-1 levels in both C and KO mice compared to the respective groups fed with the standard diet; however, the difference was statistically significant only in C mice.

3.5. Effects of standard diet and high-fat diet on the tibial insulin receptor and Akt phosphorylation

To determine whether the increased serum insulin levels in mice fed with high-fat diet may have activated the IR and, in turn, Akt (one of the intracellular mediators of insulin action) in the mouse tibial growth plate, we analyzed phosphorylated-IR and phosphorylated-Akt expression by Western at the end of the 4-week in vivo study. High-fat diet was associated with greater phosphorylated-IR and Akt expression in C mice compared to KO mice, while no difference was observed between standard-diet-fed C and KO mice (Fig. 6). We found greater phosphorylated-IR and Akt expression in high-fat-fed C mice compared to standard-diet-fed C mice. No difference was found between high-fat-fed and standard-diet-fed KO mice. To determine whether the different effects induced by high-fat diet in C and KO mice were due to a different IGF1-R expression, we analyzed the IGF-1R mRNA expression in the tibial growth plate. We found no difference when we compared high-fat-fed C and KO mice or standard-diet-fed C and KO mice (Fig. 6D).

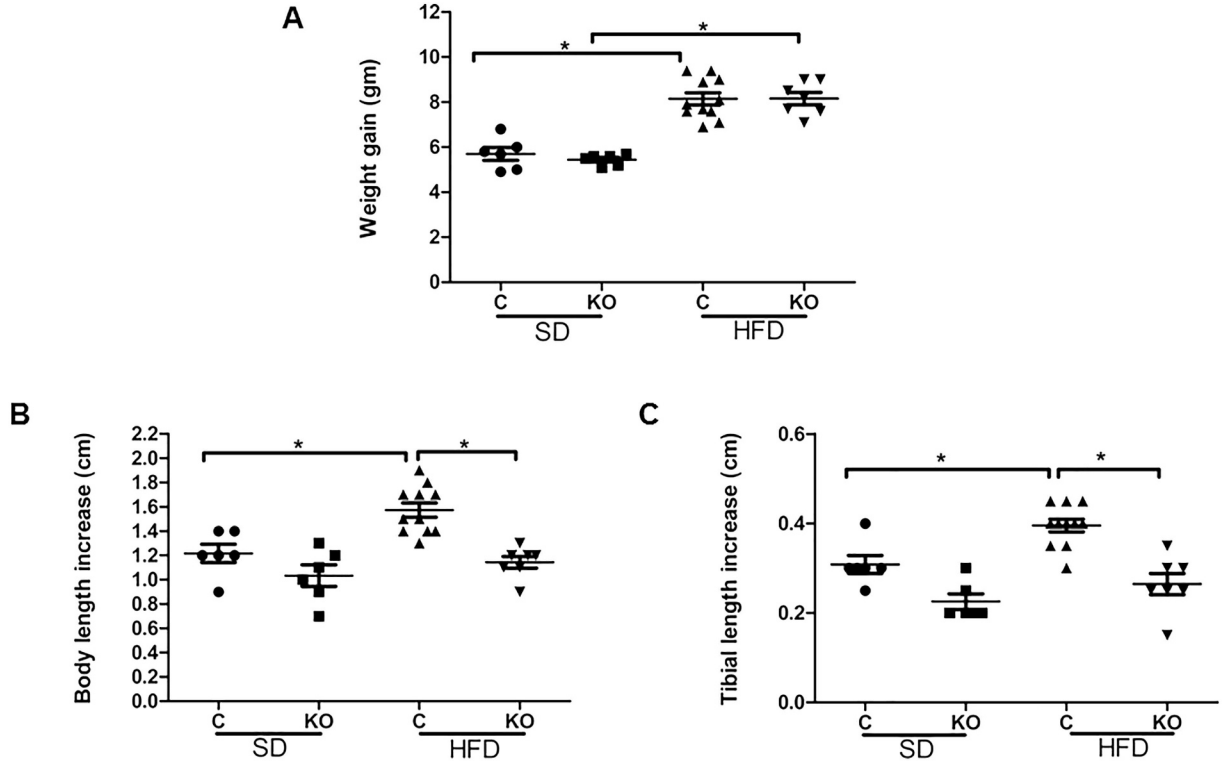

Fig. 2. Effects of high-fat diet on weight gain, body length increase, and tibial length increase of control and KO mice.
At the beginning of the 4-weeks study period, three-week old KO (knock-out) and C (control) mice were assigned to four groups: two "SD" (Standard Diet) groups (C, 3 males and 3 females. KO, 3 males and 3 females) and two "HFD" (High-Fat Diet) groups (C, 6 males and 5 females. KO, 3 males and 4 females). The Standard Diet consisted of standard chow (3.85 kcal/g), while the High-Fat-Diet had 5.24 kcal/g. Both diets were provided ad lib. Body weight, whole body length (from nose to anus) and tibial length (average length of both tibiae measured on radiographs) were obtained weekly. The difference in body weight (in g), body length (in cm) and tibial length (in cm) measured at the beginning and at the end of the 4-week study period represents the body weight gain (panel A), whole body length increase (panel B) and tibial length increase (panel C). *P $<$ 0.05.

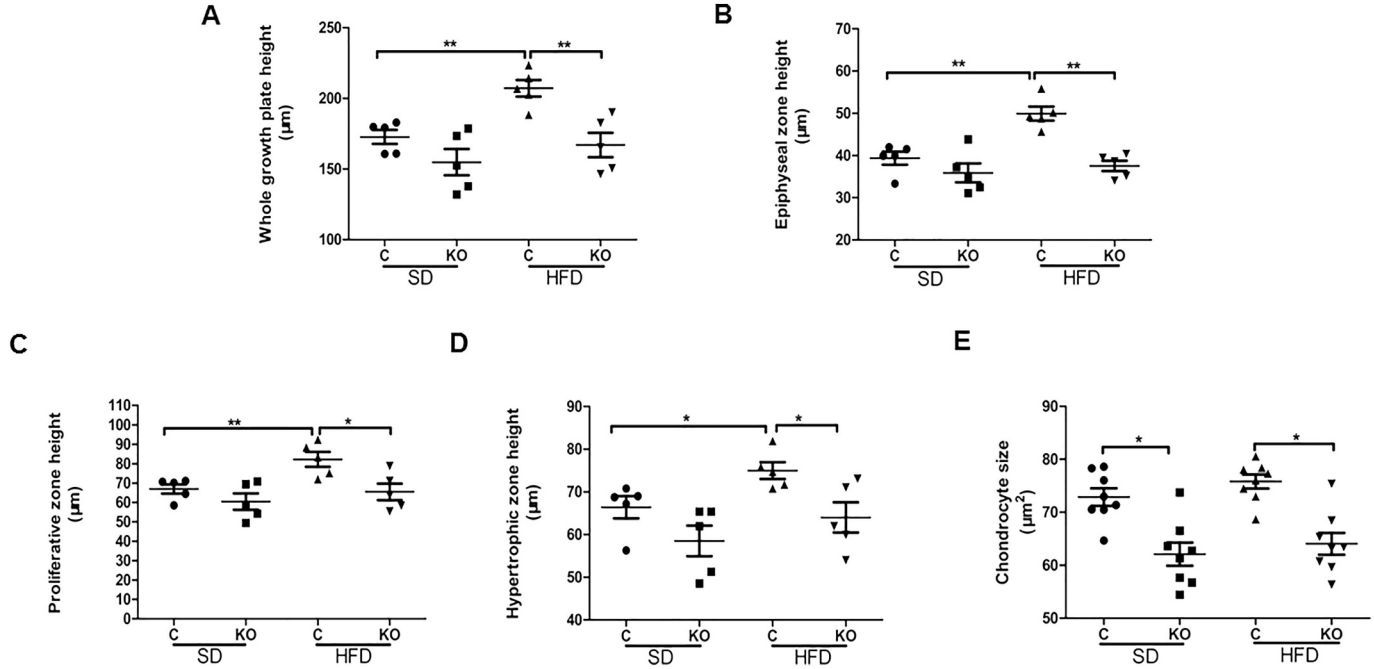

Fig. 3. Effects of high-fat diet on the growth plate morphology of C and KO mice.
At the end of the 4-week experimental period, tibiae were dissected from C and KO mice assigned to four groups (C-standard diet, C-high-fat diet, KO-standard diet, and KO-high-fat diet). After dissection, tibiae were fixed, decalcified, and paraffin-embedded. Three 5–7 μm thick longitudinal sections were obtained and stained with hematoxylin/phloxine/saffron. The tibial growth plate zone heights were measured by one observer blinded to the treatment groups. A, epiphyseal zone; B, proliferative zone; C, hypertrophic zone; D, whole growth plate. E: **S**ize of hypertrophic chondrocyte. Chondrocyte size was measured as described in the Experimental Procedures. Data are expressed as means ± SE in each scatter plots. *P $<$ 0.05,**P $<$ 0.01.

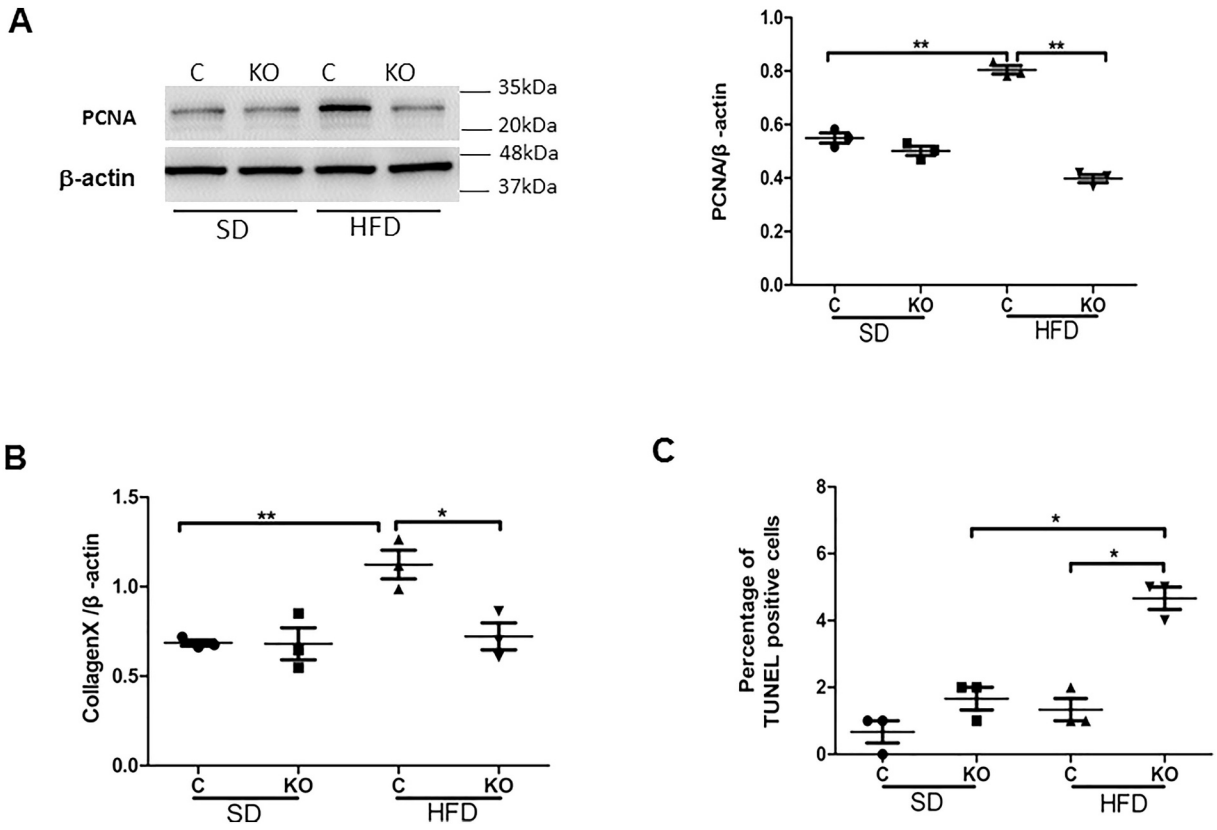

Fig. 4. Effects of high-fat diet on the growth plate chondrocyte proliferation, differentiation, and apoptosis of C and KO mice.
Chondrocyte proliferation and differentiation were assessed by protein expression of PCNA and mRNA expression of Collagen X, respectively. Protein extracted from the mouse tibial growth plates at the end of the 4-week study period was electrophoresed and immunoblotted for PCNA and the loading control β-actin. The intensity of the bands on Western blots was analyzed by NIH Image J. Results are expressed as the ratio of PCNA to β-actin (Mean ± S.E). A: left panel, a representative blot from three independent experiments is presented. SD, standard-diet-fed mice. HFD, high-fat-fed mice. Right panel, quantitation of PCNA was normalized by b-actin. B: Expression of Collagen X mRNA was detected by real-time PCR. The mRNA level was normalized by β-actin in the same samples. C: The percentage of TUNEL-positive cells in the whole growth plate was quantitated as described in the Experimental Procedures (n = 6/group). Data are expressed as means ± SE in each scatter plots. *P < 0.05,**P < 0.01.

3.6. Role of Akt signaling in the insulin-mediated stimulation of proliferation and differentiation in cultured chondrocytes

To determine whether Akt mediates the effects of insulin on chondrocyte function, we transfected cultured chondrocytes with specific Akt siRNA. Insulin significantly promoted chondrocyte proliferation and differentiation in chondrocytes transfected with control (scramble) siRNA (as evaluated by BrdU incorporation (Fig. 7A) and collagen X expression (Fig. 7B); however, these stimulating effects were prevented by Akt siRNA transfection.

4. Discussion

Children with obesity secondary to excessive caloric intake are typically tall for their age; such tall stature is due to an accelerated statural growth rate and is associated with an advanced skeletal maturation. The fact that obese children often experience an earlier onset of puberty explains why they do not achieve an adult height in excess of their genetic target. With respect to the biological mechanisms responsible for the obesity-associate acceleration of linear growth and skeletal maturation, hyperinsulinemia has been implicated as one of the causative factors.

Multiple lines of evidence support a regulatory role for insulin in statural growth. Newborns affected by pancreatic agenesis or mutations of the insulin receptor exhibit severe intrauterine growth retardation (IUGR) [31]. Conversely, insulin resistance and secondary hyperinsulinemia have been linked with obesity-related tall stature [10,11]. Pre-pubertal obese children have significantly higher serum insulin levels than lean controls, with insulin levels being positively correlated and their insulin sensitivity inversely correlated with their height [32]. The stimulatory effects of hyperinsulinemia on bone growth, despite its underlying insulin resistance, may be explained by the fact that insulin resistance does not uniformly affect all mammalian organs and tissues. Indeed, it is known that obesity-related insulin resistance in mammals is typically detected in the liver, while keratinocytes and sex-steroid secreting cells in the ovaries (and possibly the growth plate) remain insulin-sensitive and, thus, may be affected by increased circulating insulin levels.

Experimental work in animals also suggests a functional role for insulin on body growth. It has been shown in rats that the systemic injection of insulin induces body growth [33,34]. Furthermore, in vitro studies have demonstrated a stimulatory effect of insulin on chondrocyte proliferation [35–38] and variable effects on chondrocyte differentiation [25,35–38]. On the other hand, such findings do not necessarily demonstrate a specific, direct effect of insulin on bone growth and skeletal maturation. We have previously shown that a hypercaloric, high-fat diet in mice induces an accelerated body and tibial growth, with the concomitant administration of an insulin sensitizer and the consequent correction of the animals' hyperinsulinemia preventing such growth acceleration. In the same study, we also demonstrated an in vitro stimulatory effect of insulin on cultured metatarsal linear growth and growth plate chondrocyte proliferation and differentiation. These insulin effects were prevented by the targeted deletion of the insulin receptor in cultured chondrocytes. Thus, these observations indicate

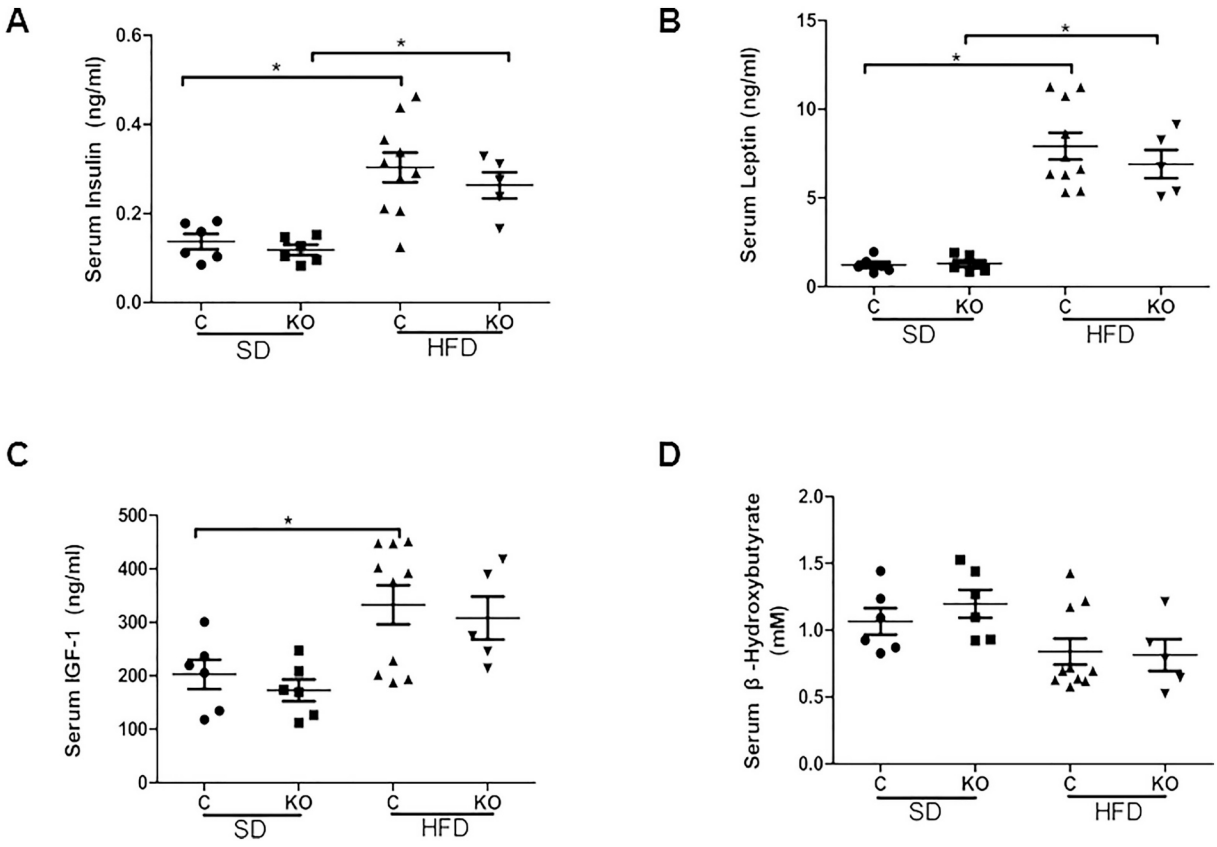

Fig. 5. Serum levels of insulin, leptin, IGF-1, and β-hydroxybutyrate in C and KO mice.
At the end of the study period, blood was collected from control (C) and knock-out (KO) mice assigned to four experimental groups: two "SD" (Standard Diet) groups (C, 3 males and 3 females. KO, 3 males and 3 females) and two "HFD" (High-Fat Diet) groups. After collection, serum was separated and stored at −80 °C before analysis. Serum levels of insulin (A), leptin (B), IGF-1 (C) and β-hydroxybutyrate (D) were determined by an ultrasensitive mouse-specific ELISA (insulin, IGF-1, and leptin) and an enzyme colorimetric assay (β-hydroxybutyrate). Results are expressed as mean ± S.E. *P < 0.05.

that the stimulatory effects of insulin on cultured chondrocyte proliferation and differentiation are primarily mediated by the insulin receptor. Yet, this evidence is not sufficient to support the notion that obesity-associated hyperinsulinemia due to insulin resistance accelerates statural growth via a direct activation of the insulin receptor expressed in the long bones' growth plate.

In this study, mice with the targeted deletion of the insulin receptor in chondrocytes and exposed to standard diet regimen exhibited a somewhat reduced body and tibial growth, as well as reduced growth plate height and hypertrophic chondrocyte size compared to standard-diet-fed control mice. However, all these effects (with the exception of the reduced hypertrophic chondrocyte size) were not statistically significant. Control mice fed with a high-fat diet experienced a significant acceleration of body and tibial growth, as well as growth plate chondrocyte proliferation and differentiation. Such effects were statistically significant when compared to control mice fed with a standard diet. In contrast, KO mice exposed to a high-fat diet did not experience any induction of growth acceleration or chondrocyte proliferation and differentiation; in addition, their body and tibial bone growth, as well growth plate chondrocyte proliferation and differentiation were significantly smaller, and chondrocyte apoptosis greater, than those observed in high-fat-fed control mice. These findings indicate that a high-fat diet induces bone growth and growth plate chondrogenesis in mice by activating the insulin receptor in the growth plate. Although immunohistochemistry has detected the expression of the insulin receptor primarily in the growth plate hypertrophic zone, the lack of increased chondrocyte proliferation and differentiation in KO mice fed with a high-fat diet suggests that insulin receptor activation does modulate both cell proliferation and differentiation, and that insulin receptor is also expressed in the growth plate proliferative zone. It is difficult to explain the mechanisms underlying the increased cell apoptosis detected in the growth plate of KO mice fed with a high-fat diet. Such finding would suggest that high-fat and/or high-calorie intake may potentially induce chondrocyte apoptosis (despite the overall induction of growth plate chondrogenesis and bone growth), which is normally prevented by insulin receptor activation in the growth plate. Yet, at this time we are unable to speculate on the nature of this potential pro-apoptotic factor.

The effects on bone growth and on the growth plate were associated with an increased phosphorylation of the insulin receptor and intracellular Akt in high-fat-fed C mice, which suggests that these animals' high serum insulin levels led to the activation of the insulin receptor and its intracellular signaling cascade. In contrast, the high-fat-diet-associated hyperinsulinemia in KO mice did not induce Akt activation in the growth plate; thus, these findings demonstrate that the activation of Akt in the growth plate of animals exposed to an excessive caloric intake exclusively depends on the activation of the insulin receptor.

In a previous publication, others have described the effects of the selective deletion of the insulin receptor in mouse chondrocytes [39]. They observed no difference in the animals' femoral length when compared to control littermates; consistent with their finding, we did not observe any significant difference in body and tibial growth between C and KO mice exposed to a standard diet. Conversely, they reported an increased number of chondrocytes in the growth plate of the knock-out mice, as well as increased Akt phosphorylation in chondrocytes lacking the insulin receptor. They interpreted many of their findings (spared long bone growth, increased cell proliferation and Akt phosphorylation) as the effects of the increased IGF1-R expression in

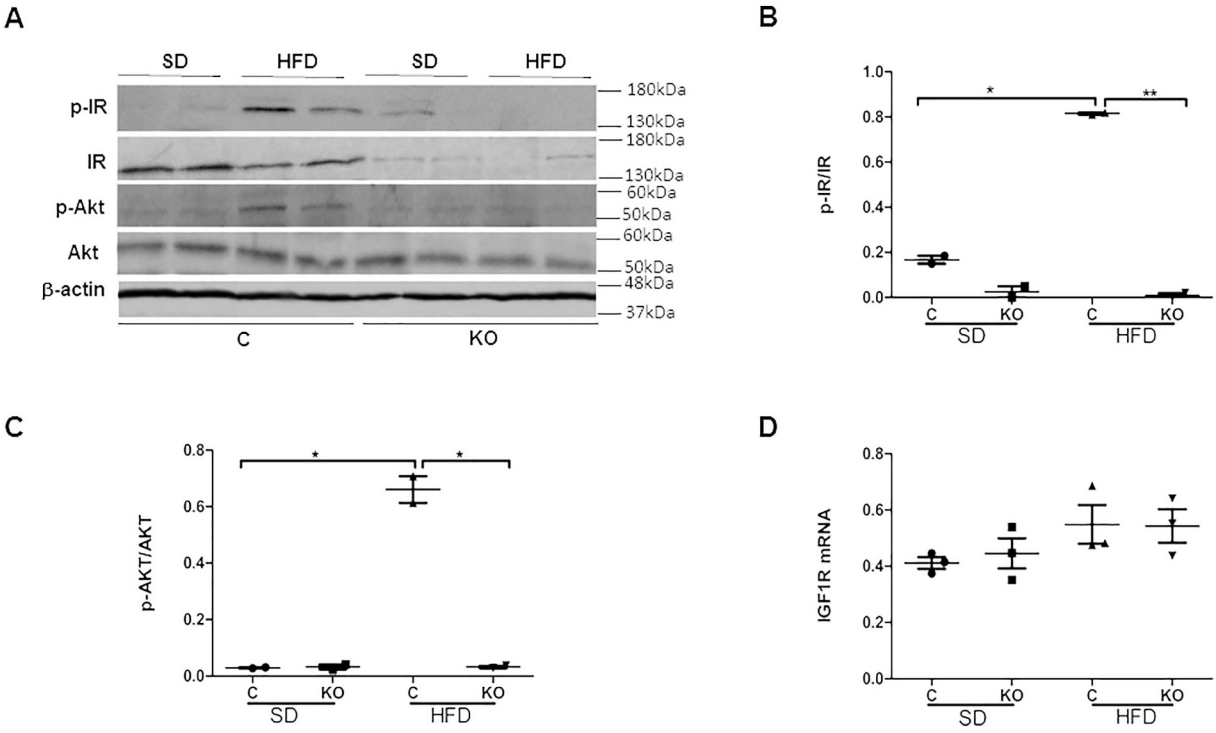

Fig. 6. Effects of high-fat diet on the tibial growth plate IR and Akt phosphorylation in C and KO mice.
Protein extracted from the mouse tibial growth plates at the end of the 4-week study period was electrophoresed and immunoblotted for IR, phosphorylated IR (p-IR), Akt, phosphorylated Akt (p-Akt) and the loading control β-actin. The intensity of the bands on Western blots was analyzed by NIH Image J. Results are expressed as the ratio of p-IR to total IR and p-Akt to total Akt (Mean ± S.E), respectively. A: A representative blot from three independent experiments is presented. SD, standard-diet-fed mice. HFD, high-fat-fed mice. B–C: Quantitation of phosphorylated IR and Akt normalized by total IR and Akt, respectively. D: At the end of 4-week study period, total RNA was extracted from the growth plate of KO and C mice (n = 6–11/group) and expression of *IGF-1R* mRNA was detected by real-time PCR. The mRNA level was normalized by β-actin in the same samples. *P < 0.05, **P < 0.01.

chondrocytes lacking the IR. In contrast with this study, we have not found any change in IGF1-R expression in chondrocytes isolated from KO mice, which may be explain the reduced longitudinal bone growth, decreased cell proliferation and differentiation, and decreased Akt phosphorylation in our KO mice exposed to a high-fat diet. The discordant findings may also depend on the different experimental design of the two studies: ours was based on the effects of a postnatal insulin receptor deletion in the growth plate, while in the other study IR deletion in chondrocytes was induced prenatally. In addition, we focused on the effects of high-fat diet and secondary hyperinsulinemia on bone growth and growth plate chondrogenesis of mice lacking the insulin receptor in chondrocytes, while the other study described the effects of insulin receptor deletion in chondrocytes of animals exposed to a standard diet regimen.

In another publication [40], the authors generated mice with an osteoprogenitor-selective (osterix-Cre) ablation of the insulin receptor

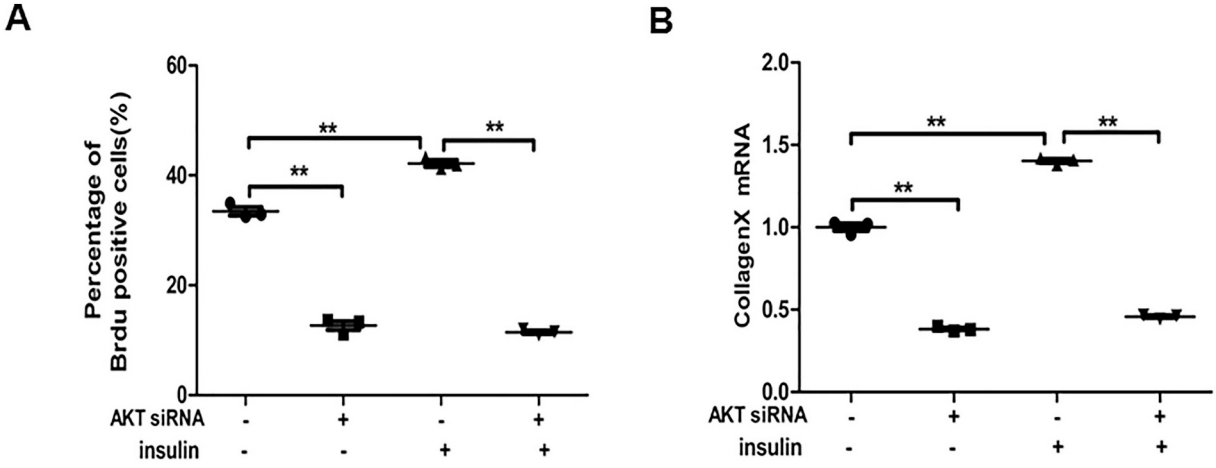

Fig. 7. Role of Akt signaling in mediating insulin effects on chondrocyte proliferation and differentiation
Primary cultured chondrocytes isolated from fetal metatarsal bones (dpc 20) were transfected with Akt siRNA and then cultured in the absence or in the presence of insulin (100 nM) for 72 h. A: Chondrocyte proliferation was evaluated by BrdU labeling, and the number of BrdU-positive cells was quantitatively analyzed (n = 4/group). B: Expression of *Collagen X* mRNA was detected by real-time PCR. The mRNA level was normalized by β-actin in the same samples. Data are expressed as means ± SE in each scatter plots. **P < 0.01.

(IR), which resulted in an exclusive and significant decrease in IR expression in osteoblasts. At the age of 10–11 wks (males) or 12–13 wks (females) the knock-out mice exhibited decreased bone strength and shorter femurs compared to control mice. No apparent morphological change of the KO mouse growth plate was noted. Compared to our work, these authors examined the prenatal and postnatal effects of the loss of IR in osteoblasts rather than in chondrocytes, focusing their attention on structural changes in the bone rather than on any specific effects on postnatal longitudinal bone growth.

With respect to the role of the intracellular signaling pathways activated by the insulin receptor in growth plate chondrocytes, it has been shown that overexpression of Akt in the cartilage of transgenic mice promotes chondrocyte differentiation whereas overexpression of a dominant-negative form delays this process [41]. While Akt1-deficient mice are smaller than controls, they only show very subtle changes in their growth plates [42], suggesting that Akt2 and/or Akt3 partially compensate for Akt1 loss in chondrocyte differentiation. Overall, the phenotype of these mice supports a model where Akt signaling promotes chondrocyte differentiation and longitudinal bone growth.

With respect to additional mechanisms possibly inducing statural growth during excessive weight gain, the increased levels of estradiol [20], leptin [14,15], prolactin [16], and adrenal androgens [12] in obese children may suggest a causative role for these hormones in growth acceleration. Furthermore, changes of circulating IGF-1 and IGF binding protein (IGFBP) levels may also be implicated in growth promotion in obese children. In several studies, serum IGF-1 levels have been reported to be low [43,44], normal [45,46], or elevated [46–48]. Increased free IGF-1 and decreased IGFBP-1 serum levels have been described in obese children [18,19,46,47,49]: it is plausible that the increased free IGF-1 associated with obesity results from the effects of obesity-related hyperinsulinemia. Although we have not measured serum free IGF-1 and IGFBP1 in our study, the fact that the lack of the insulin receptor in the growth plate of KO mice prevented any hypercaloric-diet-related effects on body and tibial growth suggests that insulin receptor activation in the growth plate may be sufficient in mediating the high-calorie-dependent bone growth acceleration.

In conclusion, in mice fed with a high-fat, hypercaloric diet the increased activation of the insulin receptor in the growth plate (secondary to hyperinsulinemia) is sufficient to induce skeletal growth and growth plate chondrogenesis. Thus, our findings suggest that insulin is the principal mediator of the obesity-related accelerated statural growth and skeletal maturation in mammals, including humans.

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.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.02.021.

References

[1] S. Shalitin, W. Kiess, Putative effects of obesity on linear growth and puberty, Horm. Res. Paediatr. 88 (1) (2017) 101–110.
[2] S. Stanojevic, J. Kain, R. Uauy, The association between changes in height and obesity in Chilean preschool children: 1996–2004, Obesity 15 (4) (2007) 1012–1022.
[3] I.E. Buchan, P.E. Bundred, D.J. Kitchiner, T.J. Cole, Body mass index has risen more steeply in tall than in short 3-year olds: serial cross-sectional surveys 1988–2003, Int. J. Obes. 31 (1) (2007) 23–29.
[4] A. Papadimitriou, T. Gousi, O. Giannouli, P. Nicolaidou, The growth of children in relation to the timing of obesity development, Obesity 14 (12) (2006) 2173–2176.
[5] M.B. Leonard, J. Shults, B.A. Wilson, A.M. Tershakovec, B.S. Zemel, Obesity during childhood and adolescence augments bone mass and bone dimensions, Am. J. Clin. Nutr. 80 (2) (2004) 514–523.
[6] D.B. Dunger, M.L. Ahmed, K.K. Ong, Effects of obesity on growth and puberty, Best Pract. Res. Clin. Endocrinol. Metab. 19 (3) (2005) 375–390.
[7] D.S. Freedman, J.C. Thornton, Z.G. Mei, J. Wang, W.H. Dietz, R.N. Pierson, M. Horlick, Height and adiposity among children, Obes. Res. 12 (5) (2004) 846–853.
[8] D.S. Freedman, L.K. Khan, M.K. Serdula, W.H. Dietz, S.R. Srinivasan, G.S. Berenson, Inter-relationships among childhood BMI, childhood height, and adult obesity: the Bogalusa Heart Study, Int. J. Obes. 28 (1) (2004) 10–16.
[9] Q. He, J. Karlberg, BMI in childhood and its association with height gain, timing of puberty, and final height, Pediatr. Res. 49 (2) (2001) 244–251.
[10] M. Vignolo, A. Naselli, E. Dibattista, M. Mostert, G. Aicardi, Growth and development in simple obesity, Eur. J. Pediatr. 147 (3) (1988) 242–244.
[11] L.H. Epstein, J. Mccurley, A. Valoski, R.R. Wing, Growth in obese children treated for obesity, Am. J. Dis. Child. 144 (12) (1990) 1360–1364.
[12] M. Desimone, G. Farello, M. Palumbo, T. Gentile, M. Ciuffreda, P. Olioso, M. Cinque, F. Dematteis, Growth charts, growth velocity and bone-development in childhood obesity, Int. J. Obes. 19 (12) (1995) 851–857.
[13] K.K.L. Ong, M.L. Ahmed, P.M. Emmett, M.A. Preece, D.B. Dunger, A.L.S. Pregnancy, Association between postnatal catch-up growth and obesity in childhood: prospective cohort study, Br. Med. J. 320 (7240) (2000) 967–971.
[14] S. Shalitin, M. Phillip, Role of obesity and leptin in the pubertal process and pubertal growth - a review, Int. J. Obes. 27 (8) (2003) 869–874.
[15] G. Gat-Yablonski, M. Phillip, Leptin and regulation of linear growth, Curr. Opin. Clin. Nutr. Metab. Care 11 (3) (2008) 303–308.
[16] H. Bucher, J. Zapf, T. Torresani, A. Prader, E.R. Froesch, R. Illig, Insulin-like growth factor-I and factor-ii, prolactin, and insulin in 19 growth-hormone deficient children with excessive, normal, or decreased longitudinal growth after operation for craniopharyngioma, New Engl. J. Med. 309 (19) (1983) 1142–1146.
[17] N. Attia, W.V. Tamborlane, R. Heptulla, D. Maggs, A. Grozman, R.S. Sherwin, S. Caprio, The metabolic syndrome and insulin-like growth factor I regulation in adolescent obesity, J. Clin. Endocrinol. Metab. 83 (5) (1998) 1467–1471.
[18] J. Frystyk, E. Vestbo, C. Skjaerbaek, C.E. Mogensen, H. Orskov, Free insulin-like growth-factors in human obesity, Metabolism 44 (10) (1995) 37–44.
[19] S.Y. Nam, E.J. Lee, K.R. Kim, B.S. Cha, Y.D. Song, S.K. Lim, H.C. Lee, K.D. Huh, Effect of obesity on total and free insulin-like growth factor (IGF)-1, and their relationship to IGF-binding protein (BP)-1, IGFBP-2, IGFBP-3, insulin, and growth hormone, Int. J. Obes. 21 (5) (1997) 355–359.
[20] K.O. Klein, K.A. Larmore, E. de Lancey, J.M. Brown, R.V. Considine, S.G. Hassink, Effect of obesity on estradiol level, and its relationship to leptin, bone maturation, and bone mineral density in children, J. Clin. Endocrinol. Metab. 83 (10) (1998) 3469–3475.
[21] M. Seip, O. Trygstad, Generalized lipodystrophy, congenital and acquired (Lipoatrophy), Acta Paediatr. 85 (1996) 2–28.
[22] Z. Laron, Insulin–a growth hormone, Arch. Physiol. Biochem. 114 (1) (2008) 11–16.
[23] N. Soto, R.A. Bazaes, V. Pena, T. Salazar, A. Avila, G. Iniguez, K.K. Ong, D.B. Dunger, M.V. Mericq, Insulin sensitivity and secretion are related to catch-up growth in small-for-gestational-age infants at age 1 year: results from a prospective cohort, J. Clin. Endocrinol. Metab. 88 (8) (2003) 3645–3650.
[24] C. Gicquel, Y. Le Bouc, Hormonal regulation of fetal growth, Horm. Res. 65 (2006) 28–33.
[25] S.F. Wu, A.L. Aguilar, V. Ostrow, F. De Luca, Insulin resistance secondary to a high-fat diet stimulates longitudinal bone growth and growth plate chondrogenesis in mice, Endocrinology 152 (2) (2011) 468–475.
[26] J.C. Bruning, M.D. Michael, J.N. Winnay, T. Hayashi, D. Horsch, D. Accili, L.J. Goodyear, C.R. Kahn, A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance, Mol. Cell 2 (5) (1998) 559–569.
[27] D. Metzger, M. Li, P. Chambon, Targeted somatic mutagenesis in the mouse epidermis, Methods Mol. Biol. 289 (2005) 329–340.
[28] E. Nakamura, M.T. Nguyen, S. Mackem, Kinetics of tamoxifen-regulated Cre activity in mice using a cartilage-specific CreER(T) to assay temporal activity windows along the proximodistal limb skeleton, Dev. Dyn. 235 (9) (2006) 2603–2612.
[29] R.N. Kulkarni, J.C. Bruning, J.N. Winnay, C. Postic, M.A. Magnuson, C.R. Kahn, Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes, Cell 96 (3) (1999) 329–339.
[30] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C-T method, Nat. Protoc. 3 (6) (2008) 1101–1108.
[31] F.A.M. Baumeister, I. Engelsberger, A. Schulze, Pancreatic agenesis as cause for neonatal diabetes mellitus, Klin. Padiatr. 217 (2) (2005) 76–81.
[32] Ong KK, Petry CJ, Emmett PM et al.; ALSPAC study team (2004) - Insulin sensitivity and secretion in normal children related to size at birth, postnatal growth, and plasma insulin-like growth factor-I levels. Diabetologia 47: 1064–1070, Diabetologia 47(11) (2004) 2046-2046.
[33] E. Heinze, U. Vetter, K.H. Voigt, Insulin stimulates skeletal growth in vivo and in vitro–comparison with growth hormone in rats, Diabetologia 32 (3) (1989) 198–202.
[34] J. Salter, C.H. Best, Insulin as a Growth Hormone, Brit Med J 2(4832) (1953) 353-&.
[35] D.J. Hill, D. Desousa, Insulin is a mitogen for isolated epiphyseal growth plate chondrocytes from the fetal lamb, Endocrinology 126 (5) (1990) 2661–2670.

[36] G. Maor, M. Silbermann, K. Vondermark, D. Heingard, Z. Laron, Insulin enhances the growth of cartilage in organ and tissue-cultures of mouse neonatal mandibular condyle, Calcif. Tissue Int. 52 (4) (1993) 291–299.
[37] E.S. Torres, C.V. Andrade, E.C. Fonseca, M.A. Mello, M.E.L. Duarte, Insulin impairs the maturation of chondrocytes in vitro, Braz. J. Med. Biol. Res. 36 (9) (2003) 1185–1192.
[38] C. Phornphutkul, K.Y. Wu, P.A. Gruppuso, The role of insulin in chondrogenesis, Mol. Cell. Endocrinol. 249 (1–2) (2006) 107–115.
[39] F.J. Zhang, Q.L. He, W.P. Tsang, W.T. Garvey, W.Y. Chan, C. Wan, Insulin Exerts Direct, IGF-1 Independent Actions in Growth Plate Chondrocytes, Bone Res 2, (2014).
[40] K. Thrailkill, R.C. Bunn, C. Lumpkin, E. Wahl, G. Cockrell, L. Morris, C.R. Kahn, J. Fowlkes, J.S. Nyman, Loss of insulin receptor in Osteoprogenitor cells impairs structural strength of bone, J. Diabetes Res. 2014 (2014) 703589.
[41] S. Rokutanda, T. Fujita, N. Kanatani, C.A. Yoshida, H. Komori, W.G. Liu, A. Mizuno, T. Komori, Akt regulates skeletal development through GSK3, mTOR, and FoxOs, Dev. Biol. 328 (1) (2009) 78–93.
[42] V. Ulici, K.D. Hoenselaar, H. Agoston, D.D. McErlain, J. Umoh, S. Chakrabarti, D.W. Holdsworth, F. Beier, The role of Akt1 in terminal stages of endochondral bone formation: angiogenesis and ossification, Bone 45 (6) (2009) 1133–1145.
[43] M. Boni-Schnetzler, C. Schmid, P.J. Meier, E.R. Froesch, Insulin regulates insulin-like growth factor I mRNA in rat hepatocytes, Am. J. Phys. 260 (6 Pt 1) (1991) E846–E851.
[44] K.C. Copeland, R.B. Colletti, J.T. Devlin, T.L. Mcauliffe, The relationship between insulin-like growth factor-I, Adiposity Aging Metab. 39 (6) (1990) 584–587.
[45] R. Gama, J.D. Teale, V. Marks, The effect of synthetic very low calorie diets on the Gh-Igf-1 Axis in obese subjects, Clin. Chim. Acta 188 (1) (1990) 31–38.
[46] G. Radetti, M. Bozzola, B. Pasquino, C. Paganini, A. Aglialoro, C. Livieri, A. Barreca, Growth hormone bioactivity, insulin-like growth factors (IGFs), and IGF binding proteins in obese children, Metabolism 47 (12) (1998) 1490–1493.
[47] H. Saitoh, T. Kamoda, S. Nakahara, T. Hirano, N. Nakamura, Serum concentrations of insulin, insulin-like growth factor (IGF)-I, IGF binding protein (IGFBP)-1 and -3 and growth hormone binding protein in obese children: fasting IGFBP-1 is suppressed in normoinsulinaemic obese children, Clin. Endocrinol. 48 (4) (1998) 487–492.
[48] G. Vanvliet, D. Bosson, E. Rummens, C. Robyn, R. Wolter, Evidence against growth hormone-releasing factor deficiency in children with idiopathic obesity, Acta Endocrinol. 113 (1986) 403–410.
[49] S. Loche, M. Cappa, P. Borrelli, A. Faedda, A. Crino, S.G. Cella, R. Corda, E.E. Muller, C. Pintor, Reduced growth-hormone response to growth hormone-releasing hormone in children with simple obesity - evidence for somatomedin-C mediated inhibition, Clin. Endocrinol. 27 (2) (1987) 145–153.