Leucine restriction inhibits chondrocyte proliferation and differentiation through mechanisms both dependent and independent of mTOR signaling

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Undernutrition is a well-documented cause of poor linear growth, whereas obesity produces accelerated linear growth in children. Primary causes of undernutrition are complex. They can range from inadequate availability of calories to inadequate specific nutrients, such as protein or amino acids. Despite the many causes and forms of undernutrition, one universal outcome is poor long bone growth, which can be presumed to be an effect on endochondral bone elongation (1, 6). Over the last several decades, the mechanisms by which nutritional status affects bone growth have focused on indirect effects via changes in insulin-like growth factor I (IGF-I) biological effect (3, 7). Impaired IGF-I production in undernutrition is in part associated with impaired hepatic growth hormone (GH) sensitivity, which is associated with decreased hepatic GH receptor expression, hepatic IGF-I mRNA, and circulating IGF-I levels (31). Similarly, overnutrition is associated with upregulation of the GH/IGF-I axis (23).

The effect of IGF-I on chondrocyte growth and differentiation within the growth plate has been well documented (14, 24). We and others have demonstrated that IGF-I has an important role in chondrocyte proliferation and differentiation (25, 28). In addition to the effects of IGF-I on chondrocytes, we have demonstrated that insulin at physiological concentration has a direct effect on chondrocyte differentiation (27), thus providing for another mechanism by which nutritional status can modulate bone growth.

In recent years, mechanisms by which nutrients exert a direct effect on cell growth and function have been elucidated (17). Although restriction of essential amino acids has been viewed as limiting because of their requirement as substrates for protein synthesis, essential amino acids also act as signaling factors in several regulatory pathways (15, 16). Perhaps the most well-characterized signaling pathway regulated by amino acids has at its center the mammalian target of rapamycin (mTOR) (17). mTOR is a nutrient-sensing kinase that integrates input from amino acids, growth factors, and the energy status of the cell (15). It acts as a central controller of translation, controlling ribosomal biogenesis and global protein synthesis (4). The TOR protein is highly conserved from yeast to mammals (5). Rapamycin, a widely used immunosuppressive agent, directly inhibits mTOR activity and has been key to understanding the role of mTOR in cell regulation (22, 35).

We have recently demonstrated the effect of rapamycin on chondrocyte growth and differentiation in ATDC5 cells (26), fetal rat metatarsal explants (26), and the rabbit growth plate (unpublished observation). mTOR inhibition with rapamycin results in significantly decreased chondrocyte differentiation, a modest decrease in chondrocyte proliferation, and decreased total bone growth in physiological systems.

Leucine is the most potent nutrient regulator of mTOR signaling (29, 30). We therefore hypothesized that leucine availability would have a direct effect on chondrocyte growth and differentiation, resulting in decreased longitudinal bone growth when restricted. We further hypothesized that the effect of leucine restriction would be a direct result of mTOR inhibition, although mTOR-independent pathways have been described. One of these involves the mammalian general control nonrepressible 2 (GCN2) kinase (32). GCN2 is a stress kinase that is activated by amino acid starvation to modulate protein synthesis. GCN2 phosphorylates the α-subunit of the eukaryotic initiation factor (eIF2). The response that allows organisms to tolerate amino acid deprivation in states of malnutrition and starvation involves repression of protein syn-
thesis and upregulation of amino acid biosynthesis and transport (10) with a net effect of decreased cell growth.

Based on our hypothesis and well-defined mechanisms that control chondrocyte growth and differentiation, we have performed studies using embryonic day 19 (E19) fetal rat metatarsal explants. The benefit of this model is the intact bone maintains its cell-cell and cell-matrix interaction. We have also used the ATDC5 chondrogenic cell line to extend our observations to an in vitro model. Last, using microarray analysis, we have explored other potential mechanisms accounting for the effect of leucine restriction on chondrocyte growth and differentiation.

MATERIALS AND METHODS

Materials. Antibodies directed toward phosphorylated S6 (phospho-S6; Ser235/236) and monoclonal antibodies against phospho-eIF2α and total S6 were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against bromodeoxyuridine (BrdU) and immunohistochemistry reagents (Vectastain Elite ABC kit, DAB Substrate kit and Avidin/Biotin blocking reagents) were obtained from Vector Laboratories (Burlingame, CA). Antibodies against total 4E-BP1, total eIF2α, and Indian hedgehog (Ihh) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against mouse type X collagen were kindly provided by Drs. William Horton and Greg Lunstrum (Oregon Health & Science University, Portland, OR). Purified pork insulin was obtained from Elanco Animal Health (Greenfield, IN). Enhanced chemiluminescence plus reagents were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Custom α-minimum essential medium (MEM), standard α-MEM, and DMEM-nutrient mixture F-12 (DMEM-F-12) were obtained from Invitrogen (Carlsbad, CA). The TdT-FragEL DNA Fragmentation kit was obtained from Calbiochem (San Diego, CA). The bicinchoninic acid protein assay was obtained from Pierce Biotechnology (Rockford, IL). Thapsigargin, BSA, leucine, l-glutamine, l-arginine, protease, hyaluronidase, human transferrin, sodium selenite, and all other chemical reagents were obtained from Sigma (St. Louis, MO).

Organ culture studies. All animal studies were approved by the Rhode Island Hospital Institutional Animal Care and Use Committee. Timed-pregnant female rats (Charles River Laboratories) were killed on E19 with intraperitoneal Nembutal (35 mg/kg). Fetal metatarsal bones were obtained and cultured individually in 12-well plates, as described previously (27). Each well contained 2 ml serum-free α-MEM supplemented with 0.2% BSA, 2.2 g/l NaHCO3, and 2% antibiotic/antimycotic (pH 7.4). Custom α-MEM devoid of glucose, arginine, glutamine, and leucine was used to modulate leucine concentrations. The medium was supplemented with 1 g/l d-glucose, 127 mg/l l-arginine, 292 mg/l l-glutamine, and 1,600 nM insulin. Experimental conditions included leucine in varying concentrations (0.40 mM as control and 0.1, 0.04, and 0.02 mM) and 50 nM rapamycin in 0.40 mM leucine. The explants were cultured for 3 days. Where indicated, BrdU (1 mg/ml) was added to the culture medium 24 h before the end of experiment.

Fig. 1. Effect of leucine restriction and rapamycin on the growth of fetal rat metatarsal explants. A: total length of metatarsal explants was measured on days 0, 1, 2, and 3 in culture. Growth was measured as the cumulative increase in length, expressed as a percentage of starting length. Results for control (0.40 mM leucine; ●), 0.10 mM leucine (■), 0.04 mM leucine (○), and 0.02 mM leucine (▲) are shown as means ± SE for 7–8 samples/data point. *P < 0.01 vs. all other groups. B: experiment in A was repeated using control conditions (0.40 mM leucine; ●), 0.02 mM leucine (▲), and 50 nM rapamycin (▲). Data are shown as means ± SE for 7–8 samples/data point. *P < 0.01 vs. all other groups. C: effect of leucine restriction on zone-specific growth of the explants. Formalin-fixed explants were sectioned and stained using hematoxylin and eosin. The lengths of the explant and of the hypertrophic zone were measured. Results for the length of the hypertrophic zone are expressed as a percentage of total length (mean ± SE for 4–5 samples/data point). *P < 0.01 vs. all other groups.

Fig. 2. Effect of leucine restriction on chondrocyte proliferation within the metatarsal explant growth plate. A: explants were cultured in either 0.02 or 0.40 mM leucine for 3 days. Bromodeoxyuridine (BrdU) was added during the last 12 h of the experiment. BrdU-labeling index was calculated as the no. of BrdU-labeled cells within a fixed area divided by the total number of cells in that area. The areas analyzed contained 100 cells on average. The analysis was carried out on 8–10 explants/group. *P < 0.001. B: representative photomicrograph showing intranuclear BrdU staining of chondrocyte explants cultured in 0.02 mM leucine (left) and 0.40 mM leucine (right). Scale bar: 50 μm.
Fig. 3. Effect of leucine restriction on chondrocyte apoptosis in metatarsal explants. Apoptosis analysis of explant sections was performed after 72 h in culture, either in 0.02 or 0.40 mM leucine. A: apoptotic index was calculated as the no. of labeled cells within a fixed area divided by the total no. of cells in that same area. The areas analyzed contained 100 cells on average. The analysis was carried out on 8–10 explants/group. B: representative photomicrograph showing terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling staining of explant chondrocytes cultured in 0.02 (left) or 0.40 (right) mM leucine. Scale bar: 100 μm.

Fig. 4. Effect of leucine restriction on mammalian target of rapamycin (mTOR) signaling in metatarsal explants. A: mTOR signaling was assessed by immunohistochemical staining for S6 phosphorylation p-S6. The analysis was carried out on 7–8 explants/group. Rapa, rapamycin. *P < 0.01 vs. all other groups. B: representative photomicrograph showing staining for phosphorylated S6 (phospho-S6) of explants cultured in 0.02 mM leucine (left), 0.40 mM leucine (center) or rapamycin (right). Scale bar: 20× = 50 μm. Top: proliferative zone of the growth plate. Bottom: hypertrophic zone of the growth plate.
Metatarsal length measurements were performed daily during the culture period using IPLab software (version 3.70; Scanalytics, Fairfax, VA). At the end of the 3-day incubation period, the explants were fixed overnight in 10% buffered formalin, embedded in paraffin, and cut in 5-μm longitudinal sections for further studies.

Histologic analysis of explants. Sections were processed for morphometric analysis of total length and length of the hypertrophic zone using previously described methods (27). Images were calibrated using a stage micrometer to express results in micrometers. BrdU staining was performed as previously described (26). Cell counts were performed by a blinded observer, with calculation of the labeling index as the ratio of labeled cells to total number of cells within a fixed area. Areas analyzed contained an average of 100 cells.

For immunohistochemical analysis of phospho-S6, Ihh, and collagen X, sections were deparaffinized and endogenous peroxidase activity quenched as follows. After preincubation with blocking serum for 20 min, sections were blocked with avidin-biotin. For collagen X staining, sections were also incubated with 0.5% hyaluronidase for 20 min at 37°C. Incubation with primary antibody (phospho-S6 1:100 in PBS; Ihh 1:600 in serum; collagen X 1:300 in serum) was performed either overnight at 4°C (phospho-S6) or for 30 min at room temperature (Ihh, collagen X). Sections were incubated with secondary antibody (1:200 in PBS) for 30 min at room temperature before color development with Vectastain Elite ABC reagents, after which counterstaining was performed with hematoxylin QS.

Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) of paraffin-embedded sections according to the TdT-FragEL DNA Fragmentation Detection kit instructions, with slight modifications. The proteinase K treatment was shortened to 10 min. Cell counts were performed by a blinded observer, with calculation of the apoptotic index as the ratio of apoptotic cells to total number of cells within a fixed area. Areas analyzed contained an average of 100 cells.

Cell culture studies. The ATDC5 cell line was obtained from the Riken Cell Bank (Tsukuba, Japan). Cells were cultured as described previously (2). Differentiation was induced by changing to α-MEM containing 0.40, 0.1, 0.04, or 0.02 mM leucine or 0.40 mM leucine with 20 nM rapamycin. It should be noted that ATDC5 cells differentiate in α-MEM without the addition of insulin. Media were supplemented with 5% FBS, 10 μg/ml human transferrin, 30 mM sodium selenite, and 2% antibiotic-antimycotic, (pH 7.45) and replaced every 2–3 days for the duration of the experiment. As a positive control for phosphorylation of eIF2α, thapsigargin was added at 0.3 μM before collection of cell lysate.

ATDC5 cell differentiation was assessed using Alcian Blue and Neutral Red staining as previously described (19). To determine the expression level for collagen X, parathyroid hormone-related protein (PTHrP), Ihh, β-actin in ATDC5 cells, and total RNA was prepared, and semiquantitative PCR was performed (26). Previously described methods (26) were used for preparation of cell lysates and immunoblotting for phosphorylated eIF2α and total eIF2α, primary antibodies were diluted to 1:500.

Microarray analyses. ATDC5 cells were grown to confluence in DNEM-F-12 media and then changed to α-MEM containing 0.40 mM leucine, 0.02 mM leucine, or 0.40 mM leucine plus 20 nM rapamycin. After 5 days under these conditions, total RNA was prepared using Tri Reagent. Samples underwent gene expression analysis using Illumina Beadchips (mouse 6 V2, BD 26–213), which assay >45,200 transcripts. These analyses were carried out at the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University School of Medicine (New Haven, CT).

Statistical analyses. All data are expressed as means and SE. The significance of differences between groups was determined using GraphPad Prism software (GraphPad Software, San Diego, CA) by one-way ANOVA followed by a Tukey post hoc test. Differences

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**Fig. 5.** Effect of leucine restriction on ATDC5 cells. A: cells were harvested after being maintained for 12 days in 0.02 or 0.40 mM leucine. At the end of that period, they were stained for proteoglycan accumulation using Alcian Blue. The absorbance of the dye extracted from each well is shown as the mean ± 1 SD of triplicate measures for each condition. Similar results were obtained in a replicate experiment. *P < 0.0002 vs. 0.40 mM leucine. B: Neutral Red staining was used to estimate the total cell mass using cells that were treated as in A. The absorbance of the dye extracted from each well is shown as the mean ± 1 SD of triplicate measures for each condition. ColX, collagen X. *P < 0.0001 vs. 0.40 mM leucine. C: ATDC5 cells were allowed to differentiate in 0.02 or 0.40 mM leucine. Total RNA was isolated at 8 days and analyzed by semi-quantitative RT-PCR for collagen X. Densitometry results are shown as the mean ± 1 SD of triplicate analyses. *P < 0.001 vs. 0.40 mM leucine. D: expression of collagen X, Indian hedgehog (Ihh), and parathyroid hormone-related protein (PTHrP) was assessed using semi-quantitative RT-PCR. Triplicate samples were analyzed for each time-point. Panel on bottom shows β-actin expression, which was used as a control.
were considered significant at $P \leq 0.05$. For microarray analysis, statistical analysis was performed at the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University School of Medicine. Data were analyzed using gene clustering and gene ontogeny software. The significance of differences between groups was determined by one-way ANOVA followed by a Tukey post hoc test. Differences were considered significant at $P \leq 0.05$.

RESULTS

Effects of leucine restriction on metatarsal explants. To study the effect of leucine restriction on longitudinal bone growth, E19 fetal rat metatarsals were harvested and then cultured in α-MEM media containing varying concentrations of leucine. Explant growth was determined as the daily percent change in length. Leucine restriction inhibited growth in a dose-dependent manner, with lengths decreased by approximately one-half in the 0.02 mM group relative to the 0.40 mM group (Fig. 1A). A separate experiment was performed to compare the relative effect of leucine restriction (0.02 mM) and rapamycin on linear growth of the explants (Fig. 1B). Both resulted in growth inhibition. However, leucine restriction had a significantly more marked inhibitory effect than rapamycin.

At the end of the 3-day culture period, bones were fixed and stained using hematoxylin and eosin. The lengths of the hypertrophic, proliferative, and mineralizing zones within the growth plate were assessed as a percentage of total length for each bone. There was a significant difference in the hypertrophic zone lengths between bones exposed to 0.02 mM leucine and all other leucine concentrations (Fig. 1C). The lengths of the proliferative and mineralizing zones were not significantly different between groups (data not shown).

To study the effect of leucine restriction on explant chondrocyte proliferation, BrdU incorporation was determined for the last 24 h of the 3-day culture period. BrdU incorporation was reduced significantly in explants grown in 0.02 mM leucine relative to the control condition of 0.40 mM (Fig. 2).

Staining of explant sections using the TUNEL method revealed a small percentage of apoptotic cells in each experimental group. There were no significant differences in apoptosis between the groups (Fig. 3).

Metatarsal explants were analyzed for collagen X and I1h content using immunohistochemistry. Staining for these two differentiation markers was unaffected by leucine concentration (0.02 vs. 0.40 mM) or rapamycin. However, variability in the level of staining prevented precise quantification. These results (data not shown) were useful in verifying our identification of the chondrocyte zones.

Metatarsal explant sections were stained for phospho-S6 as an indirect indicator of mTOR activity (Fig. 4). Rapamycin-treated explants showed a complete loss of staining by day 3 in culture. In contrast, staining for phospho-S6 was only partially inhibited by leucine restriction to 0.02 mM.

Immunohistochemistry for phospho-eIF2α was performed on metatarsals grown in 0.02 and 0.40 mM leucine. There were no differences in the phospho-eIF2α staining among the three groups (data not shown).

Effects of leucine restriction on ATDC5 cells. ATDC5 cells were allowed to differentiate in α-MEM containing 0.40 or 0.02 mM of leucine for 12 days. At the end of this period, Alcian Blue staining was used to assess the degree of proteoglycan accumulation. The lower leucine concentration was associated with a 65% reduction in Alcian Blue staining (Fig. 5A). Neutral Red staining, an indicator of cell number, showed a modest decrease under conditions of leucine restriction (Fig. 5B). Correction of Alcian Blue staining for Neutral Red indicated a disproportionate effect on the former, consistent with inhibition of proteoglycan accumulation in cells grown under conditions of leucine restriction. The effect of leucine restriction on the expression of collagen X, a marker of hypertrophic chondrocytes was examined (Fig. 5C).

RNA was isolated from cells grown in 0.40 or 0.02 mM leucine for 8 days. Leucine restriction was associated with a marked reduction in expression of collagen X. The effect on
differentiation was assessed by examining, in addition to collagen X, Ihh and PTHrP (Fig. 5). In this experiment, expression of collagen X was again significantly lower in cells grown in low leucine. The induction of Ihh was also inhibited while PTHrP was unaffected.

To determine whether the inhibition of ATDC5 growth and differentiation by leucine restriction is through inhibition of mTOR signaling, we performed immunoblotting for phospho-S6, an indicator of mTOR activity. ATDC5 cells were differentiated in H9251-MEM media containing 0.02 or 0.40 mM leucine with or without 20 nM rapamycin, for 1, 3, and 6 h and 1, 6, and 12 days. At the end of the experiment, cell lysates were collected and analyzed by immunoblotting for phospho-S6 and total S6 (Fig. 6, A and B). Although rapamycin was associated with a complete loss of phospho-S6, the effect of leucine restriction was minimal at all time points (1, 3, and 6 h). This partial inhibition of S6 phosphorylation under conditions of leucine restriction was similar to that observed in the metatarsal explants.

mTOR has also been shown to be an upstream kinase that phosphorylates 4E-BP1 protein. Immunoblotting for total 4E-BP1 was used as another indicator of mTOR activity. Again, ATDC5 cells were differentiated in α-MEM media containing 0.02 or 0.40 mM leucine with or without 20 nM rapamycin, for 1, 6, and 12 days. At the end of the experiment, cell lysates were collected and analyzed (Fig. 6C). Rapamycin was associated with the appearance of a third, faster-migrating 4E-BP1 band. This corresponds to the hyperphosphorylated α-4E-BP1 form. Leucine restriction did not induce an increase in α-4E-BP1 as was seen in cells exposed to rapamycin.

Given the modest change in mTOR signaling seen in response to leucine restriction, we questioned whether an mTOR independent pathway could be involved. GCN2 kinase pathway was assessed by examining the phosphorylation of eIF2α. Lysates from differentiating ATDC5 cells grown in 0.02 mM leucine were collected on days 1, 2, 4, 7, 10, and 14 and analyzed by Western immunoblot for phospho- and total eIF2α. Thapsigargin was used as a positive control for GCN2 activation. Results (Fig. 7A) showed a modest increase in the phosphorylation of eIF2α in cells grown in 0.02 mM leucine at all time points. Quantification of eIF2α on day 1 confirmed an increase in cells cultured under conditions of leucine restriction (Fig. 7B), thus indicating that this pathway is active in these cells.

To further examine the hypothesis that leucine restriction differed from rapamycin-mediated mTOR inhibition with regard to biological effects on ATDC5 cells, we used microarray...
analysis to characterize the effects of the two conditions on gene expression (Fig. 8). Confluent ATDC5 cells were switched to media containing 0.40 mM leucine, 0.02 mM leucine, or 0.40 mM leucine with 20 nM rapamycin. Total RNA prepared after 5 days was analyzed by microarray. Cluster analysis of the resulting data revealed dramatic differences among the three groups. Relative to cells grown in 0.40 mM leucine, the expression of 1,571 genes was altered in cells grown under conditions of leucine restriction. In contrast, 535 genes were affected by exposure to rapamycin. Only 176 genes were affected by both leucine restriction and rapamycin. Gene ontology and pathway analyses were performed for genes for which change was significant at the \( P < 0.001 \) level (Table 1). These analyses revealed significant changes in both groups among genes involved in carbohydrate metabolism, extracellular matrix formation, and cell adhesion. However, many more biological processes, cellular components, molecular functions, and pathways were affected by leucine restriction relative to rapamycin. These analyses further supported the conclusion that leucine restriction affects gene expression in a manner that is clearly distinguished from rapamycin-induced inhibition of mTOR signaling.

**DISCUSSION**

The present studies were aimed at characterizing the effect of restricting a key nutrient, leucine, on two models of bone growth: metatarsal explants and growth and a chondrogenic cell line. Amino acids, particularly the branched-chain amino acids, regulate protein synthesis beyond the level of their own availability as the substrate for peptide-chain elongation (15, 17). They do so by functioning as signaling molecules. Leucine appears to be the most potent of the branched-chain amino acids in this regard, having a potent effect on signaling via two important signaling kinases, mTOR and GCN2 (8, 10, 32, 33).

The mTOR signaling pathway has been shown to mediate the effects of leucine on mRNA translation initiation. We recently demonstrated the important role of mTOR in the regulation of chondrocyte growth and differentiation (26). Our previous findings provided for a mechanism whereby nutrients, acting through mTOR, can directly modify linear growth. In the present study, we performed analogous experiments comparing the effect of leucine restriction in the fetal metatarsal explant model as well as ATDC5 cells.

The present studies were undertaken to test the hypothesis that leucine restriction would exert its effects on bone growth through its ability to signal via mTOR. Using the more physiological bone explant model, we confirmed that leucine affected growth in a dose-dependent manner. The observed growth inhibition was associated with decreased chondrocyte proliferation as measured by decreased BrdU incorporation. Decreased chondrocyte proliferation presumably results in fewer chondrocytes that are available to differentiate and become hypertrophic cells, consistent with the decreased hypertrophic zone height that we observed.

Chondrocytes that differentiate into prehypertrophic and hypertrophic chondrocytes undergo a 4- to 10-fold increase in

**Table 1. Gene ontology and pathway analysis of the effects of rapamycin and leucine restriction on gene expression**

<table>
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<th>Category</th>
<th>No. of Affected Genes</th>
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<td>Cell communication: cell-cell signaling</td>
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<tr>
<td>Development: growth</td>
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<tr>
<td>Physiological processes</td>
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<td>Domain of Cellular Component</td>
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<td>Apoptosis regulator activity</td>
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<tr>
<td>Cell cycle</td>
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</tr>
<tr>
<td>p53 signaling pathway</td>
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</tr>
<tr>
<td>Angiotensin-converting enzyme 2 regulates heart function pathway</td>
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</tr>
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</table>

Data are shown as the no. of genes changed under conditions of rapamycin (Rapa), leucine restriction, or both (numerator) relative to the total number of genes in the ontology or pathway category (denominator). Only genes for which changes were significant at the \( P < 0.001 \) level are included.
cytoplasmic volume, making distal hypertrophic cells a significant component of longitudinal bone growth. In rapidly growing bones, ~10% of bone length is contributed by proliferating cells, a one-third by matrix synthesis throughout the growth plate, and nearly two-thirds by the contribution of hypertrophic cells (34).

Although leucine restriction appears to have an effect on chondrocyte proliferation and differentiation, we did not observe an increase in apoptosis as assessed by TUNEL staining. Premature cell death can result in reduced bone growth, as observed in humans with skeletal dysplasia (12) and in mice with disruption of genes important to chondrogenesis, including those encoding filamin B, matrilin-3, or components of the β-catenin signaling pathway (11, 21). Our results indicate that the effect of leucine restriction occurs during the early phase of the chondrocyte growth and differentiation process, not as a result of increased apoptosis.

Because mTOR is a signaling target for leucine, we examined the effect of leucine restriction on mTOR signaling in metatarsals by assessing the phosphorylation state of ribosomal protein S6. We observed only a modest decrease in phospho-S6 staining in the explants grown in 0.02 mM leucine. This was in sharp contrast to rapamycin, which abolished phospho-S6 staining. This raised the possibility that leucine response was mediated through a mechanism other than one involving modulation of mTOR activity.

Using the ATDC5 chondrogenic cell line, we extended our observations in an attempt to support or refute conclusions drawn from the metatarsal explant studies. Leucine restriction decreased chondrocyte differentiation as measured by accumulation of proteoglycan and expression of collagen X. Total cell mass measured by Neutral Red accumulation was also decreased. Again, markers of mTOR activity, S6 phosphorylation and the pattern of 4E-BP1 phosphorylation, were modestly inhibited under conditions of leucine restriction relative to the marked effect of rapamycin. The expression of Ihh, a key contributor to chondrocyte proliferation and differentiation (9, 18, 20), was also decreased under conditions of leucine restriction. We previously demonstrated that mTOR inhibition may directly regulate Ihh expression (26).

In an effort to identify an mTOR-independent pathway to account for the effects of leucine restriction, we examined the regulation of GCN2 in the ATDC5 cell line. The GCN2 pathway is activated by the accumulation of uncharged tRNAs during amino acid starvation as shown in myoblasts (10). This leads to the phosphorylation of eIF2α, resulting in inhibition of translation initiation of cellular proteins and a global reduction in protein synthesis (10, 13). We observed only a modest increase in the phosphorylation of eIF2α under conditions of leucine restriction.

Microarray analysis of the effects of leucine restriction vs. rapamycin on gene expression in ATDC5 cells revealed that only a small proportion of genes was affected by both leucine restriction and rapamycin. These genes, which numbered 176, accounted for 11.2% of the genes that were affected in leucine restriction. The large effect of leucine restriction relative to rapamycin (1,571 vs. 535 genes) may indicate that leucine restriction has a broader effect on chondrocyte growth and differentiation than does targeted inhibition of mTOR. Using very stringent criteria for significance, gene ontology and pathway analysis further supported marked differences in the effects of the two conditions.

In summary, our studies show that leucine restriction affects proliferation and differentiation in the ATDC5 chondrogenic cell line. Results using the fetal metatarsal explant model are consistent with the ATDC5 studies. Our findings support the conclusion that both mTOR and GCN2 signaling may contribute to the effect of leucine restriction on chondrocyte proliferation and differentiation and, therefore, on long bone growth. However, our studies are also consistent with the possibility that the effects of leucine restriction are mediated by pathways that are independent of effects on these two signaling kinases. We are left to conclude that the mechanism by which leucine restriction inhibits chondrogenesis and attenuates linear bone growth is complex, likely involving modulation of multiple pathways that may involve mTOR and GCN2, but that may also be independent of both of these well-characterized pathways.

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