A novel spontaneous mutation of Irs1 in mice results in hyperinsulinemia, reduced growth, low bone mass and impaired adipogenesis

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Abstract

A spontaneous mouse mutant, designated ‘small’ (sml), was recognized by reduced body size suggesting a defect in the IGF1/GH axis. The mutation was mapped to the chromosome 1 region containing Irs1, a viable candidate gene whose sequence revealed a single nucleotide deletion resulting in a premature stop codon. Despite normal mRNA levels in mutant and control littermate livers, western blot analysis revealed no detectable protein in mutant liver lysates. When compared with the control littersates, Irs\textsuperscript{1ml}/Irs\textsuperscript{1ml} (Irs\textsuperscript{sml}/Irs\textsuperscript{sml}) mice were small, lean, hearing impaired; had 20\% less serum IGF1; were hyperinsulinemic; and were mildly insulin resistant. Irs\textsuperscript{1ml}/Irs\textsuperscript{1ml} mice had low bone mineral density, reduced trabecular and cortical thicknesses, and low bone formation rates, while osteoblast and osteoclast numbers were increased in the females but not different in the males compared with the Irs\textsuperscript{1+/+} controls.\textit{In vitro}, Irs\textsuperscript{1ml/ml} bone marrow stromal cell cultures showed decreased alkaline phosphatase-positive colony forming units (pre-osteoblasts; CFU-AP\textsuperscript{+}) and normal numbers of tartrate-resistant acid phosphatase-positive osteoclasts. Irs\textsuperscript{1ml/ml} stromal cells treated with IGF1 exhibited a 50\% decrease in AKT phosphorylation, indicative of defective downstream signaling. Similarities between engineered knockouts and the spontaneous mutation of Irs\textsuperscript{1ml} were identified as well as significant differences with respect to heterozygosity and gender. In sum, we have identified a spontaneous mutation in the Irs1 gene associated with a major skeletal phenotype. Changes in the heterozygous Irs\textsuperscript{1+/m} mice raise the possibility that similar mutations in humans are associated with short stature or osteoporosis. \textit{Journal of Endocrinology} (2010) \textbf{204}, 241–253

Introduction

subunit (Igfals) in mice promotes marked thinning of the cortical bone compartment, despite minimal changes in linear growth (Yakar et al. 2009). Hence, skeletal and circulating IGF1 are essential for optimal peak bone acquisition.

Insulin and IGF1 initiate a chain of intracellular responses and signaling cascades upon binding to tyrosine kinase receptors (IR, and IGF1R). The first substrates phosphorylated after ligand binding are the insulin receptor substrate (IRS) proteins. Once phosphorylated by their cognate receptors, these substrates bind to proteins containing Src homology-2 domains, which in turn activate a variety of signaling pathways, including activation of phosphatidylinositol 3-kinase and mitogen-activated protein kinase (Lienhard 1994, White 2003, Niu & Rosen 2005). These intracellular signaling pathways are essential for bone acquisition, since they impact the recruitment, differentiation, and death of osteoblasts (Cornish et al. 1996, Niu & Rosen 2005).

Deletion of Ins 1 (either the Irs1tm1Tka or the Irs1tm1Jos allele) on a mixed B6/CBA hybrid mouse background results in growth retardation; however, both female and male mice are relatively healthy and fertile (Araki et al. 1994, Tamemoto et al. 1994). Adult Irs1tm1Tka/Irs1tm1Tka (Irs1tm1Tka/Irs1tm1Tka) mice have low aBMD and delayed fracture healing, with reductions in osteoblast and osteoclast numbers and function, resulting in decreased bone turnover. These null mice also exhibit impaired anabolic response to intermittent parathyroid hormone administration (Ogata et al. 2000, Hoshi et al. 2004, Shimoaka et al. 2004, Yamaguchi et al. 2005). Both the Irs1tm1Tka/Irs1tm1Tka and the Irs1tm1Jos/Irs1tm1Jos (Irs1tm1Jos/Irs1tm1Jos) mice have high serum insulin levels and are insulin resistant despite a lean phenotype, but circulating IGF1 levels were reported not to differ significantly from those of the controls (Araki et al. 1994, Tamemoto et al. 1994, Shirakami et al. 2002). Thus, several animal models have confirmed that insulin and IGF1 signaling are critical for skeletal acquisition and maintenance.

There are no reports of a spontaneous mutation in the Ins 1 gene leading to complete loss of function in mice or in humans. However, there are numerous studies on humans demonstrating polymorphisms in the Ins 1 gene associated with metabolic disease, some of which induce amino acid changes and variable responsiveness to insulin and/or IGF1 signaling (Imai et al. 1994, Ura et al. 1996, Le Fur et al. 2002). The most common polymorphism, the G972R variant, has been associated with type II diabetes mellitus (Almind et al. 1993, Sesti 2000, Tok et al. 2006). In contrast, there is no data examining the relationship between these polymorphisms and BMD or fracture risk.

Recently, in the Mouse Mutant Resource at The Jackson Laboratory, we discovered a small mouse phenotype that arose as a spontaneous autosomal recessive mutation on a congenic C3.SW-H2b/SnJ inbred background. Molecular genetic studies revealed that this mutation, designated small (allele symbol sml), was due to a frameshift mutation in the Ins 1 gene. This report details the molecular and phenotypic characterization of this mutant and the cellular changes that occur in response to altered IRS-1 signaling. Findings from this study add to insights gained from previous work using genetic engineering, and raise important questions about the inter-relationship between the IGF1 regulatory system and bone acquisition.

### Materials and Methods

#### Mouse husbandry

The Irs1tm1 allele is a spontaneous mutation that occurred in an inbred C3.SW-H2b/SnJ mouse strain. All mice used in this study were produced and maintained in our research colony at The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in groups of 4 or 5 of the same sex within polycarbonate boxes of 324 cm² in area on sterilized shavings of Northern White Pine. Colony environmental conditions included 14 h light:10 h darkness cycles, with free access to acidified water (pH 2.5 with HCl to retard bacterial growth) and irradiated NIH-31 diet containing 6% fat, 19% protein, Ca:P 1:15:0:85, plus vitamin and mineral fortified (Purina Mills International, Gray Summit, MO, USA). All studies were conducted using groups of mutant, heterozygous, and wild-type male and female mice. All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee of The Jackson Laboratory.

#### Genetic mapping

To map the sml mutation, 204 F2 mice were produced from an intercross between (C3.SW-H2b/SnJ × CAST/Ei) F1 hybrid mice. Genomic DNA from F2 mice was prepared and genotyped using Mit marker primer pairs as described previously (Gagnon et al. 2006). The sml mutation was mapped utilizing recombination frequencies and the Map Manager Program (Manly et al. 2001).

#### Sequencing of the Irs1 gene

PCR primers used to amplify exons 1 and 2 of the mouse Irs 1 gene for sequence comparisons between mutant and control are given in Table 1. PCR conditions were described previously (Gagnon et al. 2006). PCR-amplified products were purified using the Qiaquick PCR Purification Kit (Qiagen Inc). DNA was sequenced using an Applied Biosystems 373A DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The same primers that were used for PCR amplification were also used for sequencing.

#### Genotyping of the Irs1tm1 sml colony

To genotype this mouse colony, we recognized that the deletion of the one adenine nucleotide in exon 1 created a restriction enzyme recognition site, which TaqI recognizes (T-CGA). We designed primers (Ins 1 F 5’-CAA GGA GGT
CTG GCA GGT TA-3' and Irs1 R 5'-CCC ACC TCG ATG AAG AAG AA-3' to amplify the region of interest and then digested them with TaqI restriction enzyme as per the manufacturer's instructions. The digested products yielded a control band of 190 bp, with a mutant band of 171 bp.

**Quantitative real-time PCR**

RNA was extracted from the femurs of four 8-week-old Irs1<sup>+/+</sup> and Irs1<sup>-/-</sup> control mice as described previously (DeMambro et al. 2008). Briefly, femurs were isolated and snap frozen in liquid nitrogen, and RNA was then isolated using the Total RNA isolation system (Promega) as per the manufacturer's instructions. DNA was then removed from the RNA samples using the DNA-free DNase Treatment & Removal Reagents (Ambion, Inc., Austin, TX, USA). RNA quality and quantity were assessed using an Agilent bioanalyzer (Caliper Technologies Corp., Hopkinton, MA, USA). Four hundred nanograms of RNA were then converted to cDNA in a reverse transcription reaction using the MessageSensor RT Kit (Ambion, Inc.) and random decamers as primers. The cDNA was then diluted 1:5 with water. Quantification of mRNA expression was carried out using an iQ SYBR Green Supermix in a iQ5 thermal cycler using an iQ SYBR Green Supermix in a iQ5 thermal cycler (Bio-Rad). GAPDH was used as an internal standard control gene for all quantifications. Primer sequences used in this study are as follows: RANKL (forward: 5'-TAC TTT CGA GCC CAG ATG GAT-3' and reverse: 5'-TCT CGT TTT CAT GGA GTC TCA-3'), osteoprotegerin (forward: 5'-TTG GCC GTG GTG CAA G-3' and reverse: 5'-AGA ACC CAT CTG GAC ATT TTG TG-3'), and GADPH (forward: 5'-TGA ACG AAG AGA TCA CTG G-3' and reverse: 5'-CAG ACC CTG TTG CTG TA-3').

**Western blotting**

Mouse livers were collected, frozen in liquid nitrogen, and stored at -80°C until processing. Total cellular protein lysates were prepared in a buffer consisting of 50 mM Tris base (pH 8.2), 150 mM NaCl, 1% Igepal, and complete protease inhibitor cocktail tablets (Roche) for 30 min at 4°C, followed by centrifugation at 10 000 g for 20 min at 4°C. For immunoprecipitation, cell lysates were incubated with a rabbit anti-C-terminus IRS-1 antibody (Upstate #06-248, Millipore, Billerica, MA, USA) for 1 h at 4°C. The immunocomplexes were then collected using an Immunoprecipitation Kit Protein A (#1719394 Roche) as per the manufacturer's instructions. Proteins were resolved on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were blocked with 5% non-fat dry milk (Bio-Rad), and were then incubated with the same anti-C-terminus IRS-1 antibody followed by a HRP-conjugated donkey anti-rabbit antibody (Santa Cruz #sc-2317). Signal was detected using an enhanced chemiluminescence kit (Amersham).

The calvarial cells post treatment were washed twice with ice-cold PBS and resuspended in a lysis radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors (Sigma–Aldrich). Cell lysates were homogenized by rotating at 4°C for 30 min and then centrifuged at 10 000 g for 20 min at 4°C. Protein concentrations were measured in the supernatant using Bradford’s reagent (Bio-Rad). Proteins were resolved on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were incubated with antibodies for phosphorylated AKT (pAKT; Cell Signaling #9271, Danvers, MA, USA) or total AKT (tAKT; Cell Signaling #9272) followed by HRP-conjugated goat anti-rabbit antibody (Cell Signaling #7074). In a separate set of experiments, calvarial cells post treatment were immunoprecipitated with an IRS-2 (Millipore # 06-506) antibody subjected to SDS-PAGE as described above and were then incubated with a phosphotyrosine-specific antibody (Millipore #05-321).

**Assessment of hearing by auditory brainstem response**

Groups of female and male Irs1<sup>+/+</sup> and littermate Irs1<sup>-/-</sup> mice (n=7-10) between 8 and 12 weeks of age were anesthetized with Avertin (tribromoethanol stabilized in tertiary amyl hydrate) given at a dose of 5 mg tribromoethanol/10 g body weight. Body temperature was maintained at 37–38°C by placing the mice on an isothermal pad in a sound-attenuating chamber. Sub-dermal needles were used as electrodes, which were inserted at the vertex and ventrolaterally to each ear. Stimulus presentation, auditory brainstem response (ABR) acquisition, equipment control, and data management were coordinated using the computerized Intelligent Hearing Systems (IHS, Miami, FL, USA). A pair of high frequency transducers was coupled with the IHS to

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**Table 1 Irs1 gene amplification and sequencing primers**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5' to 3'</th>
<th>Product size</th>
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<tbody>
<tr>
<td>I1-1F</td>
<td>GACTGGGGGAGACATAGTCC</td>
<td>804</td>
</tr>
<tr>
<td>I1-1R</td>
<td>TCCAGAGGAGACAAAAACGTGA</td>
<td>794</td>
</tr>
<tr>
<td>I1-2F</td>
<td>CTTGTCGAGCTGAGGATGGA</td>
<td>856</td>
</tr>
<tr>
<td>I1-3F</td>
<td>TTCATTGCCGTTATAGCAG</td>
<td>930</td>
</tr>
<tr>
<td>I1-4F</td>
<td>CTATTGACCGTATGAGCAGGT</td>
<td>818</td>
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<tr>
<td>I1-5F</td>
<td>CAGGAGACGGGCGAACAGGAGG</td>
<td>826</td>
</tr>
<tr>
<td>I1-6F</td>
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<tr>
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<tr>
<td>I1-9F</td>
<td>CAAATTCTAAGGCAGACATTG</td>
<td>804</td>
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<tr>
<td>I1-10F</td>
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<td>804</td>
</tr>
<tr>
<td>I1-11F</td>
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<td>804</td>
</tr>
<tr>
<td>I1-12F</td>
<td>CAACTTACAGGTCTGAGCA</td>
<td>725</td>
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<tr>
<td>I1-13F</td>
<td>ACCAGCTCGGGAATGAATG</td>
<td>433</td>
</tr>
</tbody>
</table>

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[Note: The table and the rest of the text are from the journal article. The text is a part of the discussion on the methods used for quantifying insulin receptor substrate-1 (Irs1) gene expression and assessing hearing in mice. The methods include quantitative real-time PCR and Western blotting, along with a description of the experimental setup and procedures used for auditory brainstem response assessment.]
generate specific acoustic stimuli. Clicks and 8, 16, and 32 kHz tone bursts were respectively channeled through plastic tubes into the animals' ear canals. The amplified brainstem responses were averaged by a computer and displayed on the computer screen. Auditory thresholds were obtained for each stimulus by reducing the sound pressure level at 10 dB steps and finally at 5 dB steps up and down to identify the lowest level at which an ABR pattern can be recognized (Gagnon et al. 2006).

Sample collection for phenotypic studies
For body composition and bone phenotyping (dual energy X-ray absorptiometry (DEXA), pQCT, and micro-computed tomography (MicroCT)), groups of female and male Irs1^sml/sml, littermate Irs1^+/sml, and Irs1^+/+ control mice (n = 10) were necropsied and measured at 4, 8, 12, and 16 weeks of age. All time points showed the same pattern and statistical significance, thus for the simplicity of presentation, only the 16-week data are reported here. For each mouse, whole body weight was recorded, whole body DEXA scans were then measured for density using the SA Plus densitometer (Orthometrics, White Plains, NY, USA). The MicroCT40 unit was calibrated weekly, and femurs were scanned under conditions described previously (DeMambro et al. 2008).

Bone histomorphometry
To determine whether the in vivo histomorphometry differences seen between the Irs1^sml/sml and the Irs1^tm1Tka/tm1Tka mice were the result of gender differences, we studied groups of Irs1^sml/sml and Irs1^+/+ females and males (n = 6) at 13 weeks of age. Mice were injected with 20 mg/kg calcine i.p. and with 50 mg/kg demeclocycline 7 days later. Mice were sacrificed 48 h following the demeclocycline injection. Femurs were then analyzed as described previously (DeMambro et al. 2008). A separate experiment in which Irs1^sml/sml and Irs1^+/+ females were evaluated was then used for comparison with the Irs1^+/+ females for the presence of any heterozygous effects. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (Parfitt et al. 1987).

Osteoblast and osteoclast cultures
Bone marrow cells were harvested from femurs and tibias of 8-week-old mice. Osteoclast-like cells were generated by plating bone marrow stromal cells at 10×10^6/well on six-well plates in α-MEM (Invitrogen) and 10% fetal bovine serum (FBS). Osteoclast-like cells were generated by plating bone marrow stromal cells at 1×10^6 cells/well in 48-well plates in α-MEM supplemented with 10% FCS and M-CSF (30 ng/ml, PeproTech Inc., Rocky Hill, NJ, USA) and RANKL (50 ng/ml PeproTech). Cultures were maintained, fixed, stained, and analyzed as described previously (DeMambro et al. 2008).

Mouse calvarial osteoblasts were harvested from 3- to 6-day-old pups using standard methods (http://skeletalbiology.uchc.edu/30_ResearchProgram/304_gap/index.htm). The cells were cultured in 10% FBS α-MEM to 90% confluence and were then serum starved in 0-1% FBS for 24 h to reduce cellular activity to quiescent levels. Cells were treated with insulin (100 nM) or IGF1 (100 ng/ml) for 15 min and were then harvested for western blot analysis. Data presented for cell culture experiments correspond to three independent experiments with at least three replicate cultures within each experiment.
Serum IGF1 and insulin

Serum IGF1 levels were measured by RIA (ALPCO, Windham, NH, USA) as reported previously (Rosen et al. 2000, 2004; Delahunty et al. 2006). Serum insulin was measured by RIA (LINCO Research, St Charles, MO, USA) as per the manufacturer’s instructions. The sensitivities of the assays were 0.01 ng/ml for IGF1 and 0.1 ng/ml for insulin. The intra-assay coefficient of variation for the assays was 4.5–4.6%. All samples were analyzed within the same assay.

Glucose and insulin tolerance tests

Female and male Irs1sml/sml, Irs1C/sml, and Irs1C/Cmice were tested at 8 and 12 weeks of age for glucose and insulin tolerance. For the glucose tolerance test (GTT), mice were placed in a clean cage with water and fasted overnight (16 h). A 1 g/kg dose of glucose was administered i.p., and blood glucose levels were measured at 0, 20, 40, 60, and 120 min post injection. For the insulin tolerance test (ITT), mice were fed ad libitum and injected i.p. with insulin at a dose of 1 U/kg. Glucose levels were then measured at 0, 20, 40, 60, and 120 min post injection.

Glucose levels were measured using the OneTouch Ultra Glucometer (LifeScan, Inc., Milpitas, CA, USA) as per the manufacturer’s instructions (Messier & Kent 1995, Weitgasser et al. 1999). In a separate experiment, we verified the accuracy and precision of the most recently manufactured OneTouch Ultra portable glucometers against Beckman Synchron CX5 Delta Clinical System (Beckman Coulter, Brea, CA, USA) using blood samples from 8- to 12-week-old C57BL6/J mice (n = 16). This comparison confirmed that glucose levels reported using portable glucometers correlated highly (r² = 0.92) against the glucose levels reported by the Beckman Synchron CX5 Delta Clinical System (data not shown).

Statistical assessment

Statistical tests were performed using JMP version 6.0 software (SAS, Cary, NC, USA) and StatView version 5.0.1 software (SAS). For the DEXA, pQCT, and MicroCT40 data, differences among Irs1sml/sml, Irs1+/+, and Irs1+/+/ mice were taken into account by including body weight and femur length as covariates in an ANCOVA model. Neither covariate was found to contribute to any of the mutant phenotypes. Data are expressed as mean ± S.E.M. in all figures. Differences between means were tested by ANOVA, with significance being declared when a P < 0.05 was observed.

Table 2 Auditory brainstem response in Irs1+/sml and Irs1sml/sml mice

<table>
<thead>
<tr>
<th>Frequency (kHz)</th>
<th>Irs1+/sml Males</th>
<th>Irs1sml/sml Males</th>
<th>Irs1+/+ Males</th>
<th>Irs1sml/sml Females</th>
<th>Irs1+/+ Females</th>
</tr>
</thead>
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<tr>
<td>n=7</td>
<td>34.3 ± 4.7</td>
<td>51.1 ± 5.9*</td>
<td>33.5 ± 0.8</td>
<td>53.8 ± 5.3*</td>
<td>41.9 ± 0.1</td>
</tr>
<tr>
<td>n=9</td>
<td>27.9 ± 6.4</td>
<td>50.0 ± 4.5*</td>
<td>23.5 ± 1.1</td>
<td>55.0 ± 6.2*</td>
<td>41.9 ± 0.1</td>
</tr>
<tr>
<td>n=10</td>
<td>15.7 ± 4.6</td>
<td>34.4 ± 6.4*</td>
<td>16.0 ± 1.0</td>
<td>36.7 ± 5.4*</td>
<td>41.9 ± 0.1</td>
</tr>
<tr>
<td>n=12</td>
<td>40.0 ± 4.2</td>
<td>61.7 ± 6.1*</td>
<td>41.0 ± 1.0</td>
<td>66.3 ± 5.4*</td>
<td>41.9 ± 0.1</td>
</tr>
</tbody>
</table>

*P < 0.05 +/sml versus sml/sml.

Figure 1 Western blots of IRS-1 (A) and AKT (B). (A) Liver lysates from Irs1sml/sml mice (lanes 1–2) and an Irs1+/+ control (lane 3) were immunoprecipitated with an IRS-1 C-terminus-specific antibody as described in the Materials and Methods. Following SDS-PAGE, the amount of IRS-1 was determined by immunoblotting. The arrow denotes the position of the IRS-1 band which is lacking in Irs1sml/sml lysates. (B) Mouse calvarial osteoblast cultures from Irs1sml/sml, Irs1+/+, and Irs1+/+ mice were treated with IGF1 (IGF1) and insulin (INS) or were given no treatment (NT), harvested, and lysed as described in the Materials and Methods. Following SDS-PAGE, membranes were immunoblotted for phosphorylated AKT (pAKT) and total AKT (tAKT).
Results

History of the Irs1sml mutation

The original Irs1sml mutant was backcrossed to the C3.SW-H2b/SnJ parental strain resulting in no mutants being observed in the F1 progeny. However, the expected Mendelian ratio of 3:1 unaffected:affected for a recessive mutation was observed in the F2 progeny. Gross histological examination found no lesions in any major organs other than decreased size (data not shown). No eye defects were detected. Hearing was assessed by ABR, and both male and female mutant mice exhibited significantly higher thresholds at 8, 16, and 32 kHz than the control littermates, indicative of hearing impairment in these mice (Table 2). Histological examination of the inner ear found no obvious defects.

Genetic mapping and mutation analysis of the Irs1sml mutation

The F2 progeny from an intercross with CAST/Ei were used to map the sml mutation to chromosome 1 between D1Mit216 (79.8 Mb) and D1Mit440 (90.7 Mb). Analysis of 204 F2 mice (408 meioses) positioned sml between D1Mit216 (79.8 Mb, 1.23% recombination) and D1Mit440 (90.7 Mb, 2.45% recombination). Irs1 was analyzed for the (see Introduction).

Genomic DNA from Irs1sml mice and Irs1+/+ controls was analyzed for the Irs1 gene by PCR amplification using overlapping primer pairs (Table 1) and sequence analysis. We discovered a deletion of one adenine nucleotide in exon 1 at position 1559 bp. This deletion results in a frameshift mutation changing a glutamine to an arginine residue, which produces a premature stop codon, predicted to result in a truncated protein of 211 amino acids instead of the full-length 1233-amino acid protein.

To confirm this mutation, we recognized that the deletion of the one adenine nucleotide created a restriction enzyme recognition site, which Taq1 recognizes (T-CGA). Thus, the region of interest was amplified, and Taq1 digestion yielded the control product of 190 bp, with a mutant product of 171 bp, confirming the deletion. Quantification of Irs1 mRNA levels by real-time RT-PCR revealed no differences in expression between the Irs1+/+ and the Irs1sml/sml mice (data not shown). Western blot analysis of proteins from the livers of the Irs1sml/sml and Irs1+/+ control mice using a C-terminus-specific IRS-1 antibody revealed that the Irs1sml/sml mice had no detectable IRS-1 protein (Fig. 1A).

Body composition by DEXA

Homozygous male and female Irs1sml/sml mice are phenotypically recognizable at ~2 weeks of age by their smaller size and thin short tail, a condition that persists throughout their lives despite normal GH levels. After weaning, mutants are ~60% the size of their Irs1+/+ control littermates, including a 20% reduction in tail length (Table 3). Whole body DEXA analysis at 16 weeks of age revealed that Irs1sml/sml male and female mice had reduced percent fat and aBMD compared with the Irs1+/+ controls. Irs1+/+ heterozygous mice had lower body weight, aBMD, and percent fat than the Irs1+/+ mice, but these parameters were greater than those of the Irs1sml/sml mutants (Table 3).

Skeletal microstructure by pQCT and MicroCT

Analysis of femurs isolated from 16-week-old Irs1+/+, Irs1sml/sml, and Irs1sml/sml mice revealed that Irs1sml/sml male and female mice had significant reductions in femur length, with an overall reduction in vbMD as measured by pQCT when compared with the Irs1+/+ control mice. Irs1sml/sml femurs also exhibited a reduction in cortical thickness as well as in periosteal circumference (Table 3). MicroCT analysis confirmed the reduction in cortical thickness with a smaller cortical bone area/total area (percent BA/TA). The distal femoral trabecular bone exhibited a significant reduction in

Table 3 Body composition and bone phenotype of Irs1+/+, Irs1sml/sml, and Irs1sml/sml mice by DEXA, pQCT, and micro-computed tomography at 16 weeks of age

<table>
<thead>
<tr>
<th></th>
<th>+/+ Males</th>
<th>+/sml Males</th>
<th>sml/sml Males</th>
<th>+/+ Females</th>
<th>+/sml Females</th>
<th>sml/sml Females</th>
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<tr>
<td>Body weight (g)</td>
<td>37.9±1.0</td>
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<td>16.5±0.7†‡</td>
<td>35.6±1.1</td>
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<tr>
<td>F. length (mm)</td>
<td>15.58±0.07</td>
<td>15.08±0.11*</td>
<td>12.55±0.04†‡</td>
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<tr>
<td>Percent fat (%)</td>
<td>32.8±0.8</td>
<td>28.5±1.2*</td>
<td>20.6±1.9†‡</td>
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<td>aBMD (g/cm²)</td>
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<td>75.2±1.1*</td>
<td>71.3±0.8†‡</td>
<td>81.3±0.4‡</td>
<td>79.2±0.6*</td>
<td>73.9±0.4†‡</td>
</tr>
<tr>
<td>CortTh (mm)</td>
<td>37.14±0.66</td>
<td>33.26±0.75*</td>
<td>25.14±0.44†‡</td>
<td>38.32±0.43</td>
<td>34.14±0.47*</td>
<td>25.3±0.20†‡</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>22.0±1.4</td>
<td>21.0±0.8‡</td>
<td>15.4±0.9†‡</td>
<td>28.3±1.2‡</td>
<td>26.2±0.6‡</td>
<td>7.5±0.7†‡</td>
</tr>
<tr>
<td>TbN (mm)</td>
<td>4.4±0.1</td>
<td>4.8±0.1</td>
<td>5.3±0.2‡</td>
<td>4.2±0.1‡</td>
<td>4.1±0.1‡</td>
<td>2.8±0.2†‡</td>
</tr>
<tr>
<td>TbTh (mm)</td>
<td>73.9±2.7</td>
<td>66.3±1.3*</td>
<td>49.3±0.7†‡</td>
<td>89.6±1.1‡</td>
<td>85.5±1.7*</td>
<td>51.8±0.8†‡</td>
</tr>
</tbody>
</table>

n=10. *P<0.05 †versus +/+sml, ‡P<0.0001 †versus +/+sml, §P<0.0001 †versus sml/sml. F. length, femur length; Peri. circ., periosteal circumference; BA/TA, bone area/total area; CortTh, cortical thickness; BV/TV, bone volume/total volume; TbN, trabecular number; TbTh, trabecular thickness.
bone volume/total volume (percent BV/TV) in both the female and male *Irs1* \textit{sml/sml} mice compared with the controls (Table 3). For the female mutants, this 73% reduction in BV/TV was accompanied by a 33% decrease in trabecular number and a 42% reduction in trabecular thickness. However, the male mutants’ distal femoral percent BV/TV was only reduced by 29% when compared with the controls. This gender difference was attributed to a 20% increase in trabecular number in the *Irs1* \textit{sml/sml} male femurs. Although, increased in number, these trabeculae were 34% thinner than those of the controls (Table 3).

Comparison of the *Irs1* \textit{+/+} female and male bones with those of the *Irs1* \textit{+/+} and *Irs1* \textit{sml/sml} mice revealed a significant intermediate phenotype for femur length, vBMD, and periosteal circumference (Table 3). Likewise, a significant intermediate phenotype was found at the mid-shaft for cortical thickness and percent BA/TA as measured by MicroCT. Interestingly, the heterozygous genotype did not affect percent BV/TV or trabecular number in the distal femur compartment, although a slight but significant reduction in trabecular thickness was observed when compared with the *Irs1* \textit{+/+} controls (Table 3).

**Histomorphometry**

Histomorphometry from the distal femur of male and female *Irs1* \textit{+/+} and *Irs1* \textit{sml/sml} mice at 13 weeks of age showed a marked and significant decrease in the percent BV/TV analogous to what was observed by MicroCT. For both genders, the *Irs1* \textit{sml/sml} mice had markedly reduced mineralizing surface/bone surface (percent MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR). Furthermore, the numbers of osteoblasts and osteoclasts per bone perimeter (Nob/BPm and Noc/BPm) were increased in both genders of *Irs1* \textit{sml/sml} mice compared with the *Irs1* \textit{+/+} controls. However, this increase was only significant for *Irs1* \textit{sml/sml} females (Table 4). Remarkably, there was no evidence of marrow adiposity in the histological sections of the *Irs1* \textit{sml/sml} femurs compared with the *Irs1* \textit{+/+} femurs (Supplementary Figure 1, see section on supplementary data given at the end of this article). Inspection of the growth plate in the proximal femur revealed a marked reduction in thickness in the *Irs1* \textit{sml/sml} mice compared with the *Irs1* \textit{+/+} mice, which was consistent with their reduced bone length (Fig. 2). The *Irs1* \textit{sml/sml} growth plate was also mildly reduced compared with the *Irs1* \textit{+/+} mice.

Bone volume fraction by histomorphometry in the *Irs1* \textit{+/+} female mice at 13 weeks of age was not different from percent BV/TV in the *Irs1* \textit{+/+} mice, akin to the findings by MicroCT. However, osteoclast numbers per bone perimeter were significantly increased (*P*<0.05) in the *Irs1* \textit{sml/sml} heterozygote mice compared with the *Irs1* \textit{+/+} females, and there was a significant (*P*<0.05) reduction in MAR, although no statistical differences were noted in the overall BFRs nor eroded surfaces/bone surface (see Supplementary Table S1, see section on supplementary data given at the end of this article).

**Cell culture**

Alkaline phosphatase (ALP) staining of bone marrow stromal cell cultures of both male and female *Irs1* \textit{sml/sml} mice on day 7 in culture revealed reductions in the number of CFU-ALP+ pre-OBs compared with the *Irs1* \textit{+/+} cultures. Similarly, on days 18 and 24, the amount of ALP staining and mineral visually detected by von Kossa staining were less in the *Irs1* \textit{sml/sml} mice than in the *Irs1* \textit{+/+} heterozygotes, and both were less than those detected in the *Irs1* \textit{+/+} control cultures (Fig. 3). When non-adherent marrow cells were cultured in m-CSF and RANKL, no differences in tartrate-resistant acid phosphatase-positive multinucleated cells were seen among mutant, heterozygote, or control mice (Fig. 3).

To determine if the increased osteoclast number in the *Irs1* \textit{sml/sml} mice as noted by histomorphometry was related to

### Table 4 Histomorphometry of *Irs1*\textit{+/+} and *Irs1*\textit{sml/sml} distal femurs at 13 weeks of age

<table>
<thead>
<tr>
<th></th>
<th>\textit{+/+} Males</th>
<th>\textit{sml} Males</th>
<th>\textit{+/+} Females</th>
<th>\textit{sml} Females</th>
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<tr>
<td>Percent BV/TV</td>
<td>12.09±0.63</td>
<td>7.80±0.08*</td>
<td>13.47±1.63</td>
<td>6.94±1.24*</td>
</tr>
<tr>
<td>Nob/BPm (mm)</td>
<td>28.00±1.23</td>
<td>29.82±2.12</td>
<td>27.28±1.52</td>
<td>31.83±1.05*</td>
</tr>
<tr>
<td>Noc/BPm (mm)</td>
<td>3.70±0.37</td>
<td>4.32±0.77</td>
<td>3.90±0.35</td>
<td>5.75±0.38*</td>
</tr>
<tr>
<td>ES/BS (%)</td>
<td>12.5±1.29</td>
<td>13.58±1.69</td>
<td>12.87±1.22</td>
<td>17.06±0.67*</td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>6.89±0.51</td>
<td>2.41±0.38*</td>
<td>7.14±0.84</td>
<td>4.10±0.32*</td>
</tr>
<tr>
<td>MAR (µm/day)</td>
<td>0.041±0.026</td>
<td>0.290±0.024*</td>
<td>0.046±0.006</td>
<td>0.049±0.047*</td>
</tr>
<tr>
<td>BFR/BS (µm²/µm² per day)</td>
<td>0.031±0.003</td>
<td>0.007±0.001*</td>
<td>0.006±0.008</td>
<td>0.017±0.003*</td>
</tr>
</tbody>
</table>

\(n=6, \ P \leq 0.05\). Percent BV/TV, bone volume/total volume; Tb. Th, trabecular thickness; Nob/BPm, number of osteoblasts/bone perimeter; Noc/BPm, number of osteoclasts/bone perimeter; ES/BS, eroded surface/bone surface; MS/BS, mineral surface/bone surface; MAR, mineral apposition rate; BFR/BS, bone formation rate/bone surface.
the secretion of osteoclastogenic cytokines, we measured RANKL and OPG (listed as TNFRSF11B in the MGI Database) expression in calvarial osteoblasts and in 8-week-old femoral samples from the Irs1<sup>tml/ml</sup> and Irs1<sup>+/+</sup> controls. There were no statistical differences in RANKL expression by real-time PCR between mutants and controls from either site. Similarly for OPG, no strain differences were noted in calvarial osteoblast expression of Opg (Trfisf11b) mRNA, although in the femoral samples from the Irs1<sup>tml/ml</sup> mice, there was a modest but non-significant (~10%) reduction in OPG expression by real-time PCR compared with the controls.

Western blot analysis of the protein isolated from calvarial osteoblast cultures revealed that at baseline, both the Irs1<sup>tml/ml</sup> and Irs1<sup>+/+</sup> cultures had significantly less pAKT compared with the Irs1<sup>+/+</sup> control cultures (Fig. 1B). Upon stimulation with IGF1 or insulin, tAKT protein was the same across the genotypes, but the Irs1<sup>tml/ml</sup> cultures showed higher levels of AKT phosphorylation than the Irs1<sup>tml/ml</sup> and the Irs1<sup>+/+</sup> cells. Finally, we examined the amount of IRS2 in calvarial osteoblasts from Irs1<sup>tml/ml</sup>, Irs1<sup>+/+</sup>, and Irs1<sup>tml/ml</sup> mice. Although total protein was the same for all the three genotypes, both Irs1<sup>tml/ml</sup> and Irs1<sup>+/+</sup> calvarial osteoblasts showed enhanced phosphorylation of IRS-2 in response to IGF1 but not to insulin (Fig. 4) compared with the Irs1<sup>+/+</sup> osteoblasts.

**Serum analysis**

Circulating levels of insulin and IGF1 were analyzed under fasting conditions. Irs1<sup>tml/ml</sup> mice were found to be relatively hyperinsulinemic, with 3.5- to 4-fold higher insulin levels compared with the Irs1<sup>+/+</sup> controls. Notably, heterozygous Irs1<sup>tml/ml</sup> mice of both genders exhibit an intermediate phenotype marked by significantly higher serum levels of insulin compared with the Irs1<sup>+/+</sup> controls, but lower insulin levels compared with the Irs1<sup>tml/ml</sup> mice (Fig. 5A). Serum IGF1 levels were modestly, but significantly reduced in the Irs1<sup>tml/ml</sup> mice relative to the controls. In addition, a gender effect was observed in the serum IGF1 levels such that the heterozygous Irs1<sup>+/+</sup> and Irs1<sup>tml/ml</sup> serum IGF1 levels were significantly lower in the females than in the males of the same genotype (P<0.0005). Unexpectedly, there was no intermediate phenotype for the Irs1<sup>+/+</sup> males. However, in the female Irs1<sup>+/+</sup> mice, we observed the same intermediate phenotype for serum IGF1 seen in the previous phenotypic datasets (Fig. 5B).

**GTT and ITT**

To investigate whether the mild hyperinsulinemia in Irs1<sup>tml/ml</sup> mice was indicative of a pre-diabetic state, GTTs were performed on fasted Irs1<sup>+/+</sup>, Irs1<sup>tml/ml</sup>, and Irs1<sup>+/+</sup> males and females at 8 weeks of age (Fig. 5C and D). At baseline, Irs1<sup>tml/ml</sup> mice exhibited significantly lower (30–40%) fasting glucose levels compared with the controls (Fig. 5C). When challenged with glucose, Irs1<sup>tml/ml</sup> glucose values rose as expected, but remained significantly lower than either Irs1<sup>+/+</sup> or Irs1<sup>tml/ml</sup> levels throughout the study. Although baseline glucose levels differed, glucose levels peaked 20 min post injection in Irs1<sup>tml/ml</sup> mice, as in both Irs1<sup>+/+</sup> or Irs1<sup>tml/ml</sup> mice, and the rate of glucose metabolism was similar among all the three genotypes of mice.

For the ITT under non-fasting conditions, the Irs1<sup>tml/ml</sup> glucose levels decreased, but not to the full extent as seen in
during the glucose and insulin tolerance tests. At 16 weeks of age, genotype * and they showed a similar pattern of significance (*P < 0.05). Male *Irs1*+/+, *Irs1*sml/sml, and *Irs1*+/sml mice at 8 weeks of age were fasted overnight and then subjected to a C GTT (Fig. 5D), revealed a mild insulin resistance. Comparison of the spontaneous mutant to *Irs1*sml/sml mice is due to the inability of osteoblasts to form new bone coupled to an increase in osteoclastic activity, as evident by the larger eroded surfaces and the greater number of osteoclasts in the *Irs1*sml/sml mice. The absence of an in vitro effect on osteoclast differentiation from this mutation (see Fig. 3B) suggests that the osteoclastic changes are due to a non-cell autonomous process. In preliminary studies using real-time PCR on femoral and calvarial samples, we were unable to detect differences in gene expression for *RANKL* and *Opg* finding slightly lower *Opg* mRNA in the *Irs1*sml/sml bones versus *Irs1*+/+ controls. Whether the enhanced osteoblast recruitment is a compensatory response to a major defect in osteoclast function or another process remains to be determined. But, interestingly, Zhang et al. (2002) reported that mice with a conditional *Igf1r* deletion in osteoblasts developed defective mineralization, and had markedly increased osteoblast numbers. This would imply there must be feedback signals that operate during terminal differentiation that attempt to compensate for impaired skeletal function.

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**Discussion**

In this study, we have characterized for the first time a spontaneous mutation in the *Irs1* gene that results in a pronounced metabolic and skeletal phenotype. The *Irs1* mutation is a spontaneous frameshift mutation that results from a single nucleotide deletion in the *Irs1* gene leading to a premature stop codon and a truncated protein. This results in a hyperinsulinemic, lean, small mouse with reductions in serum IGF1 levels, BMD, and hearing. Mutant mice, although hyperinsulinemic, were only mildly insulin resistant as exogenous insulin treatment was effective in reducing circulating glucose levels. This partial ability of *Irs1*sml/sml mice to respond to exogenous insulin most likely prevents the development of overt hyperglycemia or diabetes.

The skeletal phenotype of the *Irs1*sml/sml mice is remarkable and differs from that of the genetically engineered *Irs1* null mice. *Irs1*sml/sml bones exhibited reductions in femur length, cortical and trabecular thicknesses, and trabecular number. In addition, *Irs1*sml/sml mice had a pronounced reduction in MAR and BFRs. But unexpectedly, these mutants had more osteoclasts and more osteoblasts per bone perimeter than the controls. To understand the mechanism for this surprising finding, we performed in vitro studies which demonstrated that *Irs1*sml/sml osteoblasts had impaired cell proliferation and differentiation, but no differences were found in osteoclast recruitment, differentiation, or appearance. Histological sections demonstrated that there was adequate recruitment of osteoblast precursors; hence the defect in bone formation was likely a result of poor osteoblast function due to reduced IRS-1 signaling. Taken together, we postulate that the markedly reduced bone volume fraction in the *Irs1*sml/sml mice is due to differences in osteoblast differentiation. The engineered *Irs1*outl/*outl* mice have reduced aBMD. Differences in phenotypes between the spontaneous *Irs1*sml/sml and the engineered *Irs1*outl/*outl* null mice include the lack of gender and heterozygous effects, as well as the degree of insulin resistance. Comparison of the spontaneous mutant to the *Irs1*outl/*outl* allele also revealed differences in osteoblast and osteoclast numbers in vivo versus +/+ controls (Araki et al. 1994, Tanemoto et al. 1994, Ogata et al. 2000).

Phenotypic differences between the *Irs1*outl/*outl* and the *Irs1*sml/sml mice may be the result of...
the different background strains. Reports have alluded to the importance of genetic background–contributing alleles that alter bone and growth phenotypes in mutants (Gonzalez et al. 1992, Bouxsein et al. 2002, Martelli et al. 2005). The genetically engineered Irs1 null models are reported to be on a hybrid B6/CBA background strain, whereas the Irs1tm1Jos nulls reveal a hypomorphic phenotype. A significant drop in IGF1 levels was observed in the Irs1tm1Jos/null mice compared with the controls. This decrease is related to the lack of IRS1 that leads to reduced IGF1 synthesis. How does this decrease occur? Perhaps locally by alterations in GH secretion, or due to the lack of IRS-2 or IRS-1. Furthermore, closer examination of the bone microstructure revealed a decrease in bone mineral density (BMD) and reduced bone turnover. It is interesting to speculate as to why the cortical compartment, but not the trabecular component of the skeleton is affected by IGF1 levels. The increase in resorption in the heterozygotes is not as dramatic as that in the mutants, and this combined with minimal alterations in BFRs results in barely detectable changes in the trabecular bone volume fraction of Irs1+/−/+ mice. In contrast, despite body composition change in the Irs1+/−/+ mice, there was no intermediate metabolic phenotype when the mice were challenged with glucose or insulin. Although no heterozygous genotype was reported for the bones of Irs1+/−/+ mice, a study done by Pete et al. (1999) did report small but significant reductions in body and organ weight in heterozygous Irs1+/−/+ mice. On the other hand, exhibited a compensatory hyperinsulinemia due to the lack of IRS1, but retained some insulin sensitivity, as shown by their nearly intact response to the glucose-lowering effects of exogenous insulin. Furthermore, the Irs1+/−/+ mice exhibited one-third lower glucose levels after fasting and throughout the GTT. This attenuated glucose response can be explained by the moderate hyperinsulinemia found in Irs1+/−/+ mice.

Initial characterizations of the Irs1+/−/+ mice reported no significant differences in serum IGF1 levels (Tamemoto et al. 1994). However, Pete et al. (1999) reported a decrease in IGF1 levels in the Irs1+/−/+ mice compared with the controls, but the change did not reach significance, most likely due to low sampling numbers. In the spontaneous Irs1+/−/+ mice, there was a consistent 20% decrease in IGF1 levels compared with the Irs1+/−/+ control mice. Dong et al. (2006) also reported a 20% decrease in IGF1 levels in the Irs1+/−/+ mouse compared with the controls. The 20% reduction in the IGF1 serum levels in the Irs1+/−/+ mice is an unexpected observation. A defect in signaling should result in increased IGF1 levels rather than a decrease, presumably as a compensatory mechanism. It is probable that the over-expression of IGF1 could partially compensate for the lack of IRS1 in postnatal growth (Pete et al. 1999). However, we postulate the emergence of a negative feedback loop in the absence of IRS1 that leads to reduced IGF1 synthesis. How that inhibition occurs (perhaps locally by alterations in GH signaling) is a subject of further investigations.

We assume that the Irs1+/−/+ mutation results in complete loss of function, but cannot rule out the possibility of a hypomorphic mutation. Indeed, it is possible that a
211-amino acid truncated peptide is produced and may bind to the IGF1 receptor by its phosphotyrosine-binding (PTB) domain. If so, all the sites of binding and phosphorylation downstream of the PTB domain are missing resulting in defective signaling as evidenced by the lack of AKT phosphorylation in mouse calvarial osteoblast cells when stimulated with IGF1 or insulin. This deleted region includes serine residues, which when phosphorylated, trigger protein degradation. Thus, it is possible that the truncated peptide may attach to the IGF1 receptor and perturb the system, resulting in the phenotypic differences observed between our spontaneous mutant and the engineered mutants of the Irs1 gene. Repeated attempts to use the only commercially available N-terminus-specific antibody were unsuccessful. However, since the PTB domain of the IRS1 protein comprises residues 144–316, our predicted protein would contain <50% of the PTB domain, and thus binding to the receptor is unlikely.

Reduced circulating IGF1 and mutations in the Igf1 gene have been directly linked to hearing loss in both mice and humans (Wood et al. 1996, Camarero et al. 2001, Barrena et al. 2003, Bonapace et al. 2003, Cediel et al. 2006). Mice lacking the Igf1 gene have been shown to lose many auditory neurons and exhibit increased auditory thresholds indicative of hearing impairment (Camarero et al. 2001, Cediel et al. 2006). It has also been shown that IGF1 is critical in the development of the cochleovestibular ganglion in the inner ear (Varela-Nieto et al. 2004). Furthermore, lack of IGF1, or mutations in the IGF1 gene, has been associated with sensorineural hearing loss in humans (Wood et al. 1996, Bonapace et al. 2003). Thus, the fact that Irs1<sub>sml</sub> mice exhibit hearing loss further supports the existence of a major defect in the IGF1 signaling pathway.

Recently, a SNP located adjacent to the Irs1 gene has been associated with type 2 diabetes, insulin resistance, and hyperinsulinemia in a human cohort of more than 14,000 individuals. The investigators reported a 40% reduction in the basal levels and function of IRS1 protein in the skeletal muscle of patients carrying this variant (Rung et al. 2009). Likewise, there are numerous studies on humans demonstrating polymorphisms in the Irs1 gene associated with variable responsiveness to insulin signaling (Imai et al. 1994, Ura et al. 1996, Le Fur et al. 2002). The most common polymorphism, the G972R variant, has also been associated with type 2 diabetes mellitus (Almind et al. 1993, Sesti 2000, Tok et al. 2006). However, there are no data examining the relationship between these polymorphisms and BMD or fracture risk. Based on our work combined with previous studies performed on the Irs1 knockout models, we predict that patients with IRS1 polymorphisms could have a subclinical but important skeletal phenotype.

There are several limitations of our studies. First, the mechanism responsible for the low serum IGF1 levels in the Irs1<sub>sml</sub> mice is not readily apparent from our data. Secondly, the degree of compensation between IRS1 and IRS2 in the mutant and heterozygote models requires further study. Thirdly, the hyperinsulinemia along with the mild insulin resistance phenotype is intriguing and raises new questions about the importance of IRS1 in glucose homeostasis. Fourthly, the heterozygous phenotype is particularly provocative as it lends credence to the theory that similar mutations in the Irs1 gene may exist in humans. These are likely to be characterized by relatively modest reductions of IGF1, low fat mass, hyperinsulinemia, reduced BMD, and short stature. However, this theory will need to be verified in large population studies. Finally, the increase in osteoclastogenesis in both the mutant and heterozygote mice requires further study to determine if the relatively modest reduction in Opg mRNA in the pre-osteoblasts of these mice is partly responsible for the skeletal phenotype. Notwithstanding, this paper reports the first spontaneous ‘loss of function’ mutation in the Irs1 gene. The unique phenotypic presentation of this mutant raises new questions about the role of IRS1 in both skeletal acquisition and glucose homeostasis.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/JOE-09-0328.

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 work was supported by the National Institute of Health (grants: RR01183, ARMYLRD, DK042424, DK073267, AR46544, and AR043618).

Acknowledgements

The authors would like to thank Lindsay G Horton, Colleen Kane, Belinda Harris, Leona Gagnon, Krista Delahunty, Lisa Carney, Pat Ward-Bailey, and Cheryl Ackert-Bicknell for assistance with this research.

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