Insulin receptor substrate 1 regulates the cellular differentiation and the matrix metalloproteinase expression of preosteoblastic cells

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Abstract

Insulin receptor substrate 1 (IRS1) is an essential molecule for the intracellular signaling of IGF1 and insulin, which are potent anabolic regulators of bone metabolism. Osteoblastic IRS1 is essential for maintaining bone turnover; however, the mechanism underlying this regulation remains unclear. To clarify the role of IRS1 in bone metabolism, we employed RNA interference to inhibit *IRS1* gene expression and observed the effects of silencing this gene on the proliferation and differentiation of and the expression of matrix metalloproteinase (MMP) and tumor necrosis factor receptor superfamily, member 11b (TNFRSF11B) in MC3T3-E1 cells. Our results showed that *IRS1* short hairpin RNAs can effectively suppress the expression of IRS1, and inhibit the

phosphorylation of AKT in IRS1 pathway; reduce the expression of MMP2, MMP3, MMP13, and MMP14, decrease the expression of TNFRSF11B and RANKL (also known as tumor necrosis factor (ligand) superfamily, member 11), however increase the RANKL/TNFRSF11B ratio; decrease cell survival, proliferation, and mineralization, and impair the differentiation of MC3T3-E1 cells. The downregulation of IRS1 had no effect on the expression of MMP1. Our findings suggest that IRS1 not only promotes bone formation and mineralization but also might play roles in bone resorption partly via the regulation of MMPs and RANKL/TNFRSF11B ratio, thus regulates the bone turnover.

Journal of Endocrinology (2010) **206**, 271–277

Introduction

Insulin receptor substrates (IRSs) are essential for receptor tyrosine kinases, such as insulin and insulin-like growth factor 1 (IGF1) receptors, as well as other cytokines affecting cellular function (Burks & White 2001). Following stimulation by a ligand, the IRSs are rapidly phosphorylated on multiple tyrosine residues (Kadowaki *et al.* 1996). The phosphorylated substrates bind to proteins containing Src homology 2 domains, and then activate phosphoinositide 3-kinase and mitogen-activated protein kinase pathways to regulate cell differentiation, growth, and metabolism (Cusi *et al.* 2000). The mammalian IRS family contains at least four members: ubiquitous IRS1 and IRS2, adipose tissue-predominant IRS3, and IRS4, which is expressed in the thymus, brain, and kidney (Sun *et al.* 1991, 1995). Studies have shown that IRS1 and IRS2 are expressed in bone tissue (Akune 2004). *Irs1* gene knockout mice (*Irs1*^{−/−} mice) and *Irs2* gene knockout mice (*Irs2*^{−/−} mice) showed osteopenia that developed via different cellular mechanisms: in the case of the *Irs1*^{−/−} mice, both bone formation and resorption were decreased (Ogata *et al.* 2000), whereas in the *Irs2*^{−/−} mice,

decreased bone formation and increased bone resorption were noted (Akune *et al.* 2002). Therefore, IRS1 is indispensable for maintaining the bone turnover, while IRS2 is essential for retaining the predominance of bone formation over bone resorption, and plays important roles in 17β-estradiol induced bone formation (Bu *et al.* 2009).

Matrix metalloproteinases (MMPs) belong to a family of zinc endopeptidases that are capable of degrading most of the structural components of the extracellular matrix (Ra & Parks 2007). MMPs promote bone resorption by facilitating the attachment of osteoclasts to the bone and removing unmineralized matrix from the bone surface (Rifas *et al.* 1994, Manduca *et al.* 2009). IRS1 is expressed in osteoblasts but not in osteoclasts, the decrease in bone resorption in the *Irs1*^{−/−} mice is caused by a defect in the osteoblasts that support osteoclast differentiation, and is not due to the intrinsic abnormalities of osteoclast progenitors or the reduced activity/survival of mature osteoclasts (Ogata *et al.* 2000). MMPs function as ‘coupling factors’ that allow osteoblasts to exert a regulatory effect on osteoclast resorptive activity. Evidence has shown that IRS2 down-regulated MMP13 expression and inhibited osteoclastogenesis (Akune *et al.* 2002).

Our previous studies have shown that osteoblasts can also express the MMPs (Li *et al.* 2004); however, it is not clear whether IRS1 affects MMPs expression and regulates bone turnover. The receptor activator of NF- κ B ligand (RANKL)/RANK/tumor necrosis factor receptor superfamily, member 11b (TNFRSF11B) system is now recognized as the dominant pathway regulating bone resorption (Gallagher 2008). RANKL is a cytokine belonging to the tumor necrosis factor family and is expressed by osteoblasts; it binds to the membrane-bound receptor RANK on osteoclasts and promotes the differentiation of the marrow cells to multinucleated osteoclasts through various stages (Yamaguchi & Uchiyama 2004). TNFRSF11B is a soluble receptor that is a potent antagonist of osteoclast formation, and it exerts its action by binding and inactivating the RANKL (Boyce & Xing 2007). IRS1 regulates the bone turnover, but the effect of IRS1 on the RANKL/RANK/TNFRSF11B system remains unclear. In this study, we used RNA interference to silence the *Irs1* gene and observe the effects of *Irs1* on cell proliferation, differentiation, and the expression of MMPs and RANKL/RANK/TNFRSF11B in murine preosteoblastic MC3T3-E1 cells.

Materials and Methods

Cell culture

The murine preosteoblastic MC3T3-E1 cells were routinely cultured in phenol red-free DMEM (Sigma) containing 10% fetal bovine serum (FBS, Invitrogen) and 50 μ g/ml ascorbic acid (Sigma) at 37 °C in a humidified atmosphere containing 5% CO₂. The medium was changed two to three times per week.

Plasmid construction

Short hairpin RNA (shRNA) plasmids were constructed by and purchased from Genesil Biotechnology Corporation (Wuhan, People's Republic of China). We designed three coding sequences (target 1135–1155, 1429–1447, and 4432–4452 to murine *Irs1* mRNA; Genbank accession number: NM_010570) for the shRNAs. Subsequently, these three coding sequences were inserted into the pGenesil-1 vector, which has a neomycin- and kanamycin-resistant gene and expresses the enhanced green fluorescent protein; these sequences were designated as *Irs1* shRNA1, *Irs1* shRNA2, and *Irs1* shRNA3 respectively, and were analyzed by BLAST search to ensure that they did not have significant sequence homology with other genes. One scramble shRNA was inserted into the pGenesil-1 vector as the negative control (denoted as HK shRNA). The loop sequence for all the shRNAs was TTCAAGACG. All the shRNA plasmids can stably express the shRNA when they were stably transfected into cells.

Transfection and selection of stably transfected MC3T3-E1 cells

Before the transfections, MC3T3-E1 cells were seeded in six-well plates at a density of 1×10^5 cells/well. The transfections with the shRNAs were performed using

the Lipofectamine 2000 transfection kit (Invitrogen) as per the manufacturer's instructions. Two days after the transfection, the cells were replated at a lower density and selected using a media containing 500 μ g/ml geneticin (G418) for 21 days until discrete colonies were formed. Individual colonies were isolated and grown, and then semi-quantitative reverse transcription PCR (RT-PCR) and western blotting were used to test the inhibition efficiency of *IRS1* gene expression. Stably transfected MC3T3-E1 (ST-MC3T3-E1) cells were sustained in a medium containing 200 μ g/ml G418 and 10% FBS. Because insulin at 1.0 U/ml level has been shown to stimulate growth of cells in media containing 10% FBS (Webber 1980), no additional insulin was added to the medium in future studies.

von Kossa staining

The MC3T3-E1 cells transfected with HK shRNA and *Irs1* shRNAs were treated with 100 nM dexamethasone, 50 μ g/ml L-ascorbic acid, and 5 mM β -glycerolphosphate, and the medium was replaced every 2–3 days. The mineral deposition of the bone-specific extra cellular matrix was determined by von Kossa staining for calcium. Briefly, cells were fixed with 3% paraformaldehyde for 10 min, stained with AgNO₃, rinsed with distilled water, exposed for 1.5 h to bright light, and counterstained with nuclear fast red for 5 min, and finally observed by transmitted light microscopy; the surface area of the cell cultures occupied by mineralized nodules was compared in each well as described previously (Rowe *et al.* 1999).

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide and [³H]-thymidine incorporation assays to determine cell survival and proliferation

MC3T3-E1 cells were seeded in 96-well plates at a density of 1×10^4 /well; after 24 h of culture, the medium was removed, and 200 μ l of 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide in DMEM were added to each well and incubated for 4 h at 37 °C. Formazan crystals were dissolved in 150 μ l of dimethyl sulfoxide, and the absorbance at 570 nm was measured. The cell survival ratio was calculated according to the following formula: survival ratio = $A_{\text{treat}}/A_{\text{control}} \times 100\%$. For [³H]-thymidine incorporation assay, cells were incubated in 24-well plates at a density of 5×10^4 /well. After 24 h of culture, [³H]-thymidine (1 μ Ci/ml in the medium) was added to the medium, and the cells were cultured for a further 8 h; radioactivity was determined using a liquid scintillation counter (Wallac OY, Turku, Finland).

Flow cytometry and acridine orange/ethidium bromide staining assay to determine apoptosis

MC3T3-E1 cells transfected with HK shRNA and *Irs1* shRNAs were seeded in six-well plates at a density of 5×10^4 /well. After the cells reached about 50–60%

Figure 1 The effects of insulin receptor substrate 1 (*Irs1*) gene silencing (by *Irs1* shRNA1, 2, and 3 transfection) on the phosphorylation of AKT in MC3T3-E1 preosteoblast cells. IRS1 expression level in MC3T3-E1 cells was efficiently down-regulated by shRNA transfection as determined by reverse transcription PCR (RT-PCR) and western blot; the phosphorylation of AKT, which is the target molecule in IRS pathway induced by IGF1, was decreased by *Irs1* gene silencing.

confluence, serum was deprived for 24 h. Cells were then labeled using the nucleic acid-binding dye mix of 100 µg/ml acridine orange and 100 µg/ml ethidium bromide (Sigma) in PBS. The cells were examined by fluorescent microscopy. Viable cells had green fluorescent intact nuclei, while the apoptotic cells had orange chromatin with highly condensed or fragmented nuclei. The necrotic cells had bright orange chromatin and round nuclei. Only the cells with yellow, condensed, or fragmented nuclei samples were counted as apoptotic cells. For flow cytometric analysis, MC3T3-E1 cells transfected with HK shRNA and *Irs1* shRNAs were seeded into 25 cm² flasks at a density of 1 × 10⁵/ml and cultured to 50–60% confluence. The cells were deprived of serum for 24 h, and then digested by 0.25% trypsin–EDTA. We fixed the cells by using ice-cold 95% ethanol and measured apoptosis by flow cytometric analysis in Dingguo Biotechnologies Inc. (Beijing, People's Republic of China).

Analyses of alkaline phosphatase activity and osteocalcin

MC3T3-E1 cells transfected with HK shRNA and *Irs1* shRNAs were cultured in the medium with 10 mM β-sodium glycerophosphate and 50 µg/ml of ascorbic acid. When the cells reached confluence, they were lysed and sonicated for 20 s. Alkaline phosphatase (ALP) activity in both the cell lysate and the culture media was measured in the three independent cultures using an ALP assay kit (Sigma). Osteocalcin released into the culture media was measured in triplicate using a specific RIA kit (DiaSorin Corp., Stillwater, MN, USA). To normalize protein levels in the culture media

to the total cellular protein, the protein content in a fraction of the lysate solution was determined using the BCA protein assay reagent (Pierce, Rockford, IL, USA).

Semi-quantitative RT-PCR

Total RNA was isolated from each sample using Trizol reagent (Invitrogen) according to the manufacturer's recommended protocol. We performed RT using 1.0 µg total RNA and the RT system (Promega). The PCR primers of type I collagen were 5'-GAAGTCAGCTGCATACACAA-3' and 5'-GCTGATTTTTCATCATAGCC-3', yielding a 403-bp fragment. The PCR primers of *IRS1* were 5'-GGTAAGCTCTTGCCCTTGCAC-3' and 5'-TGTGTGAGGCTTGACTCTGG-3', yielding a 326-bp fragment. The PCR products of β-actin served as the internal control. The amplification cycles were 26, 29, and 25 respectively. The reactions were stopped at the exponential phase. The PCR products were run on a 1.2% agarose gel, stained with ethidium bromide, and photographed under an u.v. illumination system. The primers were synthesized by Shanghai BioAsia Biotechnology Company, Ltd (Shanghai, People's Republic of China). In addition, the PCR products were extracted from the agarose gels and sequenced by BioAsia Company.

Cell treatment and western blotting analysis

To detect the phosphorylation of AKT, the direct target in IRS1 pathway, after the *IRS1* shRNA transfection, the cells were starved for 24 h and then incubated in the absence or in the presence of 100 nmol/l IGF1 for 15 min at 37 °C. At the end of incubation, the cells were rapidly washed with Ca²⁺/Mg²⁺ free PBS, and then mechanically detached in ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, 0.02% sodium azide, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, and 10 µg/ml aprotinin, pH 8.0). The protein concentrate was determined using a BCA protein assay reagent (Pierce). The cell lysates

Figure 2 The effects of insulin receptor substrate 1 (*Irs1*) gene silencing on cell survival (left panel) and proliferation (right panel) in MC3T3-E1 cells. Cell survival was determined by MTT assay. Cell proliferation was determined by [³H]-TdR incorporation assay. Data are expressed as mean (bars) ± S.E.M. (error bars). Compared with untransfected (control) group and the HK group, **P* < 0.01, ***P* < 0.05.

Figure 3 Effects of *Irs1* gene silencing on the differentiation of MC3T3-E1 preosteoblast cells. von Kossa stain for mineralization, alkaline phosphatase activity, and osteocalcin secretion were detected in each group. Marked mineralization was seen in scramble shRNA-transfected (HK group) 3T3-E1 cells (black dots); however, mineralization was reduced in 3T3-E1 cells with *Irs1* gene silencing; *Irs1* short hairpin RNA (shRNA) can reduce the ALP activity and osteocalcin secretion. Data are expressed as mean (bars) $\pm$ S.E.M. (error bars). Compared with untransfected (control) group and the HK group, * $P < 0.05$.

containing an equivalent amount of protein (30 μ g) were electrophoresed by 8% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% fat-free milk, the lysates were incubated with a rabbit anti-IRS1, MMP2, MMP13, MMP14, RANKL (Santa Cruz Biotechnology, Santa Cruz, CA, USA), AKT and phosphorylated AKT (Cell Signal, Danvers, MA, USA), goat anti-collagen I, mouse anti-MMP1 and TNFRSF11B (Santa Cruz Biotechnology), and then incubated with incubated peroxidase secondary antibody. Immunoreactive bands were stained using the enzymatic chemiluminescence reaction kit (Pierce). The same membrane was stripped, and then reprobed with a mouse monoclonal antibody against human β -actin (Sigma) as an internal control. The intensity of each band was measured by densitometry (Bio-Rad Laboratories). The target protein levels were calculated after standardization to the β -actin concentration and were expressed as the mean value of the three independent experiments.

Statistical analyses

Statistical significance was determined by Statistical Product and Service Solutions 13.0 for windows. Data are expressed as the mean $\pm$ S.D. of mean. Statistical differences between mean values (derived from the same clone in several different wells for each experiment, and we repeated the experiment at least three times) were determined using one-way ANOVA and Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

IRS1 shRNA inhibits the AKT phosphorylation induced by IGF1 in MC3T3-E1 cells

The semi-quantitative RT-PCR and western blot analyses revealed that the expression levels of IRS1 mRNA and protein were significantly lower in the *Irs1* shRNA 1, 2, and 3 groups than those in the untransfected control and the HK shRNA groups (Fig. 1). All the three *Irs1* shRNAs effectively inhibited the expression and phosphorylation of AKT induced by IGF1 in MC3T3-E1 cells (Fig. 1).

The effects of IRS1 gene silencing on cell survival and proliferation in MC3T3-E1 cells

Compared with the untransfected control and the HK shRNA groups, the cell survival and proliferation rates were lower in the *Irs1* shRNA 1, 2, and 3 groups (Fig. 2) than the untransfected control and the HK shRNA groups; the percentage cell survival in *Irs1* shRNA 1, 2, and 3 groups is 42 ± 13 , 40 ± 5 , and $55 \pm 11\%$ respectively. Therefore, our results indicate that *Irs1* shRNA 1 and shRNA 2 have greater abilities to inhibit the cell survival and proliferation of murine preosteoblasts than *Irs1* shRNA 3.

Figure 4 The effects of insulin receptor substrate 1 (*Irs1*) gene silencing on collagen mRNA and protein expression in MC3T3-E1 preosteoblastic cells. The bars represent the ratio of optical density values of collagen I mRNA and protein relative to that of β -actin measured by densitometry. Data are expressed as mean (bars) $\pm$ S.E.M. (error bars). Compared with untransfected (control) group and the HK group, * $P < 0.01$.

Figure 5 Effect of insulin receptor substrate 1 (*Irs1*) gene silencing on cell apoptosis in MC3T3-E1 cells. (a and c) HK group; (b and d), shRNA1 group. Cell apoptosis was assessed by acridine orange/ethidium bromide staining assay (a and b) and flow cytometric analysis. Magnification was the same for all micrographs ($\times 400$). Arrows show the apoptotic cells with orange nuclei.

The effect of Irs1 gene silencing on cell differentiation and apoptosis in MC3T3-E1 cells

ALP activity and osteocalcin expression are important markers for osteoblast differentiation. The *Irs1* shRNA 1, 2, and 3 groups significantly decreased the ALP activity and osteocalcin levels in the MC3T3-E1 cells ($P < 0.05$, Fig. 3). The change of osteoblast differentiation potential after IRS1 silence was assessed by evaluating the mineralization of the cell cultures in the presence of osteogenic induction medium. Marked mineralization was seen in control 3T3-E1 cells; however, area of mineralized nodules was reduced in 3T3-E1 cells with *Irs1* gene silencing (Fig. 3); the mRNA and protein expressions of type I collagen were also down-regulated ($P < 0.01$, Fig. 4). *IRS1* gene silencing also induced apoptosis in the MC3T3-E1 cells; the apoptotic cells had condensed and orange nuclei (Fig. 5b). The degree of apoptosis in the HK group and *Irs1* shRNA group was 1.03 ± 0.32 and $3.57 \pm 1.80\%$ respectively (Fig. 5c and d).

The effect of Irs1 gene silencing on the expression of TNFRSF11B, RANKL, and MMPs in MC3T3-E1 cells

The expression levels of MMP2, MMP3, MMP13, and MMP14 were lower in the *Irs1* shRNA 1, 2, and 3 groups than the untransfected control and HK shRNA groups ($P < 0.05$, Fig. 6). However, no difference was observed in the expression of MMP1 among all the groups (Fig. 6). The TNFRSF11B and RANKL expression were also reduced in the *Irs1* shRNA 1, 2, and 3 groups ($P < 0.05$, Fig. 7), however, the ratio of RANKL to TNFRSF11B is up-regulated by *Irs1* gene silencing (Fig. 7).

Discussion

Bone remodeling is a spatially coordinated lifelong process, whereby old bone is removed by osteoclasts and replaced by bone-forming osteoblasts (Kearns *et al.* 2008). The TNFRSF11B/RANK/RANKL system identified in the mid-1990s was thought to be one of the most important coupling factors between bone formation and resorption, which regulates the bone turnover (Hamdy 2007). IRS1, might also be an important coupling factors, was proved to regulate the bone turnover by performing research on the *IRS1* gene knockout mice (Ogata *et al.* 2000). Unlike the TNFRSF11B/RANKL/RANK system, which regulates the bone turnover via both the osteoblast and osteoclast, the IRS1 regulates bone turnover mainly via the osteoblast, but the underlying mechanism is not clear.

To elucidate the mechanism underlying the regulation of bone turnover by the IRS1, we generated MC3T3-E1 cells stably transfected with the plasmids expressing shRNA-targeting murine *Irs1* gene, which allows the generation of stable cell lines with permanently reduced levels of the IRS1 protein. Our data showed that the three *Irs1* shRNAs targeting the nucleotides 1135–1155, 1429–1447, and 4432–4452 of the *Irs1* gene can inhibit *Irs1* mRNA and protein expression. Therefore, *Irs1* shRNAs provide a useful strategy for identifying the functions of IRS1. The down-regulation of IRS1 expression not only reduced cell survival and proliferation, but also impaired cell differentiation in MC3T3-E1 cells (ALP activity and the expression of both

Figure 6 The effects of insulin receptor substrate 1 (*Irs1*) gene silencing on matrix metalloproteinases MMP1, MMP2, MMP3, MMP13, and MMP14 protein expression in MC3T3-E1 osteoblast-like cells. The bars represent the ratio of optical density values of each protein relative to that of β -actin measured by densitometry. Data are expressed as mean (bars) $\pm$ S.E.M. (error bars). Compared with untransfected (control) group and HK group, $*P < 0.05$.

Figure 7 The effects of insulin receptor substrate 1 (*Irs1*) gene silencing on TNFRSF11B and receptor activator of NF-κB ligand (RANKL) protein levels in MC3T3-E1 preosteoblast cells. The expression of TNFRSF11B and RANKL was down-regulated simultaneously by *Irs1* silencing (A and B); however, RANKL/TNFRSF11B ratio was increased (C). The bars in panel B represent the ratio of optical density values of each protein relative to that of β-actin measured by densitometry, and bars in panel C represent the ratio of RANKL to TNFRSF11B. Data are expressed as mean (bars) ± S.E.M. (error bars). Compared with untransfected (control) group and HK group, * $P < 0.01$, ** $P < 0.05$.

osteocalcin and type I collagen mRNA and protein were all decreased), which suggested that IRS1 can increase the bone formation by promoting the proliferation and differentiation of preosteoblasts and increase the bone matrix by producing more collagen. Although the mechanism by which IRS1 increases the cell proliferation and differentiation needs to be further studied, it is known that IRS1 is the major substrate of insulin and IGF1 receptors, which play important roles in bone metabolism (Bikle 2008, Giustina *et al.* 2008), *IRS1* gene silencing in osteoblast obviously inhibited the AKT phosphorylation (Fig. 1), and AKT is the direct target of IGF1 in IRS1 signal pathway. Therefore, we hypothesized that *Irs1* gene silencing hindered insulin and IGF1 signal transduction, which led to reduced cell survival and proliferation and impairment in the differentiation of MC3T3-E1 cells.

Ogata's research (Ogata *et al.* 2000) indicated that IRS1 was expressed only in the osteoblasts and not in the osteoclasts in wild-type mice; however, bone resorption was decreased in *Irs1*^{-/-} mice. Osteoblast-derived MMPs function as a coupling factor and play important roles in the bone turnover. MMPs are believed to generate collagen fragments, which are necessary for the recruitment and activation of osteoclasts and the initiation of bone resorption (Holliday *et al.* 1997, Parikka *et al.* 2001). We conducted this study to determine whether IRS1 regulates the expression of MMP1, MMP2, MMP3, MMP13, and MMP14 in murine preosteoblastic MC3T3-E1 cells. Our results showed that knocking down *Irs1* decreased the protein levels of MMP2, MMP13, and

MMP14, but had no effect on MMP1 expression. IRS1 is an essential molecule for the intracellular signaling of IGF1 and insulin, which are potent anabolic regulators of bone metabolism, and MMPs are regulators of catabolism; it seems self-contradictory that the silencing of anabolic regulator decreased the expression of regulators of catabolism, but our results were consistent with the study in malignant H-ras transformed cells by Yoon & Hurta (2001), which showed that IGF1 up-regulated the expression of MMP2, further studies are needed to elucidate how IRS1 regulates the MMP expression in osteoblasts. Except that IRS1 regulated the bone turnover discussed below, it is possible that the absence of proper anabolic regulation will hinder the functions of osteoblasts, which include the synthesis of matrix and matrix degradation enzymes, cell proliferation and differentiation.

MMP1 (collagenase-1) and MMP13 (collagenase-3) can cleave the fibrillar collagens (Kerkvliet *et al.* 2003); MMP2 (gelatinase-A) can degrade gelatin, laminin, and nidogen, as well as native type I collagen. MMP14 (MT-MMP) acts as a collagenase, and can cleave native type I, II, and III collagens (Luo *et al.* 2004, Holmbeck *et al.* 2005). All the above-mentioned MMPs can be expressed by osteoblasts, and play important roles in bone resorption. In the present study, silencing *IRS1* decreased the expression of MMP2, MMP3, MMP13, and MMP14, which may lead to a reduction in bone resorption.

Key factors in bone remodeling include RANK ligand (RANK), which is an osteoclast differentiation factor and the ligand of TNFRSF11B (Theill *et al.* 2002) and stimulates bone resorption, and TNFRSF11B inhibits bone resorption. The ratio between these two factors regulates osteoclast formation and activity (Murthy *et al.* 2009). The expression of RANKL was decreased in the *IRS1*^{-/-} osteoblasts, which suggested that IRS1 is one of the molecules upstream of the RANKL/TNFRSF11B pathway (Ogata *et al.* 2000). In this *in vitro* study, both RANKL and TNFRSF11B were found to be decreased in cells transfected with IRS1 shRNA. Normally, TNFRSF11B plays its role by binding to RANKL and preventing it from binding to RANK (Boyce & Xing 2008), the TNFRSF11B and RANKL were down-regulated simultaneously, which may be one of the reasons that led to the decrease in bone turnover in the IRS1 knockout mice. However, bone remodeling appears to be mainly controlled by the balance of RANKL/TNFRSF11B, and it is now thought that the final step in the osteoclast regulatory pathway may be determined by the relative ratio of the RANKL/TNFRSF11B system (Boyce & Xing 2008), so we analyzed the ratio of the RANKL/TNFRSF11B after IRS1 silencing in MC3T3-E1 cells and found that *IRS1* gene silencing increased the RANKL/TNFRSF11B ratio, which can partly clarify why mice lacking the *IRS1* gene showed severe osteopenia in Ogata's research.

In conclusion, we found that the downregulation of IRS1 reduced cell survival and proliferation; decreased the ALP activity; reduced the expression levels of osteocalcin,

type I collagen, and ratio of TNFRSF11B to RANKL; and suppressed the synthesis of MMP2, MMP3, MMP13, and MMP14 in murine preosteoblastic MC3T3-E1 cells. Therefore, IRS1 not only promotes bone formation but also plays an important role in bone resorption by regulating MMPs, which maintains the normal bone turnover and remodeling.

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 grants from the National Natural Science Foundation of China (No. 30872617 and No. 30400218).

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Received in final form 23 May 2010

Accepted 4 June 2010

Made available online as an Accepted Preprint
4 June 2010