

# Insulin receptor substrate-2 maintains predominance of anabolic function over catabolic function of osteoblasts

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Insulin receptor substrates (IRS-1 and IRS-2) are essential for intracellular signaling by insulin and insulin-like growth factor-I (IGF-I), anabolic regulators of bone metabolism. Although mice lacking the *IRS-2* gene (*IRS-2*<sup>-/-</sup> mice) developed normally, they exhibited osteopenia with decreased bone formation and increased bone resorption. Cultured *IRS-2*<sup>-/-</sup> osteoblasts showed reduced differentiation and matrix synthesis compared with wild-type osteoblasts. However, they showed increased receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) expression and osteoclastogenesis in the coculture with bone marrow cells, which were restored by reintroduction of IRS-2 using an adenovirus vector. Although IRS-2 was expressed and

phosphorylated by insulin and IGF-I in both osteoblasts and osteoclastic cells, cultures in the absence of osteoblasts revealed that intrinsic IRS-2 signaling in osteoclastic cells was not important for their differentiation, function, or survival. It is concluded that IRS-2 deficiency in osteoblasts causes osteopenia through impaired anabolic function and enhanced supporting ability of osteoclastogenesis. We propose that IRS-2 is needed to maintain the predominance of bone formation over bone resorption, whereas IRS-1 maintains bone turnover, as we previously reported; the integration of these two signalings causes a potent bone anabolic action by insulin and IGF-I.

## Introduction

Bone is continually being remodelled according to physiological circumstances through bone formation by osteoblasts and resorption by osteoclasts. These two processes are closely coupled, and bone mass is maintained without change by their balance in healthy adults. A rupture of the balance causing a predominance of bone resorption over bone for-

mation results in bone loss such as that seen in osteoporosis. Among many systemic and local factors involved in the regulation of bone metabolism, insulin and insulin-like growth factor-I (IGF-I)\* are known to play important anabolic roles (Canalis, 1993; Thomas et al., 1997). Patients with insulin deficiency as exemplified by type 1 diabetes are associated with osteoporosis (Krakauer et al., 1997; Piepkorn et al., 1997); those with Laron syndrome caused by IGF-I deficiency also exhibit this condition (Laron et al., 1999). A reduction in IGF-I is also implicated as an important factor in the etiology of involutional osteoporosis, especially of age-related bone loss (Nicolas et al., 1994; Rosen, 1994; Reed et al., 1995; Canalis, 1997). However, the cellular and molecular mechanism underlying bone loss due to the deficiency of insulin and IGF-I signalings has not yet been clarified.

Insulin and IGF-I initiate cellular responses by binding to distinct cell surface receptor tyrosine kinases that regulate a variety of signaling pathways controlling metabolism, growth, and survival. Insulin receptor substrates (IRSs) are essential substrates of both of the receptor tyrosine kinases, which integrate the pleiotropic effects of insulin, IGF-I, and other cytokines on cellular function (Kadowaki et al., 1996;

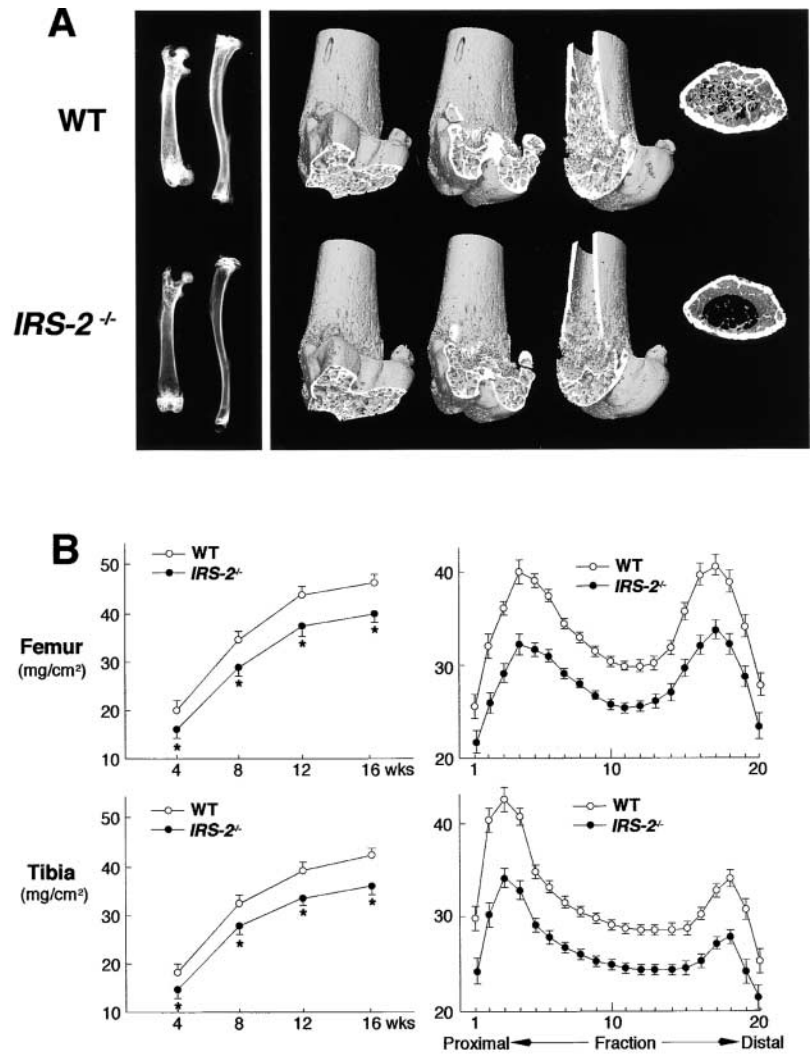
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\*Abbreviations used in this paper: ALP, alkaline phosphatase; BMD, bone mineral density; BMP-2, bone morphogenetic protein 2; Id-1, inhibitor of differentiation-1; IGF-I, insulin-like growth factor-I; IL, interleukin; IRS, insulin receptor substrate; Lrp5, LDL receptor-related protein 5; M-BMM $\phi$ , M-CSF-dependent bone marrow macrophage; M-CSF, macrophage colony-stimulating factor; MMP-13, matrix metalloproteinase-13; MOI, multiplicity of infection; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PI3, phosphatidylinositol 3; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; TRAP, tartrate-resistant acid phosphatase; WT, wild type.

Key words: insulin-like growth factor-I; osteoclast; osteoporosis; diabetes mellitus; cytokine

Figure 1. *IRS-2<sup>-/-</sup>* mice showed osteopenia both in trabecular and cortex bones. (A) Plain X-ray images of femora and tibiae (left) and three-dimensional CT images of distal femora (right) of representative WT and *IRS-2<sup>-/-</sup>* littermates (8 wk old). (B) BMD of the femora and tibiae of WT and *IRS-2<sup>-/-</sup>* littermates. (B, left) BMD of the whole femora and tibiae at 4, 8, 12, and 16 wk of age. \*, significantly different from WT mice;  $P < 0.05$ . (B, right) BMD of each of 20 equal longitudinal divisions of the femora and tibiae of 8-wk-old mice. Data are expressed as means (symbols)  $\pm$  SEMs (error bars) for 12 bones/group for WT and *IRS-2<sup>-/-</sup>* mice.



White, 1998; Burks and White, 2001). The mammalian IRS family contains at least four members, ubiquitous IRS-1 and IRS-2, adipose tissue-predominant IRS-3 (Lavan et al., 1997b), and IRS-4, which is expressed in thymus, brain, and kidney (Lavan et al., 1997a). In bone cells, IRS-1 and IRS-2 are expressed, and we previously reported that IRS-1 in osteoblasts is indispensable for maintaining bone turnover because *IRS-1<sup>-/-</sup>* mice showed osteopenia with a low bone turnover in which both bone formation and resorption were decreased (Ogata et al., 2000).

IRS-1 and IRS-2 signalings are reported to have distinct biological roles and to be differentially expressed in a variety of cells. Regarding glucose homeostasis, IRS-1 plays an important role in the metabolic actions of insulin and IGF-I mainly in skeletal muscle and adipose tissue, whereas IRS-2 does so in the liver (Yamauchi et al., 1996; Bruning et al., 1997). Most hemopoietic cells express IRS-2 but not IRS-1 (Sun et al., 1997), and in bone cells as well, IRS-1 expression was limited to osteoblastic cells, whereas IRS-2 was expressed in both osteoblastic and osteoclastic cells (Ogata et al., 2000). In addition, IRS-2 is known as a signaling molecule of interleukin (IL)-4 and IL-13, cytokines with inhibitory effects on osteoclastogenesis (Wang et al., 1998; Scopes et al., 2001; Wurster et al., 2002). Hence, there is a possibil-

ity that IRS-2 has a different function in the regulation of bone metabolism from that of IRS-1, especially in the catabolic phase by osteoclastic cells.

This study analyzed the bones of mice lacking the *IRS-2* gene (*IRS-2<sup>-/-</sup>* mice), which we generated by homologous recombination (Kubota et al., 2000). *IRS-2<sup>-/-</sup>* mice were normal in appearance with a body size similar to wild-type (WT) mice and showed no abnormalities in major organs such as brain, heart, liver, spleen, or kidney, except that their general adiposity was slightly increased and females were infertile (Withers et al., 1998; Burks et al., 2000; Kubota et al., 2000; Tobe et al., 2001). These phenotypes were also different from *IRS-1<sup>-/-</sup>* mice whose growth was significantly retarded and in which group both males and females were fertile (Araki et al., 1994; Tamemoto et al., 1994). Furthermore, *IRS-2<sup>-/-</sup>* mice displayed type 2 diabetes with peripheral insulin resistance and pancreatic  $\beta$  cell dysfunction characterized by reduction in  $\beta$  cell mass, whereas *IRS-1<sup>-/-</sup>* mice did not develop diabetes due to an enhanced  $\beta$  cell mass that compensated for the peripheral insulin resistance.

In this study, we first found osteopenia in *IRS-2<sup>-/-</sup>* mice, and sought to determine the cellular and molecular mechanism underlying the abnormality of bone metabolism using in vivo morphological analyses and in vitro cell culture systems.

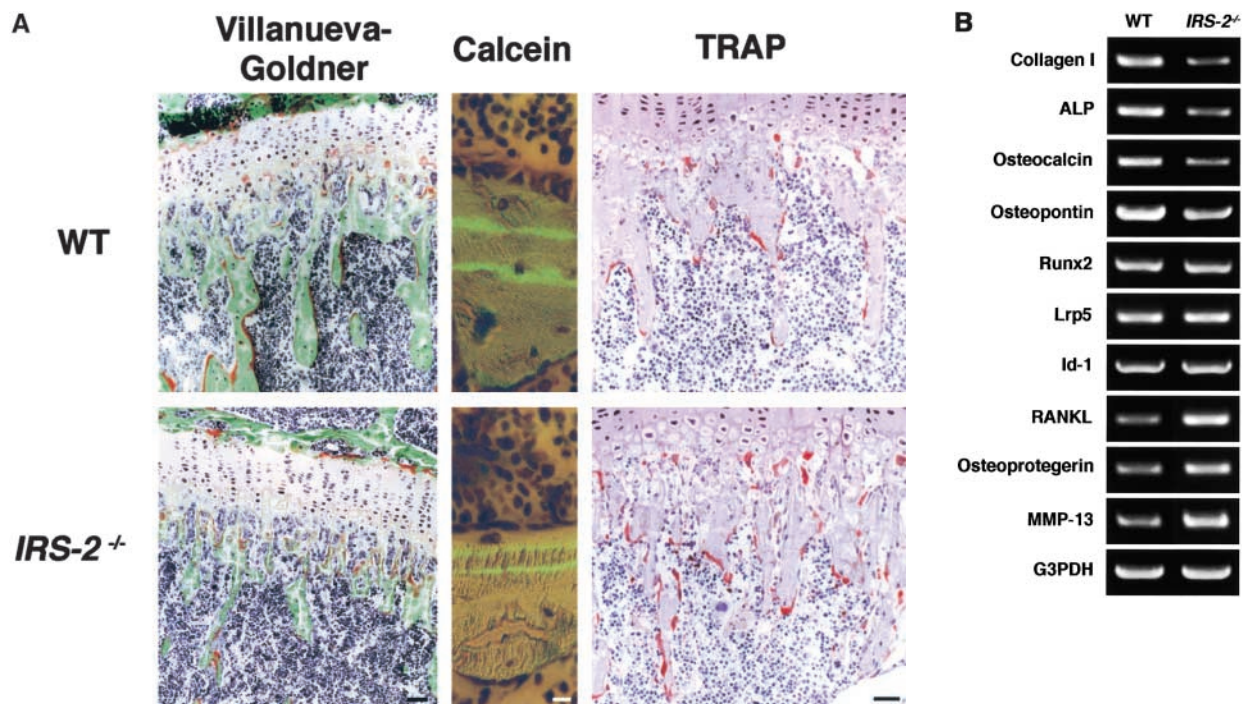


Figure 2. *IRS-2*<sup>-/-</sup> bone showed an uncoupling of decreased bone formation and increased bone resorption in vivo. (A) Histological features at proximal tibiae of WT and *IRS-2*<sup>-/-</sup> littermates (8 wk old). In Villanueva-Goldner staining (left), mineralized bone is stained green and unmineralized osteoid red. Bar, 50  $\mu$ m. In calcein double labeling (middle), mineralization front is stained as a green line. Bar, 10  $\mu$ m. In TRAP staining, TRAP-positive osteoclasts are stained red. Bar, 50  $\mu$ m. Data of histomorphometric parameters are shown in Table I. (B) Expression of matrix proteins (type I collagen, ALP, osteocalcin, and osteopontin) and key molecules for differentiations of osteoblasts (Runx2, Lrp5, and Id-1) and osteoclasts (RANKL, osteoprotegerin, and MMP-13) in long bones of WT and *IRS-2*<sup>-/-</sup> littermates (5 wk old) by semiquantitative RT-PCR.

## Results

### *IRS-2*<sup>-/-</sup> mice showed osteopenia with an uncoupling of decreased bone formation and increased bone resorption

Because the lengths of femora and tibiae did not differ between WT and *IRS-2*<sup>-/-</sup> littermates at 8 wk of age (Fig. 1 A), *IRS-2* was suggested not to be involved in the regulation of skeletal growth. However, *IRS-2*<sup>-/-</sup> mice showed osteopenia in these long bones by X-ray and three-dimensional CT analyses (Fig. 1 A). This was also found in vertebral bodies (unpublished data). Bone mineral density (BMD) of these bones was decreased by  $\sim$ 10–15% in *IRS-2*<sup>-/-</sup> mice at 4, 8, 12, and 16 wk of age compared with their WT littermates (Fig. 1 B, left). To determine the distribution of BMD of long bones at 8 wk, the femora and tibiae were analyzed by dividing them longitudinally into 20 equal regions (Fig. 1 B, right). BMD of each fraction was similarly decreased in all fractions of *IRS-2*<sup>-/-</sup> bones, suggesting that both trabecular and cortex bones were similarly affected by *IRS-2* deficiency.

Histological features of the proximal tibiae of 8-wk-old *IRS-2*<sup>-/-</sup> mice displayed a decrease in the trabecular bone volume (Fig. 2 A, left). Calcein double labeling showed that the width between the two stained labels was narrower in *IRS-2*<sup>-/-</sup> (Fig. 2 A, middle); however, the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts was higher in *IRS-2*<sup>-/-</sup> than in WT littermates (Fig. 2 A, right). Histomorphometric measurements revealed that bone volume (BV/TV) was  $\sim$ 30% decreased, and parameters for bone resorption (Oc.N/B.Pm, Oc.S/BS, and ES/BS) were 70–80% in-

creased in *IRS-2*<sup>-/-</sup> compared with WT littermates (Table I). Regarding bone formation parameters, osteoblast number (Ob.S/BS) was slightly, although not significantly, increased in *IRS-2*<sup>-/-</sup> bones. However, mineral apposition rate (MAR), reflecting the ability of an individual osteoblast to form bone, was  $\sim$ 50% decreased, and, consequently, bone formation rate (BFR/BS) was significantly lower in *IRS-2*<sup>-/-</sup> compared with WT littermates. Taking these in vivo observations together, *IRS-2*<sup>-/-</sup> mice exhibited osteopenia with increased osteoclastic bone resorption and decreased osteoblastic bone formation, suggesting that the two actions were not coupled. The thickness of the growth plate at the proximal tibiae was not different between *IRS-2*<sup>-/-</sup> and WT littermates ( $70.8 \pm 8.9$  and  $76.9 \pm 10.8$   $\mu$ m, mean  $\pm$  SEM of eight mice each, respectively), indicating that *IRS-2* signaling is not important for chondrocytes.

To further investigate the regulation of expression of genes related to bone metabolism by *IRS-2* deficiency, we compared mRNA levels of matrix proteins and key molecules for osteoblast and osteoclast differentiations in long bones between *IRS-2*<sup>-/-</sup> and WT littermates (Fig. 2 B). Expression of matrix proteins such as type I collagen, alkaline phosphatase (ALP), osteocalcin, and osteopontin was decreased in *IRS-2*<sup>-/-</sup> bones, which was consistent with the histomorphometric data showing reduced bone formation. However, expression of the putative central determinants of major pathways for osteoblast differentiation, i.e., Runx2 (Karsenty, 2001), LDL receptor-related protein 5 (Lrp5) (Patel and Karsenty, 2002), and inhibitor of differentiation-1 (Id-1) (Ogata et al., 1993), was not different between

Table 1. **Histomorphometry of trabecular and cortex bones of tibiae**

	Trabecular							Cortex
	BV/TV	Ob.S/BS	MAR	BFR	Oc.N/B.Pm	Oc.S/BS	ES/BS	C.Th
	%	%	$\mu\text{m}/\text{d}$	$\text{mm}^3/\text{cm}^2/\text{y}$	/100 mm	%	%	$\times 10^{-1} \text{ mm}$
WT	9.86 $\pm$ 0.74	8.93 $\pm$ 1.83	2.03 $\pm$ 0.25	4.06 $\pm$ 0.44	136.19 $\pm$ 19.26	4.92 $\pm$ 0.68	5.22 $\pm$ 0.89	2.59 $\pm$ 0.13
<i>IRS-2</i> <sup>-/-</sup>	6.95 $\pm$ 0.63 <sup>b</sup>	13.71 $\pm$ 2.51	1.08 $\pm$ 0.16 <sup>b</sup>	2.71 $\pm$ 0.38 <sup>a</sup>	244.27 $\pm$ 22.96 <sup>b</sup>	8.71 $\pm$ 1.03 <sup>b</sup>	9.14 $\pm$ 0.91 <sup>b</sup>	2.18 $\pm$ 0.12 <sup>a</sup>

Parameters for the trabecular bone were measured in an area 1.2 mm in length from 0.5 mm below the growth plate at the proximal metaphysis of the tibiae in Villanueva-Goldner and calcein double-labeled sections. Parameters for the cortex bone were measured at the midpoint of the tibiae. Data are expressed as means  $\pm$  SEMs for eight bones/group for WT and *IRS-2*<sup>-/-</sup> mice. BV/TV, trabecular bone volume expressed as a percentage of total tissue volume; Ob.S/BS, percentage of bone surface covered by cuboidal osteoblasts; MAR, mineral apposition rate; BFR, bone formation rate expressed by MAR  $\times$  percentage of bone surface exhibiting double labels plus one half single labels; Oc.N/B.Pm, number of mature osteoclasts in 10 cm of bone perimeter; Oc.S/BS, percentage of bone surface covered by mature osteoclasts; ES/BS, percentage of eroded surface; C.Th, cortex thickness.

<sup>a</sup>Significantly different from WT mice;  $P < 0.05$ .

<sup>b</sup>Significantly different from WT mice;  $P < 0.01$ .

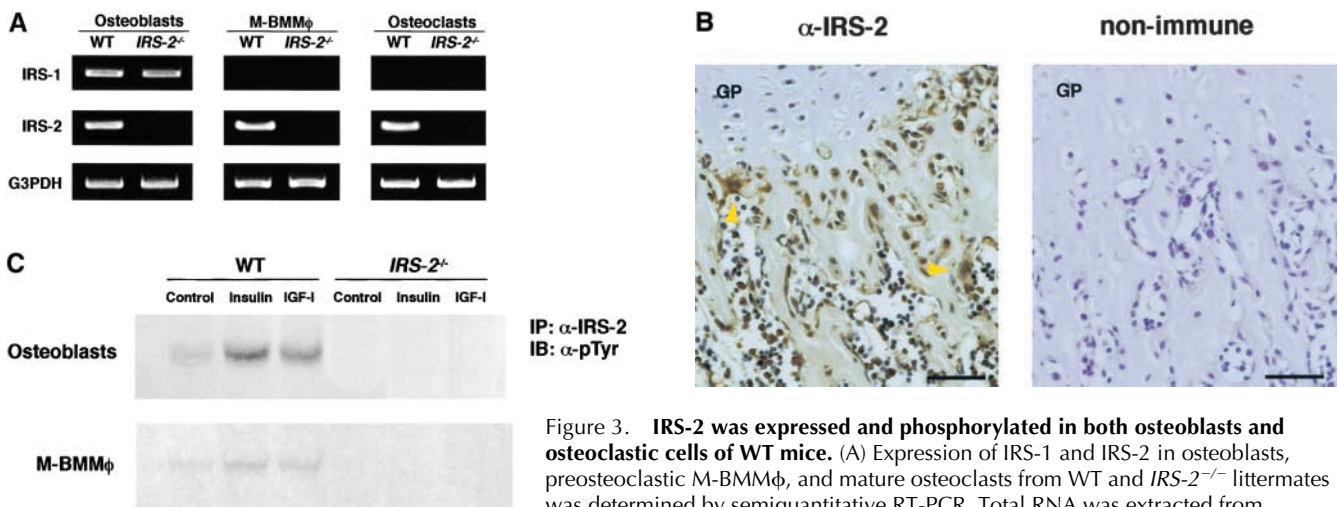
WT and *IRS-2*<sup>-/-</sup> bones, implying an independent signaling pathway of IRS-2 in osteoblasts. Positive regulatory molecules of bone resorption, such as receptor activator of nuclear factor  $\kappa\text{B}$  ligand (RANKL) (Suda et al., 1999) and matrix metalloproteinase-13 (MMP-13) (Uchida et al., 2000), were increased in *IRS-2*<sup>-/-</sup> bones, which was also consistent with the histomorphometric data showing enhanced bone resorption. The stimulation of expression of osteoprotegerin, a decoy receptor of RANKL and a negative regulator of osteoclastic bone resorption, observed in *IRS-2*<sup>-/-</sup> bones might possibly be due to a compensative response to enhanced bone resorption, as is reported in postmenopausal women (Yano et al., 1999).

The serum insulin level was significantly higher in *IRS-2*<sup>-/-</sup> mice than in WT littermates both before (0.91  $\pm$  0.08 vs. 0.25  $\pm$  0.06 ng/ml at 8 wk and 1.23  $\pm$  0.16 vs. 0.44  $\pm$  0.07 ng/ml at 16 wk, mean  $\pm$  SEM of five mice each) and after (1.64  $\pm$  0.09 vs. 0.54  $\pm$  0.04 ng/ml at 8 wk and 2.33  $\pm$  0.39 vs. 0.67  $\pm$  0.12 ng/ml at 16 wk) glucose load, as re-

ported previously (Kubota et al., 2000). Serum IGF-I level was similar between *IRS-2*<sup>-/-</sup> and WT littermates (134.21  $\pm$  11.42 vs. 132.14  $\pm$  10.65 ng/ml, respectively) at 8 wk, with no significant change at 16 wk in either type of mice.

### IRS-2 was expressed and phosphorylated in both osteoblasts and osteoclastic cells of WT mice

Because IRS-1 and IRS-2 are reported to be differentially expressed in a variety of cells and tissues having different biological roles, we compared their expression patterns in primary calvarial osteoblasts. Macrophage colony-stimulating factor (M-CSF)-dependent bone marrow macrophages (M-BMM $\phi$ ) as osteoclast precursors and mature osteoclasts isolated from the coculture of osteoblasts and bone marrow cells were taken from WT and *IRS-2*<sup>-/-</sup> littermates. Both IRS-1 and IRS-2 were expressed in WT osteoblasts, whereas in *IRS-2*<sup>-/-</sup> osteoblasts, only IRS-1 was expressed with no compensatory increase for IRS-2 deficiency (Fig. 3 A). Re-



**Figure 3. IRS-2 was expressed and phosphorylated in both osteoblasts and osteoclastic cells of WT mice.** (A) Expression of IRS-1 and IRS-2 in osteoblasts, preosteoclastic M-BMM $\phi$ , and mature osteoclasts from WT and *IRS-2*<sup>-/-</sup> littermates was determined by semiquantitative RT-PCR. Total RNA was extracted from cultured osteoblasts from neonatal calvariae, M-BMM $\phi$  in the presence of soluble

RANKL and M-CSF, osteoclasts formed in the coculture of marrow cells, and osteoblasts. IRS-1 expression was not seen in osteoclastic cells even when the amount of template cDNA or the number of amplification cycles was increased. (B) Immunohistochemical stainings with an anti-IRS-2 antibody ( $\alpha$ -IRS-2, left) and a nonimmune serum as a control (right) in the proximal tibial metaphysis of 4-wk-old WT mice. Positive and specific stainings shown in brown are seen in both osteoblasts on the bone surface and osteoclasts (arrowheads). GP, growth plate. Bar, 50  $\mu\text{m}$ . (C) Phosphorylation of IRS-2 in osteoblasts and M-BMM $\phi$  from WT and *IRS-2*<sup>-/-</sup> mice in the control culture and cultures stimulated by insulin or IGF-I. Osteoblasts and M-BMM $\phi$  from WT and *IRS-2*<sup>-/-</sup> littermates were cultured with and without insulin or IGF-I for 2 min, and extracted cellular proteins were immunoprecipitated with an anti-IRS-2 antibody ( $\alpha$ -IRS-2). After being subjected to SDS-PAGE, they were immunoblotted with an antiphosphotyrosine antibody ( $\alpha$ -pTyr).

garding cells of osteoclastic lineage, IRS-1 expression was not detected either in M-BMM $\phi$  or mature osteoclasts, and only IRS-2 was expressed in both of these cells from WT mice (Fig. 3 A).

Immunohistochemical analysis confirmed the expression pattern of IRS-2 in the proximal tibial metaphysis of 4-wk-old WT mice (Fig. 3 B). It was localized in both osteoblasts and osteoclasts on the bone surface. This staining was specific to IRS-2 because neither of these cells was stained with a nonimmune serum.

Fig. 3 C shows the immunoblotting with an antiphosphotyrosine antibody for the extracts immunoprecipitated with an anti-IRS-2 antibody in cultured osteoblasts and M-BMM $\phi$ . IRS-2 protein was confirmed to be expressed in both cells from WT mice, but not in those from *IRS-2*<sup>-/-</sup> mice. This protein was phosphorylated by insulin and IGF-I potently in osteoblasts whereas slightly in M-BMM $\phi$ .

### Differentiation and anabolic function of osteoblasts were impaired by IRS-2 deficiency

To learn the cellular mechanism underlying the abnormalities in the bone of *IRS-2*<sup>-/-</sup> mice, we first compared the proliferation, differentiation, and matrix synthesis of cultured osteoblasts from WT and *IRS-2*<sup>-/-</sup> calvariae. Cell proliferation determined by [<sup>3</sup>H]thymidine uptake was similarly seen between WT and *IRS-2*<sup>-/-</sup> cultures (Fig. 4 A); however, cell differentiation determined by ALP activity was significantly decreased in *IRS-2*<sup>-/-</sup> not only in the control culture, but also in the culture with insulin or IGF-I (Fig. 4 B). Neither potent stimulation of cell proliferation by FGF-2 nor that of cell differentiation by bone morphogenetic protein 2 (BMP-2) was decreased by IRS-2 deficiency. Alizarin red S staining also showed decreased calcified matrix synthesis by *IRS-2*<sup>-/-</sup> osteoblasts in cultures with and without insulin/IGF-I (Fig. 4 C). Here again, similar potent stimulation by BMP-2 was seen in WT and *IRS-2*<sup>-/-</sup> osteoblast cultures, suggesting that IRS-2 and BMP-2 systems are independent in osteoblasts. These results indicate that osteoblast differentiation and anabolic function, but not its proliferation, were impaired by IRS-2 deficiency.

We further compared the mRNA levels of Runx2, Lrp5, and Id-1, in addition to the matrix proteins type I collagen, ALP, osteocalcin, and osteopontin in cultured osteoblasts (Fig. 4 D). The results were similar to those seen in vivo: matrix proteins were decreased, but none of Runx2, Lrp5, and Id-1 was affected by IRS-2 deficiency. This again indicates that IRS-2 does not directly impact these major pathways for osteoblast differentiation.

### Osteoclastogenesis and RANKL expression in osteoblasts were enhanced by IRS-2 deficiency

Osteoclasts are known to be derived from hemopoietic cells and to require the cell-cell interaction with osteoblasts/stromal cells for differentiation. To investigate osteoclast formation, we measured the number of TRAP-positive multinucleated osteoclasts formed in the coculture system of osteoblasts and bone marrow cells. Osteoclastogenesis in the coculture induced by the bone resorptive factors prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), IL-11, and 1,25(OH)<sub>2</sub>D<sub>3</sub> was more in-

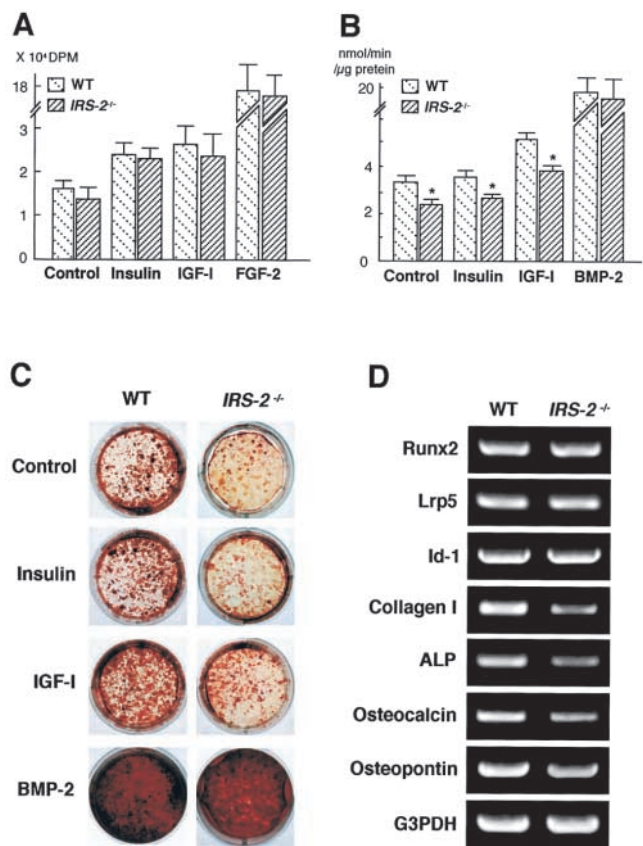
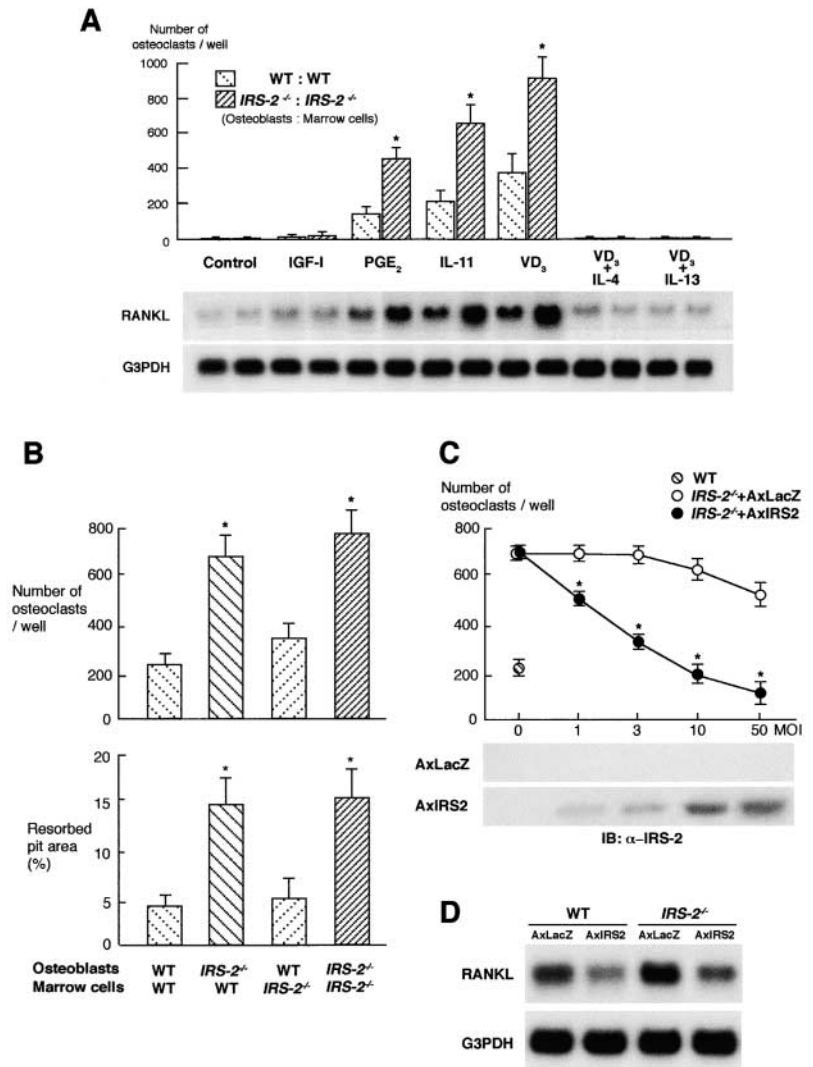


Figure 4. **Osteoblast differentiation and matrix synthesis, but not proliferation, were impaired by IRS-2 deficiency.** (A) Proliferation determined by [<sup>3</sup>H]TdR incorporation into osteoblasts from WT and *IRS-2*<sup>-/-</sup> calvariae cultured with and without insulin, IGF-I, or FGF-2 for 24 h. (B) ALP activity of osteoblasts from WT or *IRS-2*<sup>-/-</sup> calvariae cultured with and without insulin, IGF-I, or BMP-2 for 14 d. For A and B, data are expressed as means (bars)  $\pm$  SEMs (error bars) for eight wells/group. \*, significant decrease by IRS-2 deficiency;  $P < 0.05$ . (C) Calcified matrix synthesis determined by Alizarin red S staining of osteoblasts from WT or *IRS-2*<sup>-/-</sup> calvariae cultured with and without insulin, IGF-I, or BMP-2 for 21 d. (D) Expression of Runx2, Lrp5, Id-1, type I collagen, ALP, osteocalcin, and osteopontin in cultured osteoblasts by semiquantitative RT-PCR.

creased when both cells were derived from *IRS-2*<sup>-/-</sup> mice than when both cells were derived from WT mice (Fig. 5 A, top). IGF-I slightly, but not significantly, induced osteoclastogenesis in both WT and *IRS-2*<sup>-/-</sup> cocultures. IL-4 and IL-13 inhibited 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced osteoclastogenesis in the WT coculture. Although IRS-2 is known as a signaling molecule of IL-4 and IL-13, neither of the inhibitory actions was different between WT and *IRS-2*<sup>-/-</sup> cocultures. These results indicate that IRS-2 signaling acts inhibitorily on the induction of osteoclastogenesis by PGE<sub>2</sub>, IL-11, and 1,25(OH)<sub>2</sub>D<sub>3</sub>, but is not related to its inhibition by IL-4 or IL-13. The results were reproducible when spleen cells were used instead of bone marrow cells as a source of hemopoietic cells (unpublished data). Because RANKL is known to be a key membrane-associated molecule expressed on osteoblasts/stromal cells inducing osteoclastogenesis (Suda et al., 1999), we examined the regulation of RANKL expression in osteoblasts from WT and *IRS-2*<sup>-/-</sup> mice by these factors (Fig. 5 A, bottom). The regulation of RANKL expression showed

**Figure 5. Osteoclastogenesis was increased through the upregulation of RANKL expression in osteoblasts by IRS-2 deficiency.**

(A) The number of TRAP-positive multinucleated osteoclasts formed in the coculture of osteoblasts and bone marrow cells from WT or *IRS-2*<sup>-/-</sup> littermates was counted after 6 d of culture with and without IGF-I, PGE<sub>2</sub>, IL-11, 1,25(OH)<sub>2</sub>D<sub>3</sub> (VD<sub>3</sub>), IL-4, or IL-13. Data are expressed as means (bars) ± SEMs (error bars) for eight wells/group. \*, significantly different from WT cultures; P < 0.01 (top). Messenger RNA levels of RANKL in cultured WT and *IRS-2*<sup>-/-</sup> osteoblasts were determined by Northern blot analysis after 24 h of culture with and without the factors above (bottom). (B) The number of osteoclasts formed in the coculture of osteoblasts and bone marrow cells from WT and *IRS-2*<sup>-/-</sup> littermates in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> (10 nM) (top) and the pit area resorbed by osteoclasts for an additional 48 h of the coculture on a dentine slice (bottom). Data are expressed as means (bars) ± SEMs (error bars) for 12 wells/group. \*, significantly different from WT: WT culture; P < 0.01. (C) Restoration of osteoclastogenesis by reintroduction of IRS-2 in *IRS-2*<sup>-/-</sup> osteoblasts using an adenovirus vector carrying *IRS-2* gene (AxIRS2). *IRS-2*<sup>-/-</sup> osteoblasts infected with AxIRS2 or the control AxLacZ at indicated MOIs were cocultured with WT marrow cells in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> for 6 d, and the number of TRAP-positive osteoclasts was counted (top). Data are expressed as means (symbols) ± SEMs (error bars) for eight wells/group. \*, significantly different from AxLacZ-infected cultures; P < 0.01. IRS-2 protein level in *IRS-2*<sup>-/-</sup> cultured osteoblasts was determined by Western blotting after 2 d of infection of AxLacZ or AxIRS2 (bottom). (D) RANKL expression by introduction of IRS-2 in WT and *IRS-2*<sup>-/-</sup> osteoblasts. Osteoblasts infected with AxIRS2 or AxLacZ at 10 MOI were cultured in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> for 24 h. RANKL mRNA level was determined by Northern blotting.



good correlation with that of osteoclastogenesis: RANKL induction by the resorptive factors was increased in *IRS-2*<sup>-/-</sup> osteoblasts compared with WT osteoblasts, whereas the inhibition by IL-4 and IL-13 was identical in both osteoblasts.

In the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub>, enhanced osteoclastogenesis in the coculture of *IRS-2*<sup>-/-</sup> osteoblasts and *IRS-2*<sup>-/-</sup> marrow cells (*IRS-2*<sup>-/-</sup>:*IRS-2*<sup>-/-</sup>) was normalized by replacing *IRS-2*<sup>-/-</sup> osteoblasts with WT osteoblasts (WT:*IRS-2*<sup>-/-</sup>), but not by replacing *IRS-2*<sup>-/-</sup> marrow cells with WT marrow cells (*IRS-2*<sup>-/-</sup>:WT) (Fig. 5 B, top). When the coculture was further performed on a dentine slice for 2 d, the area of resorption pits was correlated with the number of osteoclasts formed, indicating no difference of bone resorptive function between individual WT and *IRS-2*<sup>-/-</sup> osteoclasts (Fig. 5 B, bottom). These results indicate that the increase in bone resorption in *IRS-2*<sup>-/-</sup> mice is caused by the abnormality of osteoblasts to support osteoclastogenesis by inducing RANKL, but is not due to abnormalities of osteoclast progenitors or osteoclast function.

To further investigate the association between IRS-2 deficiency and the upregulation of osteoclastogenesis/RANKL expression, we examined their restoration by reintroduction of IRS-2 in *IRS-2*<sup>-/-</sup> osteoblasts using an adenovirus vector.

When *IRS-2*<sup>-/-</sup> osteoblasts infected with an adenovirus vector carrying the *IRS-2* gene (AxIRS2) were cocultured with WT marrow cells in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub>, osteoclastogenesis was decreased in a dose-dependent manner based on the AxIRS2 multiplicity of infection (MOI) and on the IRS-2 protein level determined by Western blotting, whereas the control adenovirus vector carrying the *LacZ* gene (AxLacZ) did not affect it (Fig. 5 C). Enhanced RANKL expression in cultured *IRS-2*<sup>-/-</sup> osteoblasts determined by Northern blotting was also decreased by AxIRS2 infection, but not by AxLacZ (Fig. 5 D). These results were reproducible in the stimulation of other bone resorptive factors, PGE<sub>2</sub> and IL-11 (unpublished data). It was therefore confirmed that the IRS-2 signaling pathway acts inhibitorily on enhanced osteoclastogenesis and RANKL expression in osteoblasts by these resorptive factors.

To confirm the role of IRS-2 intrinsic to osteoclastic cells, we investigated the differentiation and survival of osteoclasts using the M-BMM $\phi$  culture system without supporting cells such as osteoblasts/stromal cells. Osteoclastogenesis from cultured M-BMM $\phi$  in the presence of soluble RANKL for 3 d was similar between WT and *IRS-2*<sup>-/-</sup> cultures (674 ± 71 and 641 ± 83 cells/well, mean ± SEM of

12 wells, respectively). In addition, the survival of mature osteoclasts isolated from the M-BMM $\phi$  culture did not differ between WT and *IRS-2*<sup>-/-</sup> osteoclasts (the average half life of 12 wells was 6.4 and 5.7 h, respectively). Hence, it was concluded that intrinsic IRS-2 signaling is not important for osteoclast function.

## Discussion

In vivo morphological analyses in this study demonstrated that *IRS-2*<sup>-/-</sup> mice exhibited osteopenia with decreased bone formation and increased bone resorption. In vitro cell culture analyses revealed that *IRS-2*<sup>-/-</sup> osteoblasts showed reduced ability of their differentiation and matrix synthesis. The increased osteoclastogenesis was due to the upregulation of RANKL in osteoblasts by IRS-2 deficiency. Although IRS-2, but not IRS-1, was expressed and phosphorylated by insulin/IGF-I in osteoclastic cells, intrinsic IRS-2 signaling in these cells was not important for their differentiation, function, or survival.

The present study revealed the difference in the physiological roles of IRS-1 and IRS-2 in bone metabolism. *IRS-2*<sup>-/-</sup> mice exhibited an uncoupling state with decreased bone formation and increased bone resorption, whereas our previous study found that *IRS-1*<sup>-/-</sup> mice displayed a decrease in both bone formation and bone resorption, indicating a low turnover osteopenia (Ogata et al., 2000). Hence, IRS-1 and IRS-2 seem to have opposite effects on osteoclastogenesis and RANKL expression in osteoblasts. The signaling specificity through the two IRSs may be accomplished by distinct phosphorylation patterns during interaction with various activated receptors (Sun et al., 1997). For example, phosphatidylinositol 3 (PI3) kinase activities in muscle and liver cells have functional differences in the ability of the two IRSs (Kadowaki et al., 1996; Burks and White, 2001). In this study, effects of bone resorptive factors, i.e., PGE<sub>2</sub>, IL-11, or 1,25(OH)<sub>2</sub>D<sub>3</sub>, on osteoclastogenesis and RANKL expression were shown to be partly inhibited by IRS-2 signaling, suggesting some convergence of signaling pathways between these factors and IRS-2. Because the main signaling pathways downstream of IRS-2 are known to be those of PI3 kinase and MAPK (Kadowaki et al., 1996; Withers et al., 1998; Burks and White, 2001), either pathway could conceivably be connected with the signaling of these resorptive factors. In fact, signalings of PGE<sub>2</sub> and IL-11 through their specific prostaglandin E and gp130 receptors, respectively, are known to utilize both PI3 kinase and MAPK (Fukada et al., 1999; Breyer et al., 2001). 1,25(OH)<sub>2</sub>D<sub>3</sub> signaling is also reported to be inhibited by MAPK pathway by the phosphorylation of retinoid X receptor  $\alpha$ , the heterodimer of the nuclear vitamin D receptor, in human keratinocytes (Solomon et al., 1999). However, our preliminary study on the mechanism of RANKL induction failed to show the involvement of PI3 kinase, p42/44 MAPK, and p38 MAPK pathways because none of their inhibitors, LY294002, PD98059, or SB203580, altered the upregulation of the RANKL mRNA level by IRS-2 deficiency in cultured osteoblasts (unpublished data). We therefore speculate that signaling(s) other than PI3 kinase and these MAPKs may be involved in the suppression of RANKL expression and osteoclastogenesis by

IRS-2. The next task ahead of us will be to elucidate the difference of signaling through IRS-1 and IRS-2 in osteoblasts.

IGF-I is a potent autocrine/paracrine factor for osteoblast proliferation and differentiation (Canalis, 1993). A relationship between bone density and IGF-I is suggested by a study showing a correlation of serum and skeletal IGF-I levels with bone density between two healthy inbred strains of mice (Rosen et al., 1997). In humans, accumulating evidence has suggested a positive correlation between the serum IGF-I level and bone density in postmenopausal women (Nicolas et al., 1994; Rosen, 1994; Reed et al., 1995; Canalis, 1997). The anabolic effect of insulin on bone formation may be primarily related to its ability to stimulate cell proliferation (Thomas et al., 1997). Streptozotocin-induced diabetic animals with impaired pancreatic insulin production lose bone rapidly, and this loss is offset by insulin replacement (Hough et al., 1981). Clinically, type 1 diabetes with insulin deficiency is associated with decreased bone mass; however, the change of bone mass in type 2 diabetic patients is controversial. Although previous reports demonstrated a decrease in bone mass in these latter patients, accumulating evidence has revealed that obese type 2 diabetic patients have normal or even increased bone mass (Kao et al., 1993; van Daele et al., 1995; Krakauer et al., 1997; Piepkorn et al., 1997). This might be due to the increased physical stress on the skeleton, or hyperinsulinemia seen in the earlier stage of type 2 diabetes. In fact, we previously demonstrated that patients with hyperinsulinemia are associated with the increased ossification of the spinal ligaments (Akune et al., 2001). Whether or not the response of bone cells to insulin is affected similarly to that of cells responsible for glucose metabolism, such as muscle, liver, and adipose tissue, in type 2 diabetic patients is an important point to be elucidated. Although the *IRS-2*<sup>-/-</sup> mice develop type 2 diabetes characterized by defects in both insulin action and insulin secretion, clinical studies on genetic polymorphisms have so far failed to show a pathogenic contribution of IRS-2 to the development of this condition (Sesti et al., 2001; Wang et al., 2001). Hence, we do not regard the *IRS-2*<sup>-/-</sup> mice as a model to define mechanisms of abnormalities of bone metabolism by type 2 diabetes. The mice may speak for themselves as a rare model showing an uncoupling state between bone formation and resorption.

In the histomorphometric analysis, osteoblast function was shown to be decreased by IRS-2 deficiency; however, the number of osteoblasts (Ob.S/BS) was either not affected or was increased (Table I). We speculate that this increase may be secondary to the upregulation of osteoclastic bone resorption through the coupling mechanism between osteoclasts and osteoblasts. In fact, in the osteoblast culture system in which osteoclastic cells were absent, cell proliferation was not different between WT and *IRS-2*<sup>-/-</sup> (Fig. 4 A), whereas cell differentiation and matrix synthesis were decreased in the *IRS-2*<sup>-/-</sup> culture. Hence, IRS-2 in osteoblasts seems to be related to the signaling of their differentiation and function, but not to their mitogenic or anti-apoptotic signaling directly.

The decrease in anabolic function of *IRS-2*<sup>-/-</sup> osteoblasts was seen not only in the culture with insulin or IGF-I but also in the control culture (Fig. 4, B, C, and D). This may possibly be due to the blockage of signals of endogenous

IGF-I acting as an autocrine/paracrine factor in the culture. The concentrations of IGF-I in the culture medium were  $0.39 \pm 0.12$  and  $0.48 \pm 0.10$  nM (mean  $\pm$  SEM) in the control cultures of WT and *IRS-2*<sup>-/-</sup> osteoblasts, respectively. In addition, serum IGF-I levels were similar between WT and *IRS-2*<sup>-/-</sup> mice in the age from 8 to 16 wk, suggesting the absence of systemic compensation for impaired IGF-I bioactivity. Hence, the decreased bone formation in vivo in *IRS-2*<sup>-/-</sup> mice may be at least in part due to the deficit of anabolic signaling of endogenous IGF-I produced by osteoblasts acting as an autocrine/paracrine factor.

Intrinsic *IRS-2* in osteoclastic cells was proven not to be important for their differentiation, function, or survival despite the fact that *IRS-2* and receptors of IGF-I and insulin are expressed in these cells (Fiorelli et al., 1996; Hou et al., 1997; Thomas et al., 1998). Whether or not IGF-I or insulin directly acts on osteoclastic cells is controversial (Mochizuki et al., 1992; Hill et al., 1995), and we, in fact, failed to show the direct action of IGF-I or insulin in the culture of M-BMM $\phi$  in the absence of osteoblasts/stromal cells (unpublished data). Furthermore, *IRS-1* and *IRS-2* are reported to regulate cell apoptosis through phosphorylation of Bcl-2 in a myeloma cell line (Ueno et al., 2000), however, these proteins did not affect osteoclast survival in our previous and present studies (Ogata et al., 2000). Hence, IGF-I/insulin and *IRS* signalings are likely to regulate bone resorption more predominantly by their indirect actions through osteoblasts/stromal cells than by their direct actions on osteoclastic cells.

Clinically, loss of anabolic actions of insulin and IGF-I on bone metabolism is implied to cause various bone metabolic diseases. Taken together with our previous study, we hereby propose that these anabolic actions are maintained as an integration of two proteins that have distinct biological roles in osteoblasts, *IRS-1* and *IRS-2*. The former is essential to maintain bone turnover by upregulating anabolic function and RANKL expression, whereas the latter is needed to retain the predominance of the anabolic function over the catabolic function. Distinct regulation of these two *IRS* proteins in osteoblasts may play a key role in controlling bone metabolism and maintaining bone homeostasis.

## Materials and methods

### Animals

In each experiment, homozygous WT and *IRS-2*<sup>-/-</sup> mice that were littermates generated from the intercross between heterozygous mice were compared. All experiments were performed on male mice unless otherwise described. All experiments were performed according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

### Analysis of skeletal morphology

Bone radiograph was taken with a soft X-ray apparatus (SOFTX; CMB-2). Three-dimensional CT scan was taken using a composite X-ray analyzing system (NX-HCP; NS-ELEX Inc.). BMD was measured by single energy X-ray absorptiometry using a bone mineral analyzer (DCS-600R; Aloka Co.). All histological analyses were performed using 8-wk-old WT and *IRS-2*<sup>-/-</sup> littermates. For Villanueva-Goldner staining, tibiae were fixed with ethanol, embedded in methyl methacrylate, and sectioned in 6- $\mu$ m slices. For double labeling, mice were injected subcutaneously with 8 mg/kg body weight of calcein at 10 and 3 d before sacrifice. TRAP-positive cells were stained at pH 5.0 in the presence of L(+)-tartaric acid using naphthol AS-MX phosphate (Sigma-Aldrich) in *N,N*-dimethyl formamide as the substrate. The specimens were subjected to histomorphometric analyses using an image analyzer (System Supply Co.). Parameters for the trabecular bone

were measured in an area 1.2 mm in length from 0.5 mm below the growth plate at the proximal metaphysis of the tibiae. Parameters for the cortex bone were measured at the midshaft of the tibiae. The thickness of the growth plate was measured at the proximal tibiae.

### Immunohistochemistry

After perfusion of 4-wk-old WT mice with 4% buffered paraformaldehyde, tibiae were removed and immersed in the same fixative for paraffin sections. 3-mm-thick paraffin sections were treated with 0.3% hydrogen peroxide in PBS. After blocking with 1% albumin in PBS, they were incubated with a rabbit polyclonal anti-mouse *IRS-2* antibody (Upstate Biotechnology) at a dilution of 1:50 for 24 h at 4°C. The sections were incubated with HRP-conjugated goat antibodies against rabbit IgG (Dakopatts) for 1 h at room temperature. After washing with PBS, they were immersed in a diaminobenzidine solution for 10 min at room temperature to observe any immunoreactivity. As a control, we used a rabbit nonimmune serum (Upstate Biotechnology) of the same dilution instead of the primary antibody.

### In vitro osteoblast cultures

Osteoblasts were isolated from calvariae of neonatal WT and *IRS-2*<sup>-/-</sup> littermates. Calvariae were digested for 10 min at 37°C in an enzyme solution containing 0.1% collagenase and 0.2% dispase five times. Cells isolated by the last four digestions were combined as an osteoblast population and cultured in  $\alpha$ MEM containing 10% FBS. For cell proliferation assay, primary osteoblasts were inoculated at a density of  $1 \times 10^4$  cells/well in a 24-multiwell plate, cultured in the same medium for 48 h, and deprived of serum for 12 h before adding the experimental medium with and without IGF-I (10 nM), insulin (100 nM), or FGF-2 (1 nM). Incorporation of [<sup>3</sup>H]thymidine (1  $\mu$ Ci/ml in the medium) added for the final 3 h was measured after 24 h of culture. For ALP activity measurement, primary osteoblasts were inoculated at a density of  $1 \times 10^4$  cells/well in a 24-multiwell plate and cultured in  $\alpha$ MEM containing 10% FBS and 50  $\mu$ g/ml ascorbic acid with and without IGF-I (10 nM), insulin (100 nM), or BMP-2 (30 ng/ml). At 14 d of culture, cells were sonicated in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl<sub>2</sub> and 0.5% Triton X-100. ALP activity in the lysate was measured using a Wako ALP kit (Wako Pure Chemical Industry, Ltd.). The protein content was determined using BCA protein assay reagent (Pierce Chemical Co.). For Alizarin red S staining, osteoblasts were inoculated at a density of  $5 \times 10^4$  cells/well in a six-multiwell plate in  $\alpha$ MEM containing 10% FBS and 50  $\mu$ g/ml ascorbic acid and 10 mM  $\beta$ -glycerophosphate. At day 21 after confluency, cultured cells were fixed in 10% buffered formalin and stained for 10 min with 2% Alizarin red S (pH 4.0) (Sigma-Aldrich).

### Coculture of osteoblasts and bone marrow cells

Bone marrow cells were collected from long bones of 8-wk-old WT or *IRS-2*<sup>-/-</sup> littermates. TRAP-positive multinucleated osteoclasts were generated by coculturing osteoblasts ( $1 \times 10^4$  cells/well) and bone marrow cells ( $5 \times 10^5$  cells/well) derived from either WT or *IRS-2*<sup>-/-</sup> littermates in a 24-multiwell plate in  $\alpha$ MEM containing 10% FBS for 6 d with and without IGF-I (10 nM), 1,25(OH)<sub>2</sub>D<sub>3</sub> (10 nM), PGE<sub>2</sub> (100 nM), IL-11 (10 ng/ml), IL-4 (100 ng/ml), or IL-13 (100 ng/ml). Cells positively stained for TRAP containing more than three nuclei were counted as osteoclasts. To determine bone resorption activity, osteoclasts formed by the coculture on 0.24% collagen gel coated on 100-mm dishes were digested with 0.2% collagenase solution, and a 1/50 aliquot including osteoclasts was seeded on a dentine slice. After 48 h of culture in  $\alpha$ MEM containing 10% FBS, the total area of pits stained with 0.5% toluidine blue was evaluated using an image analyzer.

### M-BMM $\phi$ culture

We used the M-BMM $\phi$  culture system as described previously (Kobayashi et al., 2000). In brief, bone marrow cells from WT or *IRS-2*<sup>-/-</sup> mice were seeded at a density of  $3 \times 10^5$  cells/well in a 24-multiwell plate and cultured in  $\alpha$ MEM containing 10% FBS with M-CSF (100 ng/ml). After culturing for 3 d, adherent cells (M-BMM $\phi$ ) were further cultured with M-CSF (100 ng/ml) and soluble RANKL (100 ng/ml) for 3 d. TRAP-positive osteoclasts were counted. To determine the survival, osteoclasts generated as above were deprived of M-CSF/soluble RANKL and cultured for an additional 48 h. At 3, 6, 12, 24, and 48 h, the number of TRAP-positive and trypan blue-negative osteoclasts was counted.

### Generation of adenoviruses and gene transfer

The recombinant adenovirus vector carrying *IRS-2* gene containing CAG (cytomegalovirus immediate early enhancer + chicken actin promoter + rabbit globin poly A signal) promoter was constructed using the Adenovi-



rus Expression Vector Kit (Takara Shuzo Co., Ltd.) following the manufacturer's protocol. The adenovirus vector carrying the  $\beta$ -galactosidase gene (AxLacZ) was provided by Dr. I. Saito (University of Tokyo). *IRS-2*<sup>-/-</sup> osteoblasts were seeded at a density of  $1 \times 10^4$  cells/well in a 24-multiwell plate and treated with viral suspensions for 1 h at 37°C at a concentration indicated as the MOI for 2 d. Infection efficiency was confirmed by  $\beta$ -galactosidase assay using X-gal as a substrate, and Western blot analysis of IRS-2. IRS-2 protein level was also confirmed by Western blotting. For the experiment of osteoclastogenesis, they were then washed three times with PBS, and WT bone marrow cells were added at a density of  $5 \times 10^5$  cells/well with and without 1,25(OH)<sub>2</sub>D<sub>3</sub> (10 nM), PGE<sub>2</sub> (100 nM), or IL-11 (10 ng/ml) and cocultured for 6 d. The number of TRAP-positive osteoclasts was counted. For the experiment of RANKL expression, osteoblasts infected with 10 MOI of AxIRS2 or AxLacZ were further cultured with and without the resorptive factors above for 24 h, and the mRNA level was determined by Northern blotting.

### Immunoprecipitation and Western blot analysis

After stimulation by IGF-I (10 nM) or insulin (100 nM), cultured cells were lysed with TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM aminoethyl-benzenesulfonyl fluoride, and 10  $\mu$ g/ml aprotinin). Immunoprecipitation was performed using antibodies either noncovalently bound or conjugated to protein G-Sepharose (GIBCO BRL). Equivalent amounts (20  $\mu$ g) of cell lysate were immunoprecipitated with a polyclonal anti-mouse IRS-2 antibody (Upstate Biotechnology) for 4 h at 4°C. Each cell lysate or immunoprecipitated protein containing an equivalent amount of protein was electrophoresed by 8% SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 5% albumin solution, they were incubated with an antiphosphotyrosine antibody (Upstate Biotechnology). Immunoreactive bands were stained using the ECL chemiluminescence reaction (Amersham Biosciences).

### Northern blotting and semiquantitative RT-PCR

For Northern blot analysis, osteoblasts were cultured in  $\alpha$ MEM containing 10% FBS with and without IGF-I (10 nM), 1,25(OH)<sub>2</sub>D<sub>3</sub> (10 nM), PGE<sub>2</sub> (100 nM), IL-11 (10 ng/ml), IL-4 (100 ng/ml), or IL-13 (100 ng/ml). Total RNA was extracted using an ISOGEN kit (Wako Pure Chemical). 30  $\mu$ g of total RNA was electrophoresed in 1.2% agarose-formaldehyde gels and transferred onto nylon membrane filters (Hybond-N; Amersham Biosciences). The membranes were hybridized for 12 h at 42°C with cDNA probes for mouse RANKL, which were labeled using a multirandom primer oligonucleotide labeling kit (Boehringer) and [<sup>32</sup>P]dCTP. Semiquantitative RT-PCR was performed within an exponential phase of the amplification. Total mRNA (1  $\mu$ g) was reverse transcribed using Super Script reverse transcriptase (Takara Shuzo Co., Ltd.) with random hexamer (Takara Shuzo Co., Ltd.), and 5% of the reaction mixture was amplified with LA-Taq DNA polymerase (Takara Shuzo Co., Ltd.) using the following primer pairs: 5'-GCAGCCCCACCTGCTCGAAAGGTAGACAC-3' and 5'-CAGCAATGCCTGTCCGCATGTCAGCATAGC-3' for IRS-1; 5'-GAAGACAGTGGTACATGCGAATG-3' and 5'-CCTCATGGAGGAAGGCACTGCTG-3' for IRS-2; 5'-CATGTAGGCCATGAGGTCCACCAC-3' and 5'-TGAAGGTCGGTGTGAACGGATTGGC-3' for G3PDH; 5'-GCACCGCCAGATGGACTGCTGAA-3' and 5'-GGCGGAAAGAGGATGGAGGTGACG-3' for Runx2; 5'-CCTGCTGCCTCCCTGTAA-3' and 5'-CTTACCGCTCACATTTCTC-3' for Lrp5; 5'-ACTGAGG-GACCAGATGGACTCCAG-3' and 5'-GCCAGTGATCATTGTAATATAACA-3' for Id-1; 5'-CTGGGATGGACAAGGAGAGT-3' and 5'-AAGGCTGAGAG-GCTATGGTG-3' for type I collagen; 5'-GCCCTCTCCAAGACATATA-3' and 5'-CCATGATCACGTCGATATCC-3' for ALP; 5'-AGTCCGACATGAGATTG-GCAGTGATTTGC-3' and 5'-ACTCGAGGCCTCTTCTTAGTTGACCTC-3' for osteopontin; 5'-TCGTTCTTTCTGCTGGTCA-3' and 5'-CTTATGTCCTCTGCTTGG-3' for osteocalcin; 5'-GCATCGCTCTGTTCTCTGTA-3' and 5'-GTGCTCCCTCCTTCATCA-3' for RANKL; 5'-TCCTGGCACCTACCTAAAACAGCA-3' and 5'-CTACACTCTCGGCATTCACCTTTGG-3' for osteoprotegerin; and 5'-CATTGACATCTGCTGGCCACCTTC-3' and 5'-CATC-CACATGGTTGGGAAGTTCTG-3' for MMP-13. Up to 25 cycles of amplification were performed with a PerkinElmer PCR thermal cycler (PE-2400) at 94°C for 30 s, at 52–60°C for 60 s, and at 72°C for 90 s.

### Statistical analysis

Means of groups were compared by ANOVA and significance of differences was determined by post-hoc testing using Bonferroni's method.

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