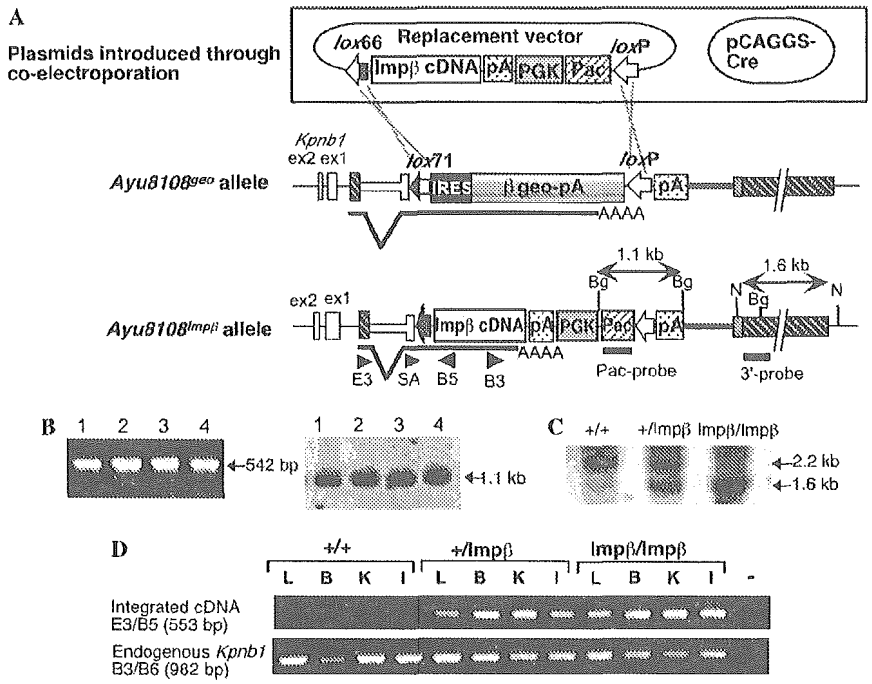


Fig. 5. Production and analysis of the *Ayu8108*$^{Impβ}$ mouse line. (A) Site-specific replacement of the *βgeo* gene with a 2.6-kb Impβ cDNA sequence through Cre-mediated recombination. The replacement vector contains *lox*66-Impβ cDNA-pA-PGK promoter-*pac*-*lox*66 (top). Ayu8108 ES cells were co-electroporated together with the replacement vector and the Cre-expressing vector, pCAGGS-Cre. Through recombination between *lox* sites, the *βgeo* gene was replaced with the Impβ cDNA sequence (bottom). Striped boxes indicate transcribed genomic DNA regions for the 1.3-kb transcript. Replaced clones were selected in the presence of Puromycin (2 μg/ml). The integrated cDNA sequence was expressed as endogenous promoter activity which had driven the *βgeo* gene before replacement. Splicing patterns of transcripts for *βgeo* and integrated cDNA are shown under the maps. Primers used for confirmation of recombination and RT-PCR are indicated by arrowheads. Bg, *Bgl*II; N, *Nco*I. (B) Confirmation of targeted recombination at the *lox* sites. Results of representative 4 clones (1–4) are shown. Left, PCR with SA and B5 primers to detect the 5′-side junction. Right, Southern blot analysis with the Pac-probe and *Bgl*II digestion to detect the 3′-side junction. Bands with expected sizes were detected in both analyses. (C) Genotyping of the *Ayu8108*$^{Impβ}$ mouse line. Tail DNA was digested with *Nco*I and hybridized with a 3′-probe. From the wild and *Ayu8108*$^{Impβ}$ allele, a 2.2-kb band (Fig. 1B) and a 1.6-kb band were detected, respectively. (D) RT-PCR analysis of expression of the inserted or endogenous Impβ transcripts in each genotype. RT-PCR was performed using total RNAs from adult brain (B), kidneys (K), intestines (I), and lungs (L). To detect transcripts from the integrated Impβ cDNA and the endogenous *Kpnb1* gene, E3/B5 primer pair, and B3/B6 primer pair were used, respectively. B6 primer was in the 3′-untranslated region, therefore the B3/B6 primer pair can specifically detect endogenous *Kpnb1* expression. In homozygous *Ayu8108*$^{Impβ/Impβ}$ mice, both inserted Impβ cDNA and endogenous *Kpnb1* gene were expressed. −, template without RT reaction.

probably because of maternal accumulation of Impβ protein. In the case of importinβ-deficient eggs derived from germline chimeras carrying Impβ-deficient female germ cells, embryogenesis did not start at all [13]. Post-implantation lethality in our homozygous mutants for the Ayu8108 allele suggested that maternal Impβ mRNA or protein was present in mouse embryos, and that these might become depleted at the time of implantation.

In the Ayu8108 trap line, the trap vector was inserted in the promoter region of the *Kpnb1* gene in the reverse orientation. Recent genome and expression studies have revealed that many pairs of genes were driven by the same promoter sequence but in reverse direction [21,22], and that there were also non-coding RNA genes of unknown functions [23,24]. We identified a 1.3-kb product as the transcript of the trapped gene in Ayu8108 clones, but no ORF was found, and its expression was under detectable levels using northern blotting. As *Ayu8108*$^{Impβ/Impβ}$ homozygous mice were viable and healthy by expressing Impβ, but not the 1.3-kb transcript, the 1.3-kb transcript might not have a significant function in development. Yet, the 1.3-kb transcript might be a non-coding RNA of unknown function.

pU-hachi was constructed as an exchangeable gene trap vector using the Cre/mutant lox system. In this study, we used this system to insert the Impβ cDNA sequence, and we successfully identified the responsible gene for the early embryonic lethal phenotype. We clearly showed that Impβ was essential for early development in mice. Since there are many overlapping genes, insertion of a targeting vector may often result in disruptions of two genes. Our exchangeable system is expected to be a useful tool for analysis of such overlapping genes.

At present, we do not know why endogenous expression of the *Kpnb1* gene in the *Ayu8108*$^{Impβ}$ allele recovered. We presume that there may be inhibitory element(s) for transcription in the IRES-βgeo sequence. It is known that the IRES sequence is quite GC rich and forms a complex secondary structure [25]. We have also produced the IRES-βgeo deleted allele by mating a Cre-expressing transgenic mouse line [26]. Homozygous mice for the IRES-βgeo

deleted allele were also viable and expressed the endogenous *Kpnb1* gene (data not shown). This supported the hypothesis of the existence of inhibitory elements. However, Impβ expression level from the $Ayu8108^{Impβ}$allele seemed to be lower than that of the wild-type allele, because we could not obtain compound heterozygous mice carrying both the $Ayu8108^{geo}$ and the $Ayu8108^{Impβ}$ alleles. Since we produced several alleles showing different expression levels of the *Kpnb1* gene, further analyses using these alleles would reveal the minimum requirements of Impβ expression level for development.

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# Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast

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# Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast

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**Abstract**

Adiponectin, an adipose-derived hormone, exhibits various biological functions, such as increasing insulin sensitivity, protecting hypertension, and suppression of atherosclerosis, liver fibrosis, and tumor growth. Here, we report the role of adiponectin on bone metabolism. C57BL/6J mice were treated with adenovirus expressing lacZ or adiponectin, and their bones were analyzed by three-dimensional microcomputed tomography. Adiponectin-adenovirus treatment increased trabecular bone mass, accompanied by decreased number of osteoclasts and levels of plasma NTx, a bone-resorption marker. In vitro studies showed that adiponectin inhibited M-CSF- and RANKL-induced differentiation of mouse bone marrow macrophages and human CD14-positive mononuclear cells into osteoclasts and also suppressed the bone-resorption activity of osteoclasts. Furthermore, adiponectin enhanced mRNA expression of alkaline phosphatase and mineralization activity of MC3T3-E1 osteoblasts. Our results indicate that adiponectin exerts an activity to increase bone mass by suppressing osteoclastogenesis and by activating osteoblastogenesis, suggesting that adiponectin manipulation could be therapeutically beneficial for patients with osteopenia.


*Keywords:* Bone mass; Adiponectin; Adipose tissue; Adipocytokines; Osteoclasts; Osteoblasts; Bone metabolism; Osteopenia

Osteoporosis and related bone fractures are growing medical problems especially with the enhanced longevity in industrial countries [1,2]. Therefore, it is important to know the factors that regulate bone mass and to develop effective therapeutic methods. Bone and bone marrow consist of various cells, including osteoblasts, osteoclasts, hematopoietic cells, and adipocytes. Bone adipocytes share a common mesenchymal precursor with osteoblasts and chondrocytes, and their numbers in bone marrow are altered in various pathophysiological conditions [3,4], but their roles in bone biology have not been clarified.

Several studies of adipocyte function have revealed that adipose tissue is not merely an energy-storing organ but it secretes a variety of biologically active molecules, which we conceptualized as "adipocytokines," including plasminogen activator inhibitor-1, tumor necrosis factor-α, resistin, leptin, and adiponectin [5–8]. Recent studies suggested that leptin, an anti-satiety adipocytokine, might have enhancing effects on bone mass

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[9,10]. Administration of leptin partially prevented bone loss in ovariectomized rats [11] and increased bone mineral density in leptin-deficient mice [12,13].

Adiponectin is a fat-specific secretory factor that was identified by our group in human fat cDNA [14]. The mouse homologue of adiponectin was independently cloned as adipoQ and Acrp30 [15,16]. We and others have shown that adiponectin has various biological functions, such as increasing insulin sensitivity in the liver and skeletal muscle, and protecting vascular walls from atherosclerosis, hence low plasma adiponectin levels in obesity might contribute to insulin resistance, diabetes, and atherosclerosis [17–21]. Furthermore, we recently showed that adiponectin inhibited liver fibrosis by suppressing proliferation and activity of hepatic stellate cells producing fibrotic collagen and transforming growth factor-β [22]. Others also reported that adiponectin have tumor growth inhibitory properties [23]. On the other hand, receptors of adiponectin were cloned and found to be expressed ubiquitously [24–26], suggesting that adiponectin might also play certain roles in bone biology.

The present study was designed to determine the effects of adiponectin on bone metabolism. The results showed that adiponectin increased bone mass by suppressing osteoclastogenesis and by activating osteoblastogenesis, suggesting that adiponectin could be potentially useful therapeutically for patients with reduced bone mass.

## Materials and methods

*Animals.* All animals were purchased from Clea Japan (Tokyo, Japan) and housed in a room under controlled temperature (23 ± 1 °C) and humidity (45–65%), and had free access to water and chow (Oriental Yeast). All animal experiments were conducted in accordance with the Institutional Guidelines for the Care and Use of laboratory animals.

*Adiponectin adenovirus.* Adenovirus producing the full-length mouse adiponectin was prepared as described previously [27]. Then, $2 \times 10^8$ plaque-forming units of adenovirus-adiponectin (Ad-adipo) or adenovirus-lacZ (Ad-lacZ) were injected into the jugular vein of 8-week-old C57BL/6J male mice. Mice were sacrificed on day 14 after virus injection.

*Skeletal morphology.* Three-dimensional microcomputed tomography (3D-μCT) scan was undertaken and the trabecular bone area (percentage of bone volume [BV] per tissue volume [TV]) was measured using a composite X-ray analysis system (Shimadzu, SMX-100CT-SV, Kyoto, Japan). Bones were fixed in 10% buffered formalin, decalcified in 14% ethylenediaminetetraacetic acid (EDTA), and embedded in paraffin. The sections were stained with tartrate-resistant acid phosphate (TRAP) and the number of TRAP-positive cells was counted in five sections per mouse.

*Bone marker measurement.* The blood samples were collected on day 14 after virus injection and processed within 30 min of collection, and aliquots of plasma were kept frozen at −80 °C until analyzed. Plasma cross-linked N-telopeptide of type I collagen (NTx), which is a marker of bone resorption, was measured by using an enzyme immunoassay (EIA). NTx concentrations were expressed as nanomoles of bone collagen equivalents (BCE) per liter (nM BCE/L).

*Culture of mouse bone marrow macrophages.* Mouse bone marrow macrophages (M-BMMs) were obtained as reported previously [28]. Briefly, whole bone marrow cells prepared from the femur and tibia of 5-week-old C57BL/6J male mice were suspended in α-minimal essential medium (αMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and recombinant human macrophage colony-stimulating factor (M-CSF, 100 ng/ml) at $5 \times 10^6$ cells in a 10-cm culture dish. After 3 days in culture, the cells were washed, harvested with 0.02% EDTA in phosphate-buffered saline (PBS), and seeded at $3 \times 10^5$ cells into another 10-cm culture dish. After a further 3 days in culture, the cells were harvested, plated at a density of $1.5 \times 10^4$ cells/cm$^2$ in 48-well plates, and maintained for 5 days in the presence of recombinant human soluble receptor activator of nuclear factor-κB ligand (RANKL, 50 ng/ml) and M-CSF (100 ng/ml) with or without recombinant human adiponectin [29].

*Culture of human CD14$^+$ peripheral blood mononuclear cells.* Human CD14-positive peripheral blood mononuclear cells (PBMCs) were prepared as reported previously [30]. Briefly, peripheral blood was obtained from healthy male volunteers and was carefully layered on the Ficoll–Paque PLUS solution (Amersham Biosciences, USA), and centrifuged at 1500 rpm for 30 min. PBMC layer was collected and washed twice in PBS. CD14-positive cells were selected using a magnetic isolation procedure (MACS CD14 Microbeads, Miltenyi Biotec, Germany). The cells were plated at a density of $1 \times 10^5$ cells/cm$^2$ in 48-well plates in αMEM containing 10% FBS and 1% penicillin/streptomycin, and cultured for 7 days in the presence of M-CSF (25 ng/ml) and RANKL (40 ng/ml) with or without recombinant human adiponectin.

*Tartrate-resistant acid phosphate staining.* TRAP staining was performed using a commercial TRAP staining kit (Hokudo, Sapporo, Japan). The number of TRAP-positive multinuclear (>3 nuclei) cells in each well was counted.

*Calcium resorption assay.* Calcified matrix-resorption activity of osteoclasts was determined using BD BioCoat osteologic calcium hydroxyapatite-coated 16-well chamber slides (BD Biosciences, Bedford, MA). Human CD14-positive monocytes were seeded at a density of $1 \times 10^5$ cells/cm$^2$ with M-CSF (25 ng/ml) and RANKL (40 ng/ml). At day 0, 3 (pre-osteoclasts) or day 7 (mature osteoclasts), recombinant human adiponectin was added to the culture medium. At day 14 cells were removed by vigorous washing, and resorption area was measured using Win Roof software version 3.5 (Mitani, Fukui, Japan).

*Cell culture.* Mouse pre-osteoblast MC3T3-E1 cells were cultured in αMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For cell differentiation study, the cells were seeded at a density of $2 \times 10^4$ per well in 12-well plates. After the cultures reached confluence, the medium was changed to αMEM with 10% FBS containing 50 μg/ml L-ascorbic acid phosphate magnesium (Wako Pure Chemical, Osaka, Japan) and 10 mM β-glycerophosphate (Wako Pure Chemical). Then, the cells were further cultured for indicated time with or without recombinant mouse adiponectin, which was generated in a manner similar to recombinant human adiponectin as described previously [31].

*RNA analysis.* Total RNAs were extracted from cells with an RNA-STAT-60 kit (Tel-Test "B"). The first-strand cDNA was synthesized using ThermoScript RT-PCR System (Invitrogen, San Diego, CA). Real-time polymerase chain reaction (PCR) was performed on a Light Cycler using the Fast Start DNA Master SYBR Green I (Roche Diagnostics) according to the protocol provided by the manufacturer [32]. Sequences of primers used for real-time PCR were as follows: alkaline phosphatase (ALP), 5′-GCC CTC TCC AAG ACA TAT A-3′ and 5′-CCA TGA TCA CGT CGA TAT CC-3′, 18S, 5′-CGG CTA CCA CAT CCA AGG AA-3′ and 5′-GCT GGA ATT ACC GCG GCT-3′.

*Mineralization assay.* MC3T3-E1 cells were incubated at 34 °C for 24 h in culture medium, and then the medium was refreshed with culture medium with or without adiponectin. The degree of mineralization was determined in the 12-well plates using Alizarin Red staining, as described previously [33]. Briefly, the differentiated MC3T3-E1 cells were rinsed twice with PBS followed by fixation with 70% ethanol for 1 h at room temperature. Then cells were stained with 40 mM

Alizarin Red S (Wako Pure Chemical) at pH 4.0 for 10 min at room temperature, and were washed five times with deionized water and twice with PBS. Then, staining was released from the cell matrix by incubation in 10% cetylpyridinium chloride at pH 7.0 for 15 min. The degree of mineralization was determined by measuring the absorbance of supernatants at 562 nm.

*Statistical analysis and ethical considerations.* Results were expressed as means ± SEM. Differences between groups were examined for statistical significance using Student's $t$ test or analysis of variance with Fisher's protected least significant difference test. A $P$ value less than 0.05 denoted the presence of a statistically significant difference. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

## Results

### *Adenovirus-mediated overexpression of adiponectin in vivo*

To investigate the in vivo role of adiponectin in bone metabolism, we treated C57BL/6J mice with adenovirus producing adiponectin (Ad-adipo) or lacZ (Ad-lacZ). Two weeks after injection, we estimated structural changes in the bone by analyzing cortical and trabecular bones with 3D-μCT. Fig. 1A shows representative 3D-μCT images of the proximal tibia of Ad-lacZ- and Ad-adipo-treated mice, demonstrating a significantly larger trabecular bone volume in Ad-adipo-treated mice than in Ad-lacZ-treated mice. The trabecular bone volume of the proximal tibia at two different areas located 50–250 and 500–700 μm from the growth plates of the proximal tibia (representing the most active areas for bone remodeling, Fig. 1B) was significantly higher in Ad-adipo-treated mice than in the control Ad-lacZ-treated mice (Fig. 1B). Similar findings were observed in the femur, by 3D-μCT analysis (data not shown).

To investigate whether adiponectin modulates osteoclastogenesis, we analyzed TRAP-stained sections of the distal femur in Ad-lacZ- and Ad-adipo-treated mice. Examination of these sections demonstrated fewer TRAP-positive osteoclasts in Ad-adipo-treated mice than in Ad-lacZ-treated mice (Fig. 2A). Quantitative analysis confirmed that the number of TRAP-positive osteoclasts was significantly lower in adiponectin-adenovirus-treated mice than in Ad-lacZ-treated mice (Fig. 2B). Furthermore, in Ad-adipo-treated mice, plasma levels of NTx, a marker of bone resorption, were significantly lower than in Ad-lacZ-treated mice (Fig. 2C). Taken together, our in vivo data indicated that adiponectin suppresses osteoclastogenesis.

### *Effects of adiponectin on osteoclast differentiation and activity*

Next, we investigated the effects of adiponectin on the differentiation of osteoclasts in vitro by treating primary cultures of bone marrow stromal cells, which contain

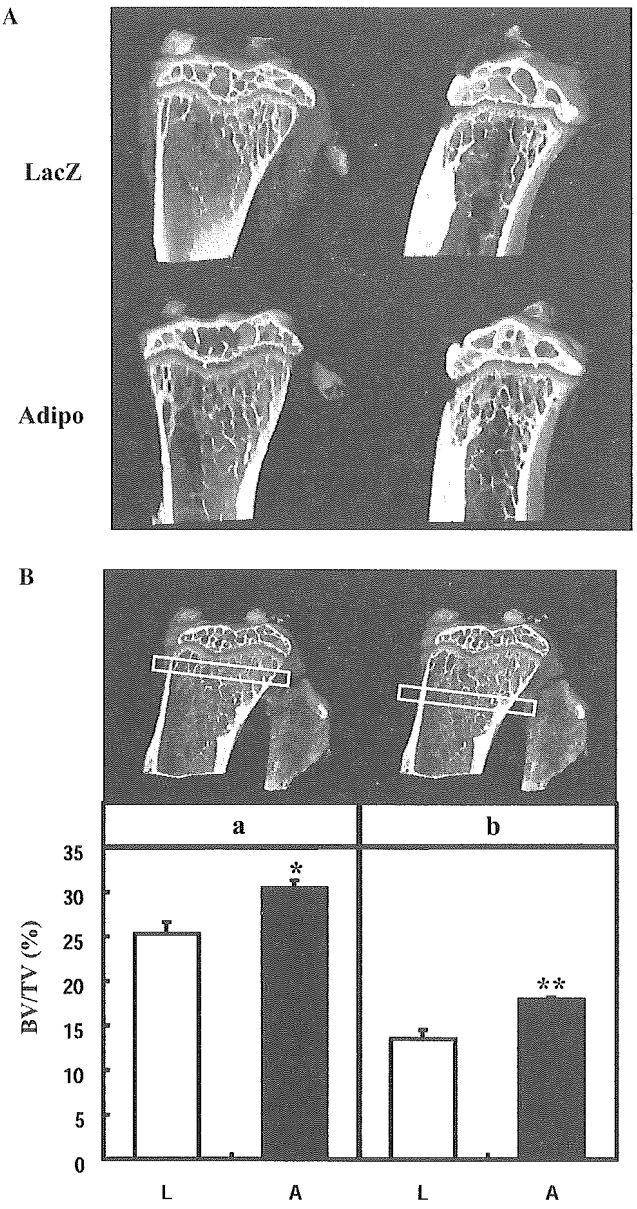


Fig. 1. Increased trabecular bone mass in adiponectin-adenovirus-treated mice. (A) Three-dimensional μCT scan images of the proximal tibia of C57BL/6J mice treated with lacZ- (upper panels) or adiponectin-adenovirus (lower panels). The left-side panels show anterior–posterior view and the right-side panels show lateral-medial view. (B) Quantitative data of trabecular bone volume at indicated two areas; 50–250 μm (a) and 500–700 μm (b) from the distal end of the growth plate, in the proximal tibia of C57BL/6J mice treated with lacZ- (L, $n = 8$) and adiponectin-adenovirus (A, $n = 8$). Data are expressed as percentage of total tissue volume (BV/TV [%]). Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with lacZ-adenovirus-treated mice.

osteoclast progenitor cells, with or without recombinant adiponectin. Previous studies reported that M-CSF and RANKL induced differentiation of progenitor cells into TRAP-positive osteoclasts [34,35]. In the present study, treatment of bone marrow stromal cells with adiponectin suppressed M-CSF/RANKL-induced differentiation of these cells into osteoclasts, in a dose-dependent

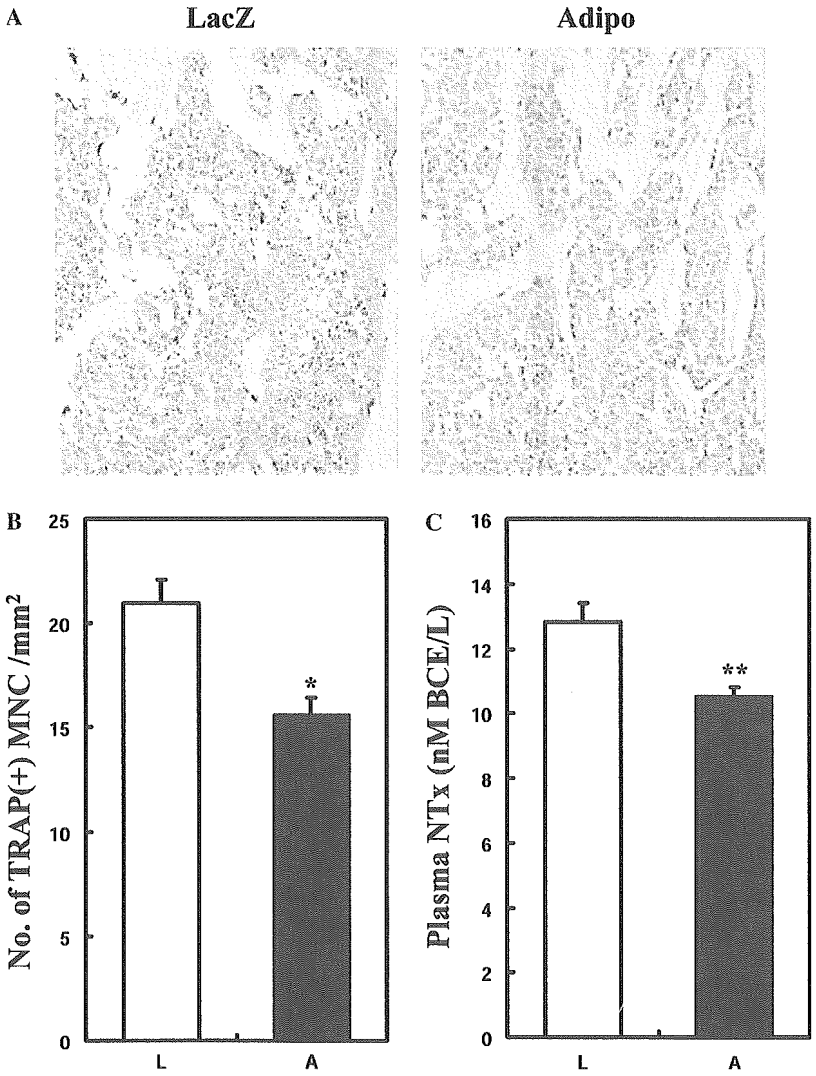


Fig. 2. Reduction of TRAP-positive osteoclasts in mice treated with adiponectin-adenovirus. (A) Histological examination of the distal femur with TRAP staining (left; lacZ, right; adiponectin). TRAP-positive osteoclasts are stained red. (B) Quantitative analysis of the number of TRAP-positive osteoclasts in C57BL/6J mice treated with lacZ- (L, $n = 8$) and adiponectin-adenovirus (A, $n = 8$). Data are means ± SEM. $^{*}P < 0.05$, compared with lacZ-adenovirus-treated mice. (C) Plasma NTx levels of C57BL/6J mice treated with lacZ- (L, $n = 8$) and adiponectin-adenovirus (A, $n = 8$). Data are means ± SEM. $^{**}P < 0.01$, compared with lacZ-adenovirus-treated mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

manner (Fig. 3A). Similarly, adiponectin also dose-dependently suppressed differentiation of human CD14-positive PBMCs into osteoclasts (Fig. 3B).

Next, we performed bone-resorption analyses with or without adiponectin treatment, using human CD14-positive cells. Treatment of these cells with adiponectin for 14 days (days 0–14) after M-CSF- and RANKL-induced differentiation resulted in a significant reduction of the resorption area in a dose-dependent manner (Fig. 3C). Furthermore, a significant reduction of the resorption area was also noted with adiponectin treatment from day 3 or 7 when human CD14-positive cells were already differentiated (days 3–14, 7–14) (Figs. 3D and E). These results suggest that adiponectin could suppress osteoclastogenesis and bone-resorption activity of osteoclasts.

*Effects of adiponectin on MC3T3-E1 osteoblasts*

To investigate the effects of adiponectin on osteoblasts, we treated MC3T3-E1 osteoblasts with adiponectin for 18 days and measured mRNA levels of alkaline phosphatase (ALP) and the mineralization activity of MC3T3-E1 osteoblasts. Treatment with adiponectin significantly increased ALP mRNA level on day 12 and 18 compared with the control (Fig. 4A), suggesting that adiponectin might enhance the differentiation of osteoblasts. Previous reports indicated increased mineralization activity of MC3T3-E1 cell matrix in the presence of ascorbic acid [33,36]. Treatment of these cells with adiponectin significantly increased mineralization of the matrix (Fig. 4B).

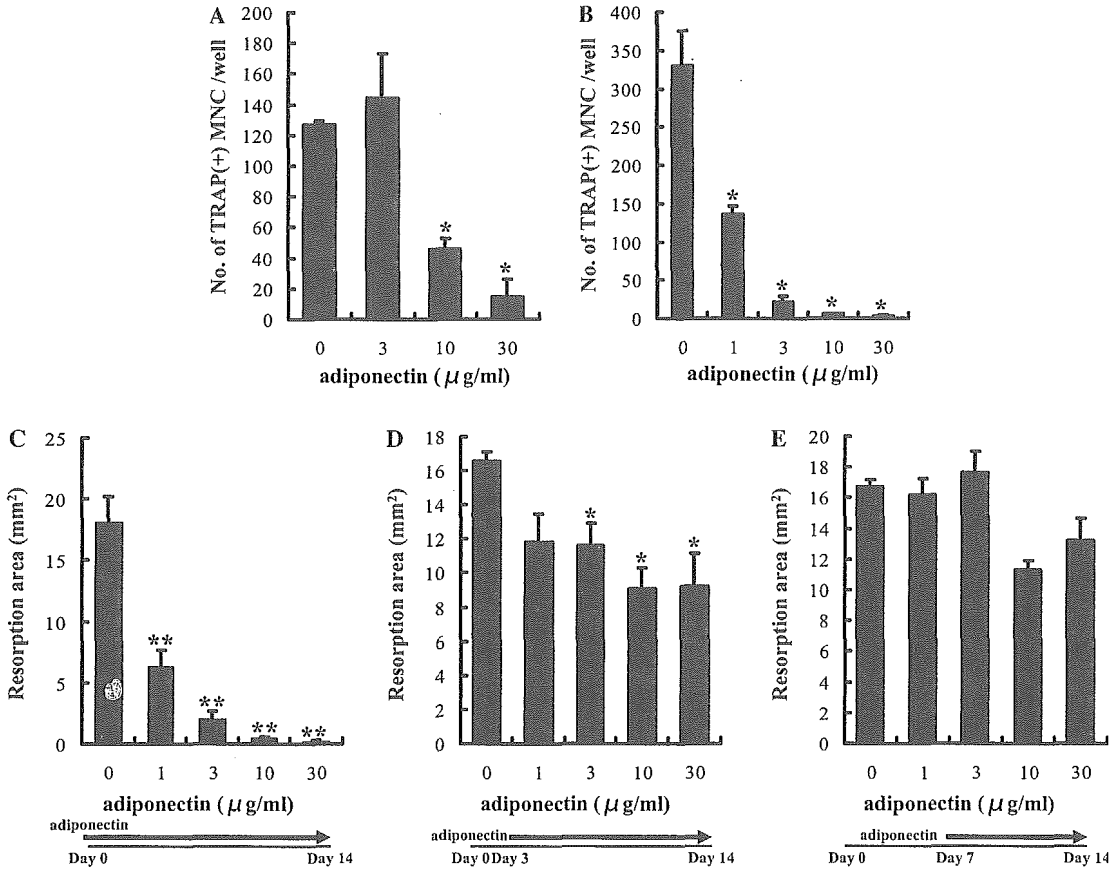


Fig. 3. Effects of adiponectin on primary cultures of osteoclasts. (A,B) TRAP assay of primary cultures of mouse bone marrow macrophages (A) and human CD14-positive PBMCs (B) stimulated by M-CSF and RANKL and treated with the indicated amounts of adiponectin. Data are expressed as means ± SEM. $^{*}P < 0.05$, compared with cells that were not treated with adiponectin (0–30 μg/ml). (C, D, and E) Bone-resorption assay of human CD14-positive PBMCs stimulated by M-CSF and RANKL, and treated with the indicated amounts of adiponectin from day 0 to 14 (C), from day 3 to 14 (D), and from day 7 to 14 (E). Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with cells that were not treated with adiponectin (0–30 μg/ml).

## Discussion

In the present study, we investigated the role of adiponectin on bone formation in vivo, using an adiponectin-producing adenovirus. The major finding of the present study was that adiponectin supplement increased bone mass in trabecular bone. Analysis of the mechanism of this action revealed that adiponectin acts by reducing the differentiation and bone-resorption activity of osteoclasts, and possibly by enhancing the differentiation and mineralization activity of osteoblasts.

We observed the increase of bone mass only in trabecular bone, but not in cortical bone, of adiponectin-adenovirus-treated mice. The duration of adiponectin overproduction by adenovirus treatment is at most 2 weeks. This could explain, at least in part, the effect of adiponectin on trabecular bone and the lack of such effect on cortical bone, since the former has a higher remodeling activity than the latter [37]. We also observed a significant decrease in osteoclast count and plasma NTx levels in adiponectin-adenovirus-treated mice, indicating that adiponectin inhibits bone resorption. These findings were confirmed in tissue culture experiments. In mouse bone marrow macrophages (M-BMMs) and CD14-positive human peripheral blood mononuclear cells (PBMCs), adiponectin inhibited the M-CSF- and RANKL-induced differentiation of these cells into osteoclasts, as well as the bone-resorption activity of mature osteoclasts. Furthermore, we found that both bone tissue and primary osteoclasts expressed both adipoR1 and R2 (Supplemental Fig. 1), suggesting that adiponectin directly targets octeoclast cells. These results demonstrate that adiponectin suppresses bone-resorption activity by inhibiting osteoclastogenesis.

In adiponectin-adenovirus infected mice, plasma glucose and insulin levels did not change compared to control-adenovirus infected mice, indicating that this adenovirus treatment did not affect glucose metabolism systemically (data not shown). This is consistent with our previous report, in which we demonstrated that adiponectin-KO mice showed normal glucose and insulin levels in plasma, and adiponectin-adenovirus has no

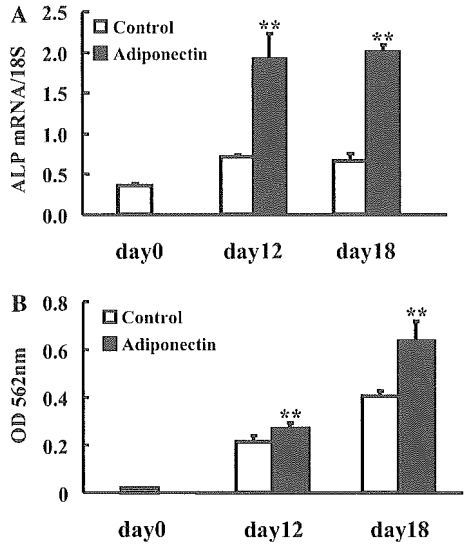


Fig. 4. Effects of adiponectin on MC3T3-E1 osteoblasts. (A) ALP mRNAs in MC3T3-E1 cells treated with or without adiponectin for 12 or 18 days were measured by real-time quantitative reverse transcriptase-PCR as described in Materials and methods. Data are normalized with 18S RNAs and expressed as means ± SEM of three independent experiments. $^{**}P < 0.01$, compared with the control. (B) The mineralization activity of MC3T3-E1 cells treated with or without adiponectin for 12 or 18 days was measured as described in Materials and methods. Data are expressed as means ± SEM of three independent experiments. $^{**}P < 0.01$, compared with the control.

effect on plasma glucose and insulin levels under control diet, although when adiponectin-KO mice are fed with high fat diet, their plasma glucose and insulin levels significantly elevate compared to wild-type mice and adiponectin-adenovirus reverses them to the level of wild-type mice [19]. Therefore, this effect of adiponectin should not be mediated by its systemic insulin-sensitizing effect in normal mice. However, whether it is mediated by local insulin-sensitizing effect remains to be elucidated.

The bone-forming activity of osteoblasts is also important in determining bone mass [37,38]. Our study revealed that adiponectin increased mRNA expression of ALP and mineralization activity of mouse MC3T3-E1 osteoblasts. We also investigated the effect of adiponectin on the osteoblastogenesis of primary mouse osteoblasts and mouse bone marrow cells. However, the effect was not significant on these cells (data not shown), indicating that adiponectin may have the potential to activate osteoblasts, but its effect may be dependent on the cell types.

In conclusion, we have demonstrated in the present study that adiponectin increases bone mass by suppressing osteoclastogenesis and possibly by activating osteoblastogenesis. These results suggest that increasing the concentration and/or enhancing the activity of adiponectin might be therapeutically beneficial for patients with reduced bone mass.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.03.210.

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# 骨粗鬆症治療

別　刷

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# アディポサイトカイン

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## はじめに

脂質の過剰摂取・運動不足という現代の生活習慣は，過剰な脂肪蓄積を惹起し，メタボリックシンドローム発症の基盤となる．メタボリックシンドロームとは，腹部肥満すなわち内臓脂肪蓄積を必須に，高血圧，脂質代謝異常，耐糖能異常を伴う動脈硬化易発症病態を包括的にとらえた疾患概念である．一方，全身の白色脂肪組織が極端に減少する全身性脂肪萎縮症（脂肪萎縮性糖尿病）も，よく似た代謝異常症候群，すなわちインスリン抵抗性，糖尿病，脂肪肝，高脂血症を呈する．これは全身の糖・脂質代謝の恒常性を保つうえで脂肪組織が重要な役割を担うことを示唆している．

脂肪組織は重量として身体の10%以上を占め，人体における主要なエネルギー備蓄臓器と考えられてきたが，近年の分子生物学的アプローチにより，アディポサイトカインと総称されるさまざまな生理活性物質を産生・分泌する生体内最大の内分泌臓器であることが知られ，肥満，とくに内臓脂肪蓄積により惹起されるアディポサイトカインの分泌異常が，肥満症，脂肪萎縮症，両疾病における病態やメタボリックシンドロームと密接にかかわることが明らかとなった．

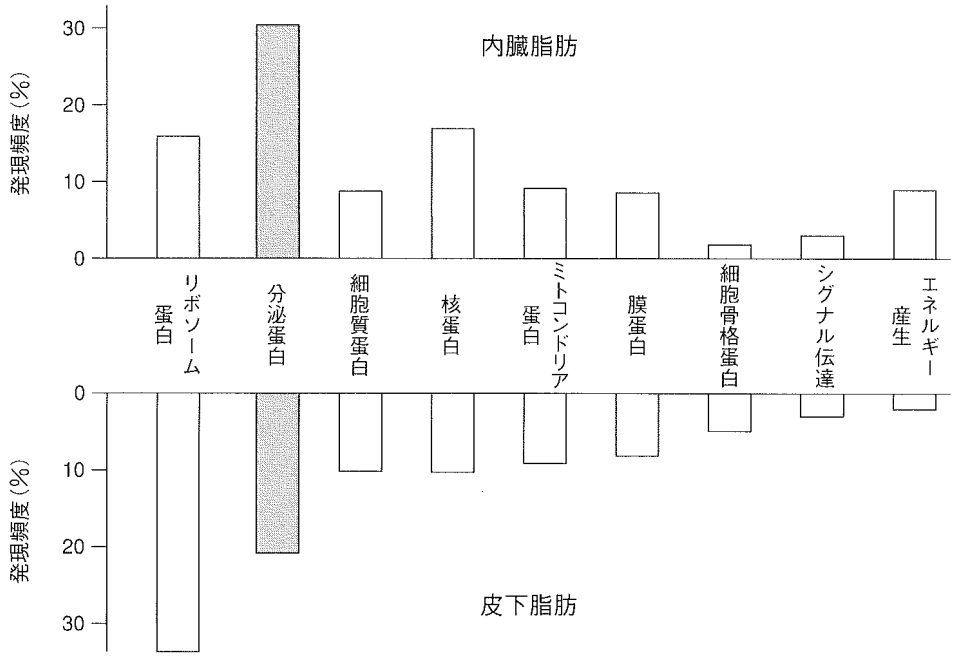
本稿では，生活習慣病あるいはメタボリックシンドロームを考えるうえで重要な4つのアディポサイトカインについて述べる（図❶，❷）．

**関連語**
・メタボリックシンドローム/生活習慣病
・脂肪組織/生理活性物質
・アディポネクチン/レプチン
・PAI-1/TNF-α

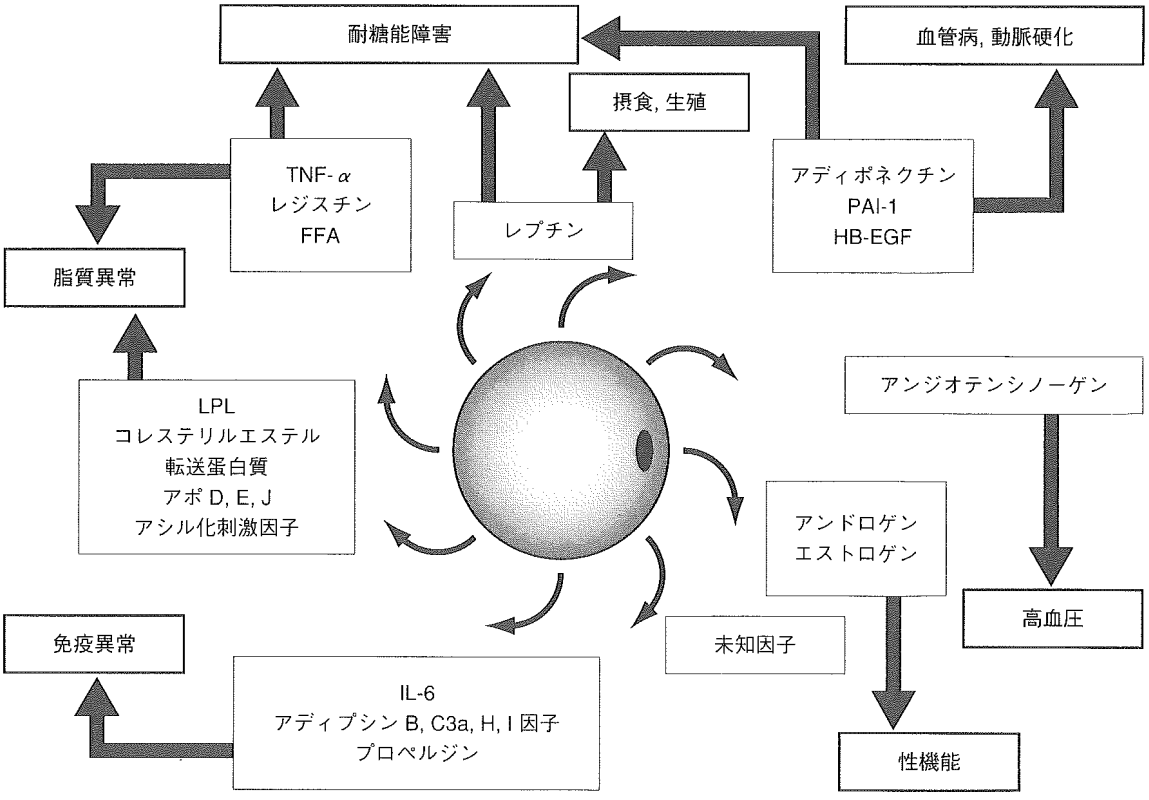
## アディポサイトカイン

### 1）Plasminogen activator inhibitor-1（PAI-1）

生体内における血液の凝固と線溶は種々の因子によりバランスが保たれるが，plasminogen activator inhibitor-1（PAI-1）はプラスミノゲンアクチベータを抑制し，プラスミン生成を妨げ，フィブリンからのフィブリノーゲン分解産物生成を低下させる．つまり，PAI-1の増加は線溶活性を低下させ，血栓形成傾向に傾く．肥満者で静脈血栓症や心筋梗塞など，血栓性疾患の頻度が高いことは周知の事実であり，実際，脂肪蓄積，とくに内臓脂肪蓄積に伴い，脂肪組織PAI-1遺伝子発現量は上昇し，この上昇に連関して血中PAI-1濃度も上昇する[1]．血中PAI-1濃度は，肥満者，2型糖尿病患者で上昇し，血清トリグリセリド値とも相関する一方で，静脈血栓症や心筋梗塞患者でも上昇を認める．また，以前は血中PAI-1のおもな産生場所は肝臓であるとされていたが，脂肪蓄積に伴うPAI-1発現量は肝臓にて変化を認めず，内臓脂肪にて肝臓のレベルを凌駕する上昇を認めた[2)3)]．これにより，肥満形成に伴い，蓄積脂肪から直接分泌される血中PAI-1の上昇が，肥満と血栓性疾患とを直接結びつける因子




図❶ ヒト脂肪組織発現遺伝子解析(Body map project)




図❷ 脂肪組織由来生理活性物質(アディポサイトカイン)とその作用

FFA : free fatty acid, HB-EGF : heparin binding-EGF like growth factor, LPL : lipoprotein lipase, TNF-α : tumor necrosis factor-α , IL-6 : Interleukin-6, PAI-1 : plasminogen activator inhibitor-1.

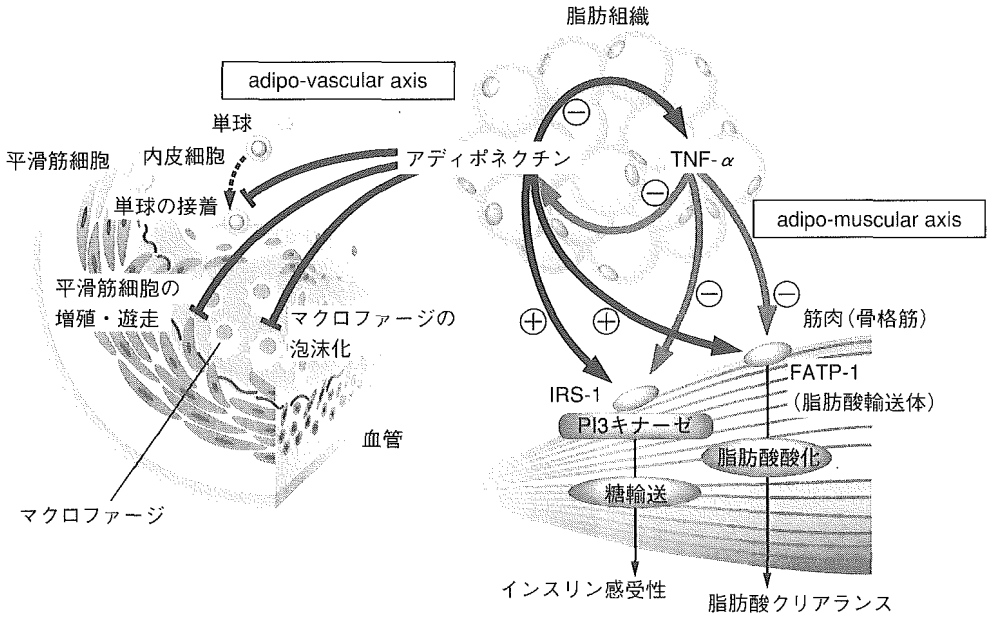
であることが示された．ヒトにおいて血中PAI-1濃度と脂肪分布の関連を調べたところ，血中PAI-1値は内臓脂肪面積と正相関を示すが，皮下脂肪面積とは相関しないことが明らかになった[4]．以上の知見から，内臓脂肪から直接分泌されるPAI-1が血中レベルに影響を与え，血栓性疾患の発症に関与するという，内臓脂肪蓄積と血管病変をつなぐ新たな道(adipo-vascular axis)の存在が明らかになった．また，最近，高脂肪食負荷による肥満・インスリン抵抗性の出現に対してPAI-1欠損マウスが抵抗性であること[5]，PAI-1がインスリンシグナルを抑制することが報告され[6]，肥満時の血中PAI-1レベルの上昇は肥満・インスリン抵抗性の病態発症そのものにかかわっている可能性もあり，興味深い．

### 2) Tumor necrosis factor (TNF)-α

Spiegelmanら[7]は遺伝性肥満動物の脂肪組織におけるサイトカイン遺伝子発現において，腫瘍壊死因子(tumor necrosis factor：TNF)-αが著明に増加していることを見出した．これら動物にTNF-αの中和抗体を投与したところ，インスリン抵抗性・糖尿病の改善がみられた．脂肪細胞や肝細胞においてTNF-αは，スフィンゴミエリナーゼの活性化を介して，インスリン受容体の基質であるインスリン受容体基質(insulin receptor substrate：IRS)-1のセリン残基をリン酸化し，チロシン残基のリン酸化を抑制することで，インスリン作用を減弱させる[8]．蓄積した脂肪組織より分泌されたTNF-αが筋肉，脂肪組織，肝臓での糖利用亢進を抑制し，インスリン抵抗性を介して，糖・脂質代謝異常をもたらすと考えられている．また，TNF-αは後述するアディポネクチンの遺伝子転写を抑制することによりアディポネクチンの産生・分泌を減少させる作用を有する(アディポネクチンの項参照)[9][10]．さらに，TNF-α，インターロイキン(IL)-1を介して核内で増えたNF-κBが，直接 peroxisome proliferator-activated receptor(PPAR)γに作用し，その転写活性を不活性化することが，脂肪細胞分化抑制につながることが明らかとなった[11]．TNF-α，IL-1のような肥大脂肪細胞から産生・分泌される因子が，未分化細胞の脂肪細胞への分化を抑制するという点は，アディポサイトカインが脂肪組織そのものに作用して代謝の恒常性・病態にかかわることを示唆しており，興味深い．

### 3) レプチン

Friedmanら[12]は肥満の遺伝的基盤を明らかにするため，ポジショナル・クローニングを用いて遺伝性肥満マウスの原因遺伝子の単離を試み，脂肪組織特異的な分泌蛋白質で，脂肪蓄積とともに脂肪細胞より分泌されるレプチンを同定した．その作用はおもに視床下部食欲中枢に作用し，食欲の抑制作用，エネルギー消費増強作用を介して，体重を減少させる．全身性脂肪萎縮症モデルマウスやレプチン欠損マウス(ob/ob)において認められるレプチンの欠乏は，糖・脂質代謝異常を招き，重篤なインスリン抵抗性，糖尿病，脂肪肝，高脂血症を発症するが，レプチン補充にて，改善もしくは正常化する[13]．レプチンの全身性脂肪萎縮症への有効性はすでにヒトにおいても証明され，糖尿病，高脂血症，脂肪肝の劇的な改善に至っている[14]．その作用機序として，レプチン欠乏が引き起こす慢性的な高インスリン血症が，肝臓の脂肪酸・中性脂肪合成を上昇させる転写因子SREBP-1cを上昇させ，脂肪肝，高脂血症を引き起こすこと，高インスリン血症が肝臓の糖代謝に重要なアダプター蛋白IRS-2の絶対量を減少させることでインスリン抵抗性を引き起こし，定常的に糖の産生・放出が上昇し高血糖へとつながることが考えられている[15]．また，レプチンには交感神経系を介した昇圧作用があり，肥満時には脂肪組織からのレプチン産生が上昇することから，肥満者の高血圧には高レプチン血症の関与が考えられる．さらに，レプチンは視床下部—交感神経系を介して，かつ骨格筋に直接作用して，骨格筋での脂肪酸酸化を亢進させることが報告され[16]，この作用がAMP-activated protein kinase (AMPK)を介して惹起されることを見出し，Leptin-AMPK axisが骨格筋におけるエネルギー消費の重要な制御機構であることを示した．レプチンは，骨代謝に及ぼす作用も報告されている．中枢性に骨形成を抑制すること[17]，逆に，骨芽細胞に直接作用して，石灰化や増殖分化を促進すること[18][19]や破骨細胞前駆細胞に作用してその分化を抑制することなどが報告されている[20]．




図❸ アディポネクチンの作用(adipo-vascular, adipo-muscular axis)

### 4) アディポネクチン

#### a. アディポネクチンの発見と血中濃度変化

アディポネクチンは，Body map project[21]におけるヒト脂肪組織遺伝子ライブラリーに最も頻回に出現し，脂肪組織特異的に発現した遺伝子adipose most abundant gene transcript 1(apM1)の産物である[22]．血中アディポネクチン濃度は5～30 $\mu g/ml$と高濃度に存在し，肥満者や男性において低下し，減量によって増加する[23][24]．さらに，心筋梗塞や狭心症といった動脈硬化性疾患，糖尿病で低下する[25][26] (図❸)．

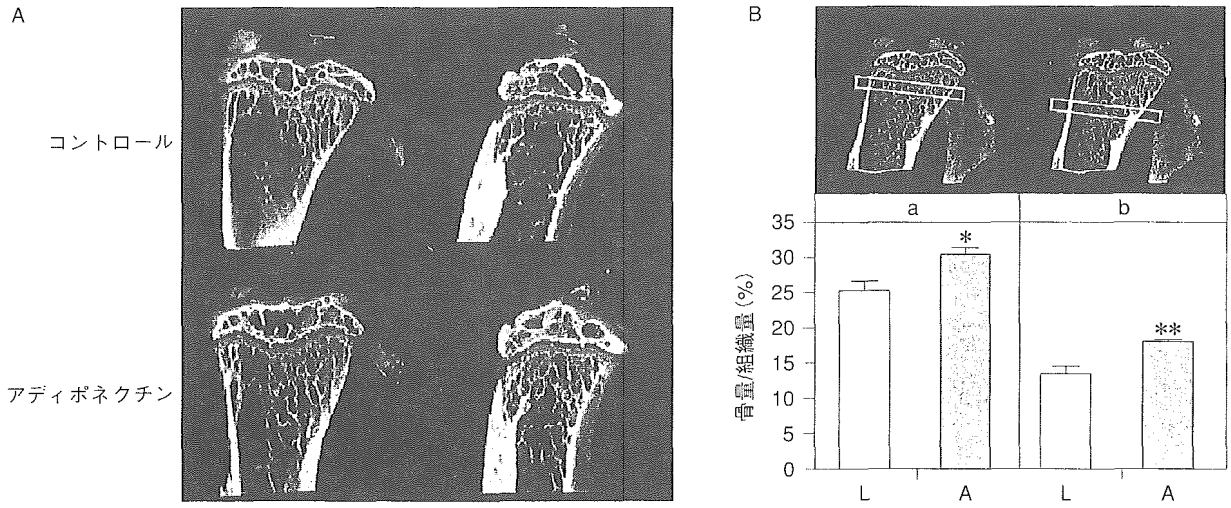
#### b. アディポネクチンの抗動脈硬化作用 (adipo-vascular axis)

アディポネクチンは，血管内皮細胞においてTNF-$\alpha$依存性に上昇する接着分子VCAM-1，ICAM-1，E-セレクチンの発現を抑制し，血管内皮細胞と単球との接着を阻害する[25]．また，マクロファージの泡沫化や血管平滑筋細胞の増殖を抑制する[27][28]．これらはアディポネクチンが抗動脈硬化ホルモンであることを示す．

最近報告されたcase-control studyでは，入院時急性心筋梗塞や急性冠症候群と診断された症例は，安定型狭心症と診断された症例にくらべ，血中アディポネクチン濃度が有意に低値であり[29]，マクロファージにアディポネクチンを添加したところ，プラーク攻撃因子であるmatrix metalloproteinase-9(MMP-9)の発現は変化しないが，これに拮抗する防御因子であるtissue inhibitor of metalloproteinase-1(TIMP-1)の著明な増加がみられた．これは，アディポネクチンが動脈硬化の発症進展を抑制するだけでなく，プラーク破綻に対しても抑制的に作用していることを示唆する[30]．

#### c. アディポネクチンの抗糖尿病作用(インスリン感受性増強作用)

血中アディポネクチン濃度は全身のインスリン感受性と強く相関する[31]．アディポネクチンは，筋肉細胞にはたらいて，IRS-1シグナリングを介したPI3-kinaseの活性および糖輸送を上昇させ，インスリン感受性を増強させる．さらに，アディポネクチンは脂肪酸輸送蛋白1型(fatty acid transport protein 1：FATP-1)の遺伝子発現増強を介して脂肪酸の酸化およびクリアランスを高め，インスリン感受性を上昇させる[32]．そして，脂肪蓄積で脂肪組織より過分泌されるTNF-$\alpha$は同経路に作用してアディポネクチンと逆の作用を示す．一方，アディポネクチンとTNF-$\alpha$は，互いの作用を抑制しあうだけでなく，その産生場所である脂肪組織において転写レベルでの調節によりお互いの産生を抑制しあうことがわかった．つまり，




**図❹ アデノウイルスを用いたアディポネクチン強発現マウスの3D-μCT解析**
A：3D-μCT画像．B：海綿骨量（脛骨），成長板より50～250μm(a)，500～700μm(b)の領域．L：コントロール，A：アディポネクチン．

アディポネクチンは，インスリン抵抗性惹起因子であるTNF-αの産生と機能を抑制する．実際，糖尿病モデルマウスにアディポネクチンを補充すると血糖値が下がることが報告され[33][34]，アディポネクチンのインスリン抵抗性改善作用は，骨格筋，肝臓で脂肪酸酸化に重要なはたらきをしているAMP kinaseを介したものであることが明らかにされた[35]．

### d．遺伝的低アディポネクチン血症からの知見

アディポネクチン欠損マウスは，高脂肪・高ショ糖食2週間負荷にて，強いインスリン抵抗性・糖尿病を呈し，アデノウイルスによる血中アディポネクチン補充により野生型レベルまで改善した[32]．血管病変については[36]，大腿動脈擦過後の血管内膜平滑筋の増殖すなわち内膜肥厚の進行を認めるも，補充にて野生型レベルまで改善した．アディポネクチン欠損マウスは，外観上正常と変化ないが，環境負荷や傷害に対して強く反応が起こるという，生体侵襲に対する防御因子としての本分子の一面がうかがえる．

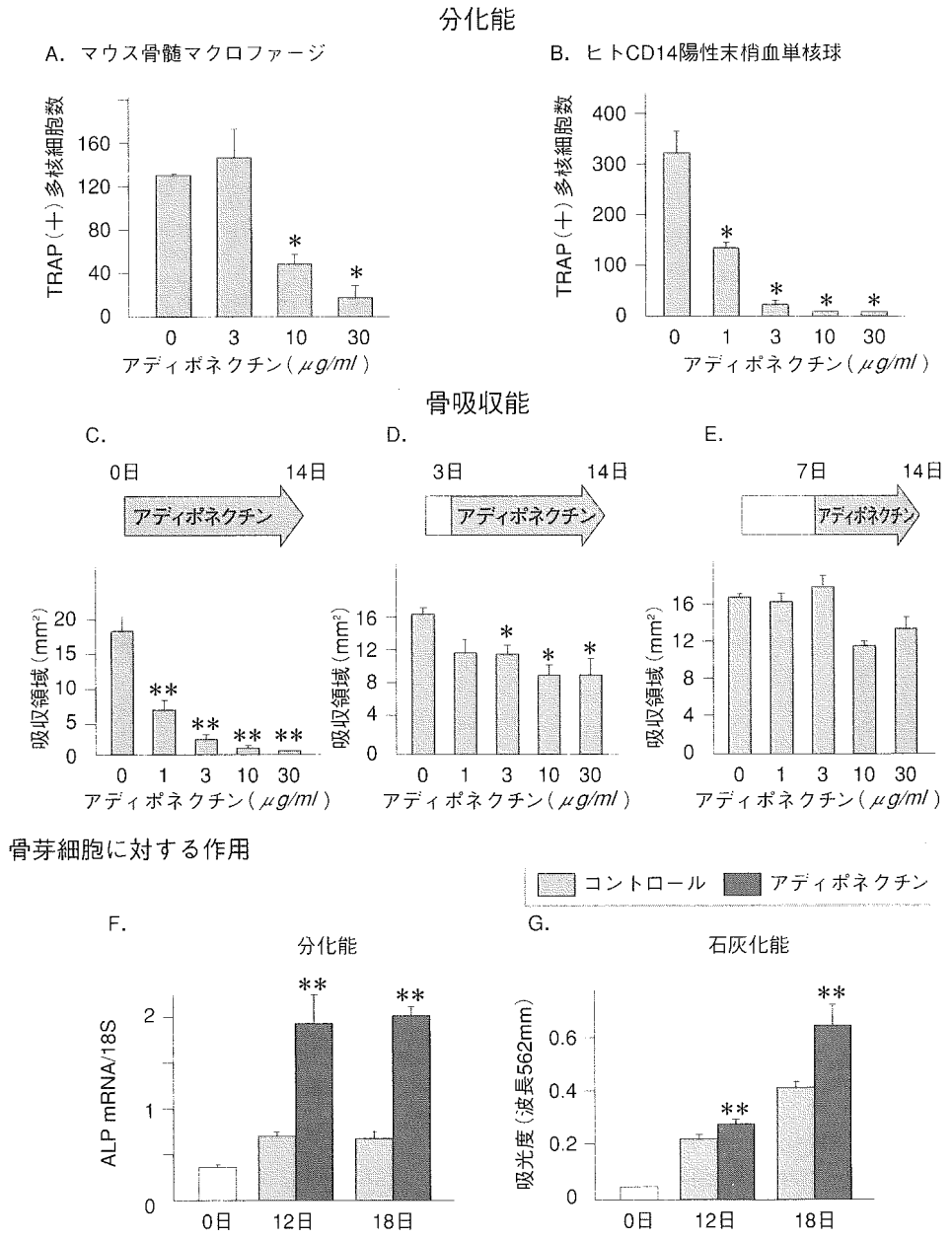
現在までヒトにおいて本分子を完全に欠損する遺伝子異常は報告されていないが，興味深いのは，血中アディポネクチン濃度の低下を伴うI164T変異で，耐糖能異常，高脂血症，高血圧や動脈硬化疾患を高率に合併し，まさにメタボリックシンドロームの表現型を示す[37]ことである．

### e．炎症，高血圧，肝線維症-生活習慣による多彩な病態への関与

近年，動脈硬化疾患や2型糖尿病発症の基盤として，炎症が注目されている．アディポネクチンはTNF-α分泌を介して炎症と関連するが，血中high sensitive CRP (hsCRP)濃度はアディポネクチン濃度と弱い逆相関関係にある．アディポネクチン欠損マウスでは脂肪組織CRP発現量が増加しており[38]，この組織が多くの補体分子を発現していることから，過栄養に対するバッファー機能のみでなく，炎症に対する防御機能を有していると考えられる．

高血圧はメタボリックシンドロームの重要な病態である．一般集団で血中アディポネクチンとの相関関係をみると，HDLコレステロール，トリグリセリド濃度やインスリン抵抗性指標にくらべ，血圧との関連は低い．しかし，本態性高血圧群をみると血中アディポネクチン濃度は血圧と逆相関する．アディポネクチン欠損マウスに高脂肪高食塩負荷をおこなうと，血管内皮機能が障害され血圧は上昇する[39]．アディポネクチンは，血管内皮機能を介し高血圧へも関与していると考えられる．

動脈硬化は一種の過剰な創傷治癒反応である．アディポネクチン欠損マウスに四塩化炭素を投与した肝臓障害実験では[40]，野生型にくらべ著明な肝線維化が起こり，線維合成にあずかる肝星細胞（α-smooth muscle actin陽




**図❻ 培養細胞に対するアディポネクチンの作用**
アディポネクチンによる破骨細胞の分化(A, B)・活性化(C, D, E)抑制作用および骨芽細胞の分化(F)・活性化(G)促進作用を示す.
A-E：誘導破骨細胞初代培養(マウスおよびヒト細胞), F-G：誘導骨芽細胞培養(MC3T3-E1細胞), TRAP：酒石酸抵抗性酸ホスファターゼ, ALP：アルカリホスファターゼ.
Studentのt検定によるP値：＊$p<0.05$, ＊＊$p<0.01$.

性細胞)が活性化され, TGF-$\beta$1, connective tissue growth factorなどの線維化関連遺伝子の発現が上昇していた.

### f. アディポネクチンの骨代謝に及ぼす影響

アディポネクチンレセプターが同定され, その発現がユビキタスであったことから, レプチンのみならずアディポネクチンも骨代謝に何らかの影響を及ぼすことが容易に想像された. 最近, *in vivo*の検討にて, アデノウイルスを用いたアディポネクチン強発現マウスの海綿骨量が著明に増加し, 破骨細胞数の減少とNTxの減少を伴うことが明らかとなり[41], *in vitro*では, アディポネクチンが

破骨細胞の分化，吸収活性を抑制すること，骨芽細胞の分化，石灰化を促進することが明らかにされた[41]（図❹，❺）．また，骨芽細胞の増殖・分化促進作用は，MAPKシグナルを介したものであることがわかった[42]．

## おわりに

近年における臨床的，基礎的両側面からの研究アプローチにより，メタボリックシンドロームの発症基盤として，脂肪組織，とくに内臓脂肪蓄積の意義は確立した．今後，内臓脂肪蓄積とそれに引き続くアディポサイトカイン産生・分泌異常を病態マーカーとして早期にとらえ，治療標的として臨床応用することが，個々のリスクファクターに対する治療を超えたメタボリックシンドロームの包括的な予防および治療につながると考えられる．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# Identification of an Alternatively Spliced Variant of $Ca^{2+}$-promoted Ras Inactivator as a Possible Regulator of RANKL Shedding*

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**The receptor activator of NF-κB ligand (RANKL), a critical regulator of osteoclastogenesis, is synthesized as a membrane-anchored protein and cleaved into a soluble form by ectodomain shedding. We developed an assay system to identify molecules regulating the RANKL shedding. Using this system, we found that a splice variant of $Ca^{2+}$-promoted Ras inactivator (CAPRI), ΔCAPRI, which is expressed in primary osteoblasts, promoted the RANKL shedding. The wild type CAPRI is a member of the Ras GTPase-activating protein (GAP) family and suppresses $Ca^{2+}$-dependent Ras activation, whereas ΔCAPRI, which lacks one exon in the GAP-related domain, activated the Ras pathway. Overexpression of ΔCAPRI or a constitutive active form of Ras up-regulated the expression level of matrix-metalloproteinase 14 (MMP14), which directly cleaves the ectodomain of RANKL, whereas Erk activation by expressing the constitutive active Mek1 did not affect the MMP14 expression or RANKL shedding. These results suggest that ΔCAPRI is a possible regulator of RANKL shedding by modulating MMP14 expression through Ras signaling cascades other than the Erk pathway.**

The receptor activator of NF-κB ligand, RANKL (also known as TNF-related activation-induced cytokine, TRANCE, osteoprotegrin ligand, OPGL, and osteoclast differentiation factor, ODF) is a type II transmembrane glycoprotein with a molecular mass of ~45 kDa, which belongs to the tumor necrosis factor (TNF)[2] ligand family (1–5). RANKL is expressed on the membrane of osteoblasts and bone marrow stromal cells and binds to and activates TNF family receptor RANK expressed on monocyte-macrophage lineage osteoclast precursors (3, 4). Upon binding to RANKL, RANK activates the intracellular signaling pathways including NF-κB, Erk, JNK, and NFATc1, and leads to osteoclast differentiation, activation, and survival (6, 7). The essential role of RANKL in normal bone turnover was further established by a series of knock-out mice, *i.e.* both RANKL- and RANK-deficient animals exhibited severe osteopetrosis because of the lack of osteoclast differentiation (8, 9), whereas the targeted disruption of osteoprotegerin, a natural inhibitor of RANKL, developed severe osteoporosis because of enhanced osteoclastogenesis (10).

Some transmembrane proteins are extracellularly cleaved and released into the surrounding environment. RANKL is also made as a membrane-bound protein, cleaved by some proteinases, and converted to the soluble RANKL (11, 12). This process, known as ectodomain shedding, has a diverse effect on a wide variety of membrane-bound proteins. For example, when TNF-α is cleaved by the TNF-α converting enzyme and released into the circulatory system, it exhibits strong systemic effects (13, 14). In contrast, the Fas ligand is a strong apoptosis inducer in its membrane-bound form, but the soluble Fas ligand has fewer effects on apoptosis induction (15).

Although some proteinases have been demonstrated to have the RANKL shedding activity, no definite RANKL sheddase(s) have yet been identified. TNF-α converting enzyme was reported to be a candidate of RANKL sheddase (11); however, a more recent study has shown that TNF-α converting enzyme had no apparent effect on the RANKL shedding and that the RANKL shedding in TNF-α converting enzyme-deficient cells was indistinguishable (16). A disintegrin and metalloproteinase domain family (ADAM)19 has also been reported to exhibit RANKL shedding activity (17), but embryonic fibroblasts from ADAM19 knock-out mice showed almost the same RANKL shedding activity as the cells from the wild type animals (16). Matrix metalloproteinase 14 (MMP14, also called the membrane-type 1 matrix metalloproteinase, MT1-MMP) can also cleave RANKL, although its cleavage site differs from that previously reported (16). These results suggest that there are other molecules implicated in RANKL shedding.

To identify molecules involved in the regulation of RANKL shedding, we developed a novel screening system, in which expression plasmids encoding secreted placental alkaline phosphatase (SEAP) fused with mouse C-terminally truncated RANKL (tRANKL-SEAP) were co-transfected with cDNA library pools of ST2 cells. Utilizing this screening system, we found that an alternatively spliced variant of $Ca^{2+}$-promoted Ras inactivator (CAPRI), ΔCAPRI led to an increase in the RANKL shedding.

## MATERIALS AND METHODS

*Reagents*—*p*-Nitrophenyl phosphate was purchased from Sigma-Aldrich. DNA polymerase, Pyrobest (for subcloning), was purchased from Takara biochemicals (Shiga, Japan), KOD plus (for reverse transcription-PCR) was from Toyobo (Osaka, Japan), and ionomycin was from Merck. Antibodies were purchased as follows: for the His tag, Erk, phosphorylated Erk, integrin-β and HSP-90 from Santa Cruz Biotechnology,

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[2] The abbreviations used are: TNF, tumor necrosis factor; Erk, extracellular signal-regulated kinase; JNK, c-Jun $NH_2$-terminal kinase; CAPRI, $Ca^{2+}$-promoted Ras inactivator; GAP, GTPase-activating protein; MMP14, matrix metalloproteinase 14; SEAP, secreted placental alkaline phosphatase; wt, wild type; Mek, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.