Caveolin-1 regulates osteoclastogenesis and bone metabolism in a sex-dependent manner

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Running title: Caveolin-1 regulates osteoclastogenesis

**Background:** Caveolin-1 plays important roles in the regulation of diverse cellular responses.  

**Results:** Caveolin-1 knockdown reduced osteoclastogenesis in vitro while caveolin-1 knockout resulted in an increased and decreased osteoclastogenesis in male and female mice, respectively.  

**Conclusion:** Regulation of osteoclastogenesis by caveolin-1 was dependent on sex.  

**Significance:** This indicates complicated, but critical, role of caveolin-1 in the regulation of bone metabolism.

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Abstract

Lipid raft microdomains have important roles in various cellular responses. Caveolae are a specialized type of lipid rafts that are stabilized by oligomers of caveolin proteins. Here we show that caveolin-1 (Cav-1) plays a crucial role in the regulation of osteoclastogenesis. We found that caveolin-1 was dramatically up-regulated by receptor activator of nuclear factor κB ligand (RANKL), the osteoclast differentiation factor. Knockdown of Cav-1 reduced osteoclastogenesis and induction of NFATc1, the master transcription factor for osteoclastogenesis, by RANKL. Consistent with in vitro results, injection of caveolin-1 siRNA onto mice calvariae showed reduction in RANKL-induced bone resorption and osteoclast formation. Moreover, Cav-1-/- female mice had higher bone volume and lower osteoclast number compared to wild type mice. However, Cav-1-/- male mice had both osteoclast and osteoblast numbers higher than wild type mice with no difference in bone volume. The sex dependency in the effect of Cav-1 deficiency was partly attributed to decreased RANK and increased cFms expression in osteoclast precursors of female and male mice, respectively. Taken together, these data demonstrate that Cav-1 has a complicated, but critical role, for osteoclastogenesis.

Introduction

Bone homeostasis is maintained by the balanced activity of bone-resorbing osteoclasts (OC) and bone-forming osteoblasts (OB) during bone remodeling, a physiological process of bone turnover. These two cell types are derived from different origin of stem cells via multiple steps governed by differentiation factors. The differentiation of OC from monocyte/macrophage lineage precursor cells requires two major cytokines, macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). M-CSF supports survival and proliferation of cells during osteoclastogenesis, while RANKL drives the differentiation program. RANKL interaction with its receptor RANK evokes recruitment of tumor necrosis factor receptor associated factor 6 to assemble signaling molecules that are required for the activation of mitogen-activated protein kinase signaling pathways (1,2). RANKL also stimulates the calcium signaling pathway and NFκB and c-Fos transcription factors (2-4). Ultimately, these signaling pathways are integrated by nuclear factor of activated T cells c1 (NFATc1), the transcription factor regarded as a master regulator of osteoclastogenesis, for the expression of osteoclast marker genes such as trarate-resistant acid phosphatase (TRAP) (5,6).

Lipid rafts are membrane microdomains which consist of cholesterol, glycosphingolipids, and glycosylphosphatidylinositol-anchored proteins (7,8). These microdomains have been implicated in regulation of various cellular functions. Especially, lipid rafts in the plasma membrane serve as organizing center for assembly of signaling molecules that respond to extracellular stimuli. Caveolae, a specialized type of lipid rafts, are 50- to 100 nm flask-shaped cell-surface membrane invagination (9). Like lipid rafts, caveolae have important roles in diverse cellular functions including endocytosis and signal transduction (10,11).

Caveolin, a component of caveolae together with cholesterol and sphingolipids, has three isoforms: Cav-1, Cav-2, and Cav-3 (10,12,13). Cav-1 and Cav-2 are present in various cell types, most abundant in epithelial cells, fibroblasts, adipocytes, and endothelial cells (12,14-17). Cav-3 expression is restricted to muscle cells (13). Cav-1 has been most extensively studied and suggested to be an essential component for the membrane microdomain stability and signal transduction. The receptors of which signaling is regulated by caveolae include G-protein coupled receptors, non-receptor tyrosine kinases, ion channels, Toll-like receptor 4, and T cell receptor (18-22). The relationship between caveolin and estrogen receptor alpha (ERα) signaling has also been a subject of great interest. While potentiation of ERα signaling by the interaction of Cav-1 with ERα was reported (23), other investigators found inhibition of estrogen signaling by the
interaction (24). As ERα is an important regulator of differentiation, activation, and survival of both osteoclasts and osteoblasts (25–32), critical involvement of Cav-1 in bone cell regulation may be projected.

We found that Cav-1 was greatly increased by RANKL during osteocalstogenesis. Cav-1 knockdown using siRNA evidently inhibited the differentiation of OC and the induction of c-Fos and NFATc1 by RANKL. However, osteoclastogenesis and bone metabolism was differentially affected by in vivo Cav-1 deficiency between male and female mice.

Method

Mice

Cav-1−/− mice in C57BL/6 background was purchased from Jackson Laboratory and backcrossed with wild type C57BL/6 for more than 6 generations. The heterogenic mice were mated and littermates were used for experiments. Animal experimental protocols were approved by the Committee on the Care and Use of Animals in Research at Seoul National University.

Reagents and antibodies

Anti-NFATc1 and anti-c-Fos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Cav-2 was purchased from Abcam (Cambridge, UK). All other antibodies were purchased from Cell Signaling Technology (Beverly, MA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Human soluble RANKL and M-CSF were from Pepro-Tech (Rocky Hill, NJ).

In vitro osteoclast differentiation

Bone marrow derived-macrophages (BMMs) were generated using long bones from 5-week-old female ICR mice and from 8-week-old WT and Cav-1−/− male and female mice. Flushed bone marrow cells were plated with α-MEM to remove adherent cells. Next day, non-adherent cells were transferred to new dishes and cultured in α-MEM with M-CSF (30 ng/ml) for 3 days. Cells at this stage (BMMs) were used for osteoclastogenesis by culturing with M-CSF (30 ng/ml) and RANKL (200 ng/ml) for 4 days.

RT-PCR and real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instruction and cDNA synthesis was carried out using SuperscriptII reverse transcriptase (Invitrogen). PCR was performed with target gene specific primers. Real-time PCR was performed with an ABI 7500 instrument (Life Technologies), for 40 cycles with SYBR Green Master Mix. Gene expression was calculated using the 2−ΔΔCT method and normalized to the level of β-actin. All samples analyzed in Fig. 1B were harvested at the same time (at day 3) and β-actin mRNA was stable in our experimental conditions.

Western blotting

Cells were washed with cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, proteinase inhibitor cocktail (Roche, Germany), 100 mM sodium varnade, 0.5 M sodium floride). After protein quantification, cell extracts were separated on polyacrylamide gel and transferred onto nitrocellulose membrane. After blocking with 5% skim-milk in TBS-T, membranes were incubated overnight with primary antibody at 4°C. Membranes were washed and incubated with HRP-conjugated secondary antibody, and the immune complexes developed were detected using enhanced chemiluminescence reagents.

In vitro TRAP staining

Cells were fixed in 3.7% formaldehyde solution and permeabilized with 0.1% Triton X-100. After washing with PBS, cells were stained using the Leukocyte Acid Phosphatase Assay Kit (Sigma, St. Louis, MO) following the manufacturer’s instruction. TRAP-positive multinuclear osteoclasts containing three or
more nuclei were counted under a light microscope.

In vitro resorption pit formation assay

BMMs were cultured on dentin slices for 9 days in the presence of M-CSF and RANKL. After removing the cells by sonication, dentin slices were stained with hematoxylin and observed under a light microscope.

Gene knockdown by small interfering RNA (siRNA)

Target-specific 21-mer nucleotide siRNA and control siRNA were purchased from Invitrogen. BMMs were transfected with siRNA (40 nM) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction. At 2 days after transfection, the expression level of protein or mRNA was determined.

Retroviral transduction

Mouse Cav-1 gene was cloned into pMX-IRES vector. Retroviral particles were packaged by transfecting Plat-E cells with DNA plasmids using Lipofectamine 2000 according to the manufacturer’s instructions. BMMs were infected with viral supernatants in the presence of polybrene (10 μg/ml) and M-CSF for 12 hr.

Confocal microscopy

Cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. Fixed cells were blocked with 1% BSA in PBS, incubated with primary antibody at room temperature for 1 hr and washed with PBS. Cells were then incubated with secondary antibody for 30 min and counterstained with 4’,6-diamidino-2-phenylindole. Cells were observed under a Zeiss LSM 5 PASCAL laser-scanning microscope (Carl Zeiss Microimaging GmbH, Goettingen, Germany) with an X400 objective (CApochromat /1.2 WCorr).

Histology and histomorphometry

Tibias from 8-week-old wild type (WT) and Cav-1<sup>-/-</sup> mice (n = 5 per group) were fixed in 4% paraformaldehyde and decalcified in 12% EDTA for 4 weeks. Then, tibias were dehydrated in 70% to 100% ethanol and embedded in paraffin. Sections of 5 μm thickness were used for hematoxylin/eosin (H&E) and TRAP stainings. For von Kossa staining, undecalcified femurs were embedded in methyl methacrylate and sectioned into 5 μm slices. Histomorphometric analysis was performed as described (33) using the Osteomex program (OsteoMetrics, Inc., GA).

Determination of C-terminal peptide of type I collagen (CTX-I) and N-terminal propeptide of type I collagen (PINP)

CTX-I levels in 8-week-old WT and Cav-1<sup>-/-</sup> mice serum were measured using CTX-I and PINP ELISA kits (Immunodiagnostic System, Boldon, UK) following manufacturer’s instruction.

Calcein-xylene orange double labeling

8-week-old WT and Cav-1<sup>-/-</sup> mice were injected intraperitoneally with calcein (25 mg/kg). After 7 days, 90 mg/kg xylene orange was injected. Mice were sacrificed at day 10 and femurs were fixed and embedded in methyl methacrylate. Using a LSM5 PASCAL laser scanning microscope (Zeiss), sectioned femurs (5 μm) were observed. The distance between calcein and xylene orange deposition was determined from five different regions of a image using 5 slides per group.

μCT analysis

Femurs of 8-week-old WT and Cav-1<sup>-/-</sup> mice were analyzed by micro-computed tomography (μCT) using SkyScan 1172 system (SkyScan, Aartselaar, Belgium; 40 Kv, 250 μA, 7.61 pixel size). Results were obtained with 1 mm thickness area of distal femurs, starting from 0.7 mm below the growth plate at thresholds minimum 70 and maximum 255. 3D images were constituted using the CT vol software.

In vivo calvariae bone resorption model

Specific or control siRNA (20 μM; 30 μl) were mixed with Lipofectamine 2000 (10 μl) and injected onto 5-week-old female mice (n = 5 per group) calvariae 3 times with 2-day intervals.
day after the first injection, collagen sheet soaked with RANKL (10 μg) or PBS was implanted into the center of calvariae. At day 6, mice calvariae were collected for TRAP-staining and μCT analyses (SkyScan; thresholds maximum 210 and minimum 100).

Statistical analysis
All in vitro experiments were repeated at least three times. All quantitative experiments were performed at least in triplicate. Student’s t test was used to determine the significance between two groups.

Results
Cav-1 expression was induced by RANKL during osteoclastogenesis
In a microarray analysis to discover new genes involved in the regulation of osteoclast differentiation, we found that the Cav-1 mRNA level was increased by RANKL (data not shown). We next analyzed the expression level of all isotypes of caveolin by real-time PCR. The mRNA level of Cav-1 was strongly increased at day 1 after RANKL treatment and maintained at high levels for 3 days during RANKL-stimulation (Fig. 1A). Cav-2 mRNA level was slightly increased at day 3 after RANKL treatment. RT-PCR analysis also showed the same expression pattern of caveolin gene family (Fig. 1B). Cav-3 mRNA was not detected. Next, we determined protein levels of Cav-1 and Cav-2 during RANKL-mediated osteoclastogenesis. As shown in Fig. 1C, both Cav-1 and Cav-2 proteins were induced by RANKL. The up-regulation of Cav-1 protein was earlier and stronger than Cav-2. Since caveolins are components of caveolae, a special microdomain in the plasma membrane, we investigated whether Cav-1 localizes to plasma membrane microdomains. Immunostaining with a Cav-1 specific antibody showed that Cav-1 expression was induced by RANKL in pre-fusion osteoclasts (pOC) and mature OC. Moreover, Cav-1 was localized with FITC-conjugated cholera toxin B subunit (CTxB) that stained plasma membrane microdomains (Fig. 1D). These results indicate that Cav-1 is induced by RANKL and may play a role as a component of membrane microdomains in pOC and mature OC.

RANKL-induced osteoclasogenesis was attenuated by Cav-1 silencing
To define the role of caveolin family during osteoclastogenesis, we designed siRNA oligonucleotides against Cav-1 and Cav-2. BMMs were transfected with scrambled or isotype-specific caveolin siRNA. Knockdown of Cav-1 strongly inhibited the formation of OC (Fig. 2A). The resorption of dentin slice was also reduced by Cav-1 knockdown (Fig. 2B). The induction of NFATc1 and c-Fos is essential for OC differentiation by RANK (2,3). The RANKL-induced NFATc1 and c-fos expression was attenuated by Cav-1 silencing in BMMs (Fig. 2C). It has been reported that the Cav-2 expression is regulated by the presence of Cav-1 (34). Consistent with the previous report, we observed that both mRNA and protein levels of Cav-2 were reduced in Cav-1 knockdown BMMs (Fig. 2D). To examine whether the decreased osteoclastogenesis from Cav-1 knockdown BMMs was influenced by the accompanying reduction in Cav-2 reduction, we directly silenced Cav-2 and assessed the osteoclastogenic potential. Unlike Cav-1 siRNA, Cav-2 siRNA did not alter formation of OC or induction of NFATc1 and c-Fos (Fig. 2E). These results suggest that Cav-1 is the major caveolin involved in the regulation of osteoclastogenesis.

Cav-1 knockdown reduced osteoclastogenesis and bone resorption in vivo
We next examined whether the anti-osteoclastogenic effect of Cav-1 siRNA could be confirmed under in vivo conditions. To this end, PBS- or RANKL-soaked collagen sponges were implanted onto mice calvariae that received injections of scrambled or Cav-1-targeted siRNA. In vivo knockdown was verified by RT-PCR (Fig. 3A). The RANKL treatment induced TRAP mRNA expression in vivo and this induction was significantly attenuated by Cav-1 silencing (Fig. 3A). When we stained the whole calvariae for TRAP activity, Cav-1 siRNA-injected mice showed reduced TRAP-positive
area (Fig. 3B). In agreement with this result, micro-computed tomography (μCT) analyses of the calvariae from Cav-1 siRNA-injected mice revealed lower level of bone resorption compared to control siRNA-injected mice in response to RANKL (Fig. 3C). Taken together, these results showed that Cav-1 silencing prevents RANKL-induced bone loss in mice calvariae by inhibiting OC formation.

**Cav-1 deficient female mice are osteopetrotic**

Next, we characterized the bone phenotype of Cav-1−/− mice. Femurs of 8-week-old female and male mice were subjected to μCT analyses. There was no statistically significant difference in trabecular bone volume of metaphyseal areas between WT and Cav-1−/− male mice (Fig. 4A). In contrast, Cav-1−/− female mice exhibited dramatically increased bone volume compared to wild type mice (Fig. 4A). Consistent with this result, Cav-1−/− female mice showed increased trabecular number and reduced trabecular separation. Trabecular thickness was not significantly different between WT and Cav-1−/− mice (Fig. 4B). For histological analysis, decalcified tibia sections were stained with hematoxylin/eosin and for TRAP activity. Stained bone slices revealed that osteoclast number was higher in Cav-1−/− male mice (Fig. 5A). BV/TV was increased in Cav-1−/− female mice, but not in Cav-1−/− male mice (Fig. 5A). All parameters related to OC [OC number (N.Oc/B.Pm), OC surface (Oc.S/BS), and eroded surface (ES/BS)] were significantly increased in Cav-1−/− male mice (Fig. 5B). In a striking contrast, female mice showed increased N.Oc/B.Pm, Oc.S/BS, and ES/BS in Cav-1−/− than in WT (Fig. 5B). For OB-related parameters, OB number (N.Ob/B.Pm) and OB surface (Ob.S/BS) were significantly increased in Cav-1−/− female mice (Fig. 5C). These OB parameters were not different between WT and Cav-1−/− female mice (Fig. 5C). Both CTX-I and PINP levels were increased in Cav-1−/− male mice (Fig. 5D). However, serum CTX-I level was lower in Cav-1−/− than in WT female mice and PINP was not different between WT and Cav-1−/− female mice (Fig. 5D). Accordingly, in vivo calcein/xylene orange labeling experiments showed increased bone formation rate (BFR/BS), mineral apposition rate (MAR), and mineralized surface (MS/BS) of Cav-1−/− male mice (Fig. 5E). Female mice did not show a statistically significant difference in BFR, MAR, and MS/BS between WT and Cav-1−/− (Fig. 5E).

**Cav-1−/− female BMMs, but not male BMMS, have defects in osteoclastogenesis in vitro**

As the number of OC on bone was observed to be affected by Cav-1 knockout in different ways between male and female mice, we next compared the osteoclastogenic potential of BMMs from male and female Cav-1−/− mice. In in vitro cultures, OC formation from BMMs of Cav-1−/− male mice was greater than that from WT male mice (Fig. 6A). In contrast, BMMs of Cav-1−/− female mice showed strong defect in RANKL-induced OC differentiation (Fig. 6B). The induction of NFATc1 was also attenuated in BMMs from Cav-1−/− female mice (data not shown). Whether this defect in osteoclastogenesis could be rescued by Cav-1 restoration was then tested. Cav-1−/− female BMMs were infected with control (pMX) or Cav-1 (pMX-Cav-1)-containing retroviruses. As shown in Fig. 6C, Cav-1 overexpression increased TRAP-positive OC formation. The RANKL induction of NFATc1 was also restored by Cav-1 overexpression. To investigate a potential mechanism for the gender difference in the effect of Cav-1 deficiency, we next determined the protein levels of cFms (the receptor for M-CSF) and RANK in Cav-1−/− BMMs. In cells from male mice, the basal cFms levels, both at mRNA and protein, were higher in Cav-1−/− BMMs than in WT BMMs (Fig. 6D, E). However, the expression pattern of RANK was not different between Cav-1−/− and WT cells (Fig. 6D, E). In female mice, the RANK level was increased during osteoclastogenesis from WT BMMs, which was not observed in Cav-1−/− BMMs (Fig. 6D, E). cFms expression was not altered both in Cav-1−/− and in WT cells from female mice (Fig. 6D, E). These data suggest that Cav-1 deficiency affected the property of OC precursor cells in a manner dependent on the sex of mice.
DISCUSSION

Caveolin has been implicated in many disorders including cancer, cardiac diseases, diabetes, and atherosclerosis (35-38). Our results obtained in the present study suggest a link between caveolin and bone metabolism, adding another disorder to the list. Cav-1, a major structural protein of caveolin, was selectively induced at a great extent among three caveolin isoforms by RANKL (Fig.1). Despite lower but constitutive presence of Cav-2 protein in OC precursors, in vitro knockdown of Cav-1 by using siRNA, but not that of Cav-2, led to a decreased potential of osteoclastogenesis (Fig. 2). These results suggest a specific role of Cav-1 for the regulation of OC differentiation. Results of these in vitro siRNA experiments performed with BMMs from female ICR mice are consistent with the osteoclastogenesis data obtained with cells from female Cav-1-/- mice (Fig. 6B). Therefore, Cav-1 plays a positive role in OC differentiation of female mice.

Radiological and histomorphometric analyses of Cav-1-/- mice in our study revealed that the regulation of bone cells, and consequently that of bone mass, was different between female and male mice. In 8-week-old Cav-1-/- female mice, femoral trabecular bone volume was significantly higher with lower number of OC in bone in comparison with WT control mice (Figs. 4 and 5). As OB number and activity (BFR and MAR) were not different between Cav-1-/- and WT (Fig. 5), the osteopetrotic phenotype of female Cav-1-/- mice could be attributed solely to decreased bone resorption by OC. Consistent with this, female Cav-1-/- mice had lower CTX-1 level in the blood than WT mice (Fig. 5). In a marked difference, male mice showed similar bone volume between Cav-1-/- and WT (Fig. 4). This indifference could be explained by a balanced stimulation of both OC and OB activities in Cav-1-/- male mice as evidenced by bone sections from Cav-1-/- mice displaying an increase in both OC and OB numbers and a higher BFR and MAR compared to those in WT mice (Fig. 5).

Some of our results are in agreement and disagreement with data shown in a couple of previous studies in which effects of Cav1 deficiency on bone were investigated (39,40). In the paper by Rubin et al. (39), distal femur metaphysis of 8-week-old Cav1-/- male mice showed increased trabecular BV/TV with an apparently paradoxical decrease in MAR compared to WT mice. This increase in BV/TV was postulated to be due to increased bone formation by OB at an earlier stage (5 week). As histological analyses were not shown in this paper, we do not know whether data on OC and OB numbers in femur sections of male mice were also different from our data. While it is not easy to answer the question that why the data of femur trabecular BV/TV and MAR are in discrepancy between our and Rubin’s studies, our µCT BV/TV results are consistent with histology and MAR data. In vitro formation of OC from marrow cells of male mice was modestly increased in the Rubin’s study, which is in agreement with our observation. As female mice bone phenotype was not analyzed in that study, we do not know whether the increased BV/TV of Cav-1-/- female mice found in our study is also in discrepancy with their study. In another paper by Hada et al. (40), in vitro osteoclastogenesis of precursor cells from Cav-1-/- male mice was not significantly different from WT cells unless Cav-2 was additionally muted by siRNA. In that study, the Cav-1/Cav-2 double deficient cells showed increased osteoclastogenesis, leading to a suggestion that Cav-1/Cav-2 complex may negatively regulate osteoclastogenesis. This result is also in disagreement with our data showing that Cav-1 deficiency was sufficient to increase osteoclastogenesis from male BMMs (Fig. 6A).

Our study clearly indicates that Cav-1 deficiency affected bone phenotype in a sex-dependent manner. What would be a potential mechanism for this difference between male and female mice in responding to Cav-1 gene deletion? Cav-1 has been suggested to regulate ER trafficking to cell membrane and estrogen-induced signaling processes (23,24). Estrogen has a proapoptotic effect on OC and ERα.
deletion abrogates this effect in the female but not in the male (41), suggesting more impact of ERα signaling in female OC. The expression levels of ERα and Cav-1 were similar between male and female OC in our study (data not shown). Therefore, it may be possible that Cav-1 deletion displayed the sex dichotomy in bone phenotype due to stronger dependency of estrogen signaling on Cav-1 in female. However, it is unreasonable to think that only a few factors are accountable for sex-dependent bone phenotype of Cav-1−/− mice. As Cav-1 is expressed in many cell types and more and more systemic factors are being discovered to influence bone cell regulation, it is likely that many factors that directly or indirectly affecting bone cell responses are changed in the Cav-1 general knockout mice used in our study. Nonetheless, it can be pinpointed that sex-dependency of Cav-1−/− bone phenotype is associated with cell-autonomous mechanisms.

As shown in Figure 6, BMMs from male and female mice had contrasting osteoclastogenesis potential in vitro and the expression patterns of c-Fms in BMMs and RANK in differentiating OC were dissimilar between males and females. Therefore, Cav-1 deficiency appears to drive changes in characteristics of OC precursors in different ways between males and females.

In summary, our results demonstrate the importance of Cav-1-mediated processes in OC differentiation and subsequently bone homeostasis. Additionally, this study arouses attention to the necessity of analyses of both sexes in studies on bone metabolism. Further investigations are required with bone cell type-specific Cav-1 knockouts to distinguish direct roles of Cav-1 in bone cells from indirect effects of Cav-1 deficiency on bone metabolism. Molecular details on how Cav-1 regulates differentiation of OC and OB are also to be unraveled.

References
1. Darnay, B. G., Ni, J., Moore, P. A., and Aggarwal, B. B. (1999) Activation of NF-κappaB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identification of a novel TRAF6 interaction motif. *J Biol Chem* **274**, 7724-7731
2. Takayanagi, H. (2005) Mechanistic insight into osteoclast differentiation in osteoimmunology. *J Mol Med (Berl)* **83**, 170-179
3. Grigoriadis, A. E., Wang, Z. Q., Cecchini, M. G., Hofstetter, W., Felix, R., Fleisch, H. A., and Wagner, E. F. (1994) c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* **266**, 443-448
4. Franzoso, G., Carlson, L., Xing, L., Poljak, L., Shores, E. W., Brown, K. D., Leonard, A., Tran, T., Boyce, B. F., and Siebenlist, U. (1997) Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev* **11**, 3482-3496
5. Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E. F., Mak, T. W., Kodama, T., and Taniuchi, T. (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell* **3**, 889-901
6. Minkin, C. (1982) Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. *Calcif Tissue Int* **34**, 285-290
7. Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31-39
8. Brown, D. A., and London, E. (1998) Functions of lipid rafts in biological membrane systems. *Annu Rev Cell Dev Biol* **14**, 111-136
9. Travis, J. (1993) Cell biologists explore 'tiny caves'. *Science* **262**, 1208-1209
10. Razani, B., Woodman, S. E., and Lisanti, M. P. (2002) Caveolae: from cell biology to animal physiology. *Pharmacol Rev* **54**, 431-467
11. Nabi, I. R., and Le, P. U. (2003) Caveolae raft-dependent endocytosis. *J Cell Biol* **161**, 673-677
12. Scherer, P. E., Lewis, R. Y., Volonte, D., Engelman, J. A., Galbiati, F., Couet, J., Kohut, D. S., van Donselaar, E., Peters, P., and Lisanti, M. P. (1997) Cell-type and tissue e-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo. *J Biol Chem* **272**, 29337-29346
13. Tang, Z., Scherer, P. E., Okamoto, T., Song, K., Chu, C., Kohtz, D. S., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996) Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *J Biol Chem* **271**, 2255-2261
14. Racine, C., Belanger, M., Hirabayashi, H., Boucher, M., Chakir, J., and Couet, J. (1999) Reduction of caveolin 1 gene expression in lung carcinoma cell lines. *Biochem Biophys Res Commun* **255**, 580-586
15. Fielding, C. J., Bist, A., and Fielding, P. E. (1997) Caveolin mRNA levels are up-regulated by free cholesterol and down-regulated by oxysterols in fibroblast monolayers. *Proc Natl Acad Sci U S A* **94**, 3753-3758
16. Nystrom, F. H., Chen, H., Cong, L. N., Li, Y., and Quon, M. J. (1999) Caveolin-1 interacts with the insulin receptor and can differentially modulate insulin signaling in trans and cells and rat adipose cells. *Mol Endocrinol* **13**, 2013-2024
17. Jiao, H., Zhang, Y., Yan, Z., Wang, Z. G., Liu, G., Minshall, R. D., Malik, A. B., and Hu, G. (2013) Caveolin-1 Tyr14 phosphorylation induces interaction with TLR4 in endothelial cells and mediates MyD88-dependent signaling and sepsis-induced lung inflammation. *J Immunol* **191**, 6191-6199
18. Ostrom, R. S., and Insel, P. A. (2004) The evolving role of lipid rafts and caveolae in N G protein-coupled receptor signaling: implications for molecular pharmacology. *Br J Pharmacol* **143**, 235-245
19. Buitrago, C., and Boland, R. (2010) Caveolae and caveolin-1 are implicated in alapha, 25(OH)2-vitamin D3-dependent modulation of Src, MAPK cascades and VDR localization in in skeletal muscle cells. *J Steroid Biochem Mol Biol* **121**, 169-175
20. Kwiatek, A. M., Minshall, R. D., Cool, D. R., Skidgel, R. A., Malik, A. B., and Tiruppathi, C. (2006) Caveolin-1 regulates store-operated Ca2+ influx by binding of its sc affolding domain to transient receptor potential channel-1 in endothelial cells. *Mol Pharmacol* **70**, 1174-1183
21. Mirza, M. K., Yuan, J., Gao, X. P., Garreau, S., Brovkovich, V., Malik, A. B., Tiruppathi, C., and Zhao, Y. Y. (2010) Caveolin-1 deficiency dampens Toll-like receptor 4 signaling through eNOS activation. *Am J Pathol* **176**, 2344-2351
22. Tomassian, T., Humphries, L. A., Liu, S. D., Silva, O., Brooks, D. G., and Miceli, M. C. (2011) Caveolin-1 orchestrates TCR synaptic polarity, signal specificity, and function in CD8 T cells. *J Immunol* **187**, 2993-3002
23. Schlegel, A., Wang, C., Katzenellenbogen, B. S., Pestell, R. G., and Lisanti, M. P. (1999) Caveolin-1 potentiates estrogen receptor alpha (ERalpha) signaling. caveolin-1 drives ligand-independent nuclear translocation and activation of ERalpha. *J Biol Chem* **274**, 33551-33556
24. Razandi, M., Oh, P., Pedram, A., Schnitzer, J., and Levin, E. R. (2002) ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. *Mol Endocrinol* **16**, 100-115
25. Srivastava, S., Toraldo, G., Weitzmann, M. N., Cenci, S., Ross, F. P., and Pacifici, R. (2001) Estrogen decreases osteoclast formation by down-regulating receptor activator
of NF-kappa B ligand (RANKL)-induced JNK activation. *J Biol Chem* **276**, 8836-8840

26. Nakamura, T., Imai, Y., Matsumoto, T., Sato, S., Takeuchi, K., Igarashi, K., Harada, Y., Azuma, Y., Krust, A., Yamamoto, Y., Nishina, H., Takeda, S., Takayanagi, H., Mettger, D., Kanno, J., Takaoka, K., Martin, T. J., Chambon, P., and Kato, S. (2007) Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. *Cell* **130**, 811-823

27. Krum, S. A., Miranda-Carboni, G. A., Hauschka, P. V., Carroll, J. S., Lane, T. F., Federman, L. P., and Brown, M. (2008) Estrogen protects bones by inducing Fas ligand in osteoclasts to regulate osteoclast survival. *EMBO J* **27**, 535-545

28. Saintier, D., Khanine, V., Uzan, B., Ea, H. K., de Vernejoul, M. C., and Cohen-Solal, M. E. (2006) Estradiol inhibits adhesion and promotes apoptosis in murine osteoclasts in vitro. *J Steroid Biochem Mol Biol* **99**, 165-173

29. Almeida, M., Martin-Millan, M., Ambrogini, E., Bradsher, R., 3rd, Han, L., Chen, X. D., Roberson, P. K., Weinstein, R. S., O’Brien, C. A., Jilka, R. L., and Manolagas, S. C. (2010) Estrogens attenuate oxidative stress and the differentiation and apoptosis of osteoblasts by DNA-binding-independent actions of the ERalpha. *J Bone Miner Res* **25**, 769-781

30. Chow, J., Tobias, J. H., Colston, K. W., and Chambers, T. J. (1992) Estrogen maintenance of trabecular bone volume in rats not only by suppression of bone resorption but also by stimulation of bone formation. *J Clin Invest* **89**, 74-78

31. Qu, Q., Peralta-Heape, M., Kapanen, A., Dahllund, J., Salo, J., Vaananen, H. K., and Harkonen, P. (1998) Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. *Bone* **22**, 201-209

32. Chang, E. J., Ha, J., Oerlemans, F., Lee, Y. J., Lee, S. W., Ryu, J., Kim, H. J., Lee, Y., Kim, H. M., Choi, J. Y., Kim, J. Y., Shin, C. S., Pak, Y. K., Tanaka, S., Wieringa, B., Lee, Z. H., and Kim, H. H. (2008) Brain-type creatine kinase has a crucial role in osteoclast-mediated bone resorption. *Nat Med* **14**, 966-972

33. Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F. C., Schedl, A., Haller, H., and Kurzchalia, T. V. (2001) Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* **293**, 2449-2452

34. Frank, P. G., Lee, H., Park, D. S., Tandon, N. N., Scherer, P. E., and Lisanti, M. P. (2004) Genetic ablation of caveolin-1 confers protection against atherosclerosis. *Arterioscler Thromb Vasc Biol* **24**, 98-105

35. Schwencke, C., Braun-Dullaeus, R. C., Wunderlich, C., and Strasser, R. H. (2006) Caveolae and caveolin-1: novel potential targets for the treatment of cardiovascular disease. *Curr Pharm Des* **13**, 1761-1769

36. Rubin, J., Schwartz, Z., Boyan, B. D., Fan, X., Case, N., Sen, B., Drab, M., Smith, D., Aleman, M., Wong, K. L., Yao, H., Jo, H., and Gross, T. S. (2007) Caveolin-1 knockout mice have increased bone size and stiffness. *J Bone Miner Res* **22**, 1408-1418

37. Hada, N., Okayasu, M., Ito, J., Nakayachi, M., Hayashida, C., Kaneda, T., Uchida, N.,
Muramatsu, T., Koike, C., Masuhara, M., Sato, T., and Hakeda, Y. (2012) Receptor activator of NF-kappaB ligand-dependent expression of caveolin-1 in osteoclast precursors, and high dependency of osteoclastogenesis on exogenous lipoprotein. *Bone* **50**, 226-236

Kameda, T., Mano, H., Yuasa, T., Mori, Y., Miyazawa, K., Shikawa, M., Nakamaru, Y., Hiroi, E., Hiura, K., Kameda, A., Yang, N. N., Hakeda, Y., and Kumegawa, M. (1997) Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. *J Exp Med* **186**, 489-495

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The abbreviations used are: Cav-1, caveolin-1; OC, osteoclast; OB, osteoblast; BMM, bone marrow-derived macrophage; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor κB ligand; NFATc1, nuclear factor of activated T cells c1; c-Fos, FBJ murine osteosarcoma viral oncogene homolog; TRAP, tartrate-resistant acid phosphatase; ER, estrogen receptor.

**Figure legends**

Figure 1. Cav-1 expression was elevated during RANKL-induced OC differentiation in female BMMs. (A) BMMs were cultured with M-CSF (30 ng/ml) and RANKL (200 ng/ml) for the indicated days. mRNA levels of Cav-1 and Cav-2 were analyzed by real-time PCR. (B) BMMs were treated with M-CSF (30 ng/ml) in the presence or absence RANKL (200 ng/ml) for the indicated days. mRNA expression of Cav-1, Cav-2 and Cav-3 was analyzed by RT-PCR. (C) BMMs were cultured in 6-well plates with M-CSF (30 ng/ml) and RANKL (200 ng/ml) for 3 days. Protein levels of Cav-1 and Cav-2 were determined by Western blotting. (D) BMMs were cultured with M-CSF (30 ng/ml) and RANKL (200 ng/ml) for 2 (pOC, pre-fusion osteoclasts) or 4 days (OC, osteoclasts). Cells were immunostained using an antibody against Cav-1 followed by Cy3-conjugated secondary antibody. Membrane was stained with FITC-conjugated cholera toxin B subunit (CTxB). DIC, differential interference contrast image; 2nd Ab, cells stained with secondary antibody only.

Figure 2. Cav-1 knockdown in female BMMs inhibited RANKL-induced TRAP-positive OC formation. (A) Cav-1 knockdown was performed in BMMs by siRNA transfection. Cells were further incubated with M-CSF (30 ng/ml) and RANKL (200 ng/ml) for 4 days and stained for TRAP activity. (B) BMMs transfected with siRNA were plated on dentin slices and cultured with M-CSF (30 ng/ml) and RANKL (200 ng/ml) for 9 days. The resorption pits were visualized by hematoxylin staining after removing the cells. The resorbed area was measured by densitometry using Image J. (C) BMMs transfected with siRNA were cultured with M-CSF (30 ng/ml) and RANKL (200 ng/ml) for the indicated days. NFATc1 and c-Fos levels were determined by Western blotting. (D) Cav-1 or Cav-2 silenced BMMs were further incubated with M-CSF (30 ng/ml) and RANKL (200 ng/ml) for 2 days. Protein and mRNA levels of Cav-1 and Cav-2 were analyzed by Western blotting and RT-PCR. (E) Cav-2 silenced BMMs were incubated with M-CSF (30 ng/ml) and RANKL (200 ng/ml). After 4 days, cells were subjected to TRAP staining. After 2 days, protein levels of NFATc1, c-Fos, and Cav-2 were determined by Western blotting. *, p < 0.05 as compared to controls. Scr, control siRNA.

Figure 3. In vivo Cav-1 knockdown suppressed osteoclastogenesis and bone resorption. Control or Cav-1 siRNA was injected onto calvariae of 5-week-old female mice, followed by implantation of a
collagen sheet soaked with PBS or RANKL. At day 6, mice were sacrificed. (A) Calvarial tissues were analyzed by RT-PCR. (B) Mice calvariae were stained for TRAP. TRAP+ area was measured by densitometry using Image J. (C) Calvariae were subjected to μCT analysis. 3D-images are shown. The resorbed area was measured by densitometry using Image J. n = 5 per group. *, p < 0.005 as compared to controls. Scr, control siRNA; Cav-1, Cav-1 siRNA, The red box indicates the analyzed area.

Figure 4. Trabecular bone volume was increased in Cav-1−/− female mice. (A) 8-week-old male and female mice femurs were subjected to μCT analyses, and representative 3D-images of femurs are shown. (B) Quantification data of trabecular bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) are presented. n = 5 per group. *, p < 0.05 ; **, p < 0.005 as compared to WT.

Figure 5. Bone formation was increased in male but not Cav-1−/− female mice. (A-C) Sections of tibia were stained with hematoxylin/eosin and for TRAP activity. (A) Representative images of stained sections are shown. Bone volume/trabecular bone (BV/TV) was quantititated from the tibia sections. (B) Quantitative analysis using the sections include: OC number/bone perimeter (N.Oc/B.Pm); OC surface/bone surface (Os.S/BS); eroded surface/bone surface (Es./BS). (C) OB number/bone perimeter (N.Ob/B.Pm) and OB surface/bone surface (Ob.S/BS) are shown. (D) Serum CTX-I and PINP levels were measured by ELISA. (E) WT and Cav-1−/− mice were intraperitoneally injected with calcein followed by xylene orange with a 7-day interval. Tibia were isolated at 3 days after final injection for von-Kossa stain and confocal microscopy imaging. Quantitative analysis using the sections include: bone formation rate/mineralized surface (BFR/MS), mineral apposition rate (MAR), and mineralized surface/bone surface (MS/BS). Fluorescence labels are indicated by arrows. n = 5 per group. *, p < 0.05 ; **, p < 0.005 as compared to WT.

Figure 6. In vitro osteoclastogenesis from BMMs was different between male and female Cav-1−/− mice. (A) Male WT and Cav-1−/− BMMs were treated with M-CSF (30 ng/ml) and RANKL (200 ng/ml) for 4 days and stained for TRAP activity. (B) Female WT and Cav-1−/− BMMs were treated with M-CSF (30 ng/ml) and RANKL (indicated dose) for 4 days and stained for TRAP activity. (C) Female Cav-1−/− BMMs were infected with empty vector (pMX) or Cav-1 (pMX-Cav-1) retroviruses. Cells were further cultured with RANKL for 4 days and stained for TRAP activity. At 2 days after RANKL treatment, NFATc1 and Cav-1 levels were determined by Western blotting. (D, E) WT and Cav-1−/− BMMs were treated with M-CSF (30 ng/ml) and RANKL (200 ng/ml). Protein and mRNA levels of cFms and RANK were determined by Western blotting and RT-PCR. *, p < 0.05 as compared to WT.
| Name  | 5’-3’ | Sequences                      |
|-------|-------|--------------------------------|
| RANK  | sense | AGAAGACGGTGCTGGAGTCT           |
|       | antisense | TAGGAGCAGTGGAACCAGTGG         |
| cFms  | sense | CTTCACCTCCGTTGTTGTTG          |
|       | antisense | GCGCACCTTGGTACTTCCGG         |
| TRAP  | sense | CGACCATTGTGTAGCCACATACCG      |
|       | antisense | TCGTCCTGAAGAFACTGCAAGT     |
| Cav-1 | sense | TATGACGCGCACAACCAAGGA          |
|       | antisense | GCCCAGATGTGCAGGAAGGA        |
| Cav-2 | sense | CGGGATCTCCACCGCTCAA           |
|       | antisense | TACCCGCAATGAAGGCAAG         |
| Cav-3 | sense | TGGAAGCTCGGATCATACAG          |
|       | antisense | AGATGTGGCTGATGCACTGG        |
| β-actin | sense | TCTGGCACCACACCACCTTCTAC       |
|       | antisense | TACGACCAGAGGCACTACAGG     |
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5 (A-D)
Figure 5 (E)
Figure 6 (A-C)
Figure 6 (D-E)
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