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FAST TRACKS

JunD Suppresses Bone Formation and Contributes to Low Bone Mass Induced by Estrogen Depletion

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Abstract JunD is an activator protein-1 (AP-1) component though its function in skeletal system is still not fully understood. To elucidate the role of JunD in the regulation of bone metabolism, we analyzed JunD-deficient mice. JunD deficiency significantly increased bone mass and trabecular number. This bone mass enhancement was due to JunD deficiency-induced increase in bone formation activities in vivo. Such augmentation of bone formation was associated with simultaneous increase in bone resorption while the former was dominant over the latter as accumulation of bone mass occurred in JunD-deficient mice. In a pathological condition relevant to postmenopausal osteoporosis, ovariectomy reduced bone mass in wild type (WT) mice as known before. Interestingly, JunD deficiency suppressed ovariectomy-induced increase in bone resorption and kept high bone mass. In addition, JunD deficiency also enhanced new bone formation after bone marrow ablation. Examination of molecular bases for these observations revealed that JunD deficiency enhanced expression levels of *c-jun, fra-1*, and *fra-2* in bone in conjunction with elevated expression levels of *runx2*, *type I collagen*, and *osteocalcin*. Thus, JunD is involved in estrogen depletion-induced osteopenia via its action to suppress bone formation and to enhance bone resorption. J. Cell. Biochem. 103: 1037–1045, 2008.

Key words: activator protein-1; JunD; ovariectomy; osteoblast; osteoclast

Osteoporosis is one of the most crucial disorders in advanced countries as it increases the risk of fractures that result in loss of QOL and threaten life in highly aged patients. Depletion of estrogen causes menopause followed by rapid bone loss. Though this phenomenon is observed regardless of mammalian species, underlying mechanisms and molecular bases have not yet been fully understood. Bone remodeling is maintained based on two major activities including bone formation by osteoblasts and resorption by osteoclasts [Wagner and Karsenty, 2001; Karsenty and Wagner, 2002]. Menopause or surgical removal of ovaries results in negative balance in bone remodeling in that estrogen depletion enhances the levels of bone resorption which would exceed bone formation to lead to bone loss.

Activator protein-1 (AP-1) is a transcription factor complex composed of Jun family proteins (c-Jun, JunB, and JunD) that form homodimers or heterodimers with Fos family proteins (c-Fos, Fra-1, Fra-2, and FosB) [Wagner, 2002;

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Eferl and Wagner, 2003]. Several members of AP-1 family proteins are known to be involved in bone metabolism in vivo. Mice overexpressing or lacking c-Fos reveal osteosarcoma or osteopetrosis respectively [Grigoriadis et al., 1994; Wagner, 2002]. Fra-1 transgenic mice show osteosclerosis due to increase in the number of osteoblasts [Jochum et al., 2000]. c-Jun deficiency leads to embryonic lethality [Eferl et al., 1999; Schorpp-Kistner et al., 1999] while chondrocyte-specific c-Jun deficiency results in malformations of axial skeleton due to inhibition of notochord and intervertebral disc formation [Behrens et al., 2003]. JunB deficiency also results in embryonic lethality and osteopenia due to cell-autonomous defects in osteoblasts and osteoclasts [Kenner et al., 2004].

However, functions of JunD in skeletons in in vivo have been unknown. We therefore examined JunD-deficient mice. We found that JunD deficiency increased bone volume and kept high bone mass even after estrogen depletion. Thus, JunD plays a critical role in the determination osteopenia due to estrogen depletion.

MATERIALS AND METHODS

Animals

JunD knockout mice were prepared as described previously [Thepot et al., 2000]. JunD knockout mice and wild type (WT) littermates in a C57BL/6 $\times$ 129/SV mixed background were used. All animal experiments were approved by the Animal Welfare Committee of Tokyo Medical and Dental University.

CT Analysis of Bone

Femora and tibiae were fixed in PBS-buffered 4% paraformaldehyde for 24–72 h, and then stored in 70% ethanol at 4°C. These bones were subjected to 3D-μCT analyses (Scan-Xmate-E090; Comscan Tecno Co., Ltd., Sagamihara, Japan) or 2D-μCT analyses (Musashi, Nittetsu-ELEX Co., Osaka, Japan). Trabecular bone volume was measured in a region between 0.2 and 0.6 mm away from the growth plate. Bone volume was quantified by using software, Tri/3D-Bon (Ratoc System Engineering Co., Ltd., Tokyo, Japan) or Luzex-F automated image analysis system (Nireco, Tokyo, Japan).

Ovariectomy (OVX) Model

Ten-week-old female mice were randomly assigned into sham operation and OVX groups. WT and JunD KO mice were ovariectomized or sham-operated, and sacrificed after 2 weeks (WT sham $n = 8$, WT OVX $n = 9$, KO Sham $n = 10$, KO OVX $n = 10$). The mice were injected with 4 mg/kg calcein 7 and 2 days before sacrifice.

Bone Marrow Ablation Model

Nine-week-old male JunD KO ($n = 5$) and WT ($n = 5$) littermates were used for the experiments. A hole was made in the intercondylar region of right femur using a 26-gauge needle and bone marrow was removed using dental files (K-file #25–#55). X-ray pictures were taken to confirm the insertion of dental file. The left femur was used as an internal control. Animals were sacrificed after 10 days. Femora were subjected to μCT analyses (Scan-Xmate-E090; Comscan Tecno Co., Ltd., Sagamihara Japan), and the levels of newly formed bone in a columnar area of ϕ0.56 mm $\times$ 1.0 mm (exact area of insertion of the file) were quantified.

Histomorphometric Analysis of Bone

Femora and tibiae were fixed in PBS-buffered 4% paraformaldehyde for 24–72 h, and then stored in 70% ethanol at 4°C. For undecalcified section, right femora were embedded in methyl methacrylate (MMA). The sections were used to examine mineral apposition rate (MAR), mineralizing surface per bone surface (MS/BS), and bone formation rate (BFR) in a square area of 0.85 mm^2 which was 0.2 mm away from the growth plate. For decalcified sections, the tibiae were placed in 20% EDTA for 7 days, embedded in paraffin, and 3 μm thick sections were prepared. The sections were stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells attached to bone were counted as osteoclasts to obtain the parameters including osteoclast number per bone surface (N.Oc/BS) and osteoclast surface per bone surface (Oc.S/BS).

Real-Time PCR Analysis

Bone marrow of femora was flashed out and RNA was isolated using TRIzol reagent (Invitrogen). Real-time PCR was performed using 1 μg total RNA, oligo (dT) 12–18 primers, and SuperScriptII trascriptase (Invitrogen).

Quantitative real-time PCR analysis was carried out using iCycler (Bio-Rad) and iQ5 data analyzing software. For PCR reactions, iQ SYBR Green Supermix was used. The primers for real-time PCR were as follows: *gapdh*, forward, 5′-AGA AGG TGG TGA AGC AGG CAT C-3′, reverse, 5′-CGA AGG TGG AAG AGT GGG AGT TG-3′; *junD*, forward, 5′-GCC TCA CGC TCT GCC TTT CC-3′, reverse, 5′-CAC ACT CAA CAC GCA ACC AAC G-3′; *runx2*, forward, 5′-TGG CTT GGG TTT CAG GTT AGG G-3′, reverse, 5′-TCG GTT TCT TAG GGT CTT GGA GTG-3′; *col1a1*, forward, 5′-CTG ACT GGA AGA GCG GAG AG-3′, reverse, 5′-GCA CAG ACG GCT GAG TAG G-3′; *ocn*, forward, 5′-CAA GCA GGA GGG CAA TAA GGT AG-3′, reverse, 5′-CTC GTC ACA AGC AGG GTT AAG C-3′; *c-jun*, forward, 5′-AGT CCC TTC TCC CGC CTT CC-3′, reverse, 5′-GGT AGC CGC TCG CCT ATT TCC-3′; *fra-1*, forward, 5′-GAG ACC GAC AAA TTG GAG GA-3′, reverse, 5′-CTC CTT CTG GGA TTT TGC AG-3′; and *fra-2*, forward, 5′-TCC AAG TTG GGT CAC AAA CA-3′, reverse, 5′-ACG TGT ACA AAG CCC TCA CC-3′.

Statistical Evaluation

The results were presented as mean values ±SD. Statistical analysis was performed based on Student's *t*-test. When the number of experimental groups exceeded three, we used single-factor analysis of variance (ANOVA) with a Tukey test for post-hoc comparisons. *P*-values less than 0.05 (*) or 0.01 (**) were considered to be statistically significant.

RESULTS

To examine the effect of JunD deficiency on morphology of bone, 3D-μCT analysis was conducted. The 3D pictures revealed that crowdedness of trabecular bone in JunD-deficient (KO) mice was more than that in WT mice (Fig. 1A). Quantification revealed that JunD deficiency enhanced the fractional bone volume (BV/TV) (Fig. 1B). Elemental analysis indicated that JunD deficiency increased 3D trabecular number (Fig. 1C) and decreased trabecular separation (Fig. 1D). These observations indicate that JunD deficiency enhances basal levels of bone mass.

To identify the mode of alteration in metabolic activities underlying JunD deficiency-induced increase in bone mass, bone formation parameters were examined based on calcein double labeling (Fig. 1E). JunD deficiency enhanced the levels of bone formation rate (BFR) (Fig. 1F). As bases for this, mineralizing surface per bone surface (MS/BS) was increased by JunD deficiency (Fig. 1G). Mineral apposition rate (MAR) tended to increase upon JunD deficiency though the difference was not statistically significant (data not shown). Thus, JunD deficiency enhances bone formation activity in vivo.

As bone mass is determined based on both osteoblastic activities and osteoclastic activities, histological sections were also subjected to analyses of osteoclasts. TRAP-positive osteoclasts covered widely the surface of secondary trabecular bone in JunD KO mice compared to WT (Fig. 1H). Quantification of TRAP-positive cell area revealed that JunD deficiency increased the osteoclast surface (Oc.S/BS) (Fig. 1I) and tended to increase the levels of the osteoclast number per bone surface (N.Oc/BS) though the difference was not statistically significant (data not shown). These results indicate that JunD deficiency also enhances basal levels of bone resorption. As final bone mass levels were enhanced by JunD deficiency, enhancement on bone formation was considered to exceed enhancement in bone resorption. Thus, JunD suppresses bone formation more than its suppression on bone resorption.

Estrogen depletion reduces bone mass in postmenopausal women while the full spectrum of molecules involved in this process has not yet been known. We therefore examined if JunD suppression of the basal levels of bone volume may have any relevance to the low bone mass state in the skeleton after estrogen depletion. Analysis of μCT images revealed that estrogen depletion due to ovariectomy (OVX) resulted in sparsity in trabecular bone patterning in WT mice (Fig. 2A). In JunD KO mice, such changes in the trabecular patterning induced by ovariectomy were not observed (Fig. 2A). Quantification of the trabecular bone mass revealed that ovariectomy caused reduction in fractional bone volume in WT (Fig. 2B). In contrast, JunD deficiency suppressed such ovariectomy-induced reduction in bone volume (Fig. 2B). Importantly, JunD-deficient mice still kept the high bone mass phenotype even after ovariectomy (Fig. 2B). As verification of estrogen deficiency in JunD KO mice, ovariectomy similarly reduced the weight of uterus in WT and JunD KO mice similarly (Fig. 2C).

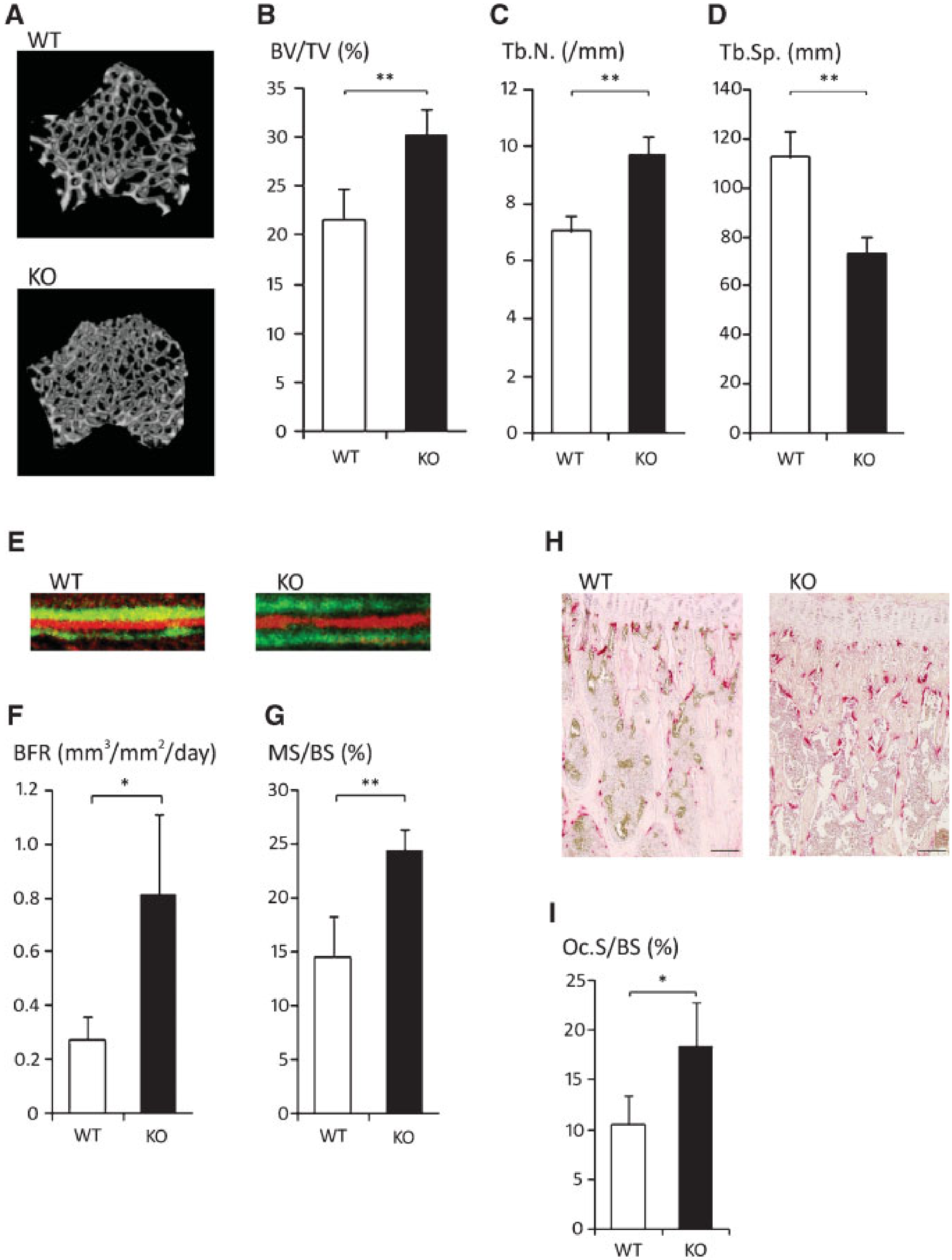

Fig. 1. JunD deficiency enhances the levels of basal bone volume. Micro-CT (μCT) pictures of trabecular bone of the metaphyseal region of femora in wild type (WT) or JunD-deficient (KO) mice. Trabecular bone patterns in JunD KO were more crowded than those of in WT mice (**A**). Quantification of the bone volume in femoral μCT pictures is shown in A (**B**). Fractional bone volume per tissue volume (BV/TV) was obtained based on the analyses of WT (n = 8) and JunD KO (n = 10) mice. 3D-μCT analysis of trabecular number (**C**) and trabecular separation (**D**) of WT and KO mice was conducted. Bone formation parameters were measured in the femora of WT (n = 4) and JunD KO (n = 4) mice (**E**). Calcein was injected as described in Materials and Methods. Undecalcified sections were subjected to confocal microscopy to obtain bone formation rate (BFR) (**F**), and mineralizing surface per bone surface (MS/BS) (**G**). Bone resorption parameters were obtained based on the analyses of the TRAP staining of bone section (**H**). Osteoclast surface per bone surface (Oc.S/BS) (**I**) in WT (n = 4) and JunD KO (n = 4) mice were obtained (**I**). $^*P < 0.05$, $^{**}P < 0.01$. Scale bar in H, 100 μm.

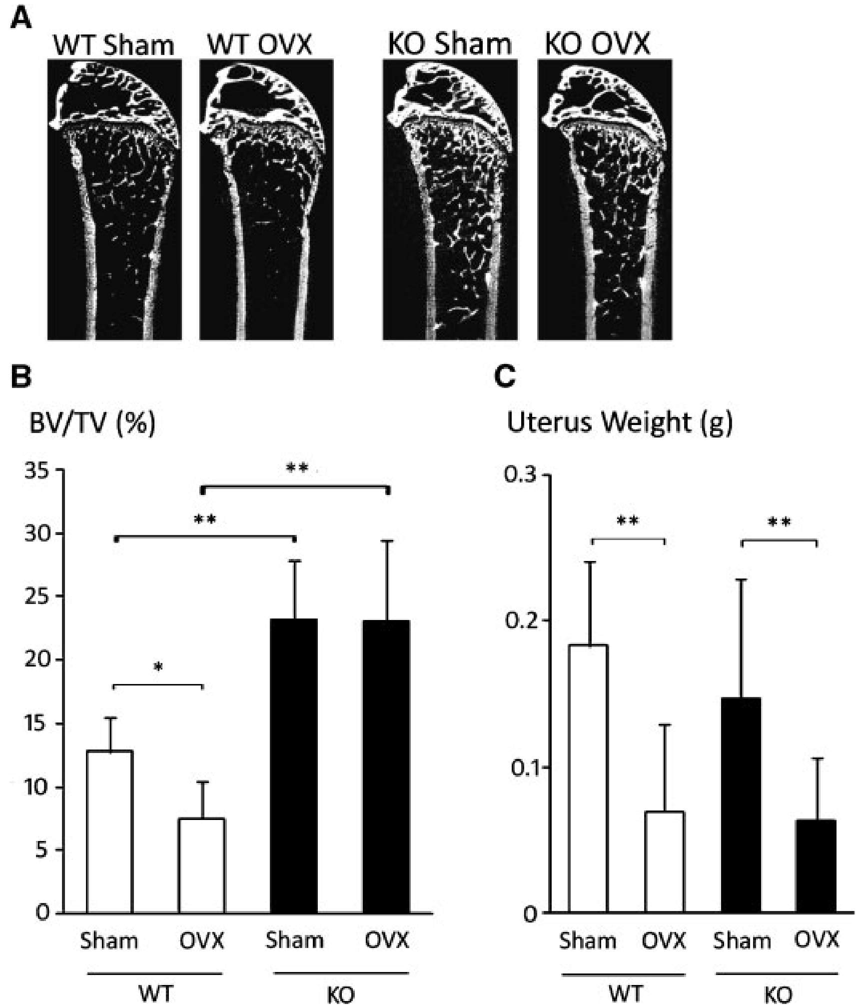

Fig. 2. JunD contributes to the low bone mass levels induced after estrogen depletion. WT or JunD KO mice were either ovariectomized (OVX) or sham-operated (Sham) (**A**). 2D-μCT pictures of trabecular bone were taken using WT or JunD KO femora. Trabecular bone patterns became sparse in WT after OVX, but those in JunD KO were similar regardless of OVX. Bone volume per tissue volume (BV/TV) in the secondary trabecular bone of the WT and JunD KO femora was obtained (**B**). WT Sham (n = 4), WT OVX (n = 4), KO Sham (n = 4), and KO OVX (n = 4). Uterus weight of sham and OVX mice was measured (**C**). $^{*}P < 0.05$, $^{**}P < 0.01$.

Thus, JunD deficiency specifically suppressed bone loss (not weight loss in uteri) induced by estrogen depletion.

To elucidate how JunD deficiency suppressed ovariectomy-induced bone loss and kept the high bone mass levels even after ovariectomy, bone formation parameters were examined in ovariectomized mice. JunD deficiency increased the width of calcein labeling in ovariectomized mice compared to that in ovariectomized WT mice (Fig. 3A). Quantification revealed that JunD deficiency enhanced the levels of BFR in ovariectomized mice compared to ovariectomized WT mice (Fig. 3B). JunD deficiency also enhanced the levels of MB/BS (Fig. 3C) and those of MAR (Fig. 3D). Thus, JunD deficiency enhanced bone formation activity in mice even under estrogen-depleted condition.

In WT mice, ovariectomy increased osteoclast number as well as osteoclast surface as known before (data not shown). As JunD deficiency increased the levels of osteoclast surface in intact mice, we assumed that JunD deficiency may exacerbate ovariectomy-induced bone resorption. Surprisingly, in the background of JunD deficiency, ovariectomy-induced increase in the levels of osteoclast parameters was no longer observed (Fig. 3E,F). Thus, JunD deficiency suppresses ovariectomy-induced bone loss via its effects on both bone formation side (enhancement) as well as bone resorption side (suppression).

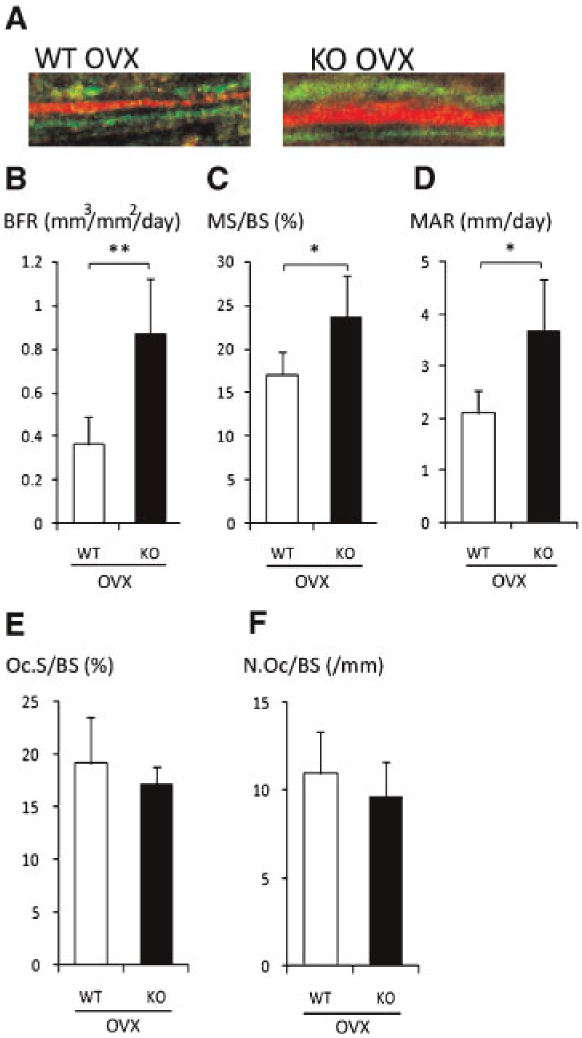

Fig. 3. JunD deficiency enhances bone formation activity even under estrogen-depleted condition. Bone formation parameters were obtained using femora of ovariectomized WT (n = 4) and JunD KO (n = 4) mice (**A**). Calcein was injected as described in Material and Methods. Undecalcified sections were subjected to confocal microscopy to obtain BFR (**B**), MS/BS (**C**), and mineral apposition rate (MAR) (**D**). TRAP-positive cells were examined in bone of the ovariectomized mice. Osteoclast surface per bone surface (Oc.S/BS) (**E**), number of osteoclast per bone surface (N.Oc/BS) (**F**) in WT (n = 4) and JunD KO (n = 4) mice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

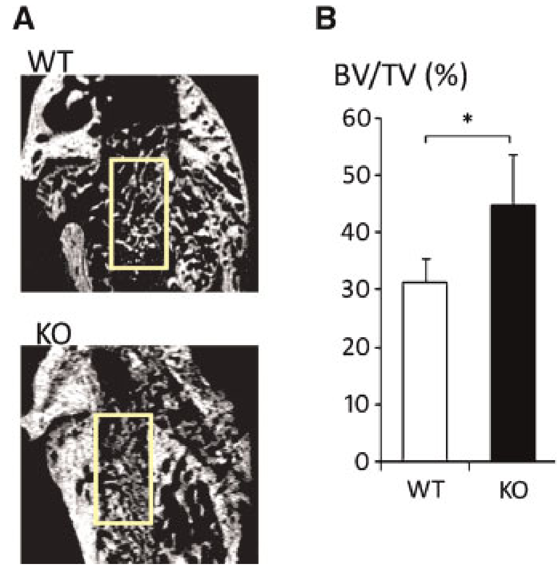

Fig. 4. JunD deficiency enhances the levels of newly formed bone during bone repair after bone marrow ablation. 2D-μCT pictures show newly formed bone 10 days after bone marrow ablation in the bone marrow cavity of femur in WT (n = 5) and JunD KO (n = 5) mice (**A**). BV/TV in the ablated area was measured (**B**). $*P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

As JunD deficiency enhanced bone formation even under high turnover conditions due to estrogen depletion, we wondered whether JunD deficiency may potentiate bone formation activity which is already triggered during repair process after injury. Bone marrow ablation model provides consistent and rapid bone repair events via bone formation after injury in vivo. In WT mice, newly formed bone was observed within 10 days in the ablated region of bone marrow cavity. JunD deficiency enhanced the levels of newly formed bone in the ablated region (Fig. 4A,B). These observations revealed that JunD deficiency enhances formation of bone even in the injury-repair model in vivo.

To identify molecular events underlying the enhancing effects of JunD deficiency on bone formation, total RNA was extracted from femora and was subjected to real-time PCR analysis. JunD deficiency enhanced the expression levels of mRNA encoding type I collagen, the most abundant protein product of osteoblasts (Fig. 5A). JunD deficiency also enhanced expression levels of the mRNA encoding osteocalcin, an osteoblast-specific maker protein (Fig. 5B). As these osteoblastic products were known to be downstream to the master regulatory genes for osteoblasts, *runx2* mRNA levels were examined. JunD deficiency enhanced the expression levels of mRNAs encoding Runx2 (Fig. 5C). These observations indicated that JunD acts as a suppressor of osteoblastic activity via targeting genes related to the phenotype of osteoblastic cells.

AP-1 transcription factors are known to regulate the expression of each other [Eferl

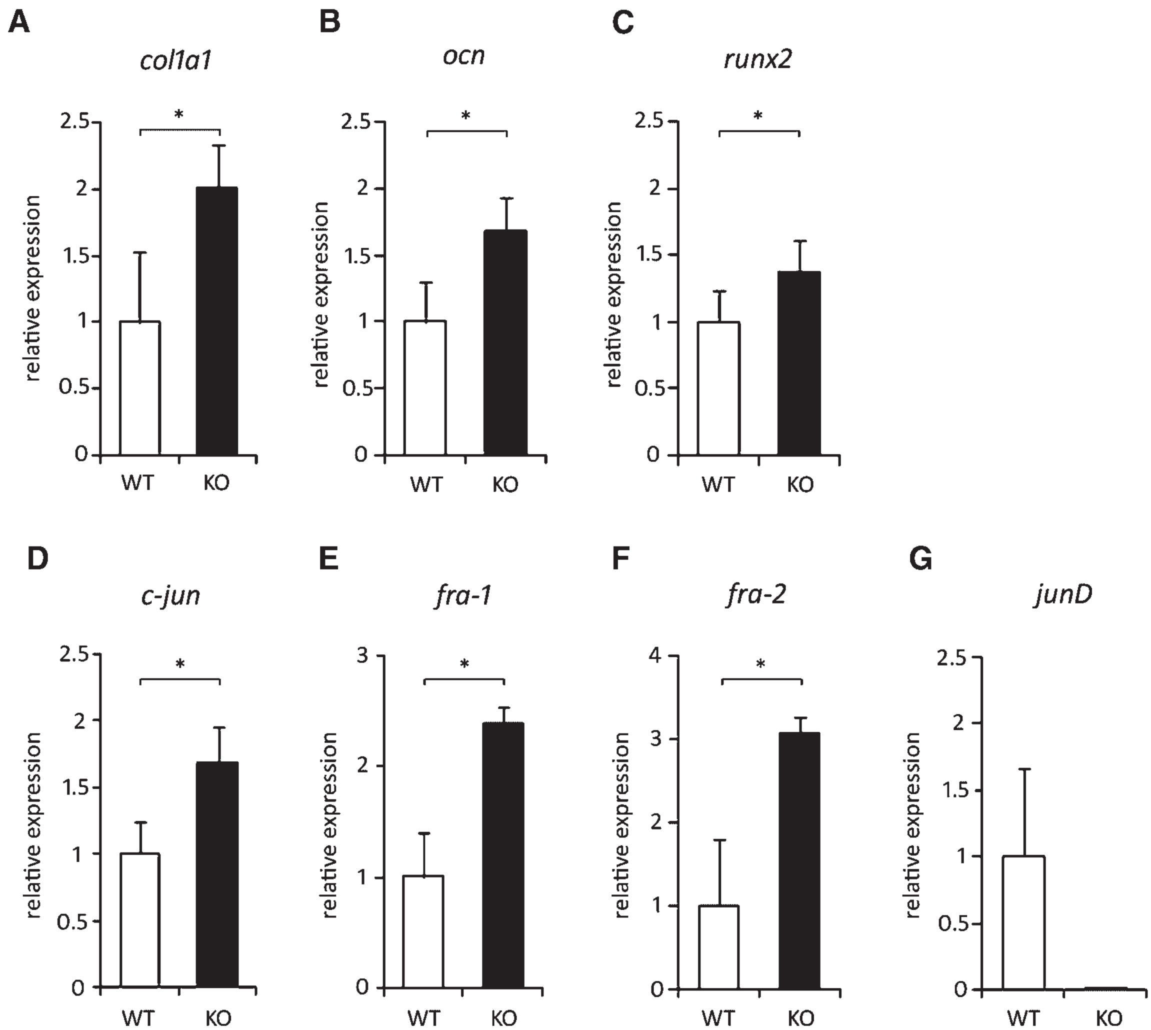

Fig. 5. JunD deficiency enhances expression of genes encoding proteins related to osteoblastic phenotype and AP-1 family members. Expression levels of mRNA encoding type I collagen (*col1a1*) (**A**), osteocalcin (*ocn*) (**B**), and Runx2 (**C**). Total RNA was extracted from bone marrow of femora and was subjected to real-time PCR analysis of WT (n = 4) and JunD KO (n = 4). Expression levels were normalized against those of *gapdh*. Relative values are presented. Expression levels of mRNA encoding c-Jun (**D**), Fra-1 (**E**), Fra-2 (**F**), and JunD (**G**). Total RNA was extracted from bone marrow of femur in WT (n = 4) and JunD KO (n = 4) mice. Expression levels were normalized against those of *gapdh*. Relative values are presented. $^*P < 0.05$.

and Wagner, 2003]. These interactions may contribute to modulate AP-1 actions when once expression of one of the family members would be activated. It was known that c-fos overexpression could result in formation of bone tumors, suggesting a "positive feedback" between AP-1 and osteoblastic activity. We therefore examined the effects of JunD deficiency on the expression levels of the mRNAs encoding other AP-1 family members. In contrast to "positive" feedback, JunD deficiency enhanced c-jun mRNA levels in bone (Fig. 5D) revealing "negative" regulation. Such "negative" regulation of AP-1 family members by JunD is not limited to *c-jun*, as JunD deficiency also enhanced the mRNA expression levels of *fra-1* and *fra-2* (Fig. 5E,F). As confirmation of the knockout, we examined *junD* expression in bone and virtually no expression of *junD* was observed (Fig. 5G). These observations indicate that JunD serves as a suppressor of expression of AP-1 family members in the skeletal system.

DISCUSSION

We discovered that JunD is an endogenous suppressor of bone mass levels and is involved in the determination of normal bone mass in basal state as well as in the state of osteopenia after estrogen depletion. Although bone mass becomes low after estrogen depletion, molecular bases involved in the bone mass reduction under this pathological condition are not fully understood. Our observations reveal that JunD contributes to the low levels of bone mass.

Under the condition of estrogen depletion, remodeling activities are elevated to form a high turnover state in adult bone. Even in the presence of high turnover state of remodeling in adults, JunD deficiency induced even higher levels of bone "formation" activity. This fact implicates that even in adults, bone formation activities possess a reservoir in its capacity to provide additional new bone accumulation. Although it is necessary to test whether such suppressive property of JunD on bone formation observed in mice can be extrapolated into the cases of humans, this would be novel aspect to understand the pathogenesis of osteopenic diseases.

Our identification of JunD as a negative determinant of bone mass provided the following unique features of this molecule. First, JunD deficiency increased basal levels of bone mass in adults. JunD is expressed in several tissues while knockout mice are born alive and survive to adult stage without exhibiting major defects in most organs [Thepot et al., 2000]. Thus, JunD exerts suppressive function almost specifically, though not exclusively, in bone.

Secondly, JunD deficiency prevented bone loss induced by estrogen depletion. Estrogen depletion reduces bone mass even in certain cases of the mutant animals with high bone mass trait. We previously observed that Tob-deficient mice exhibit high bone mass, but estrogen depletion in these mice reduces bone mass levels to those similar to the intact WT mice [Usui et al., 2004]. In contrast to Tob-deficient mice, JunD-deficient mice kept high levels of bone mass and did not lose bone after estrogen depletion at all. This was at least in part due to JunD deficiency-induced enhancement of bone formation activity. Thus, high bone mass levels were preserved regardless of estrogen levels in JunD-deficient mice.

Intriguingly, JunD deficiency increased the levels of osteoclast surface in mice without ovariectomy. However, JunD deficiency suppressed ovariectomy-induced increase in osteoclast parameters. This implies that JunD deficiency renders certain resistance against rapid pathological bone loss induced by estrogen depletion. These observations suggest a possibility that if certain inhibitory measures could be developed to suppress expression and function of JunD, it could be beneficial for the preservation of bone mass in patients with reduced bone mass.

JunD is expressed constitutively at high levels in bone. This is a unique feature of this AP-1 protein since expression of many AP-1 family members is activated mostly transiently and is rapidly reduced [McCabe et al., 1995]. For instance, Fos gene expression is enhanced immediately and transiently after treatment with growth factors and cytokines [Shaulian and Karin, 2002]. Fra-2 is also rapidly induced upon anabolic stimuli such as PTH [McCauley et al., 2001]. Therefore, the implication of the constitutively high expressions of JunD was not fully understood formerly. Our observations indicated a novel function in that JunD acts as a constitutively present "negative" modulator in bone. This inhibitory activity may reduce the base line activities of AP-1-related signaling events and thus may contribute to facilitate sensitivity of AP-1 system to be activated sharply upon positive stimuli.

Although several previous in vitro reports suggest controversial role of JunD in cultures [McCabe et al., 1996; David et al., 2001; Naito et al., 2005], our "in vivo" data provide evidence that the role of JunD in bone metabolisms is to be a negative regulator. This discrepancy suggests the mechanisms of JunD to regulate bone metabolism in vivo, that is, the high bone mass in JunD KO mice may result from secondary effect of JunD deficiency. Thus, JunD suppress the bone mass in vivo despite the positive function of JunD to enhance osteoblast differentiation.

In conclusion, we identified that JunD is a suppressor of bone mass levels in normal bone and is involved in causing the osteopenic state of bone after estrogen depletion.

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