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Bone Mass Loss Due to Estrogen Deficiency Is Compensated in Transgenic Mice Overexpressing Human Osteoblast Stimulating Factor-1[1]

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Osteoblast stimulating factor-1 (OSF-1) stimulates in vitro proliferation and differentiation of osteoblastic cells, and its gene is expressed in the bone and brain tissues in mammals and amphibians. To evaluate the in vivo function of OSF-1 in bone metabolism, transgenic mice overexpressing the human *osf-1* gene driven by the osteocalcin promoter were generated. Femoral bone mineral content was increased in transgenic mice relative to wild-type controls as estimated by ash assay, depending on the transgene copy number per cell. In ovariectomized mice, bone mass loss due to estrogen deficiency was observed in both transgenic and control mice but bone mass was still higher in transgenic mice than in controls. Bone mass in ovariectomized transgenic mice was comparable to that in wild-type mice without ovariectomy. These observations indicate that OSF-1 may direct in vivo appositional bone formation by increasing osteoblast activity rather than decreasing osteoclast activity, suggesting a new way to treat osteoporosis with OSF-1.

Bone is a complex tissue composed of cells, collagenous matrix and inorganic elements, mainly calcium, and is continuously remodeled during adulthood through the resorption of old bone by osteoclast cells and the subsequent formation of new bone by osteoblast cells [1]. cDNA clones encoding osteoblast stimulating factor-1 (OSF-1, formally called osteoblast specific factor-1 [2], and also named HB-GAM [3], PTN [4] and HBNF [5]) have been isolated from mouse and human osteoblastic cells on the assumption that at least some proteins involved in growth and differentiation of bone tissues may be produced specifically in osteoblasts. OSF-1, a heparin binding Lys-rich 18 kDa protein, purified from bovine bone powder has been shown to stimulate both proliferation and differentiation of mouse osteoblastic cells in vitro without affecting cell shape unlike 1,25-$(OH)_2$ vitamin D_3 (VD_3) or TGF-β_1 [6]. The transcription of *osf-1* was not affected by VD_3 but was markedly suppressed by TGF-β_1 in cultured mouse osteoblastic cells [7]. Developmental and differential expression of the amphibian *X-ptf-*α and *X-ptf-*β corresponding to mammalian *mk* [8] and *osf-1*, respectively, all of which belong to the same family, have been studied in *Xenopus laevis* [9]. Although *X-ptf-*α transcripts exist as maternal mRNA in oocytes and are widely expressed throughout development in various embryonic tissues, *X-ptf-*β transcripts become evident only at the later stages of larval development and are spatially restricted to the region from the hindbrain along with the spinal cord in tailbud embryos.

To elucidate the in vivo function of OSF-1 in mammalian bone metabolism, we generated transgenic mice expressing the human *osf-1* gene under the control of the human osteocalcin promoter. Analyses of the bone mass evaluated by bone mineral content using ash

[1] The nucleotide sequence of the *HPoc-Hosf-1* transgene will appear in the DDBJ databases under Accession No. AB004306.

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Abbreviations: DXA, dual energy X-ray absorptiometry; OSF-1, osteoblast stimulating factor-1; VD_3, vitamin D_3.

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assay with femora and by bone mineral density using dual energy X-ray absorptiometry (DXA) with vertebrae indicate that (i) bone mass was increased in femora of transgenic mice relative to negative controls depending on transgene copy number per cell, (ii) although bone mass loss due to estrogen deficiency was found in both transgenic and negative control mice, the bone mass increase was more evident in transgenic mice relative to negative controls when ovariectomized, and (iii) bone mass in ovariectomized transgenic mice was comparable to that in wild-type mice without ovariectomy. These results suggest that OSF-1 may play a role in vivo in stimulating osteoblasts resulting in a bone mass increase without affecting osteoclast activity unlike parathyroid hormone, interleukin-1 and tumor necrosis factor (TNF) [10].

MATERIALS AND METHODS

Transgenic mice. Construction strategy and structure of the transgenic human (*H-*) *osf-1* gene are schematically shown in Fig. 1A and B, respectively. The *HPoc-Hosf-1* transgene DNA fragment was excised by *Eco*RI and *Hind*III digestion, purified and microinjected into fertilized eggs of BDF1 mice essentially as described [15]. The eggs were transferred into the oviducts of pseudopregnant ICR female mice and the presence of the transgene in mouse chromosomes was confirmed by tail-tip Southern blotting using Probe I (Fig. 1B). Mice were kept at room temperature and had free access to a standard diet, MF (Oriental Yeast Co. Ltd., Tokyo, Japan), containing 0.88 % phosphorus, 1.15 % calcium and 1.08 IU/g VD_3. The mice used for mRNA analysis and bone mass evaluation were all female. Ovariectomy or sham-operation was carried out at the age of 14 weeks by the dorsal incision method under anesthesia induced by the intrapenitoneal injection of pentobarbital sodium salt, and the mice were allowed to survive for further 14 weeks.

Northern blotting analysis. Total RNA from the whole brain, calvaria, liver, muscle, spleen, kidney of 8- or 28-week-old mice was isolated by the acid guanidinium isothiocyanate-phenol extraction method [16], electrophoresed, transferred onto nylon membranes and hybridized with radiolabeled Probe II (Fig. 1B) as described [17].

Bone mass evaluation. Bone mineral content and density were evaluated by ash assay and DXA using femoral and vertebral bones, respectively. For ash assay, homozygous ($hosf^6/hosf^6$) and heterozygous ($hosf^6/hosf^0$) transgenic and wild-type ($hosf^0/hosf^0$) mice were produced twice by independent crossing, where $hosf^6$ and osf^0 indicate the existance of 6 and 0 copies of human *osf-1* transgene per haploid genome, respectively. Bilateral femora were excised and soft tissues including muscles surrounding the bones were completely removed. After determining the wet weight of femora, the samples were dried at 100°C for 48 hrs, treated with ethanol/ether (2:1) solution to remove fat, and ashed in a muffle (FM37, Yamato Co., Japan) at 650°C for 12 hrs. The ashed samples were weighed to determine bone mineral weight. Bone mineral content was defined as percentage of ratio of bone mineral weight to wet weight. For DXA using vertebrae, soft tissues and spinal nerves were removed and lumber spines (L2-L4) were resected off as a block, of which bone mineral density expressed by mg/cm^2 was determined using a DCS-600R instrument (Aloka Co., Japan) and bone radiodensity was photographed with soft X-rays using FR film (Fuji Co., Japan) at 30 V, 2 mA and 60 sec. The vertebral images were enhanced using an Epscan 1.40 (Epson Co, Japan) and Adobe Photoshop 2.5J (Adobe Systems Incorporated, USA.) under the same conditions. The final pictures were taken by Color Fast (GCC Technologies, USA). The precision and stability of the measurements were evaluated periodically and the coefficient of variation was $< 0.5\%$. The operators were unaware of sample identities during manipulation.

RESULTS

Generation of human osf-1 (H-osf-1) transgenic mice. Three founder mice (F_0) harboring the *H-osf-1* transgene were identified by Southern blotting analysis of tail DNA samples and the F_1 heterozygotes were analyzed for mRNA at 8 weeks of age (Fig. 1C). In two of the three lines, the transgene mRNA was detected specifically in the calvaria, whereas endogenous mouse *osf-1* transcripts were detected in the brain and calvaria but not in other tissues examined (data not shown). One of the two lines, *HPoc-Hosf1-6*, carrying 6 transgene copies per haploid cell, was used for further studies. No significant differences were found in external appearance, body weight or behavior between the transgenic and wild-type BDF1 mice. No apparent pathological conditions such as osteosarcoma and osteopetrosis were observed by X-ray photography in transgenic mice irrespective of homozygosity ($hosf^6/hosf^6$) or heterozygosity ($hosf^6/hosf^0$) for the transgene (data not shown).

Ash assay using femora. Transgenic littermates with $hosf^6/hosf^6$ and $hosf^6/hosf^0$ genotypes and wild-type BDF1 mice, all of which were bred under the same conditions from parental mating to sacrifice at 30 weeks of age, were examined to determine their bone mineral content by ash assay using bilateral femora as described in Materials and Methods. Significant bone mineral increases were found in $hosf^6/hosf^6$ mice relative to $hosf^6/hosf^0$ littermates and wild-type mice ($p<0.05$), but no significant bone mass difference was found between $hosf^6/hosf^0$ and wild-type mice ($p>0.05$) (Fig. 2A). Subsequently, the possible influence of estrogen deficiency was examined on the bone mineral increase in transgenic mice with or without ovariectomy. Homozygous transgenic and wild-type mice were ovariectomized or not operated at 14 weeks of age and sacrificed at 28 weeks of age. As shown in Fig. 2B, femoral bone mineral content was decreased by ovariectomy in both transgenic and wild-type mice, but was again higher in transgenic mice than in wild-type controls irrespective of ovariectomy or non-ovariectomy (Fig. 2B). In the ovariectomized transgenic mice, bone mineral content was comparable to that in wild-type mice without ovariectomy.

Dual energy X-ray absorptiometry (DXA) using vertebrae. To shuffle possible unknown gene(s) which might have been responsible for the bone mass increase in the transgenic mice, $hosf^6/hosf^6$ females were back-crossed to wild-type BDF1 males. The heterozygous offspring were paired and $hosf^6/hosf^6$, $hosf^6/hosf^0$ and $hosf^0/hosf^0$ pups were produced. As shown in Fig. 3, bone mass increase in transgenic mice was evident, depending on the transgene

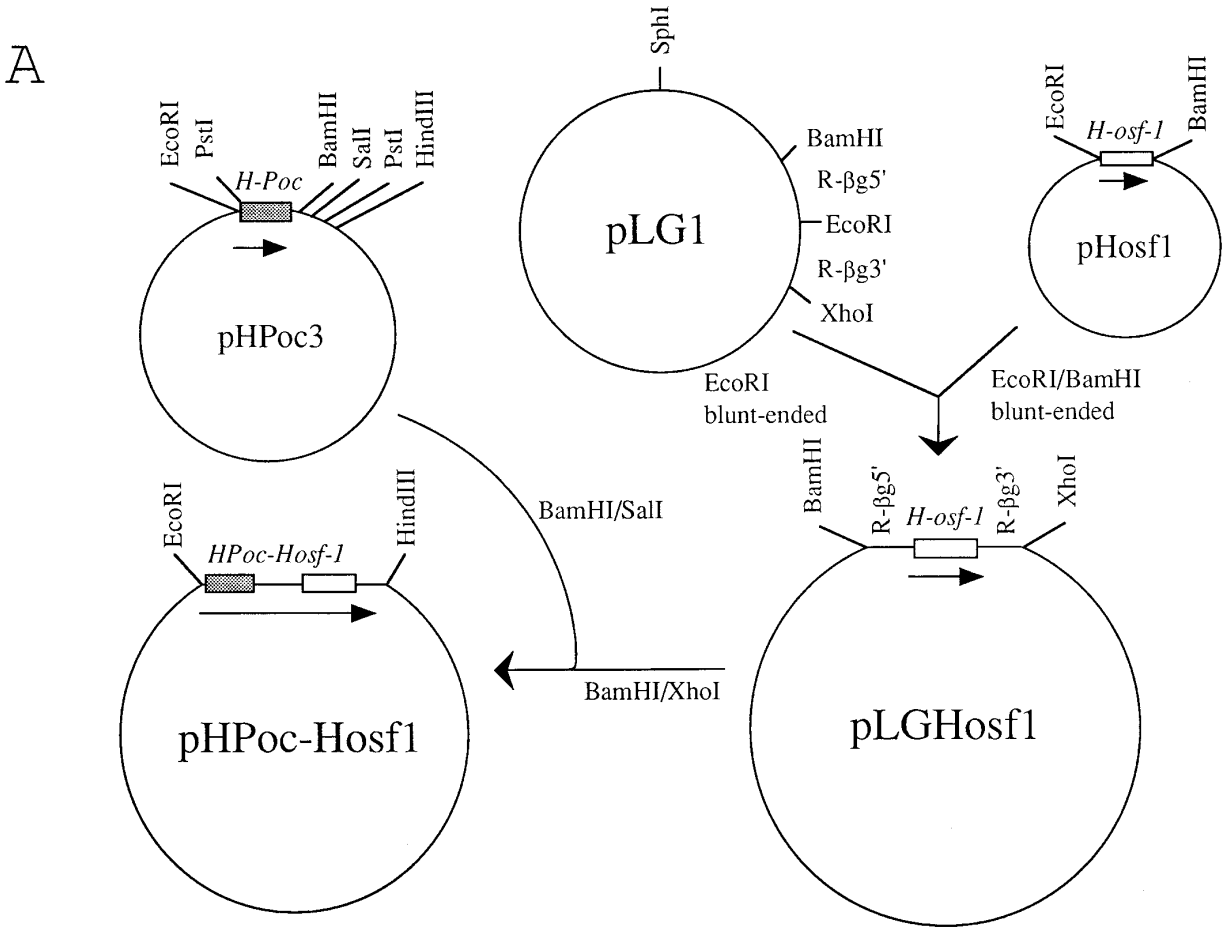

FIG. 1. Construction strategy and structure of the *H-osf-1* transgene and mRNA expression in transgenic mice. (A) The coding region for *H-osf-1* cDNA cloned between *Eco*RI and *Bam*HI sites on pKF4 [11], pHosf1, was excised, blunt-ended and recloned at blunt-ended *Eco*RI site flanked by the 5′- and 3′-untranslated regions (UTRs) of the rabbit β-globin gene on pLG1 [12], pLGHosf1. Then, the *Bam*HI and *Xho*I fragment containing the *H-osf-1* coding region flanked by the rabbit 5′- and 3′-UTRs was excised and placed under the control of the human osteocalcin promoter (*H-Poc*) [13] between *Bam*HI and *Sal*I sites on pKF18k [14], pHPoc-Hosf1. Shadowed and open boxes represent *H-Poc* promoter and *H-osf-1* coding region, respectively. Arrows inside plasmid circles indicate transcriptional direction in *H-Poc* and *H-osf-1*. R-βg5′ and R-βg3′ indicate the 5′- and 3′-UTRs. (B) The *H-osf-1* transgene structure is shown schematically with representative restriction sites. The small hatched box in the *H-Poc* promoter indicates the approximate position of the vitamin D responsive element (*VDRE*). The horizontal arrows labeled Probe I and Probe II indicate approximate extents of probe sequences (2.6 kbp and 570 bp) used for Southern and Northern hybridization experiments, respectively. The vertical arrow marked with polyA indicates the approximate position of the polyA signal sequence. Other symbols are as in (A). (C) Northern blotting analysis of total RNA isolated from the brain and calvaria of a F_1 heterozygote. Total RNAs (10 μg/lane) isolated from mouse brain and calvaria at 8 weeks of age were electrophoresed and hybridized with probe II as described in Materials and Methods. "Wt" and "He" indicate wild-type and transgenic heterozygote, respectively.

copy number per cell, in cases with ovariectomy. In sham-operated mice, consistent results with the data by ash assay in non-ovariectomized mice were obtained, although the bone mass increase in homozygous mice was less significant in vertebrae (Fig. 3). The bone mass difference in ovariectomized mice was consistently demonstrated by X-ray radiography using vertebrae, where bone density increases were visible in ovariectomized transgenic mice (Fig. 4).

DISCUSSION

A number of factors are known to be involved in development of osteoclasts, including interleukin-1, interleukin-3, interleukin-6, interleukin-11, tumor necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, leukemia inhibitory factor, stem-cell factor [1]. Including inhibitors against such factors, substances that decrease or suppress osteoclast activity may contribute to retention of higher bone mass in vivo. In fact, anti-osteoporosis reagents currently in clinical use are all included in this category. Recently, Simonet et al. [18] and Ammann et al. [19] reported bone mass increases in transgenic mice overproducing authentic and artificially manipulated soluble receptors, respectively, belonging to the TNF receptor family. In the former study, bone mineral increase or

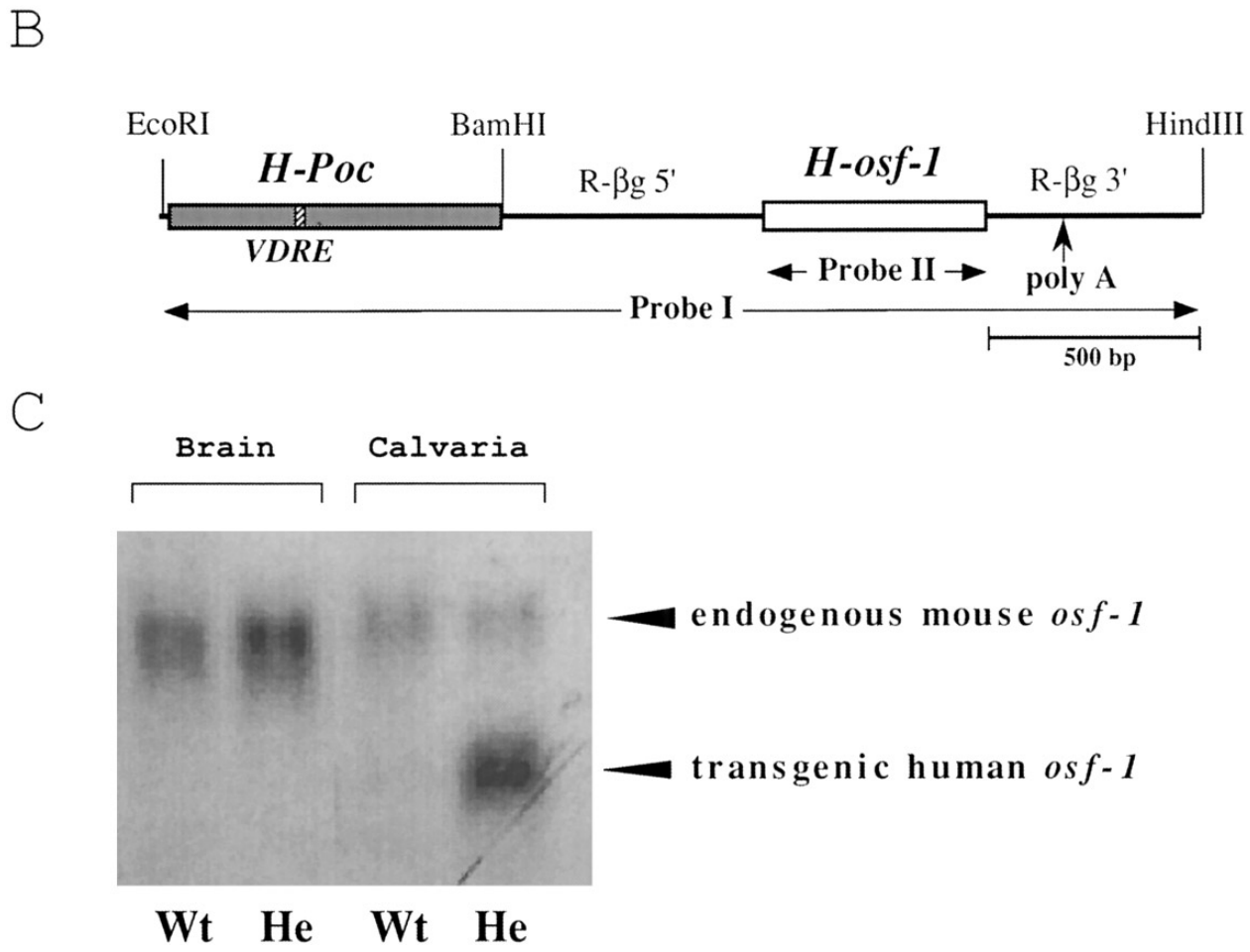

FIG. 1—*Continued*

osteopetrosis was observed without ovariectomy, whereas in the latter no bone mineral increase was observed before ovariectomy. They both found that bone mass was increased dependent on the soluble TNF receptors in their animals even after ovariectomy. Their results may be explained in terms of suppression of osteoclast development through TNFα inactivation by the soluble receptors. On the other hand, few factors, if any, involved in osteoblast development are known thus far. Bone mass increase observed in our transgenic mice relative to negative controls without ovariectomy was not as much as that in osteopetrosis observed by Simonet et al. [18]. The increase was significantly detected without ovariectomy only when evaluated by ash assay using femora but not by DXA or X-ray radiodensity. With ovariectomy, however, bone mass increase in transgenic mice was evident by either evaluation using femora or vertebrae (Figs. 2B, 3 and 4). It should be stressed that bone mass loss due to estrogen deficiency was detected in our transgenic mice (Figs. 2B and 3) in contrast to their reports described above. This suggests that the increase of osteoclast activity after ovariectomy was undergoing in our mice but appositional bone formation may still progress due to the transgene overexpression as to keep a high bone mass in our transgenic mice relative to that in wild type mice after ovariectomy. Consequently, bone mass in the ovariectomized transgenic mice was comparable to that in negative control mice without ovariectomy. Thus, we concluded that the *H-osf-1* transgene could compensate for bone mass loss due to osteoclast activation by estrogen deficiency. In fact, we observed *H-osf-1* expression in addition to that of mouse endogenous *osf-1* in bone tissues, including vertebrae, femora and calvaria, of ovariectomized transgenic mice at the time of sacrifice by Northern blotting analysis (data not shown). The role of OSF-1 in bone formation should be different from that of TGF-β related proteins such as bone morphogenetic proteins (BMPs), because the latter proteins trigger endochondral bone formation or ectopic bone morphogenesis [20] and function primarily at the early stages of embryonic development in regulation of dorsoventral pattern formation not only in vertebrates [21] but also in invertebrates [22]. It may be noteworthy to point out that *Xenopus osf-1* homologue (*X-ptf-*β) transcripts, are not present in unfer-

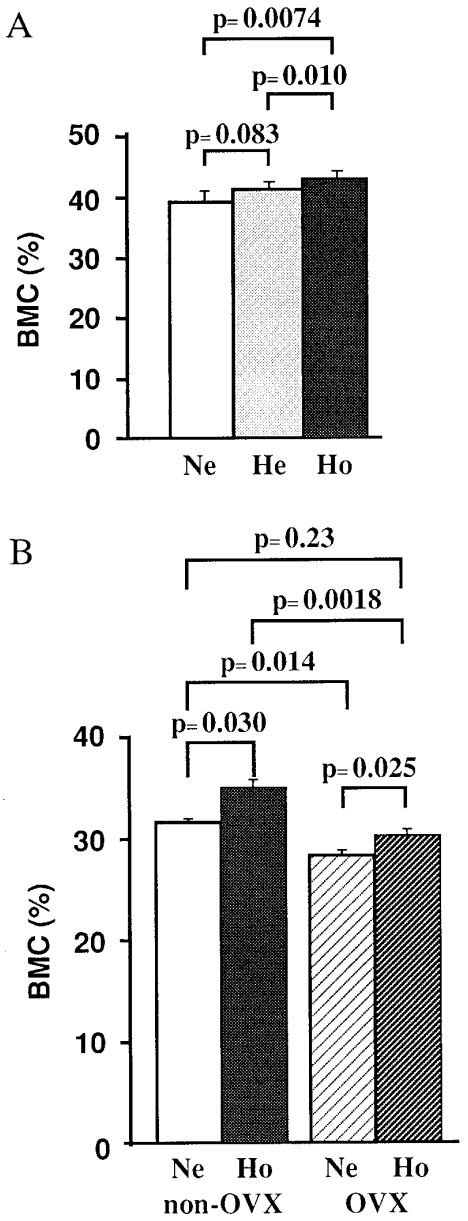

FIG. 2. Bone mineral content (BMC) in femora of *H-osf-1* transgenic mice. BMC values were evaluated by ash assay as described in Materials and Methods. Means of groups were compared by ANOVA and significance of differences was determined by post hoc test using Scheffe's method. (A) and (B) are independent experiments. (A) Mean values±SEM (%) are 39.534±3.965, 40.497±2.694 and 43.049±1.481 for Ne, He and Ho, respectively, where n=5-9. "Ne" indicates wild-type negative control mice. "He" and "Ho" indicate heterozygous and homozygous transgenic littermates, respectively. (B) Means values±SEM (%) are 31.530±0.523, 35.274±1.236, 28.255±0.177 and 30.550±0.868 for Ne/non-OVX, Ho/non-OVX, Ne/OVX and Ho/OVX, respectively, where n=2-4. "Ne" and "Ho" are as in (A). "OVX" and "non-OVX" indicate ovariectomy and no operation, respectively.

tilized eggs and in the early stages of larval development [9]. Furthermore, TGF-β and OSF-1 should function in opposite directions in bone metabolism since the former suppresses the gene expression of the latter [7]. In this context, it is of interest to point out that osteoblast-specific overexpression of TGF-β_2 in transgenic mice resulted in progressive bone mass loss resembling human osteoporosis [23]. In addition, OSF-1 did not exhibit in vivo activity for ectopic bone formation like BMPs (data not shown). OSF-1 could be one of the bone mass determinants which positively controls appositional bone formation occurring at bone remodeling sites through osteoblast activation

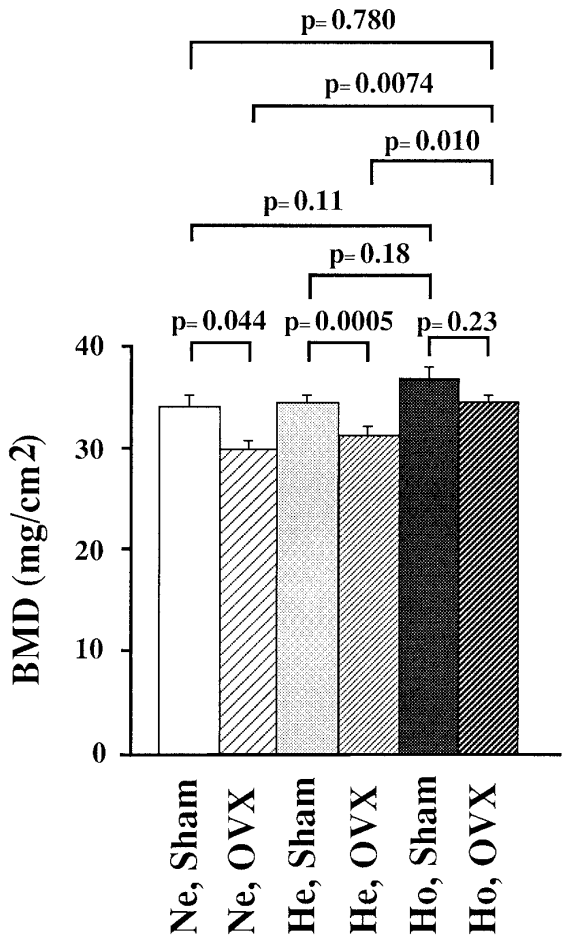

FIG. 3. Bone mineral density (BMD) in vertebrae of transgenic and negative littermates. The transgenic heterozygous offspring were paired separately and their pups from each *hosf*^{6}/*hosf*^{6}, *hosf*^{6}/*hosf*^{0} or *hosf*^{0}/*hosf*^{0} genotype were taken independently and divided into the 3 groups of 8 pups. In each group, pups were divided into two subgroups for ovariectomy and sham-operation, which were carried out at 14 weeks of age. The animals were sacrificed for BMD evaluation by DXA using vertebral bones (L2-L4) at 28 weeks of age as described in Materials and Methods. Means of groups were compared by ANOVA and significance of differences was determined by post hoc test using Bonferroni's method. Mean values±SEM of BMD (mg/cm^2) are 34.875±1.303, 29.800±1.507, 35.450±0.417, 30.150±0.659, 37.775±1.436 and 35.375±1.108 for Ne/Sham, Ne/OVX, He/Sham, He/OVX, Ho/Sham and Ho/OVX, respectively, where n=4. "He", "Ho", "Ne" indicate heterozygous (*hosf*^{6}/*hosf*^{0}) and homozygous (*hosf*^{6}/*hosf*^{6}) transgenic and negative (*hosf*^{0}/*hosf*^{0}) littermates, respectively. "OVX" and "Sham" are as in Fig. 2B.

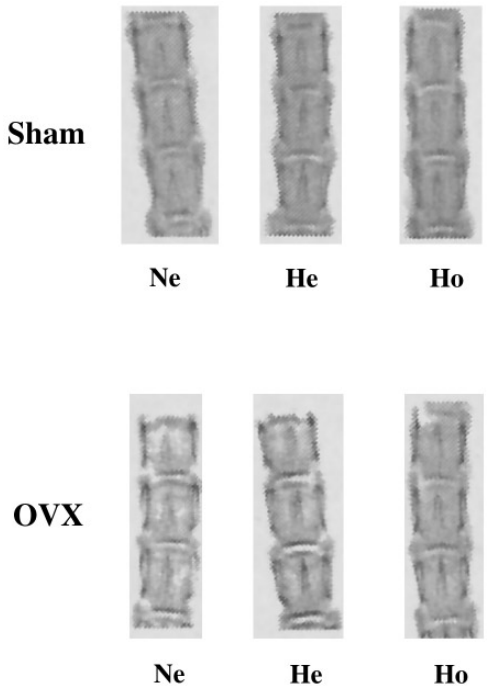

FIG. 4. Vertebral bone radiodensity of the sham-operated or ovariectomized heterozygous and homozygous transgenic and negative littermates. The vertebrae (L2-L4) of ovariectomized or sham-operated littermates were photographed by soft X-rays as described in Materials and Methods. "Sham", "OVX", "Ne", "He" and "Ho" are as in Fig. 3.

but not osteoclast suppression [24]. OSF-1 may be useful to stimulate isotopic bone formation in treatment of post-menopausal osteoporosis in addition to use of osteoclast suppresser-type substances.

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