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Bone mass increase specific to the female in a line of transgenic mice overexpressing human osteoblast stimulating factor-1

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Abstract We have reported that transgenic mice overexpressing human *osteoblast stimulating factor-1* (*osf1*) under the control of the human *osteocalcin* promoter have a significantly higher bone mineral content and density than nontransgenic littermates. Consequently, bone mass loss due to estrogen deficiency was compensated for in ovariectomized female mice. Here, we show that in this transgenic line, the bone mass increase was evident in female, but not male, mice, as evaluated using the ash assay, double-emission X-ray analysis, and calcein double-labeling to determine the bone formation rate. To elucidate a possible influence on gene expression, we analyzed genomic structures of the inserted transgene and its flanking regions in mouse chromosomes. The results revealed that the transgene was integrated in the mouse repetitive sequences, 234-bp-long γ-satellite repeats, as inverted multiple (5 + 8) copies. Twelve copies at most seemed to be functional, but no direct evidence supporting female-specific mRNA synthesis of the transgene was obtained.

Key words OSF1 · BDF1 mouse · primary hybrid strain · littermates · bone mass

Introduction

Osteoblast stimulating factor-1 (OSF1 [1], also named HB-GAM [2] and PTN [3]), a heparin-binding Lys-rich 18-kDa protein, belongs to the family that also includes midkine (MK) [4]. One of the important differences between the *osf1* and *mk* genes may be that the former is not expressed in the ovary or oocytes and thus is not expressed in early embryonic cells, whereas the latter is transcribed abundantly in those cells [5]. This finding strongly suggests that OSF1 may be responsible for rather mature processes of biological functions. Furthermore, the function of OSF1, but not MK, may be specific to nerve and osteoblast cells, because the gene expression is restricted to these cells in mature animals [2,5]. In osteoblasts, *osf1* is expressed between the early and middle stages of osteoblastic differentiation. OSF1 stimulates osteoblasts not only in differentiation but also in proliferation [6] and even recruits osteoblast cells [7]. In fact, we have proposed that OSF1 may not be relevant to embryonic bone morphogenesis but rather may function in later stages of bone metabolism such as bone remodeling [8]. This function is distinct from the case of bone morphogenetic protein-2 (BMP2), which works during early embryogenesis for ventral patterning [9] and endochondral bone morphogenesis [10].

Recently, Tare et al. have reported that our OSF1 transgenic mice increase bone mass only in males, but not females [11,12]. However, they failed to use appropriate littermate controls indispensable for quantitative experiments using transgenic mice, particularly those that are created in the primary hybrid strains. In this report, we show that bone mass increase in our transgenic mice is in fact more apparent in female than in male mice as evaluated by the ash assay, double-emission X-ray analysis (DXA), and an examination of the bone formation rate (BFR) using calcein double-labeling.

Materials and methods

Animals

The line of *osf1* transgenic mice expressing human *osf1* under the control of human osteocalcin promoter (*HPoc-Hosf1*) has been constructed in a primary hybrid strain, BDF1, between two congenic strains, female C57BL/6 and male DBA/2 [8]. The homozygous transgenic progeny were maintained by repeated inbred

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crossing, and therefore their genetic background is biased from that of BDF1. Therefore, it is most likely that one may obtain artificial observations if they are compared directly with BDF1 mice as controls. Thus, before experiments, they were crossed back three times to BDF1 mice purchased from Nihon CLEA (Tokyo, Japan). The resulting heterozygous transgenic parents were then crossed to produce transgene negative, heterozygous, and homozygous littermates. Mice were fed as described previously [8].

Measurements of bone mass and bone formation rate

Bone mineral content and density were evaluated with the ash assay using bilateral femora and by double-emission X-ray analysis (DXA) using vertebrae (L2–L4) as described previously [8]. The bone formation rate was calculated using vertebra (L3) and tibia dually labeled with calcein at 7-day intervals as previously described [13]. Histomorphometry was performed with a semiautomatic image-analyzing system linked to a light microscope. For each undecalcified vertebral body, the area of secondary spongiosa was measured, but the region within 0.3 mm from the growth plate–metaphyseal junction was not examined to exclude primary spongiosa. In the right tibia section, the metaphyseal cancellous bone area located within 0.2 mm distal to the growth plate–metaphyseal junction was analyzed. As parameters of bone formation, the followings were measured and calculated: mineralizing bone surface (MS/BS, %), mineral apposition rate (MAR, μm/day) calculated as the distance between double labels divided by interval labeling time and multiplied by π/4, and bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/day).

Genomic analysis of the transgene and flanking regions

Mouse genomic DNA was prepared from the liver and digested partially by *Sau*3AI, filled with Klenow enzyme in the presence of dGTP and dATP, and fractionated by sucrose gradient density centrifugation for isolation of DNA fragments of approximately 40 kbp. Then, the isolated DNAs were used for cloning into cosmid vector, pHSG429 [14], digested with *Xho*I and partially filled in the presence of TTP and dCTP, and introduced into *Escherichia coli* STBL4 after in vitro packaging (Invitrogen, Carlsbad, CA, USA). The library was screened by colony hybridization with human *osf1* cDNA labeled with [^{32}P]dCTP. For cloning the entire transgene repeats located between *Hin*dIII and *Acc*I sites, pHC79 [15] was used. The cloned DNAs were subcloned into pKF4 [16] for sequencing analysis.

Results

Transgene negative, heterozygous, and homozygous littermates were bred under the same conditions from parental mating to death at 30 weeks of age and examined to determine their bone mineral content and density as described in Materials and methods. It was revealed that, in females, the bone mineral content of bilateral femurs was significantly higher in homozygous transgenic mice than in nontransgenic littermates. However, in males, no significant differences were observed among negative, heterozygous, and homozygous littermates (Fig. 1A). Essentially the same results were obtained by DXA using vertebrae (Fig. 1B). Moreover, in homozygous mice, the bone mass of females was significantly higher than that of males by either method, ash assay or DXA (Fig. 1A,B).

The MAR values of the third lumbar vertebra and right tibia were higher in homozygous transgenics than in nontransgenic littermates only in females (Fig. 2A). Similarly, the BFR/BS values of lumbar and tibia were higher in homozygous transgenics than in nontransgenic littermates only in females (Fig. 2B). The MS/BS values

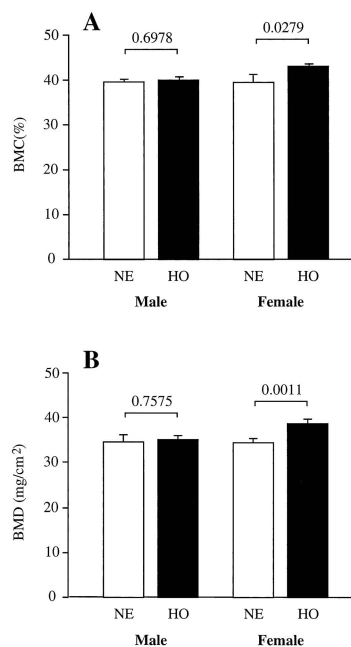

Fig. 1. Bone mineral content (*BMC*) in femur (**A**) and bone mineral density (*BMD*) in vertebra (**B**). BMC and BMD were evaluated by ash assay and double-emission X-ray analysis (DXA), respectively, as described in Materials and methods. Means of groups were compared by ANOVA, and the significance of differences was determined with the post hoc test using Bonferroni–Dunn's method. **A** n = 9 (*NE*) and n = 4 (*HO*) in male; n = 5 (*NE*) and n = 9 (*HO*) in female. **B** n = 7 (NE) and n = 7 (HO) in male; n = 8 (NE) and n = 8 (HO) in female. *NE*, *HE*, and *HO* indicate transgenic negative, heterozygous, and homozygous littermates, respectively

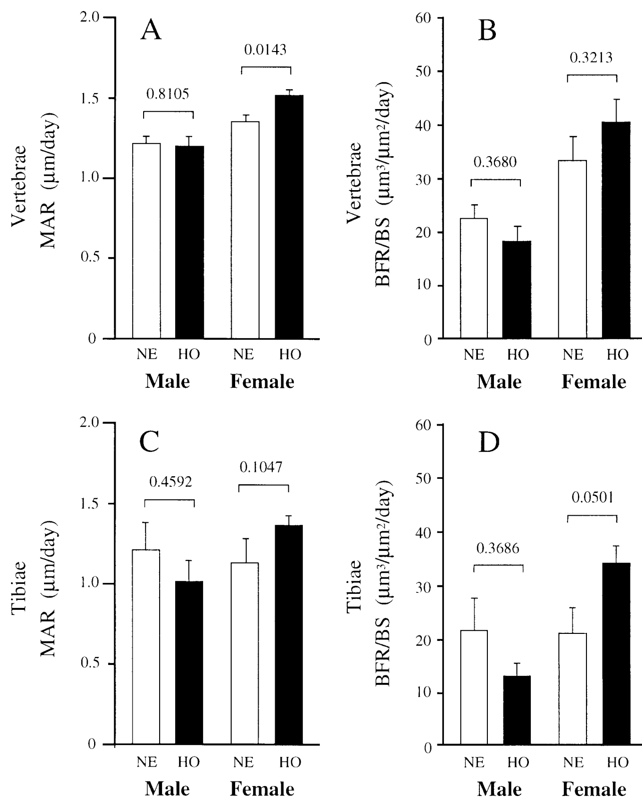

Fig. 2. Mineral appositional rates (*MAR*) and bone formation rates per bone surface (*BFR/BS*) in vertebrae and in tibiae. MAR and BFR/BS values were determined in vertebrae (**A** and **B**, respectively) and tibiae (**C** and **D**, respectively), as described in Materials and methods. The p values were calculated with Student's t test: $n = 9$ (NE) and $n = 4$ (HO) in males; $n = 4$ (NE) and $n = 9$ (HO) in females

were also higher in homozygous transgenics than in nontransgenic littermates only in females but not in males (data not shown).

We hypothesized that the chromosomal location of the transgene might have an influence on its gene expression. For example, the transgene might be inserted close to some gene controlled by enhancer sequences such as the estrogen-responsive element (ERE). Therefore, we analyzed the genomic structure of transgene copies as well as flanking regions. We constructed a cosmid library, and obtained two positive clones, E3C5 and A2F7, containing the left and right flanking regions, respectively (Fig. 3A). By using the sequence information obtained from these clones, we then obtained a single clone, H3A1, covering all the transgene copies

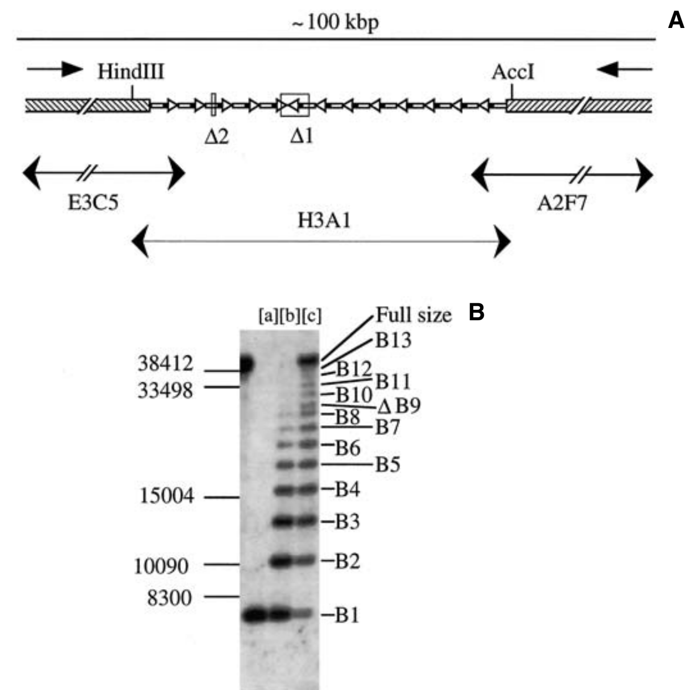

Fig. 3. Schematic structure of the human *osf1* transgene copies and flanking regions (**A**) and Southern blot analysis of multiple insertions of the transgene (**B**). **A** Cosmid clones, E3C5 and A2F7, were obtained in pHSG429 as described in Materials and methods. Another clone, H3A1, was obtained by cloning DNA fragments (~40 kbp in size) prepared by *Hin*dIII and *Acc*I (filled in) digestion between *Hin*dIII and *Bam*HI (filled in) on pHC79 [15]. DNA sequences of these clones were determined after subcloning, as described in Materials and methods. *Open arrows with small closed boxes*, representing the human *osf1* open reading frame, indicate the orientation and approximate size (2.65 kbp) of respective transgene expression units. *Two open boxes* shown by Δ1 and Δ2 indicate the regions deleted in the transgene copies. The *solid line* on the top indicates the approximate size (~100 kbp) and regions covered by the three cosmid clones. The flanking regions consisting of γ-satellite repeated sequences are shown by *hatched boxes*. Note that the γ-satellite repeats are inverted together with transgene copies, as shown by *shadows* in the boxes as well as by *arrows* on the top. Nucleotide sequences at the inversion point are . . . GGCTAA/AAGTAC . . . linked between . . . GGCTAA[TAAAGG . . .] and [. . . TAGAGA]AAGTAC . . . , in which the sequences in [ ] are deleted as shown by Δ1. The nucleotide positions of A/A at the junction correspond to A at the 2243rd and T at the 1184th nucleotide positions in the transgene (accession no. AB004306). A 248-bp-long deletion between nucleotide positions 894 and 1141, Δ[CTTCAG . . . AACGAA], contained in the 3rd copy from the left, is indicated by Δ2. The nucleotide sequences of the left and right flanking regions will appear in the DDBJ databases under accession nos. AB105218 and AB105219, respectively. **B** Because *Hin*dIII does not cut at all and *Bam*HI cuts only once in the transgene DNA, the H3A1 DNA was digested completely with *Hin*dIII and completely with an excess amount of *Bam*HI (*lane* [a]) and partially with two different dilutions of *Bam*HI (*lanes* [b] and [c]), then separated on a 0.6% agarose gel and probed with the ^{32}P-labeled *Hin*cII–*Pst*I fragment (2.2 kbp in size) of pHC79. Size marker positions are indicated on the *left* (bp). The *osf1* transgene copies are repeated 13 times as shown on the *left* by B1 to B13. The *top band* indicated by "*Full size*" in *lane* [*c*] represents the entire H3A1 DNA singly cut by *Hin*dIII. Note that ΔB9 is migrating slightly faster than expected in the ladder

and both the flanking regions, namely mouse γ-satellite repeat sequences [17]. Southern blot analysis of H3A1 DNA indicated that the transgene was inserted as multiple copies, most likely 13 repeats (Fig. 3B). Apparently, the 9th band from the bottom migrated slightly faster than expected, suggesting some deletion(s) in the transgene.

Furthermore, DNA sequencing analysis revealed that 5 and 8 copies of the transgene were invertedly repeated, and that the 8th copy from the right and the 5th copy from the left (Fig. 3A) fused as a tail-to-tail inversion with a deletion in the middle. Another deletion was also found in the 5′-untranslated region of the 3rd copy from the left (Fig. 3A). Therefore, the transgenic mice contain at most 12 functional transgene copies per haploid, twice more than the initial estimation [8]. Concerning the flanking regions, it was revealed that the transgene copies were located in the middle of the mouse repeated sequences, called γ-satellite repeats, and no signs of any enhancer-like sequences such as an ERE were obtained, at least within ~50 kbp either side of the center of the transgene copies.

Discussion

We have reported that bone mass loss resulting from estrogen deficiency was compensated for in ovariectomized female mice in our transgenic line, which overexpresses human *osf1* under the control of a regulatory promoter containing the vitamin D response element (VDRE) for the human *osteocalcin* gene [8]. In this study, we compared the bone mass increase in the transgenic mice between males and females and found that it occurred only in females as judged by three criteria: bone mineral content (BMC), bone mineral density (BMD), and bone formation rate (BFR) (see Figs. 1, 2). In principle, there may be three possible explanations for the female-specific phenotype derived from the *osf1* transgene. First, the mRNA synthesis could be more efficient in females, probably due to an ERE which is located somewhere close to the transgene (positional effect). Second, it may be due to the VDRE contained in human osteocaolcin promoter used, which was multiply repeated in the mouse chromosome and thus could partially be cross-reactive to estrogen (repetition effect). Third, OSF1 protein synthesis or function may be more active in the presence of some other protein(s), whose gene(s) is (are) under the control of an ERE.

The first possibility is unlikely because we found no ERE sequences close to the transgene (within ~50 kbp). The second possibility would be that the human *osteocalcin* promoter with VDRE inserted repeatedly would cross-react with the estrogen receptor, and thus the *HPoc-Hosf1* transgene may be expressed more efficently. In fact, the VDRE in the *osteocalcin* promoter contains features in common with that of the ERE [18]. Concerning the third hypothesis, we have no confirming evidence so far.

Recently, Tare et al. [11,12] reported, using our transgenic mice, that bone mass increase was observed only in males but not in females. However, they failed to employ appropriate control mice or littermate mice indispensable for such experiments [7,8,19]. Instead, they used directly BDF1 mice purchased from animal breeders. Although our transgenic mice were created in BDF1 embryos, they have been maintained as transgenic homozygotes by inbred crossing and must therefore be genetically biased from the original strain. It is well known that, in mouse experiments, one mutation can have markedly different phenotypes when placed on different genetic backgrounds [20]. The variation is caused by different alleles at modifying loci in various mouse inbred strains. This consideration is particularly important in the case of BDF1, because it is genetically uniform because of the nature of a primary hybrid strain between two congenic strains, from which their descendents are largely biased after repeated inbred crossings.

The transgenic mice used in this work can be obtained as "bone-rich mice" from the Center for Animal Resources and Development, Kumamoto University, Kumamoto 860-0811, Japan (http://card.medic.kumamoto-u.ac.jp).

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References

1. Tezuka K, Takeshita S, Hakeda Y, Kumegawa M, Kikuno R, Hashimoto-Gotoh T (1990) Isolation of mouse and human cDNA clones encoding a protein expressed specifically in osteoblasts and brain tissues. Biochem Biophys Res Commun 173:246–251
2. Rauvala H (1989) An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. EMBO J 8:2933–2941
3. Li YS, Milner PG, Chauhan AK, Watson MA, Hoffman RM, Kodner CM, Milbrandt J, Deuel TF (1990) Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. Science 250:1690–1694
4. Kadomatsu K, Tomomura M, Muramatsu T (1988) cDNA cloning and sequencing of a new gene intensely expressed in early differentiation stages of embryonal carcinoma cells and in mid-gestation period of mouse embryogenesis. Biochem Biophys Res Commun 151:1312–1318
5. Tsujimura A, Yasojima K, Kuboki Y, Suzuki A, Ueno N, Shiokawa K, Hashimoto-Gotoh T (1995) Developmental and differential regulations in gene expression of *Xenopus* pleiotrophic factors-α and -β. Biochem Biophys Res Commun 214:432–439

6. Zhou H-Y, Ohnuma Y, Takita H, Fujisawa R, Mizuno M, Kuboki Y (1992) Effects of a bone lysine-rich 18 KDa protein on ostoblast-like MC3T3-E1 cells. Biochem Biophys Res Commun 186:1288–1293
7. Imai S, Kadsone M, Raulo E, Kinnunen T, Fages C, Meng X, Lakso M, Rauvala H (1998) Osteoblast recruitment and bone formation enhanced by cell-matrix associated heparin-binding growth-associated molecule (HB-GAM). J Cell Biol 143:1113–1128
8. Masuda H, Tsujimura A, Yoshioka M, Arai Y, Kuboki Y, Mukai T, Nakamura T, Tsuji H, Nakagawa M, Hashimoto-Gotoh T (1997) Bone mass loss due to estrogen deficiency is compensated in transgenic mice overexpressing human osteoblast stimulating factor-1. Biochem Biophys Res Commun 238:528–533
9. Suzuki A, Thies RS, Yamaji N, Song JJ, Wozney JM, Murakami K, Ueno N (1994) A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early *Xenopus* embryo. Proc Natl Acad Sci USA 91:10255–10259
10. Mundy GR (1993) Factors which stimulate bone growth *in vivo*. Growth Regul 3:124–128
11. Tare RS, Oreffo RO, Sato K, Rauvala H, Clarke NM, Roach HI (2002) Effects of targeted overexpression of pleiotrophin on postnatal bone development. Biochem Biophys Res Commun 298:324–332
12. Tare RS, Oreffo ROC, Clarke NML, Roach HI (2002) Pleiotrophin/osteoblast-stimulating factor 1: dissecting its diverse functions in bone formation. J Bone Miner Res 17:2009–2020
13. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR (1987) Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR histomorphometry nomenclature committee. J Bone Miner Res 2:595–610
14. Brady G, Jantzen HM, Bernard HU, Brown R, Schütz G, Hashimoto-Gotoh T (1984) New cosmid vectors developed for eukaryotic DNA cloning. Gene (Amst) 27:223–232
15. Hohn B, Collins J (1980) A small cosmid for efficient cloning of large DNA fragments. Gene (Amst) 11:291–298
16. Hashimoto-Gotoh T (1995) Quantitative determination of effective nibbling activities contaminating restriction endonuclease preparations. Anal Biochem 231:230–236
17. Allen MJ, Jeffreys AJ, Surani MA, Barton S, Norris ML, Collick A (1994) Tandemly repeated transgenes of the human minisatellite MS32 (D1S8), with novel mouse γ satellite integration. Nucleic Acids Res 22:2976–2981
18. Kerner SA, Scott RA, Pike JW (1989) Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D_3. Proc Natl Acad Sci USA 86:4455–4459
19. Daluiski A, Engstrand T, Bahamonde ME, Gamer LW, Agius E, Stevenson SL, Cox K, Rosen V, Lyons KM (2001) Bone morphogenetic protein-3 is a negative regulator of bone density. Nat Genet 27:84–88
20. Erickson RP (1996) Mouse models of human genetic diseases: which mouse is more like a human? Bioessays 18:993–998