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Systemically Administered rhBMP-2 Promotes MSC Activity and Reverses Bone and Cartilage Loss in Osteopenic Mice

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Abstract Osteoporosis is a disease manifested in drastic bone loss resulting in osteopenia and high risk for fractures. This disease is generally divided into two subtypes. The first, post-menopausal (type I) osteoporosis, is primarily related to estrogen deficiency. The second, senile (type II) osteoporosis, is mostly related to aging. Decreased bone formation, as well as increased bone resorption and turnover, are thought to play roles in the pathophysiology of both types of osteoporosis. In this study, we demonstrate in murine models for both type I (estrogen deficiency) and type II (senile) osteopenia/osteoporosis that reduced bone formation is related to a decrease in adult mesenchymal stem cell (AMSC) number, osteogenic activity, and proliferation. Decreased proliferation is coupled with increased apoptosis in AMSC cultures obtained from osteopenic mice. Recombinant human bone morphogenetic protein (rhBMP-2) is a highly osteoinductive protein, promoting osteogenic differentiation of AMSCs. Systemic intra-peritoneal (i.p.) injections of rhBMP-2 into osteopenic mice were able to reverse this phenotype in the bones of these animals. Moreover, this change in bone mass was coupled to an increase in AMSCs numbers, osteogenic activity, and proliferation as well as a decrease in apoptosis. Bone formation activity was increased as well. However, the magnitude of this response to rhBMP-2 varied among different stains of mice. In old osteopenic BALB/c male mice (type II osteoporosis model), rhBMP-2 systemic treatment also restored both articular and epiphyseal cartilage width to the levels seen in young mice. In summary, our study shows that AMSCs are a good target for systemically active anabolic compounds like rhBMP-2. J. Cell. Biochem. 86: 461–474, 2002.

Key words: osteoporosis; ovariectomy; rhBMP-2; mesenchymal stem cells; osteogenesis

Osteoporosis continues to represent a major public health problem. Loss of bone mass can compromise the skeleton's structural integrity, resulting in painful and debilitating fractures of the wrist, spine, and femur. Osteoporosis is generally divided into two types (I and II), which share similar histopathological features but which differ in clinical features and epidemiology [Kassem, 1996; Notelovitz, 1997]. The rapid loss in bone mass that occurs in postmenopausal women is known as type I osteoporosis and is primarily related to the loss of circulating estrogens [Kassem, 1996]. Type II osteoporosis, on the other hand, is one of the consequences of the aging process, and is manifested in a gradual decline in bone mass [Kahn et al., 1995; Bergman et al., 1996; Kassem, 1996]. This slower loss in bone mass that occurs in both women and men is also known as senile osteoporosis.

The mechanisms underlying these two types of osteoporosis are considered to be somewhat

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different. In type I osteoporosis, rapid estrogen depletion is the primary cause for the imbalance between bone formation and resorption [Pacifici, 1996, 1998; Jilka, 1998]. This type of bone loss is caused principally by an increase in cytokines, such as IL-1, IL-6, TNF-α, M-CSF, and GM-CSF, which promote osteoclastogenesis and subsequent bone resorption [Pacifici, 1996, 1998; Jilka, 1998]. The mechanisms that are responsible for type II osteoporosis are less well understood. However, one important component of this disease appears to be a decline in osteoblast numbers, as well as a decrease in the function of these bone forming cells [Nicolas et al., 1994; Manolagos and Jilka, 1995; Parfitt et al., 1995; Quarto et al., 1995; Gazit et al., 1998, 1999; Byers et al., 2001]. Thus, in senile (type II) osteoporosis, even in the absence of increased in bone resorption, there is a slow loss in bone mass [Manolagos and Jilka, 1995].

The osteoblasts that line the endosteum and the surfaces of trabecular bone are derived from adult mesenchymal stem cells (AMSCs) that are located in the bone marrow stroma [Kahn et al., 1995; Zhang et al., 1995; Bergman et al., 1996; Simmons, 1996]. AMSCs are pluripotent cells that can differentiate into various lineages including osteogenic cells [Owen, 1988; Owen and Friedenstein, 1988; Kahn et al., 1995; Bruder et al., 1997; Jaiswal et al., 1997; Krebsach et al., 1997; Prockop, 1997; Pittenger et al., 1999; Liechty et al., 2000]. Decreased AMSC levels and osteogenic activity in the bone marrow may be related to bone loss occurring in senile (type II) osteoporosis [Tsuji et al., 1990; Kahn et al., 1995; Bergman et al., 1996; Inoue et al., 1997; Gazit et al., 1998, 1999; D'Ippolito et al., 1999; Nishida et al., 1999; Muschler et al., 2001].

Transforming Growth Factor-β1 (TGF-β1) is an autocrine growth factor that is important for the promotion of proliferation and differentiation of AMSCs to osteogenic cells [Kuznetsov et al., 1997; Gazit et al., 1998, 1999]. We have previously shown that a reduction in the amount and/or activity of TGF-β1 in old osteopenic male mice is partly responsible for these regressive changes in the AMSCs [Gazit et al., 1998]. These observations are consistent with the hypothesis that in old mice, the decrease in both AMSC levels and osteogenic activity are caused by a reduction in the synthesis of TGF-β1, and perhaps also its bioavailability [Gazit et al., 1998, 1999]. Moreover, we have also shown that systemic administration of TGF-β1 to these aged mice is able to restore AMSC levels and activity, while also restoring bone mass [Gazit et al., 1999].

Bone morphogenic protein-2 (BMP-2) is a member of the TGF-β family. Recombinant human BMP-2 (rhBMP-2) induces enchondral ectopic bone formation [Wozney et al., 1988; Wang et al., 1990; Volek-Smith and Urist, 1996] and bone defect regeneration [Bostrom et al., 1999; Schmitt et al., 1999]. It does so by primarily promoting the osteogenic differentiation of AMSCs [Volek-Smith and Urist, 1996; Yamaguchi et al., 1996; Chaudhari et al., 1997; Hanada et al., 1997; Lacenda et al., 1997; Fromigue et al., 1998; Gori et al., 1999]. Since rhBMP-2 is a potent osteoinductive agent, we hypothesized that introducing exogenous rhBMP-2 systemically into both type I and type II osteopenic mice would reverse bone loss by stimulating AMSCs, and therefore, inducing bone formation and increasing bone mass.

In this study, we show that systemic intraperitoneal (i.p.) administration of rhBMP-2 to osteopenic mice has the following effects: (i) an increase in the levels of AMSCs; (ii) an increase in the proliferation and the differentiation of AMSCs; (iii) a decrease in AMSC apoptosis; (iv) a stimulation of bone formation; (v) an increase in the trabecular bone volume (TBV); and (vi) in senile osteopenic male mice, an increase in cartilage plate thickness to levels indistinguishable from those observed in younger mice. However, we also show that the ability of systemically administered rhBMP-2 to reverse bone loss in osteopenic mice varies with the strain of mice studied.

MATERIALS AND METHODS

Tissue Culture Supplies and Mice

Recombinant human BMP-2 was obtained from the Genetics Institute (Cambridge, MA). Fetal bovine serum (FBS), MEM tissue culture medium, and other tissue culture supplements were purchased from Biological Industries (Beit HaEmek, Israel). All other reagents were purchased from Sigma (St. Louis, MO). BALB/c male mice were obtained from Charles River (Wilmington, MA), ICR female mice were obtained from Harlan Laboratories (Israel), and Swiss Webster female mice were obtained from Taconic Farms (Germantown, NY). Swiss Webster mice were housed in a facility accre-

dited by the American Association for Accreditation of Laboratory Animal Care in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals, and the study was approved by the institutional animal care and use committee of Wyeth–Ayerst Research.

Cell Culture

Isolation and culturing of bone marrow derived AMSCs was performed as previously described [Gazit et al., 1998]. Briefly, bone marrow was recovered from the tibial and femoral diaphyses. After being gently re-suspended in medium by passage through a series of 19G, 21G, and 23G needles, the marrow cells were counted, diluted, and seeded into 35 mm plastic culture dishes (Nunc, Denmark) at a density of 10^6 cells/plate. The plates were then incubated at 37°C in a 5% CO_2/95% air incubator with MEM medium supplemented by 100 U/ml of Pen-Strep and 10% FBS. The medium was replaced 48 h after plating, and twice a week thereafter. Supplements for mineralization included 10 mM β-glycerophosphate, 10^{-8} M dexamethasone (Dex), and 50 μg/ml ascorbic acid that were added to the medium with the first and all subsequent media changes. Cultures of AMSCs were generally assayed after 12 days of incubation.

Alkaline Phosphatase Histochemistry and Histomorphometry

On day 12, six 35-mm plates of each experimental group were washed with PBS, fixed with citrate-acetone-formalin, and stained for alkaline phosphatase (ALP) activity (Sigma 86-R kit). After staining, colonies were counted: $>50\%$ of the colonies typically stained positive for ALP. We defined a colony as a group or cluster containing 16 or more cells. Within each group, we averaged the scores/plate for the six plates.

Histomorphometry

The size of the ALP positive AMSCs was determined using a computerized morphometric system (Galai: CUE-3 Electro Optical Inspection and Diagnostic Laboratories Ltd., Migdal Haemek, Israel).

Cell Proliferation

AMSCs in two four-well chamber slides (Nunc, Denmark) were incubated with BrdU labeling solution at 37°C for 2 h, fixed in 70% alcohol, washed with distilled-deionized water, and stained immunohistochemically using the Streptavidin-Biotin system (BrdU Staining Kit, Zymed, Cat93-3943). BrdU-positive cells were counted in seven randomly selected fields, and data were presented as the percentage of the total cell number.

Apoptosis

AMSC cultures obtained from old osteopenic male BALB/c mice were analyzed for the presence of apoptotic cells. We detected AMSCs in both the early and late stages of apoptosis using ApoAlert Annexin V Apoptosis Kit (Clonetech) [Griffith et al., 1995; Martin et al., 1995]. In this assay, cells are incubated in the Annexin V-FITC, with either 1 or 10 μl/ml propidium iodide (PI). Early stage apoptotic cells bind Annexin V-FITC and display green staining on the plasma membrane. Late stage apoptotic cells, which have lost membrane integrity, show red (PI) staining throughout the cytoplasm as well as green staining on the plasma membrane. Late stage cells were also detected by incubating CFU-f in DAPI (4′,6′-diamidino-2-phenylindole) [Keren-Tal et al., 1995; Amsterdam et al., 1996, 1997]. The DNA of apoptotic cells includes highly dense and irregular nuclear chromatin inclusions, while the DNA of non-apoptotic cells stain homogenously throughout the entire nucleus [Amsterdam et al., 1996]. As positive controls, we pretreated AMSCs with 100 μg/ml etoposide, which is known to elicit apoptosis in cultured cells [Moran et al., 1996; Stefanelli et al., 1998]. Quantitative determination of the degree of apoptosis was done on seven randomly selected microscopic fields of each well [Keren-Tal et al., 1995]. Data for each chamber slide were expressed as the mean ± SE. The level of apoptosis in AMSCs obtained from ovariectomized and sham operated ICR mice, grown in four well chamber slides, was established using an apoptosis detection kit-Fluorescein (ApopTag, Oncor) based on the TUNEL technique. Apoptotic cells were visualized with an epifluorescent microscope (Nikon EDF-3) and Zeiss 410 LSM confocal microscope. Quantitative determination of the degree of apoptosis was done on seven randomly selected microscopic fields of each well [Keren-Tal et al., 1995]. Data for each chamber slide were expressed as the mean ± SE.

In Vivo Experiments

As a model for type I osteopenia/osteoporosis (estrogen deficiency), female ICR mice (6–7-weeks old) were either ovariectomized (OVX) or sham operated. Five months after surgery, the mice were systemically administrated with rhBMP-2, i.p., at doses of 1.0 or 5.0 μg per mouse per day (0.2 ml total volume) for 20 days. The vehicle control group received injections of 0.1% BSA/PBS. In addition, female Swiss Webster mice were also either OVX or sham operated at 2 months of age. The mice were then placed on a casein diet (Animal Specialties and Provisions, Quakertown PA). Eighty one days after surgery, treatments were initiated: rhBMP-2 was injected once daily, i.p., for 20 days, at doses of 0.5–25 μg per mouse per day. The vehicle control group received PBS containing 0.1% BSA, and the dosage volume in all cases was 0.1 ml per mouse. As an additional control, the OVX mice were also treated with 2 μg/day of hPTH 1–34, s.c., for 20 days.

As a model for type II osteopenia/osteoporosis (senile bone loss), aged male BALB/c mice (24-months old) were used. These mice were systemically administrated with rhBMP-2, i.p., at doses of 0.5, 1.0, and 5.0 μg per mouse per day (0.2 ml total volume) for 20 days. The vehicle control group received injections of 0.1% BSA/PBS.

Histomorphometry

Femora and tibiae were dissected free of soft tissue, fixed in 4% buffered formalin, decalcified, embedded in paraffin, sectioned (at 5 μm), and stained with H&E and Masson trichrome. TBV was measured in a standardized metaphyseal area (secondary spongiosa) using automated image morphometric analysis (Automatic Morphometrical Computerized system, Galai: CUE-3 Electro Optical Inspection and Diagnostic Laboratories Ltd.). For the kinetic analysis of bone formation, 2.5 mg/kg of the fluorochrome label calcein green was administered, i.p., at 7 and 2 days prior to euthanasia. The tibiae were then removed and fixed in 70% ethyl alcohol (EtOH), and embedded in plastic (Immunobed Kit, Polysciences, Inc.). Ten-micrometer unstained-sections were evaluated by fluorescent microscopy. Specifically, histomorphometric measurements of the proximal tibia metaphysis (secondary spongiosa) were performed with computerized NIH image 1.60 program interfaced via a camera lucida attached to a Nikon light/fluorescent microscope. Total and double-labeled areas were measured at a 25 × magnification. Parameters of mineral apposition rate (MAR) [Bain et al., 1993; Rubin et al., 1995] were determined at 100 × magnification. Trabecular mineral apposition rates (MARt) were calculated as described previously [Bain et al., 1993]. In addition to MAR, we have measured the length in millimeter of both double-labeled and single-labeled mineralizing fronts on top of the bone. Using these two measurements, we calculated the percent length of the double-labeled fronts of the total (both single- and double-labeled) labeled bone surfaces. This resulted value serve to indicate the number and extent of active matrix deposition sites represented by double labeling, compared to total bone surface area and non-active sites represented by single-labeled surfaces.

The quantitative analysis of the articular and epiphyseal cartilage thickness in young and old-osteopenic BALB/c male mice was performed on paraffin sections of Femur (stained with Masson-trichrome) using computerized NIH image 1.60 program interfaced via a camera lucida attached to a Nikon light/fluorescent microscope. The thickness of the cartilage was obtained from the average of four separate measurements, two made on each side and all four perpendicular to the transverse axis of the cartilage [Gonzalez-Riola et al., 1997].

pQCT and Micro-CT Analysis of OVX Swiss Webster Mice

After treatment, animals were euthanized and the peritoneal cavity was cut open to verify that all bilateral ovariectomies had been properly executed. The uterus was carefully excised, freed of adherent tissues, and weighed. Both tibias and both femurs from each mouse were excised, placed in 70% ethanol, and stored at 4°C.

For pQCT, analysis of the excised femurs was performed using a Norland Stratec XCT Research "M" bone densitometer (Model 921010). Readings of the distal femur metaphysis spongiosa were obtained using a reference line on the bone located 2.5 mm from the distal end of the femur. Analysis of variance and Dunnetts *t*-test were used for statistical evaluation of the data. There were 12 animals per group.

For micro-CT analysis, the distal portion of the right femur (about 1 cm in length) was placed in an 8-mm diameter polycarbonate tube. A total of 1.8 mm (200 slices, 9 μm each with a voxel size of 9 μm^2) was scanned at high-resolution from the growth plate proximally towards the mid-shaft region. Three-dimensional analysis was performed on X-ray images by contouring the trabecular boundaries at the cortical junctions. The area analyzed was immediately adjacent to the growth plate and 1.22 mm (135 slices) proximal to the growth plate corresponding to the VOI included in histological evaluations and pQCT analysis. Sequential analysis of X-ray images at an interval of 5–10 slices allowed the software to recreate a three-dimensional picture of VOI and derive a number of actual and calculated parameters including bone volume, trabecular number/mm (Tb.N), trabecular thickness, trabecular separation, and connectivity density.

Morphology of Internal Organs

Liver, spleen, and kidney were removed and fixed in 4% buffered formalin, dissected, embedded in paraffin, sectioned (5 μM), and stained (with H&E and Masson trichrome).

Statistical Analysis

Quantitative data were analyzed using the Mann–Whitney test or the one factor ANOVA test [Siegel, 1956]. The data were presented as the mean values ± SE.

RESULTS

The Skeletons of Old Male BALB/c Mice Are Osteopenic

The osteopenic nature of the skeleton of aged male mice is readily apparent in the hemisected femur shown in Figure 1. The femurs of these old animals contained fewer, thinner, and less well connected trabeculae (Fig. 1b) than their young mouse counterparts (Fig. 1a). In bones from young mice, the trabeculae had a nearly plate-like morphology that not only reflected a larger amount of bone but also suggested greater mechanical strength.

The Skeletons of Ovariectomized Female ICR Mice 5-Months After Surgery Are Osteopenic

Estrogen depletion in OVX ICR mice compared to sham operated animals was apparent from the decrease in uterine weight in OVX compared with sham operated animals [data not shown, see also Zhou et al., 2001]. The osteopenic/osteoporotic nature of the skeletons of OVX ICR mice was characterized previously by our group [Zhou et al., 2001], and is demonstrated again in Figure 1c,d. Sham operated mice had significantly more TBV in tibia and femur compared to OVX mice [Zhou

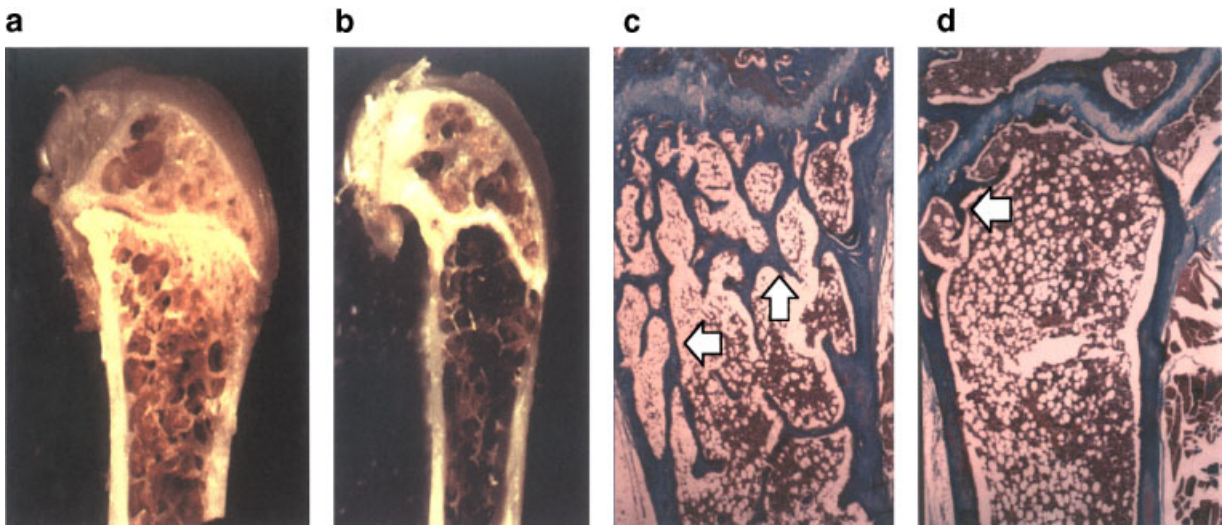
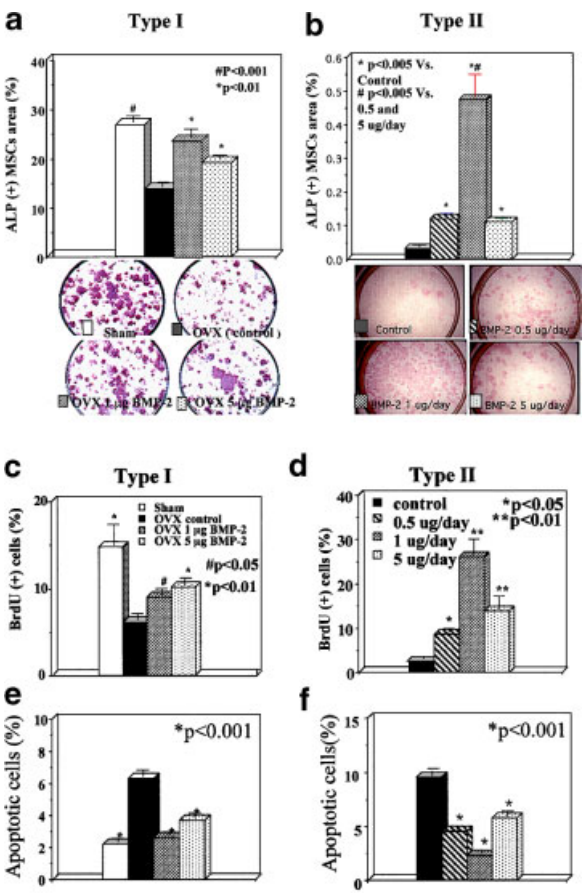


Fig. 1. The structure of mouse femurs from osteopenic mice. Fresh, non-fixed, femurs obtained from untreated BABL/c male mice: (**a**) young (4 months) and (**b**) old (24 months). In (b), note the decrease in the number of trabeculae, which are also thinner and less well connected than in (a) (original magnification × 3.5). In (**c**), a photomicrograph of sham operated female ICR mice showing normal trabecular bone, compared with the osteopenic phenotype of ovariectomized ICR mice shown in (**d**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 2001]. Bone formation parameters as evaluated by double labeling and mineral apposition rate trabecular (MARt) and cortical (MARc) were also shown to decrease in OVX animals compared with sham operated animals [Zhou et al., 2001] and (Fig. 4c).

Decreased ALP Activity in AMSCs Obtained From Ovariectomized ICR Mice

AMSCs obtained from OVX and sham ICR mice (5 months after operation) were assayed for ALP. The results show a significant decrease in ALP positive colony area ($P < 0.001$) in cultures obtained from OVX mice compared with sham operated animals (Fig. 2a). Conversely, a significant increase in the size of ALP positive colonies ($P < 0.01$) was observed in animals treated systemically with rhBMP-2 in doses of either 1 or 5 μg per day, i.p., for 20 days (Fig. 2a). Expression of murine BMP-2 mRNA in AMSCs obtained from OVX mice compared to sham was demonstrated by our group previously [Zhou et al., 2001]. BMP-2 transcripts were practically non-detectable in cultures obtained from OVX mice [Zhou et al., 2001].



Systemic rhBMP-2 Treatment Increases the Number and Proliferative Activity of Marrow AMSCs and Inhibits AMSCs Apoptosis

We have postulated that the levels and osteogenic activity of AMSCs is a function of growth factor-stimulated bone formation. In

Fig. 2. Changes in the cellular properties of cultured AMSCs after systemic treatment with rhBMP-2. (**a**, **Top**) Histogram representing the size of ALP positive AMSC colonies in primary cultures after 12 days of incubation. The cells were derived from the bone marrow of ovariectomized ICR mice (5 months after surgery) systemically treated with rhBMP-2, i.p., at doses of 0 μg (vehicle control), 1, and 5 μg per mouse per day for 20 day, as well as sham-operated control mice. Note the significant increase in ALP (+) colony area in OVX mice treated with rhBMP-2, reaching the levels of sham-operated mice. The results represent the mean ± SE. (a, **Bottom**) ALP histochemistry of AMSCs obtained from ovariectomized mice treated with rhBMP-2 as described above. Note the increase in the ALP (+) colony area in AMSCs obtained from ovariectomized rhBMP-2 treated mice. (**b**, **Top**) Same as (a) for cultures obtained from aged (24 months) BALB/c male mice treated systemically with rhBMP-2, i.p., at doses of 0 μg (vehicle control), 0.5, 1, and 5 μg per mouse per day for 20 days. Note the significant three-fold increase in ALP (+) colony area at the doses of 0.5 μg/day and 5 μg/day, and more than 10-fold increase at the dose of 1 μg/day ($P < 0.005$ vs. non-treated controls). The results represent the mean ± SE of triplicate cultures from two experiments. (b, **Bottom**) ALP histochemistry of AMSCs obtained from old male mice treated with rhBMP-2 as described above. Note the increase in the area of ALP (+) colonies from treated mice. (**c**) Histogram representing the percentage of BrdU positive cells in primary AMSC cultures after 12 days of incubation. The cells were derived from ovariectomized ICR mice (5 months after surgery) systemically treated with rhBMP-2 as described in a. Again note the significant increase in the percentage of BrdU positive cells. (**d**) Histogram representing the percentage of BrdU positive cells in primary AMSC cultures after 12 days of incubation. The cells were derived from the bone marrow of aged (24 months) BALB/c male mice treated systemically with rhBMP-2 as described in Figure 2. Note the significant increase in the percentage of BrdU-positive cells, which reflects the rate of cell proliferation in the treated animals. The results represent the mean ± SE in duplicate cultures of two experiments. (**e**) Histogram representing the percentage of apoptotic cells (DAPI) in AMSC cultures after 12 days of incubation. The cells were derived from ovariectomized-ICR mice (5 months after surgery) systemically treated with rhBMP-2 as described in (a). Again note the significant decrease in the percentage of apoptotic cells from treated animals comparable to those obtained from OVX control mice. (**f**) Histogram representing the percentage of apoptotic AMSCs after 12 days of incubation. The cells were derived from the bone marrow of aged (24 months) BALB/c male mice treated systemically with rhBMP-2 as described in (b). Note the significant decrease in the percentage of apoptotic cells among the treated AMSCs when compared to the non-treated control cells ($P < 0.001$); this difference reflects a decrease in the rate of programmed cell death. The results represent the mean ± SE of duplicate cultures from two experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

fact, we have found that treating osteopenic mice (of both type I and II osteoporosis models) systemically with rhBMP-2 increased the number and osteogenic differentiation of AMSCs isolated from their bone marrow (Fig. 2). Injections of rhBMP-2 at 0.5 μg (in type I model, Fig. 2a), 1 and 5 μg per day, i.p., for 20 days led to significant increases in the size of ALP-positive colonies as measured by the percentage of the culture plate covered by the cells (Fig. 2). This noted increase in size of ALP-positive colonies was observed in both models of osteoporosis. Given these increases in colony size, it is not unexpected that systemic treatment of rhBMP-2 also stimulated the AMSC proliferation (Fig. 2c,d). Actively dividing cells in the S-phase of mitosis are 5-bromo-2′-deoxyuridine positive (BrdU) [Porstmann et al., 1985]. The percentage of BrdU-positive cells was significantly increased in cultures obtained from old osteopenic male BALB/c or OVX ICR mice treated with rhBMP-2 compared to cultures from non-treated animals. In contrast, the fraction of cells undergoing apoptosis is markedly reduced in AMSCs cultured from rhBMP-2 treated mice when compared to those derived from control animals (Fig. 2e,f).

Systemic rhBMP-2 Treatment Increases the Volume of Trabecular Bone in Both Type I and Type II Osteopenic Mice

We have found statistically significant increased TBV in osteopenic mice treated systemically with rhBMP-2 at doses of 0.5, 1, and 5 μg per mouse per day, i.p., for 20 days (Fig. 3). However, the effect was not dose dependent, since we observed a 4–5-fold increase in bone volume for each of these doses in the type II model of osteoporosis (Fig. 3b). In the type I osteoporosis model, a reduction in TBV was observed in mice treated with the 5 μg dose of rhBMP-2 when compared to animals treated with the 1 μg dose (Fig. 3a). Note that the administration of rhBMP-2 at all three doses to aged male BALB/c mice (type II) and with the 1 μg dose to OVX ICR mice (type I) increased their TBVs to levels approximating those seen in young mice and sham operated mice, respectively.

In contrast to the robust responses observed in aged male BALB/c mice and OVX ICR mice, OVX Swiss Webster mice exhibited a smaller response to rhBMP-2 (Fig. 3c). As expected, ovariectomy of 2-month-old Swiss Webster mice for 81 days resulted in an 83% decrease in uterine wet weight and a 59–61% decrease in distal femur TBV as determined by histology and pQCT (data not shown). Systemic injection (i.p.) of 0.5, 1, and 25 μg of rhBMP-2 per mouse per day for 20 days increased distal femur trabecular bone mineral density (BMD) by only 4% at the lowest dose, while the higher doses decreased BMD (data not shown). Similarly, the 0.5 μg dose of rhBMP-2 increased distal femur mineralized area by 22.6%, while the higher doses of peptide again resulted in a decrease in this histological parameter (data not shown). However, all of these responses were not significantly different from the vehicle treated control animals. On the other hand, treatment of the OVX mice with 0.5 μg of rhBMP-2 significantly ($P < 0.05$) increased trabecular number by 18% over the vehicle treated animals as determined by μCT (Fig. 3d). Likewise, trabecular connectivity density increased 47% ($P < 0.01$) and trabecular spacing decreased 16% ($P < 0.01$) upon treatment of the OVX Swiss Webster mice with 0.5 μg of rhBMP-2 (data not shown). For comparison, subcutaneous (s.c.) injections of synthetic hPTH 1–34 at a dose of 2.0 μg per mouse per day for 20 days increased trabecular BMD by 50% ($P < 0.05$) as determined by pQCT [Alexander et al., 2001]. Thus, in OVX mice, the effect of systemically administered rhBMP-2 on the skeleton appears to be strain dependent and is not as effective as hPTH 1–34.

Systemic rhBMP-2 Treatment Stimulates Bone Formation in Both Type I and II Osteopenic Mice

Given the results reported above, it is not unexpected that systemic injection of rhBMP-2 also markedly stimulates osteoblastic activity. This is shown both qualitatively and quantitatively (Fig. 4). The extent to which the cell surfaces are labeled is a reflection of the number of osteoblasts involved in matrix synthesis [Gazit et al., 1993; Rosen et al., 1994; Manolagos and Jilka, 1995]. In both aged osteopenic male BALB/c mice and OVX female ICR mice at doses of 1 and 5 μg/mouse/day, rhBMP-2 significantly increased the extent of labeled surfaces above that of the vehicle control treated animals (Fig. 4a,b). Moreover, the administration of rhBMP-2 also augmented the MAR [Bain et al., 1993; Rubin et al., 1995] as we have shown quantitatively (Fig. 4c,d). Together, these data

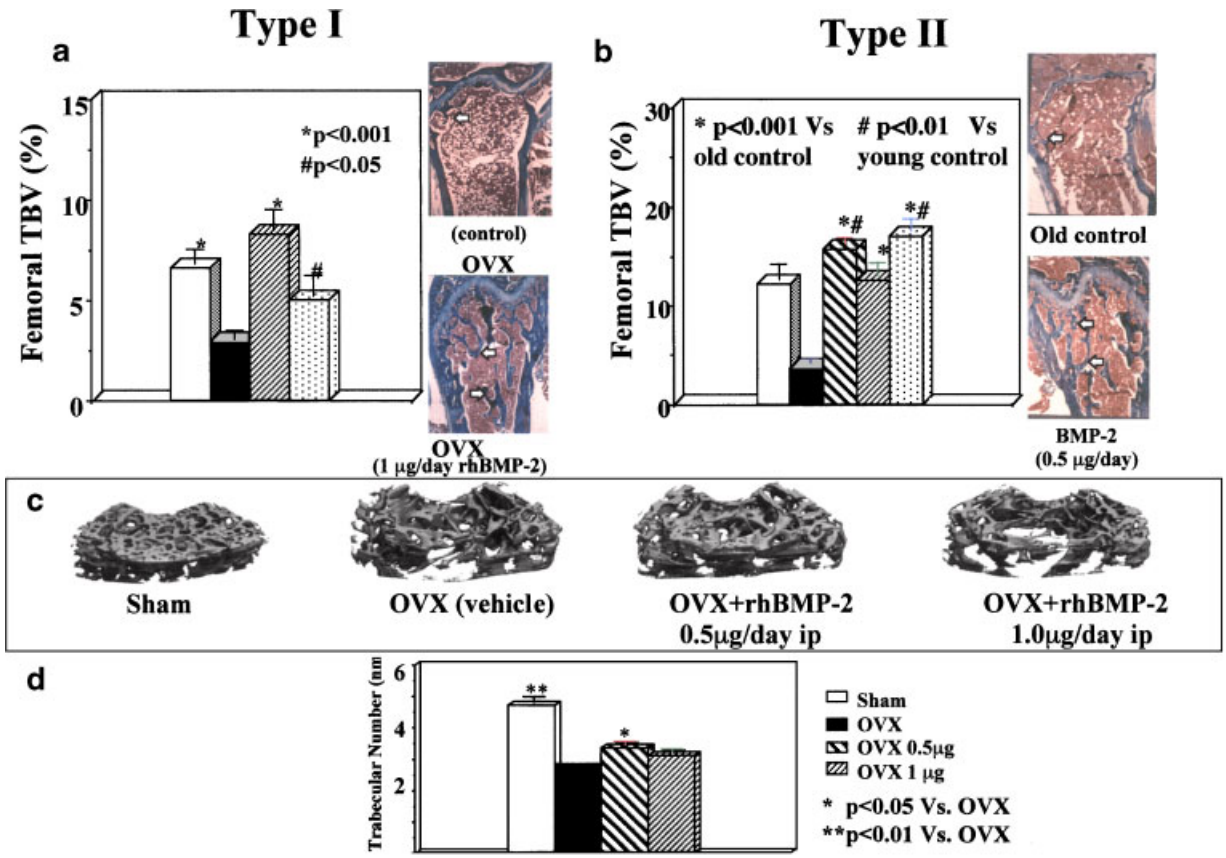


Fig. 3. Systemic treatment with rhBMP-2 leads to increased TBV. (**a**, **Left**) Histogram representing TBV in the femur metaphysis obtained from sham and ovariectomized rhBMP-2 treated ICR mice. Note the significant increase in TBV in treated animals, reaching the levels of the sham-operated mice. The results are presented as mean ± SE, and statistical significance was determined by Mann–Whitney test. (a, **Right**) Photomicrographs of sections stained with Masson Trichrome demonstrating rhBMP-2 treated and control mice. (**b**, **Left**) Histogram representing TBV in femurs obtained from young (4 months) BALB/c male non-treated mice, and old mice (24 months) treated systemically with rhBMP-2 as described in Figure 2. Note the significant increase in trabecular volume in bones from the treated animals. The results are presented as mean ± SE of 18 animals from three experiments. (b, **Right**) Photomicrographs of sections stained with Masson trichrome demonstrating rhBMP-2 treated and control mice. (**c**) Micro-CT three-dimensional reconstructions of distal femur trabecular bone from Swiss Webster mice that were sham operated, OVX treated with vehicle, OVX treated with 0.5 μg rhBMP-2, and OVX treated with 1 μg rhBMP-2 (as described in Fig. 2). Note the small increase in TBV in the OVX mouse treated with 0.5 μg of rhBMP-2 compared to the OVX (vehicle) control treated mouse. (**d**) Histogram showing the micro-CT analysis for trabecular thickness from the same experiment. Note the small but significant ($P < 0.05$) increase in trabecular number in OVX mice treated with 0.5 μg rhBMP-2 compared with OVX vehicle control treated mice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

showed that systemic treatment of mice suffering from both type I and II osteopenia with rhBMP-2 led to increases in the rates of matrix synthesis and mineralization.

Systemic rhBMP-2 Treatment Stimulates Cartilage Formation in Old Osteopenic Male Mice

Systemic i.p. injection of aged male BALB/c mice with 0.5 and 1.0 μg of rhBMP-2 per mouse per day for 20 days markedly stimulated articular (Fig. 5a) and epiphysial (Fig. 5b) cartilage regeneration in femoral bones. As shown both qualitatively (Fig. 5c) and quantitatively (Fig. 6), cartilage thickness, reflecting the rate of cartilage regeneration, and matrix synthesis, increased significantly in both the articular and epiphysial cartilage of these mice following rhBMP-2 treatment.

Systemic rhBMP-2 Treatment Does Not Appear to Have Adverse Extraskeletal Effects in Osteopenic Mice

Systemic daily i.p. injections of aged male BALB/c mice or OVX ICR mice with rhBMP-2

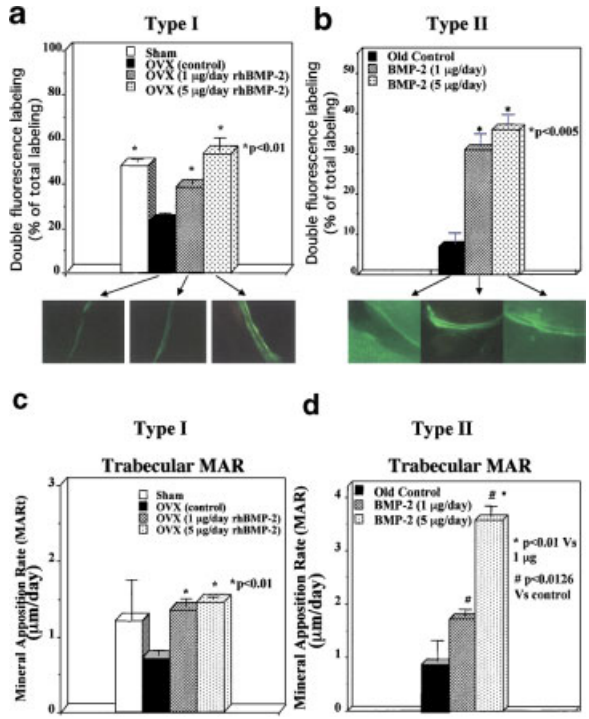


Fig. 4. Systemic treatment with rhBMP-2 leads to increased trabecular bone formation. (**a**) Histogram representing the percentage of double fluorescent labeled surfaces from the tibia metaphyseal area of ovariectomized ICR mice (5 months after surgery) systemically treated with rhBMP-2 as described in Figure 2. Note the significant increase ($P < 0.01$) in the extent of double labeled surfaces (reflective of matrix mineralization) in the rhBMP-2 treated animals compared to the levels of OVX vehicle treated control mice. Results are presented as mean ± SE. (**b**) Histogram representing the percentage of double fluorescent labeled surfaces from the tibia metaphyseal area of aged BALB/c male mice (24 months) treated systemically with rhBMP-2 as described in Figure 2. Note the significant increase ($P < 0.05$) in the extent of double labeled surfaces (reflective of matrix mineralization) in the treated animals. The results are presented as mean ± SE of 18 animals from three experiments. Bellow (a) and (b) are representative fluorescent photomicrographs. (**c**) Histogram showing the mineral apposition rate in trabecular bone (MARt) in the tibial metaphyseal area of the ovariectomized mice treated with rhBMP-2. Note the significant increase in MARt in BMP-2 treated mice reaching the levels observed in the sham animals. The results are presented as mean ± SE. (**d**) Histogram showing the mineral apposition rate of trabecular bone (MARt) in the tibial metaphyseal area of the aged male mice (24 months) treated with rhBMP-2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

for 20 days did not produce changes in the body weights of the animals. Also, no effects on kidney, liver, or spleen morphology were observed in the aged male mice treated with the highest dose (5 μg/day) of the peptide (data not shown).

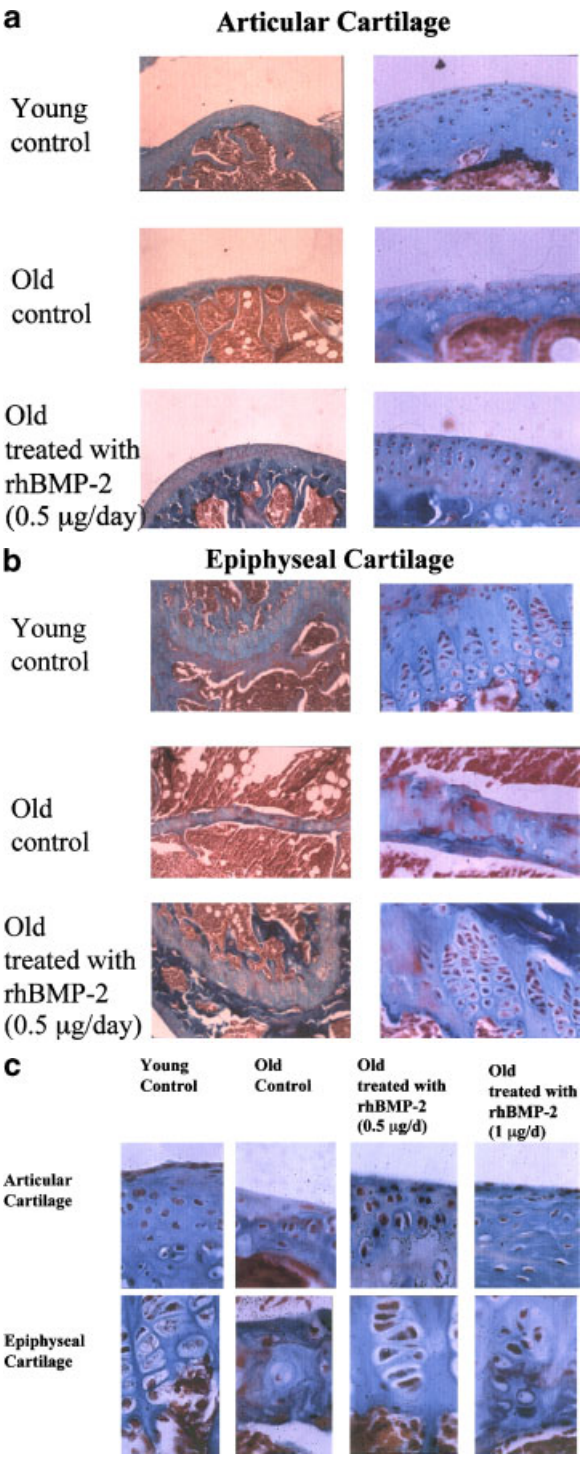
DISCUSSION

Bone marrow stromal cells represent a stem cell population that is able to differentiate into different mesenchymal lineages including bone and cartilage [Prockop, 1997; Pittenger et al., 1999; Liechty et al., 2000; Turgeman et al., 2001]. As indicated in the literature, the age-related decrease in the AMSC population results in a more limited ability of such marrow cells to undergo osteogenesis, and leads to the loss of trabecular bone and bone mass in aged male mice [Kahn et al., 1995; Gazit et al., 1999]. Previously, we confirmed that there was a lower AMSC level in the marrow of old male mice by showing that the bone marrow of such animals produces substantially smaller bone ossicles when transplanted under the renal capsule than does the marrow of young mice [Gazit et al., 1998]. Moreover, several studies reported similar observations in humans that correlate reduced levels of AMSCs with increased aging [D'Ippolito et al., 1999; Nishida et al., 1999; Byers et al., 2001]. Our observations in the current study suggest a similar effect of ovariectomy on osteogenesis. Moreover, BMP-2 mRNA expression was down-regulated in AMSCs obtained from OVX mice [Zhou et al., 2001], suggesting a decrease in the osteogenic potential of AMSCs following estrogen depletion.

BMPs are part of the TGF-β superfamily whose members are related to each other and have a large number of biological functions [Rosen and Thies, 1992; Kingsley, 1994]. The osteogenic activity of BMP-2 has been demonstrated by its ability to induce endochondral bone formation in ectopic sites in vivo [Wozney et al., 1988; Wang et al., 1990; Volek-Smith and Urist, 1996]. BMP-2 has been detected at fracture sites in rats with the highest amount in mesenchymal cells, early chondrocytes, and early osteoblasts [Bostrom et al., 1995; Bostrom, 1998]. Likewise, rhBMP-2 induces osteogenic differentiation of various cell types in vitro including osteoblast-like and chondroblast-like cells [Katagiri et al., 1990; Chen et al., 1991; Cheng et al., 1994; Hughes et al., 1995; Puleo, 1997], as well as AMSCs [Thies et al., 1992; Rickard et al., 1994; Yamaguchi et al., 1996; Hanada et al., 1997]. It is important to mention that the ability of rhBMP-2 to induce ectopic bone formation in aging rats decreases as a function of increasing age. This decrease in the response to BMP-2 may be due in part to a

decrease in the number of AMSCs present in aged rats or to a change in the responsiveness of these target cells to the peptide [Fleet et al., 1996].

At the present time, there is great interest in the possible therapeutic applications of growth factors capable of stimulating bone formation



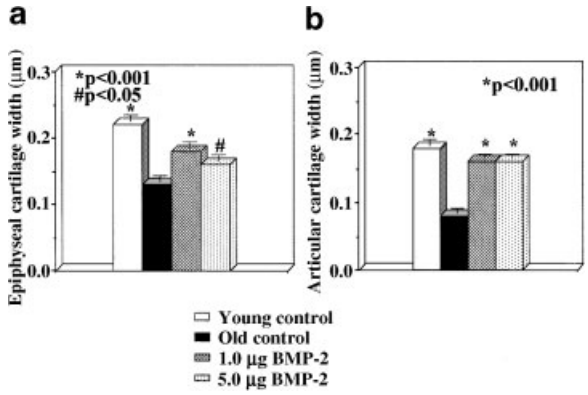


Fig. 6. Systemic treatment with rhBMP-2 leads to increased articular and epiphyseal cartilage width. Histogram representing articular and epiphyseal cartilage width measured in the distal femoral epiphysis obtained from aged (24 months) BALB/c male mice treated for 20 days with 1 and 5 μg/day of rhBMP-2, i.p. Note the significant increase in articular and epiphyseal cartilage width in rhBMP-2 treated animals.

in the treatment of age-related osteoporosis [Rodan and Martin, 2000]. Most existing therapies are thought to influence healing primarily through their inhibition of bone resorption. BMP-2 is well established as having powerful modulatory effects on bone. Moreover, our results regarding the effect of systemic rhBMP-2 on cartilage suggest that rhBMP-2 can be used to stimulate cartilage formation as was shown before with local administration of the peptide both to cartilage and bone [Glansbeek et al., 1997; Sellers et al., 1997; Turek et al., 1997].

In the current study, we have extended our previous analysis to the whole animal by administering rhBMP-2 systemically via i.p. injection. Our earlier results suggested that the reduced osteoblastic bone forming activity of

Fig. 5. Effects of systemic administration of rhBMP-2 on cartilage production. (**a**) Femoral articular cartilage obtained from young (4 months) BALB/c male non-treated (control) mice, aged (24 months) male non-treated mice, and aged (24 months) male mice treated for 20 days with 0.5 μg/day of rhBMP-2, i.p. Note the increase in cartilage plate width and chondrocyte number in the rhBMP-2 treated animals. (The photomicrographs are of decalcified paraffin embedded sections that were Masson trichrome stained; original magnification × 40). (**b**) Same as in (a) but for the epiphyseal cartilage. (**c**) Femoral articular and epiphyseal cartilage obtained from young (2 months) male non-treated mice, old (24 months) male non-treated mice and old (24 months) male mice treated for 20 days with 0.5 and 1 μg/day of rhBMP-2, i.p. Again note the increase in cartilage plate width and chondrocyte number in treated animals. (These photomicrographs are of decalcified paraffin embedded sections that were Masson trichrome stained; original magnification × 100). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

AMSCs in aged male mice was principally the result of the diminished availability of TGF-β family members in the matrix [Kahn et al., 1995; Gazit et al., 1998]. We have, therefore, tested the possible role of systemic rhBMP-2 administration in providing such aged animals with a new source of the growth factor in order to attempt to reverse senile bone loss. We found that rhBMP-2 injected into old male mice increased the amount of trabecular bone as well as epiphyseal and articular cartilage thickness. In addition, the proliferative activity and differentiation rate of AMSCs increased upon peptide administration. The suppressive effects of rhBMP-2 on apoptosis that we observed are consistent with observations by D'Alessandro et al. [1994] which showed the ability of this growth factor to induce differentiation and prevent the death of astrocyte lineage cells. Finally, we did not observe any adverse effects of rhBMP-2 on several internal organs of the aged male mice.

Interestingly, similar results were also observed when the same methodology was applied to OVX female ICR mice representing a model of type I osteoporosis. Daily systemic administration of rhBMP-2 to these mice increased the amount of trabecular bone to that seen in the sham operated control animals. In addition, the proliferative rate and ALP activity of the AMSCs also increased, while apoptosis of these cells decreased after treatment with the peptide. In contrast to these results, relatively mild effects of systemically administered rhBMP-2 were observed with the OVX Swiss Webster mice. This observation suggests that the ability of rhBMP-2 to act systemically on the skeleton varies with the strain of mice studied. Indeed it was previously shown that the bone inductive activity of demineralyzed bone matrix varies in different mice strains [Marusic et al., 1999]. The reasons for this strain variation are not clear at the present time. However, since systemically administered hPTH 1–34 produced a robust increase in bone formation in the OVX Swiss Webster mice [Alexander et al., 2001], this variation is not due to an overt skeletal defect in these animals.

The etiology of postmenopausal osteoporosis is believed to be related to increased osteoclastogenesis, and estrogens have been shown to suppress bone resorption [Pacifici, 1996, 1998; Jilka, 1998]. However, impairment of osteogenesis has also been observed in postmenopausal osteoporotic patients [Rodriguez et al., 1999]. Furthermore, we have previously shown that estrogens can stimulate the osteogenesis of mouse AMSCs in vitro [Zhou et al., 2001], and our current results indicate that osteogenesis is also affected by estrogen depletion in vivo. Thus, in view of the observation that BMP-2 mRNA expression is down-regulated in AMSCs obtained from OVX mice, one can postulate that this growth factor is an important mediator of estrogen action on AMSCs in vitro and on osteogenesis in vivo. Moreover, it is reasonable to propose that this effect can be mimicked (after estrogen depletion) by administrating rhBMP-2.

Finally, we conclude from our experiments that osteogenesis is impaired not only in type II (senile) osteoporosis, but also in type I (postmenopausal) osteoporosis. Reduction in the number and activity of AMSCs should, therefore, be seriously considered as part of the pathophysiology of type I osteoporosis. Moreover, directing therapy towards inducing osteogenesis in both types of osteoporosis can be effective in restoring bone mass.

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