Prevention of Bone Loss after Ovariectomy in Mice with Preferential Overexpression of the Transcription Factor Paired Box-5 in Osteoblasts

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We have recently shown that the transcription factor Paired box-5 (Pax5) promotes bone formation in vivo and osteoblastogenesis in vitro. Here, we demonstrated the involvement of Pax5 in bone remodeling after ovariectomy (OVX). A significant increase was seen in vertebrae bone volume in transgenic mice preferentially overexpressing Pax5 in osteoblasts by using the mouse aI(I)Collagen promoter, whereas OVX significantly reduced vertebrae bone volume in wild-type (WT) mice without significantly affecting that in Pax5 transgenic mice. Preferential osteoblastic Pax5 overexpression invariably led to significant increases in osteoblastic and osteoclastic parameters in mice with sham operation. However, OVX significantly increased osteoclastic parameters in WT mice, without additionally increasing osteoblastic and osteoclastic parameters in Pax5 transgenic mice. These results suggest that osteoblastic Pax5 would play a role in OVX-induced bone loss through a mechanism relevant to the promotion of both osteoblastic bone formation and osteoclastic bone resorption in vivo.

Key words osteoblast; osteoclast; Paired box-5; ovariectomy; transgenic mouse

The transcriptional regulation of osteoblast differentiation and function involves many endogenous players globally and locally expressed. At least three out of these three transcription factors are highly and preferentially expressed by osteoblasts: runt-related transcription factor-2 (Runx2), Osterix and activating transcription factor-4 (ATF4). The former two factors are critical determinants of the initial transition from mesenchymal stem cells into an osteoblast lineage, while the third factor ATF4 regulates terminal differentiation into functional osteoblasts able to determine bone mass. The transcription factor Paired box-5 (Pax5) is a member of Pax family comprised of at least nine homologous proteins with a highly conserved N-terminal paired domain and a C-terminal transactivation domain, and predominantly expressed at the pro-B to mature B cellular stage in the hematopoietic system to control the differentiation, function and identity of B lymphocytes. In mice globally defective of Pax5 (Pax5−/−), a low bone mass phenotype is seen along with a increased number of osteoclasts. We have demonstrated that Pax5 also modulates osteoblastic differentiation and maturation through upregulation of both Osterix and Osteocalcin expression at the transcriptional level. In transgenic mice with overexpression of Pax5 preferentially in osteoblasts, a high bone mass phenotype is found in conjunction with the increased bone formation. In the present study, therefore, we have investigated the possible pathological importance of Pax5 in bone remodeling under estrogen deficiency in ovariectomized mice, which is a preclinical model of postmenopausal osteoporosis in vivo.

The protocol employed here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University (approval number: AP-101806). All efforts were made to minimize animal suffering and to reduce the number of animals used (38 mice were used in total). Transgenic mice overexpressing Pax5 in osteoblasts by using the mouse aI(I)Collagen promoter (aI(I)Collagen-Pax5) and wild-type (WT) mice were kept in cages under a standard 12 h light/dark cycle, with access to food and water ad libitum. Eight week-old mice were anesthetized by an intraperitoneal injection of pentobarbital and subjected to ovariectomy (OVX) or sham operation under aseptic environments as described previously. Mice were also subjected to the intraperitoneal injection of 17β-estradiol dissolved in corn oil at a dose of 5µg/kg once a week for 28 consecutive days. Vertebrae were dissected 28 d after operation, followed by fixation with 10% formalin. Total RNA was extracted from long bone, followed by synthesis of cDNA with reverse transcriptase and oligo-dT primer. The cDNA samples were then used as a template for real-time polymerase chain reaction (PCR) analysis performed on an MX3005P instrument (Agilent Technologies, Santa Clara, CA, U.S.A.). Expression levels were normalized with the genes examined by using 36b4 expression levels as an internal control for each sample.

Bone histomorphometric analyses were performed on vertebrae not decalcified as previously described. Briefly, vertebrae were fixed with 10% formalin, followed by dehydration in different concentrations of ethanol and subsequent embedding in methyl methacrylate resin according to standard protocols. The bone volume over tissue volume (BV/TV) ratio was measured by Von Kossa staining on vertebral sections with a thickness of 7µm. Bone formation rate (BFR) was analyzed by the calcine double-labeling method. Calcein was injected to mice twice with an interval of 3 d, and then mice were killed 2 d after the last injection. Osteoblast and osteoclast parameters were analyzed by staining with toluidine blue and tartrate-resistant acid phosphatase (TRAP), respectively. Analyses were performed using the Osteomeasure Analysis System (Osteometrics, Atlanta, GA, U.S.A.) according to standard protocols.

Results are all expressed as the mean±S.E. and the
Statistical significance was determined by the two-tailed and unpaired Students’ t-test or the one-way analysis of variance ANOVA with Bonferroni/Dunnett post hoc test.

Since an anabolic property of Pax5 is shown for bone formation in mice upon overexpression in osteoblasts, we tested whether Pax5 impacts bone volume in the ovariectomized mice. OVX drastically decreased the uterine weight determined 28 d after operation in both WT and α1(I)Collagen-Pax5 mice (Fig. 1A). In WT mice, OVX failed to significantly affect the expression level of Pax5 in long bone (Fig. 1B), which was insensitive to the intraperitoneal injection of 17β-estradiol irrespective of OVX (Fig. 1C). In WT mice, moreover, a marked reduction of BV/TV was observed in cancellous bone stained with Von Kossa staining in vertebrae of ovariectomized mice compared with that of sham-operated mice (Fig. 1D). In vertebrae of ovariectomized α1(I)Collagen-Pax5 mice, however, no marked reduction was seen with the BV/TV ratio in cancellous bone. A significant

![Fig. 1. OVX-Induced Bone Loss Is Protected in α1(I)Collagen-Pax5 Transgenic Mice](image-url)

Twenty eight days after operation (WT-sham, n=12; WT-OVX, n=10; α1(I)Collagen-Pax5-sham, n=8; α1(I)Collagen-Pax5-OVX, n=8), mice were subjected to determination of (A) uterine weight, long bone Pax5 expression in WT mice (B) without and (C) with 17β-estradiol injection, (D) Von Kossa staining of vertebrae and (E) quantitative determination of BV/TV ratio. **p<0.01, significantly different from the control value obtained in sham-operated WT mice. ***p<0.01, significantly different from the value obtained in ovariectomized WT mice. N.S., not significant.
increase was found in BV/TV ratio in α1(I)Collagen-Pax5 mice with sham operation, while OVX failed to significantly decrease the ratio in α1(I)Collagen-Pax5 mice with a concomitant decrease in WT mice (Fig. 1E).

To further identify the responsive cells in α1(I)Collagen-Pax5 mice, histomorphometric analyses were done on osteoblastic and osteoclastic parameters with vertebrae. Osteoblast parameters, such as the number of osteoblast over bone perimeter (N.Ob/B.Pm) (Fig. 2A) and BFR (Fig. 2B), were significantly higher in vertebrae of α1(I)Collagen-Pax5 mice than those in WT mice irrespective of OVX. However, OVX failed to significantly affect these osteoblastic parameters in both α1(I)Collagen-Pax5 and WT mice. The osteoclast parameters, including the extent of osteoclast surface/bone surface (Oc.S/BS) (Fig. 3A) and the number of osteoclast over bone perimeter (N.Oc/B.Pm) (Fig. 3B), were significantly increased in α1(I)Collagen-Pax5 mice with sham operation, but not in those with OVX, compared with those of WT mice. In addition, OVX significantly increased the 2 osteoclastic parameters in WT mice without significantly affecting those in α1(I)Collagen-Pax5 mice.

The bone modeling and remodeling are coordinately regulated by two different types of bone cells, which are bone-forming osteoblasts and bone-resorbing osteoclasts. Imbalance between sophisticated regulations by these cells leads to the pathogenesis as well as the etiology of certain metabolic bone diseases including osteoporosis, Paget’s disease, and osteopetrosis. Osteoporosis is generally thought to be a common disease characterized by a systemic disruption of bone mass, strength, and microarchitecture, leading to the increased risk of bone fractures.
possibility of bone fragility to fractures.\textsuperscript{(13)} Fractures commonly occur in the spine, hip, or wrist, which often induces loss of mobility and autonomy, and subsequently major drops in the quality of life in patients with osteoporosis, in addition to increasing medical and socioeconomic threats.\textsuperscript{(12)} Postmenopausal estrogen deficiency is a critical important risk factor in the pathogenesis of osteoporosis in women. The current findings that osteoblastic Pax5 overexpression significantly increased both osteoblastic and osteoclastic parameters in mice with sham operation give rise to an idea that osteoblastic Pax5 would play a pivotal role in bone remodeling mediated by bone-forming osteoblasts and bone-resorbing osteoclasts after O VX in mice.

The increased osteoclastic parameters could be accounted for by taking into consideration the fact that the number of TRAP-positive multinucleated cells is markedly increased in co-cultures of bone marrow macrophages with calvarial osteoblasts from a(I)Collagen-Pax5 mice.\textsuperscript{(5)} In long bone of a(I)Collagen-Pax5 mice, however, no significant change is seen in levels of the osteoblast-derived critical regulators of osteoclast differentiation, Receptor activator of nuclear factor-κB ligand (Rankl) and Osteoprotegerin (Opg).\textsuperscript{(5)} It is thus unlikely that the aberrant interaction between Rankl and Opg is responsible for the promotion of osteoclastogenesis in a(I)Collagen-Pax5 mice with sham operation or OVX. Although we have already demonstrated that these a(I)Collagen-Pax5 mice show a higher bone volume phenotype under normal conditions,\textsuperscript{(5)} to our knowledge, this is the first direct demonstration of the prevention by osteoblastic Pax5 of bone loss under pathological conditions in a model of postmenopausal osteoporosis through a mechanism relevant to its constitutive anabolic property \textit{in vivo}. In addition to parathyroid hormone (1–34) currently available as a sole anabolic agent to treat osteoporosis,\textsuperscript{(13)} taken together, Pax5 could be a novel target for the discovery and development of a drug useful for the anabolic treatment and therapy of postmenopausal osteoporosis.

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