INTERNATIONAL JOURNAL OF Experimental Pathology

Int. J. Exp. Path. (2012), 93, 332–340

ORIGINAL ARTICLE

Vitamin C deficiency accelerates bone loss inducing an increase in PPAR-γ expression in SMP30 knockout mice

Jin-Kyu Park*, Eun-Mi Lee*,†, Ah-Young Kim*,†, Eun-Joo Lee*,†, Chang-Woo Min*,†, Kyung-Ku Kang*,†, Myeong-Mi Lee*,† and Kyu-Shik Jeong*,†

*Department of Pathology, College of Veterinary Medicine, Kyungpook National University, Daegu, Korea and †Stem Cell Therapeutic Research Institute, Kyungpook National University, Daegu, Korea

INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY

doi: 10.1111/j.1365-2613.2012.00820.x

Received for publication: 7 February 2012
Accepted for publication: 23 March 2012

Correspondence:
Kyu-Shik Jeong
College of Veterinary Medicine
Kyungpook National University
Daegu 702-701
Korea
Tel.: 82 53 950 5975
Fax: 82 53 950 5955
E-mail: jeongks@knu.ac.kr

Summary

Senescence marker protein (SMP) 30 knockout (KO) mice display symptoms of scurvy, including spontaneous bone fractures, and this was considered to be induced by a failure of collagen synthesis owing to vitamin C deficiency. However, low bone mineral density is also known to be associated with spontaneous bone fracture. Therefore, we investigated the effects of vitamin C deficiency on the balance between osteoblasts and osteoclasts in SMP30 KO mice as evidenced by histopathology. All mice were fed a vitamin C-free diet, and only one group (KV) mice were given water containing 1.5 g/l of vitamin C, whereas wild-type (WT) and KO mice were given normal drinking tap water without vitamin C for 16 weeks. After 16 weeks, all femur samples were removed for histopathological examination. The femurs of KO mice showed significantly reduced bone area and decreased number of osteoblasts compared with those of WT mice and KV mice. KO mice also exhibited the lowest level of alkaline phosphatase (ALP) expression in their femurs. However, KO mice showed the most elevated expression of the receptor activator of nuclear factor kappa-B ligand (RANKL). Moreover, KO mice had the strongest peroxisome proliferator-activated receptor (PPAR)-γ expression level in their osteoblasts and the highest number of TUNEL-positive bone marrow cells. Therefore, we concluded that vitamin C deficiency plays an important role in spontaneous bone fracture by inhibiting osteoblast differentiation and promoting transition of osteoblasts to adipocytes, and this could in turn be related to the increased PPAR-γ expression.

Keywords
bone, osteoblast, peroxisome proliferator-activated receptor-γ, SMP30, vitamin C

Senescence Marker Protein 30 (SMP30), a 34-kDa ageing marker protein, was first revealed to be present predominantly in the liver, and to decrease with age in an androgen-independent manner (Fujita *et al.* 1992; Mori *et al.* 2004). SMP30 was also known to play an important role in the anti-ageing process in several organs (such as lung, brain and kidney) by suppressing oxidative stress and cellular senescence (Mori *et al.* 2004; Sato *et al.* 2006; Son *et al.* 2006; Yumura *et al.* 2006; Kondo *et al.* 2008). Recently, many studies have revealed that SMP30 exhibits gluconolactonase activity, which is involved in L-ascorbic acid (vitamin C) biosynthesis in the liver. Therefore, it is believed that SMP30 can play a critical role in the anti-ageing mechanism via a role in the vitamin C biosynthesis pathway.

It is well documented that vitamin C is a powerful scavenger of free radicals and reactive oxygen species by providing hydrogen ions and electrons (Buettner 1993; Ishigami *et al.* 2002). Additionally, vitamin C plays a pivotal role in collagen synthesis as a cofactor of prolyl hydroxylase, which is essential for normal tissue structure composition in several organs (Myllylä *et al.* 1978; Padayatty *et al.* 2003). Previously, Kondo *et al.* (2006) demonstrated that SMP30 knockout (KO) mice displayed symptoms of scurvy, including spontaneous bone fracture, which was believed to be induced by a failure of collagen synthesis owing to the vitamin C deficiency. However, bone consists not only of collagen but also of minerals, non-collagenous proteins and water (Kloss & Gassner 2006). Moreover, a previous study demonstrated that the

denaturation of collagen affects age-related changes in bone only slightly (Wang *et al.* 2002; Kloss & Gassner 2006). This suggests that low bone mineral density is also closely associated with the scurvy associated spontaneous bone fracture. Moreover, SMP30 KO mice are considered to be a useful animal model of ageing which is inducible if there is vitamin C deficiency. Therefore, there is a possibility that other ageing factors, either in addition to collagen damage or in conjunction with collagen damage, may play an important role in the spontaneous bone fracture and bone loss seen in SMP30 KO mice.

Age-related bone loss is associated closely with the decrease in sexual hormones in humans. Recently, several studies have demonstrated that the number of osteoblasts is reduced, whereas the number and activity of osteoclasts increases with age in both men and women (Cao *et al.* 2003, 2005). These changes correlated with decreased sexual hormone levels. Interestingly, Hie & Tsukamoto (2011) reported that vitamin C deficiency stimulates osteoclastogenesis. On the basis of these results, we speculated that vitamin C deficiency can induce factors which interfere with the balance between osteoblasts and osteoclasts during ageing. Although Kondo *et al.* reported that spontaneous bone fracture is induced in vitamin C-deficient SMP30 KO mice by a failure of collagen synthesis (scurvy), there was no report about histopathological analysis of bone in vitamin C-deficient SMP30 KO mice. In this study, therefore, we have used a tissue pathology approach to study the question, and as a result provide new evidence into the mechanisms associated with age-related bone loss in vitamin C-deficient SMP30 KO mice.

Materials and methods

Animals

Gene-targeted SMP30 KO mice backcrossed to the C57BL/6 as previously described (Kondo *et al.* 2008) were obtained from the Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). The SMP30 KO mice were housed with wild-type (WT) C57BL/6 mice in a room at 22 ± 3 °C, relative humidity 50 ± 10%, a 12-h light/dark cycle and were fed food and water *ad libitum*. For genotyping of SMP30 KO mice, PCR was performed using genomic DNA isolated from tail-tissue as previously described (Park *et al.* 2008). Eight-week-old male, C57BL/6 WT mice and SMP30 KO mice were used in this study. The mice weighing 22–25 g were divided into three groups as follows: WT C57BL/6 mice group (WT, $n = 6$), vitamin C-deficient SMP30 KO mice group (KO, $n = 6$), vitamin C-treated SMP30 KO mice group (KV, $n = 6$). All mice were given vitamin C-free diet (PICO 5053 LabDiet, Richmond, IN, USA). Water containing vitamin C (1.5 g/l) was supplied to KV group, whereas WT group and KO group were given normal drinking tap water without vitamin C for 16 weeks. The vitamin C dose was determined according to a previous study (Kondo *et al.* 2006). All animal experiments were performed according to the NIH guidelines for the care and use of laboratory animals.

Measurement of femur bone length

All right femurs were measured with vernier callipers after the mice had been sacrificed. The longest dimension of each bone tissue (from femoral head to trochlea femoris) was measured, and the results were expressed as mean value ± SD for each group.

Histopathology

After 16 weeks, all mice were sacrificed and the right femur was removed for histopathology. The femurs were decalcified in EDTA and fixed in 10% neutral buffered formalin fixation. The fixed bone tissues were processed routinely and cut into sections of 4 μm thickness. The sections were deparaffinized in toluene and rehydrated in a graded alcohol series. For histopathological observation, the sections were stained with haematoxylin and eosin (H&E).

Histomorphometry of trabecular bone

The femurs were examined using histomorphometry. The 4-μm-sectioned bone tissues were stained with Masson's trichrome stain, and the images were captured using a Leica microscope (Leica Microsystems, Heerbrugg, Switzerland). For each sample, the whole bone matrix area of the distal femur at magnification ×50 was analysed, and the histomorphometric data of trabecular bone dimensions were obtained using image analysis software, Leica Application Suite analysis program (version 2.5.0 R1; Leica Microsystems).

Immunohistochemistry

The 4-μm-thick femur tissues were deparaffinized, rehydrated using a graded alcohol series and incubated in a solution of 3% hydrogen peroxide in methanol for 30 min, before being heated in 0.01 M citric acid buffer (pH 6.0) for 5 min using a microwave at 750 W. After being blocked for 30 min, the femur sections were immunostained with the following primary antibodies for anti-alkaline phosphatase (ALP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), receptor activator of nuclear factor kappa-B ligand (RANKL) (Santa Cruz Biotechnology) and peroxisome proliferator-activated receptor (PPAR)-γ (Santa Cruz Biotechnology). The antigen–antibody complex was detected by an avidin–biotin peroxidase complex solution, an ABC kit (Vector Laboratories, Burlingame, CA, USA) and a DAB (3,3′-diaminobenzidine tetrahydrochloride) kit (Zymed Laboratories Inc., San Francisco, CA, USA). All positive cells in five fields from each section of the femur tissue were observed and counted at ×400 magnification.

TUNEL assay

TUNEL assay was performed using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International Inc., Temecula, CA, USA), according to the manufacturer's instructions. TUNEL-positive cells in five fields from each

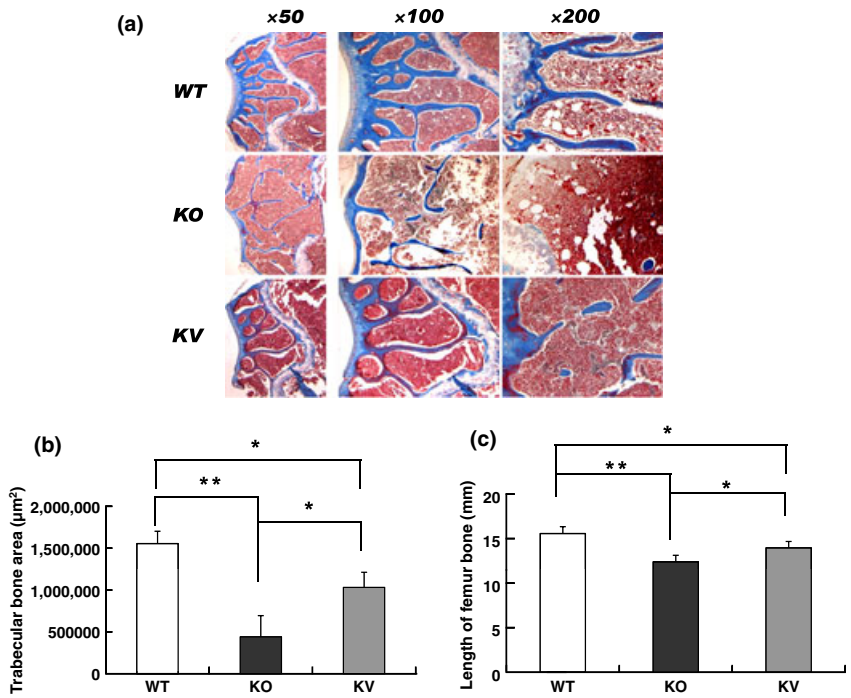

Figure 1 Measurement of bone area by Masson's trichrome stain. (a,b) Vitamin C-deficient SMP30 knockout (KO) mice exhibited significantly decreased trabecular bone area compared with that of wild-type and KV mice. Masson's trichrome stain (original magnification ×50, ×100, ×200). The histomorphometric analysis of trabecular bone areas was performed using Leica Application Suite analysis program (version 2.5.0 R1; Leica Microsystems). (c) Vitamin C-deficient SMP30 KO mice showed the shortest length values among all groups in femur length measurement. Data are shown as mean ± SD (*$P < 0.05$, **$P < 0.01$).

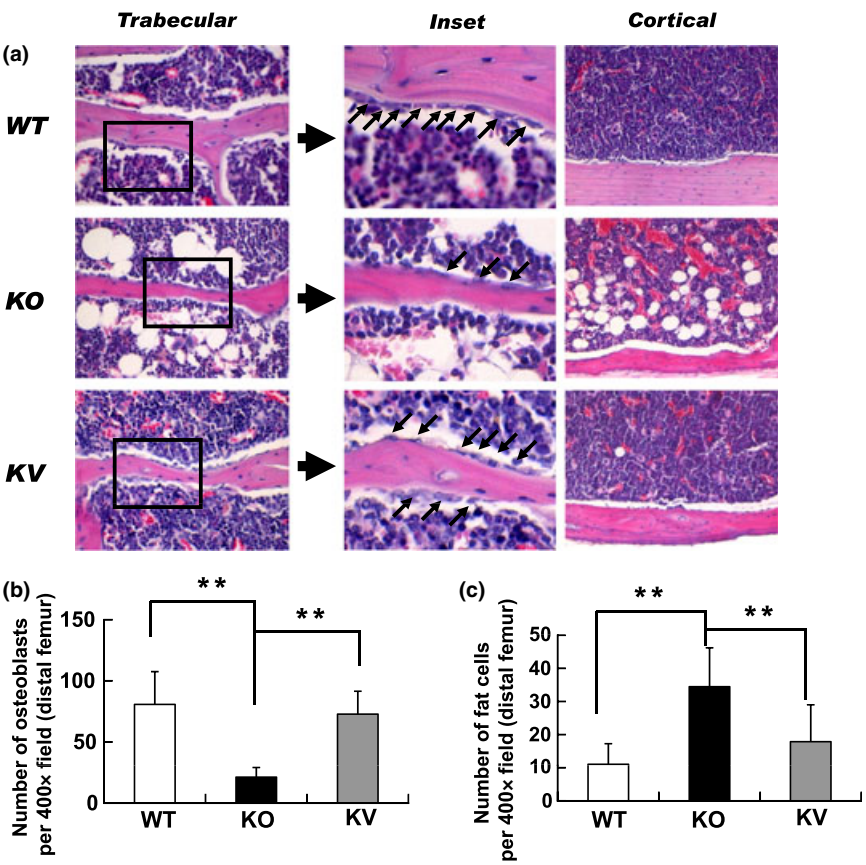

Figure 2 Histopathological analysis of femur tissues in all groups. (a) Representative microphotographs of haematoxylin and eosin stain of femur tissue after 16 weeks of vitamin C depletion. Haematoxylin and eosin stain (original magnification ×200, ×400, ×1000). (b) The number of osteoblasts in vitamin C-deficient knockout (KO) mice was significantly lower than that seen in wild-type and KV mice. (c) The number of adipocytes in bone marrow components was highest in the KO mouse group. Data are shown as mean ± SD (*$P < 0.05$, **$P < 0.01$).

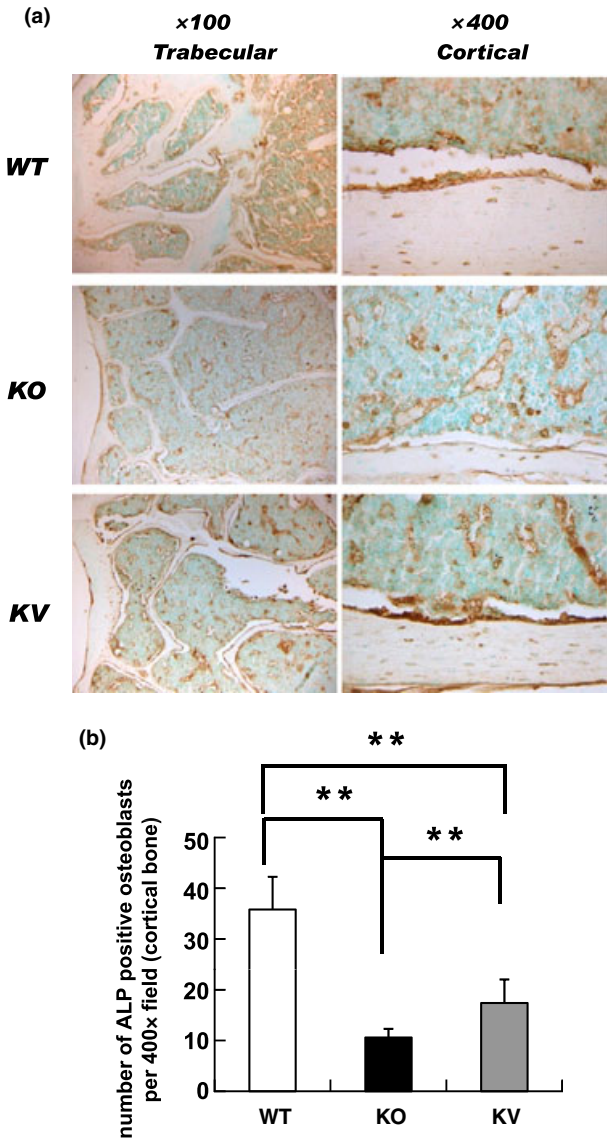

Figure 3 Significantly decreased alkaline phosphatase (ALP) expression level in femoral tissue of vitamin C knockout (KO) mice. (a) Vitamin C-deficient SMP30 KO mice exhibited considerably decreased numbers of ALP-positive cells around the bone matrix compared with those of wild-type (WT) and KV mice. Additionally, the bone marrow components of WT mice exhibited much higher expression of ALP compared with that of vitamin C-deficient mice (original magnification ×100, ×400). (b) The number of ALP-positive cells in femoral tissues of all groups. Data are shown as mean ± SD (*$P < 0.05$, **$P < 0.01$).

section of the femur tissue were observed and counted at ×400 magnification.

Statistical analysis

Results taken from each group were expressed as mean ± SD. The statistical significance between experimental groups was determined by Student's *t*-test or one-way ANOVA using GraphPad InStat (version 3.05; GraphPad Software Inc., San Diego, CA, USA) The statistical significance value was set at *$P < 0.05$ or **$P < 0.01$.

Results

Bone area significantly decreased in vitamin C-deficient SMP30 KO mice, whereas it was recovered by vitamin C treatment

To assess the changes in bone formation of vitamin C-deficient SMP30 KO mice, decalcified bone sections were stained with Masson's trichrome staining method (Figure 1). At 16 weeks after vitamin C deficiency, SMP30 KO mice showed significantly decreased trabecular bone densities compared with those of WT mice (Figure 1a). Bone formation in WT mice was 3-fold higher than in vitamin C-deficient SMP30 KO mice (Figure 1b). However, the vitamin C-treated SMP30 KO mice revealed significantly ameliorated bone loss compared with vitamin C-deficient SMP30 KO mice. Moreover, in the femur length measurements, the vitamin C-deficient SMP30 KO mice had the shortest lengths among all groups.

Osteoblastic differentiation was remarkably inhibited by vitamin C deficiency

To determine the effect of vitamin C deficiency on osteoblastic differentiation using histopathology, bone sections stained with H & E were examined (Figure 2). The number of osteoblasts was significantly lower in the femurs of vitamin C-deficient SMP30 KO mice compared with the femurs of WT and vitamin C-treated mice (Figure 2a,b). Additionally, in vitamin C-deficient SMP30 mice, osteoblasts arranged along the trabecular and cortical bone were poorly differentiated, whereas WT mice showed well-differentiated flattened osteoblasts. In the bone marrow components, the vitamin C-deficient SMP30 KO mice also revealed significant increase in adipogenesis characterized by the formation of adipocytes (Figure 2a,c). To confirm the decreased osteoblastic differentiation associated with vitamin C deficiency, we performed immunohistochemistry using an anti-ALP antibody, which is known as an osteoblastic differentiation marker. As expected, vitamin C-deficient SMP30 KO mice showed a dramatically decreased number of ALP-positive cells around the bone matrix compared with those of WT mice and vitamin C-treated SMP30 KO mice (Figure 3). Moreover, the bone marrow component of WT mice exhibited much stronger expression of ALP compared with that of vitamin C-deficient mice (Figure 3a). However, vitamin C treatment increased ALP expression in the bone tissue of SMP30 KO mice significantly.

Elevation of RANKL expression in the bones of vitamin C-deficient SMP30 KO mice

As RANKL has been reported previously to play a pivotal role in osteoclast differentiation, we explored the expression levels of RANKL as a marker of osteoclastic

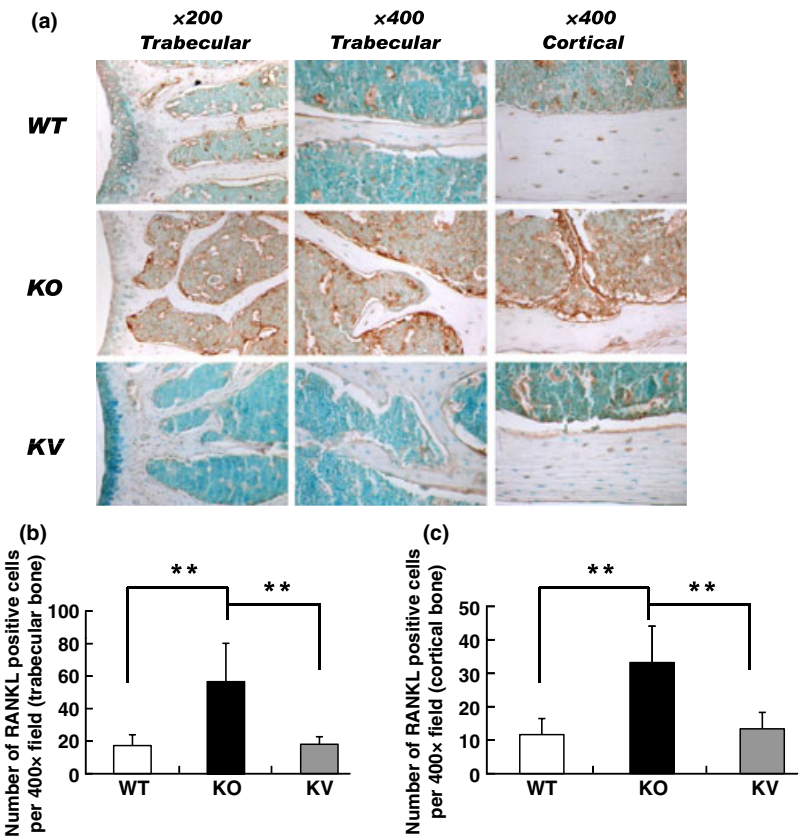

Figure 4 Dramatic elevation of receptor activator of nuclear factor kappa-B ligand (RANKL) expression level by vitamin C depletion. (a) The vitamin C-deficient SMP30 knockout mice indicated dramatically increased RANKL expression compared with wild-type and KV mice (original magnification ×200, ×400). (b) The number of RANKL-positive cells in the trabecular bone area of the femoral tissues. (c) The number of RANKL-positive cells in the cortical bone area of the femoral tissues. Data are shown as mean ± SD (*$P < 0.05$, **$P < 0.01$).

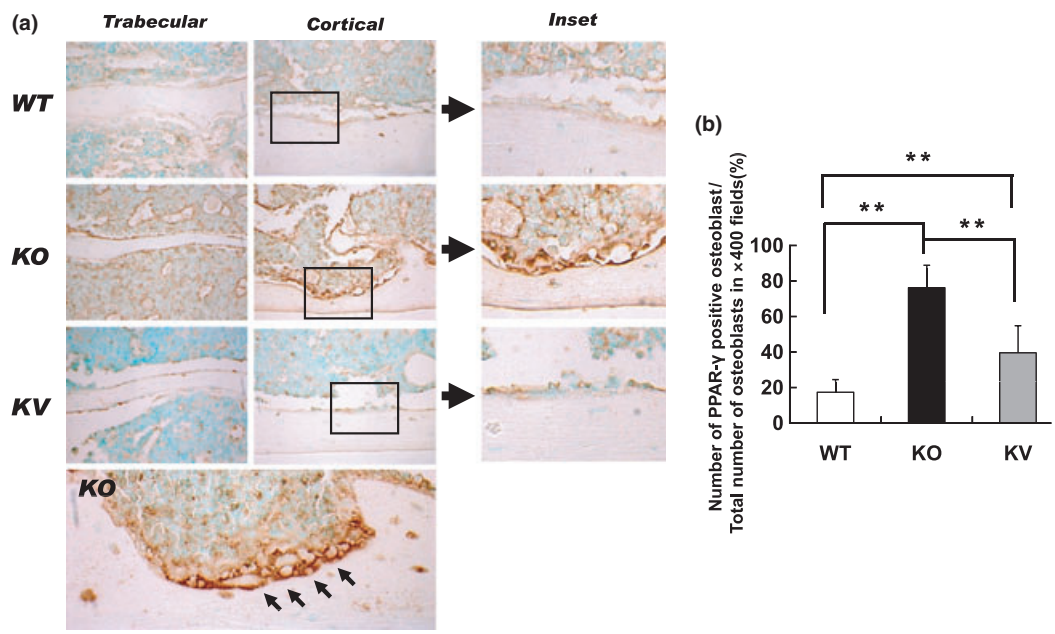

Figure 5 Remarkable increase in peroxisome proliferator-activated receptor (PPAR)-γ expression in vitamin C-deficient SMP30 knockout (KO) mice. (a) PPAR-γ expression was significantly up-regulated in vitamin C-deficient KO mice, whereas KV mice showed almost normal expression similar to the expression of WT mice. The strongest PPAR-γ expression was observed in specific bone marrow cells characterized by adipocyte-like appearance (arrows) (original magnification, ×400 and ×1000). (b) Ratio of PPAR-γ-positive osteoblasts to total osteoblasts in ×400 fields. Data are shown as mean ± SD (*$P < 0.05$, **$P < 0.01$).

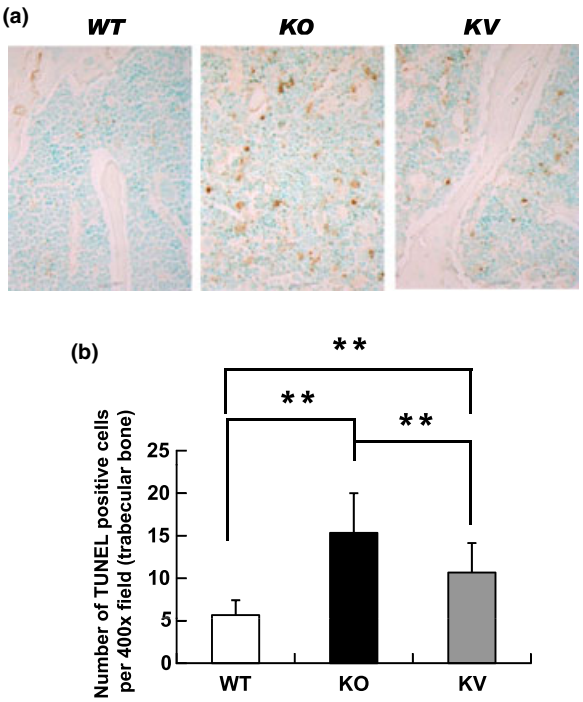

Figure 6 Increase in cell apoptosis in femoral tissues associated with vitamin C deficiency. (a) In the TUNEL assay, significantly increased numbers of apoptotic cells were observed in vitamin C-deficient KO mice compared with wild-type and KV mice (original magnification ×400). (b) Numbers of TUNEL-positive cells per field (×400). Data are shown as mean ± SD (*$P < 0.05$, **$P < 0.01$).

differentiation. Vitamin C-deficient SMP30 KO mice showed dramatically increased RANKL expression compared with that of WT mice and vitamin C-treated SMP30 KO mice (Figure 4). Although bone marrow components were diffusely positive for RANKL, specifically located cells around the trabecular and cortical bone matrix showed a strong positive reaction in vitamin C-deficient mice. The number of strongly positive cells was significantly lower in both WT mice and vitamin C-treated mice.

Significantly increased PPAR-γ expression and transition of osteoblastic cells to adipocytes

Next, we investigated PPAR-γ expression levels in vitamin C-deficient SMP30 KO mice (Figure 5). In accordance with previous reports showing remarkably increased adipogenesis in vitamin C deficiency, PPAR-γ expression was increased significantly by vitamin C deficiency. Conversely, vitamin C treatment reduced the abnormally increased PPAR-γ expression to normal levels similar to WT mice. In particular, the highest PPAR-γ expression was seen in specific bone marrow cells characterized by an adipocyte-like appearance, indicating that there was probably differentiation common precursors, or of osteoblasts, to adipocytes. Interestingly, those PPAR-γ-positive cells were mainly observed in the peripheral region of the cortical and trabecular bone, which was in the same localisation as the osteoblasts.

Vitamin C deficiency induced the increase in cell apoptosis in bone tissue

To assess apoptosis of bone marrow cells, we performed a TUNEL assay on bone tissue sections of each group. Figure 6 shows the significantly increased number of TUNEL-positive cells in vitamin C-deficient SMP30 KO mice compared with WT and vitamin C-treated SMP30 KO mice. Vitamin C treatments significantly decreased the number of TUNEL-positive bone marrow cells, indicating that vitamin C inhibits apoptotic cell death in bone tissue.

Discussion

To date it has been considered a well-established ''fact'' that vitamin C deficiency induces bone loss and spontaneous bone fracture and that this is due to a failure of appropriate collagen synthesis for bone formation (Peterkofsky 1991). However, histopathological analysis of the bone loss induced by vitamin C deficiency because of SMP30 depletion has not been reported previously. In the present study, we elucidated for the first time that increased PPAR-γ expression level induced by vitamin C deficiency may play a pivotal role in the bone loss occurring during the ageing process in SMP30 KO mice. These results also suggest that vitamin C ameliorates bone loss by inhibiting PPAR-γ expression in bone marrow cells. Further, we also demonstrated that vitamin C decreases the number of apoptotic cells in vitamin C-deficient SMP30 KO mice, showing that vitamin C suppresses the cellular senescence of bone marrow cells.

Previously several studies have shown that vitamin C-deficient SMP30 KO mice were characterized by lower body weight, shorter lifespan and depletion of serum vitamin C level (Ishigami *et al.* 2004; Kondo *et al.* 2006; Kashio *et al.* 2009). These characteristics were observed repeatedly in our own several previous studies (Park *et al.* 2010a,b), which confirms that vitamin C-deficient SMP30 mice have a less robust phenotype compared with WT mice. Probably the vitamin C level in bone becomes almost depleted in vitamin C-deficient SMP30 KO mice, although we could not confirm this.

In histopathological analysis of bone area via Masson's trichrome stain, the vitamin C-deficient KO mice showed remarkably decreased bone formation compared with WT and vitamin C-treated KO (KV) mice (Figure 1a,b). Moreover, the femurs of vitamin C-deficient KO mice were not only shorter (Figure 1a,c), but also more fragile compared to those of WT and KV mice. These results indicate that the bone loss of SMP30 KO mice was caused by vitamin C deficiency. Previously, Kondo *et al.* (2006) reported that SMP30 KO mice showed symptoms of scurvy including susceptibility to bone fracture. We also demonstrated by histopathology that vitamin C deficiency induces severe bone loss in SMP30 KO mice. As osteoblasts have been known to play a major role in bone

formation, we speculated that vitamin C deficiency may affect either the osteoblastic differentiation of bone marrow stem cells or death of well-differentiated osteoblasts.

In the histopathological sections we also observed that the number of well-differentiated osteoblasts decreased dramatically in KO compared with those of the WT and KV groups (Figure 2a,b). Moreover, the KO mice showed a significantly increased number of adipocytes in bone marrow component compared with those of WT and KV mice (Figure 2a,c). These results suggest that vitamin C deficiency may inhibit the differentiation of osteoblasts and induce adipogenesis from bone marrow stem cells. As ALP was known to be a representative marker for osteoblastic differentiation (Ongphiphadhanakul *et al.* 1993; Garcia *et al.* 2002; Chipoy *et al.* 2004), immunohistochemistry using an anti-ALP antibody was used to confirm the inhibition of osteoblast differentiation by vitamin C deficiency. As expected, we observed significantly decreased number of ALP-positive osteoblasts around the bone matrix in KO compared with those of the WT and KV groups, (Figure 3). These results suggested that vitamin C deficiency causes bone loss by the inhibition of osteoblast differentiation.

Previously, Hie & Tsukamoto (2011) reported that vitamin C deficiency stimulates osteoclastogenesis with an increase in RANK expression. Moreover, another previous study revealed that osteoclastogenesis was promoted by an increased RANKL expression level (Hofbauer *et al.* 2000). These previous studies suggest that osteoclastogenesis can be promoted via the up-regulated RANKL/RANK signalling pathway by vitamin C deficiency. Therefore, we investigated the expression level of RANKL in femoral tissues using an anti-RANKL antibody. Surprisingly, we observed dramatically increased RANKL expression in the KO group compared with the WT and KV groups. The KV group showed much weaker RANKL expression level in femoral tissues compared with the KO group, which indicated that vitamin C supplement reduced RANKL expression in SMP30 KO mice. Interestingly, RANKL expression was much higher in osteoblastic cells surrounding the bone matrix in comparison with other bone marrow cells. These results suggest that vitamin C deficiency can induce the increase in osteoclastic differentiation as well as the inhibition of osteoblastic differentiation of bone marrow cells.

Several previous studies indicated that RANKL is a homotrimeric protein that is typically membrane-bound on osteoblastic stromal cells and induces osteoclast differentiation and activation by acting on its receptor, RANK (Takayanagi 2007; Boyce & Xing 2008). These results suggest the possibility that vitamin C deficiency can increase osteoclastogenesis by enhancing the RANKL/RANK signalling pathway, although we could not detect any significant difference in the number of osteoclasts among all groups of the present study (data not shown). Hie *et al.* (2011) showed recently that vitamin C deficiency increases osteoclastic differentiation via an up-regulated RANKL/RANK signalling pathway. Moreover, other previous *in vitro* studies have shown that vitamin C is essential for osteoblastic differentiation (Franceschi *et al.* 1994; Otsuka *et al.* 1999). Taken together, these data suggest that vitamin C deficiency induces bone loss by inhibition of osteoblastic differentiation and promotion of osteoclastogenesis.

How can the differentiation of osteoblasts be inhibited by vitamin C deficiency? In the present study, we observed increased adipogenesis in bone marrow components of vitamin C-deficient SMP30 KO mice. Interestingly transition of osteoblastic cells to adipocyte-like cells containing lipid droplets was observed in the femoral tissue of vitamin C-deficient SMP30 KO mice. Therefore, we hypothesized that osteoblastic differentiation may be inhibited by transition of osteoblastic cells to adipocyte-like cells induced by vitamin C deficiency. As recent data revealed that PPAR-γ is the main factor for adipogenesis, we investigated the expression levels of PPAR-γ in all femoral tissues (Kersten *et al.* 2000; Rosen *et al.* 2000; Rosen & Spiegelman 2001). As expected, a significantly increased number of PPAR-γ-positive osteoblastic cells around the bone matrix were observed in KO mice compared to WT and KV groups (Figure 5). The bone marrow cells also showed enhanced PPAR-γ expression in the presence of vitamin C deficiency. Thus the pattern of PPAR-γ expression was consistent with the RANKL expression pattern. Therefore, osteoblastic differentiation may be inhibited by adipogenesis via increased PPAR-γ expression and osteoclastogenesis via RANKL expression in vitamin C-deficient SMP30 KO mice.

PPAR-γ, a member of the nuclear hormone receptor superfamily, is a pivotal factor in adipogenesis and is expressed mainly in adipose tissue (Nagy *et al.* 1998; Barak *et al.* 1999; Rosen *et al.* 1999). Recently, several studies have shown that PPAR-γ induces bone loss by suppressing osteoblast differentiation (Ali *et al.* 2005; Liu *et al.* 2010; Viccica *et al.* 2010; Yoshiko *et al.* 2010). Ali *et al.* (2005) demonstrated that PPAR-γ agonist causes bone loss in mice by suppressing osteoblast differentiation and bone formation. Yoshiko *et al.* (2010) also showed that a high endogenous level of PPAR-γ switches the fate of osteoblasts to adipocytes. Moreover, other studies indicated that down-regulation of PPAR-γ enhanced osteogenesis by increased osteoblast differentiation (Akune *et al.* 2004; Cock *et al.* 2004). These studies suggest that PPAR-γ plays a key role in bone loss by inhibiting osteoblast differentiation and promoting adipogenesis. Consistent with these studies, we also observed more inhibition of osteoblast differentiation (Figure 2) and poorly differentiated osteoblasts transforming to adipocytes (Figure 5) in the femoral tissues of KO mice.

As SMP30 exhibits gluconolactonase activity for vitamin C biosynthesis, SMP30 KO mice were known to have a much shorter lifespan by the promoted ageing process caused by vitamin C depletion (Kondo *et al.* 2006). Previously, we demonstrated that SMP30 KO mice displayed high expression of PPAR-γ in the liver, specifically hepatic stellate cells, which caused inhibition of liver fibrosis (Park *et al.* 2010a,b). Moerman *et al.* (2004) reported that ageing activates adipogenesis and suppresses osteogenesis in bone marrow mesenchymal stem cells with increased PPAR-γ expression. Additionally, several previous studies showed that PPAR-γ promotes

cellular senescence by increasing apoptosis and inducing cell cycle arrest (Guan *et al.* 1999; Lu *et al.* 2005; Gan *et al.* 2008). These data demonstrate that vitamin C depletion-induced bone loss can also be caused by promoted senescence of bone marrow cells. To confirm the cellular senescence of bone marrow cells, we performed the TUNEL assay using femoral tissue. To our surprise, vitamin C-deficient KO mice showed a dramatically increased number of TUNEL-positive cells in all bone components, whereas KV mice showed a significantly decreased number of TUNEL-positive cells following vitamin C treatment. Additionally, more TUNEL-positive cells were observed in the bone marrow stem cells than in the osteoblastic cells, which suggests that the bone loss of vitamin C-deficient SMP30 KO mice is induced by suppressed osteoblast differentiation and increased cellular senescence of bone marrow stem cells via the elevation of PPAR-γ expression.

In the present study, we observed significant differences (both increased and decreased) in bone morphometry and protein expression levels between WT group and KV group. This suggested that the bone tissues of vitamin C-treated KO mice were weaker than those of WT mice. Previous studies showed that WT mice had higher vitamin C level compared with vitamin C-treated KO mice in several organs (Amano *et al.* 2010; Iwama *et al.* 2011). As vitamin C is easily converted to oxidized forms even in the peripheral blood, it is suggested that biosynthesis of vitamin C via SMP30 works more efficiently than vitamin C supplementation in mice.

SMP30 is also known as regucalcin, which plays an important role in intracellular Ca^{2+} homoeostasis (Fujita *et al.* 1992; Yamaguchi 2000). Previously, Yamaguchi *et al.* reported that overexpression of regucalcin induces bone loss by increased adipogenesis and osteoclastogenesis (Yamaguchi 2010), which conflicts with our findings in the present study. They also reported that regucalcin is expressed in rat bone marrow cells (Yamaguchi *et al.* 2004). They focused on the effect of regucalcin overexpression using regucalcin transgenic rats. Therefore, it is believed that their results are not associated with endogenous vitamin C levels. In the present study, we investigated SMP30 expression in femoral tissue via immunohistochemistry, but were not able to detect any SMP30-positive cells in either WT or KO mice (data not shown), suggesting that SMP30 expression in bone tissue may be critically lower than in other organ tissues such as liver, kidney, lung, brain and testis. Moreover, the present study revealed that bone formation and osteoblast differentiation were up-regulated similarly in WT mice by vitamin C treatment in SMP30 KO mice. Therefore, based on our results, we believe strongly that bone loss in vitamin C-deficient SMP30 KO mice is more closely associated with vitamin C depletion than with SMP30 itself in bone tissue.

To summarize, our data suggest that age-related bone loss is accelerated by vitamin C deficiency, inducing an increase in PPAR-γ expression in SMP30 KO mice. To determine accurately the mechanisms associated with vitamin C deficiency and PPAR-γ expression in age-related bone loss, further studies will be needed in the future. Because SMP30 KO mice are considered a very useful, and robust, animal model of ageing we believe that the present study provides new evidence about the relationships between ageing, vitamin C deficiency, and age-related bone loss.

Acknowledgement

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0025973).

References

Akune T., Ohba S., Kamekura S. *et al.* (2004) PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. *J. Clin. Invest.* **113**, 846–855.

Ali A.A., Weinstein R.S., Stewart S.A. *et al.* (2005) Rosiglitazone causes bone loss in mice by suppressing osteoblast differentiation and bone formation. *Endocrinology* **146**, 1226–1235.

Amano A., Aigaki T., Maruyama N., Ishigami A. (2010) Ascorbic acid depletion enhances expression of the sodium-dependent vitamin C transporters, SVCT1 and SVCT2, and uptake of ascorbic acid in livers of SMP30/GNL knockout mice. *Arch. Biochem. Biophys.* **496**, 38–44.

Barak Y., Nelson M.C., Ong E.S. *et al.* (1999) PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol. Cell* **4**, 585–595.

Boyce B.F. & Xing L. (2008) Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch. Biochem. Biophys.* **473**, 139–146.

Buettner G.R. (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* **300**, 535–543.

Cao J., Venton L., Sakata T., Halloran B.P. (2003) Expression of RANKL and OPG correlates with age-related bone loss in male C57BL/6 mice. *J. Bone Miner. Res.* **18**, 270–277.

Cao J.J., Wronski T.J., Iwaniec U. *et al.* (2005) Aging increases stromal/osteoblastic cell-induced osteoclastogenesis and alters the osteoclast precursor pool in the mouse. *J. Bone Miner. Res.* **20**, 1659–1668.

Chipoy C., Berreur M., Couillaud S. *et al.* (2004) Downregulation of osteoblast markers and induction of the glial fibrillary acidic protein by oncostatin M in osteosarcoma cells require PKCdelta and STAT3. *J. Bone Miner. Res.* **19**, 1850–1861.

Cock T.A., Back J., Elefteriou F. *et al.* (2004) Enhanced bone formation in lipodystrophic PPARgamma (hyp/hyp) mice relocates haematopoiesis to the spleen. *EMBO Rep.* **5**, 1007–1012.

Franceschi R.T., Iyer B.S., Cui Y. (1994) Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J. Bone Miner. Res.* **9**, 843–854.

Fujita T., Uchida K., Maruyama N. (1992) Purification of senescence marker protein-30 (SMP30) and its androgen-independent decrease with age in the rat liver. *Biochim. Biophys. Acta* **1116**, 122–128.

Gan Q., Huang J., Zhou R. *et al.* (2008) PPAR{gamma} accelerates cellular senescence by inducing p16INK4{alpha} expression in human diploid fibroblasts. *J. Cell Sci.* **121**, 2235–2245.

Garcia T., Roman-Roman S., Jackson A. *et al.* (2002) Behavior of osteoblast, adipocyte, and myoblast markers in genome-wide expression analysis of mouse calvaria primary osteoblasts in vitro. *Bone* **31**, 205–211.

Guan Y.F., Zhang Y.H., Breyer R.M., Davis L., Breyer M.D. (1999) Expression of peroxisome proliferator-activated receptor gamma (PPARgamma) in human transitional bladder cancer and its role in inducing cell death. *Neoplasia* **4**, 330–339.

Hie M. & Tsukamoto I. (2011) Vitamin C-deficiency stimulates osteoclastogenesis with an increase in RANK expression. *J. Nutr. Biochem.* **22**, 164–171.

Hofbauer L.C., Khosla S., Dunstan C.R., Lacey D.L., Boyle W.J., Riggs B.L. (2000) The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *J. Bone Miner. Res.* **15**, 2–12.

Ishigami A., Fujita T., Handa S. *et al.* (2002) Senescence marker protein-30 knockout mouse liver is highly susceptible to tumor necrosis factor-alpha- and Fas-mediated apoptosis. *Am. J. Pathol.* **161**, 1273–1281.

Ishigami A., Kondo Y., Nanba R. *et al.* (2004) SMP30 deficiency in mice causes an accumulation of neutral lipids and phospholipids in the liver and shortens the life span. *Biochem. Biophys. Res. Commun.* **315**, 575–580.

Iwama M., Shimokado K., Maruyama N., Ishigami A. (2011) Time course of vitamin C distribution and absorption after oral administration in SMP30/GNL knockout mice. *Nutrition* **27**, 471–478.

Kashio A., Amano A., Kondo Y. *et al.* (2009) Effect of vitamin C depletion on age-related hearing loss in SMP30/GNL knockout mice. *Biochem. Biophys. Res. Commun.* **390**, 394–398.

Kersten S., Desvergne B., Wahli W. (2000) Roles of PPARs in health and disease. *Nature* **405**, 421–424.

Kloss F.R. & Gassner R. (2006) Bone and aging: effects on the maxillofacial skeleton. *Exp. Gerontol.* **41**, 123–129.

Kondo Y., Inai Y., Sato Y. *et al.* (2006) Senescence marker protein 30 functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 5723–5728.

Kondo Y., Sasaki T., Sato Y. *et al.* (2008) Vitamin C depletion increases superoxide generation in brains of SMP30/GNL knockout mice. *Biochem. Biophys. Res. Commun.* **377**, 291–296.

Liu L.F., Shen W.J., Zhang Z.H., Wang L.J., Kraemer F.B. (2010) Adipocytes decrease Runx2 expression in osteoblastic cells: roles of PPARγ and adiponectin. *J. Cell. Physiol.* **225**, 837–845.

Lu M., Kwan T., Yu C. *et al.* (2005) Peroxisome proliferator-activated receptor gamma agonists promote TRAIL-induced apoptosis by reducing survivin levels via cyclin D3 repression and cell cycle arrest. *J. Biol. Chem.* **280**, 6742–6751.

Moerman E.J., Teng K., Lipschitz D.A., Lecka-Czernik B. (2004) Aging activates adipogenic and suppresses osteogenic programs in mesenchymal marrow stroma/stem cells: the role of PPAR-gamma2 transcription factor and TGF-beta/BMP signaling pathways. *Aging Cell* **3**, 379–389.

Mori T., Ishigami A., Seyama K. *et al.* (2004) Senescence marker protein-30 knockout mouse as a novel murine model of senile lung. *Pathol. Int.* **54**, 167–173.

Myllylä R., Kuutti-Savolainen E.R., Kivirikko K.I. (1978) The role of ascorbate in the prolyl hydroxylase reaction. *Biochem. Biophys. Res. Commun.* **83**, 441–448.

Nagy L., Tontonoz P., Alvarez J.G., Chen H., Evans R.M. (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell* **93**, 229–240.

Ongphiphadhanakul B., Jenis L.G., Braverman L.E. *et al.* (1993) Etidronate inhibits the thyroid hormone-induced bone loss in rats assessed by bone mineral density and messenger ribonucleic acid markers of osteoblast and osteoclast function. *Endocrinology* **133**, 2502–2507.

Otsuka E., Yamaguchi A., Hirose S., Hagiwara H. (1999) Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. *Am. J. Physiol.* **277**, C132–C138.

Padayatty S.J., Katz A., Wang Y. *et al.* (2003) Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J. Am. Coll. Nutr.* **22**, 18–35.

Park J.K., Jeong D.H., Park H.Y. *et al.* (2008) Hepatoprotective effect of Arazyme on CCl4-induced acute hepatic injury in SMP30 knock-out mice. *Toxicology* **246**, 132–142.

Park J.K., Hong I.H., Ki M.R. *et al.* (2010a) Vitamin C deficiency increases the binucleation of hepatocytes in SMP30 knock-out mice. *J. Gastroenterol. Hepatol.* **25**, 1769–1776.

Park J.K., Ki M.R., Lee H.R. *et al.* (2010b) Vitamin C deficiency attenuates liver fibrosis by way of up-regulated peroxisome proliferator-activated receptor-gamma expression in senescence marker protein 30 knockout mice. *Hepatology* **51**, 1766–1777.

Peterkofsky B. (1991) Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. *Am. J. Clin. Nutr.* **54**, 1135S–1140S.

Rosen E.D. & Spiegelman B.M. (2001) PPARgamma : a nuclear regulator of metabolism, differentiation, and cell growth. *J. Biol. Chem.* **276**, 37731–37734.

Rosen E.D., Sarraf P., Troy A.E. *et al.* (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol. Cell* **4**, 611–617.

Rosen E.D., Walkey C.J., Puigserver P., Spiegelman B.M. (2000) Transcriptional regulation of adipogenesis. *Genes Dev.* **14**, 1293–1307.

Sato T., Seyama K., Sato Y. *et al.* (2006) Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking. *Am. J. Respir. Crit. Care Med.* **174**, 530–537.

Son T.G., Zou Y., Jung K.J. *et al.* (2006) SMP30 deficiency causes increased oxidative stress in brain. *Mech. Ageing Dev.* **127**, 451–457.

Takayanagi H. (2007) Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat. Rev. Immunol.* **7**, 292–304.

Viccica G., Francucci C.M., Marcocci C. (2010) The role of PPARγ for the osteoblastic differentiation. *J. Endocrinol. Invest.* **33**, 9–12.

Wang X., Shen X., Li X., Agrawal C.M. (2002) Age-related changes in the collagen network and toughness of bone. *Bone* **31**, 1–7.

Yamaguchi M. (2000) Role of regucalcin in calcium signaling. *Life Sci.* **66**, 1769–1780.

Yamaguchi M. (2010) Regucalcin and metabolic disorders: osteoporosis and hyperlipidemia are induced in regucalcin transgenic rats. *Mol. Cell. Biochem.* **341**, 119–133.

Yamaguchi M., Sawada N., Uchiyama S., Misawa H., Ma Z.J. (2004) Expression of regucalcin in rat bone marrow cells: involvement of osteoclastic bone resorption in regucalcin transgenic rats. *Int. J. Mol. Med.* **13**, 437–443.

Yoshiko Y., Oizumi K., Hasegawa T. *et al.* (2010) A subset of osteoblasts expressing high endogenous levels of PPARgamma switches fate to adipocytes in the rat calvaria cell culture model. *PLoS ONE* **5**, e11782.

Yumura W., Imasawa T., Suganuma S. *et al.* (2006) Accelerated tubular cell senescence in SMP30 knockout mice. *Histol. Histopathol.* **21**, 1151–1156.