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Expression and Role of Sonic Hedgehog in the Process of Fracture Healing with Aging

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Abstract. *Aging is one of the risk factors for delayed fracture healing. Sonic hedgehog (SHH) protein, an inducer of embryonic development, has been demonstrated to be activated in osteoblasts at the dynamic remodeling site of a bone fracture. Herein, we compared and examined the distribution patterns of SHH and the functional effect of SHH signaling on osteogenesis and osteoclastogenesis between young (5-week-old) and aged (60-week-old) mice during fracture healing. We found that SHH was expressed in bone marrow cells from the fractured site of the rib of young mice on day 5, but was barely detectable in the corresponding cells from the rib of aged mice. SHH was also detected in osteoblasts and bone marrow cells at the callus remodeling stage on days 14 and 28 in both young and aged mice. The number of alkaline phosphatase (ALP)-positive osteoblasts was significantly higher in young mice on days 5 and 14, whereas the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts was significantly higher in aged mice. SHH stimulated significantly more osteoblast formation in the young compared to old mice. SHH stimulated the osteoclast formation directly in the aged mice and suppressed the formation indirectly through osteoprotegerin expression in the young mice. Results indicate that an aged-related delay of fracture healing may contribute to the unbalanced bone formation and resorption, regulated by hedgehog signaling.*

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Fracture healing is a well characterized process that is influenced by many biological and mechanical factors (1). One of the risk factors for delayed fracture healing is aging (2). Age-related changes in bone characteristics can delay bone formation and cause disuse-related bone loss. The clarification of the mechanisms of aging's effects on fracture healing could make a substantial contribution to the development of new treatments for fracture healing (3-4). Fracture healing involves a delicate balance between bone formation and bone resorption. On the cellular level, osteoblast cells that have differentiated from mesenchymal precursor cells in the bone marrow migrate to the bone surface of the fracture site and form a mineralized bone matrix. Mesenchymal precursor cells then differentiate into chondrocytes and form the cartilage callus, and the callus is then removed by osteoclasts and osteoblasts (1).

Sonic hedgehog (SHH) protein is a morphogen that acts in a wide variety of tissues during embryonic development (5, 6). The hedgehog signaling is also reactive to several adult bone regenerative processes related to fracture healing (7), socket healing after tooth extraction (8), and the bone regeneration of calvaria defects (9). On the other hand, hedgehog signaling indirectly induces osteoclast formation by up-regulating parathyroid hormone-related peptide (PTHrP) (10) and receptor activator for nuclear factor-κB ligand (RANKL) (11) in osteoblasts and bone stromal cells. SHH was activated in osteoblasts at the dynamic remodeling site of a bone fracture and regulated their proliferation and differentiation, as well as osteoclast formation (12). Multiple factors could contribute to the aged-related changes in fracture healing, such as an imbalance of RANKL and osteoprotegerin (OPG) (13) and a decreased anabolic effect of PTH (3).

In the present study, we examined the distribution patterns of SHH during fracture healing between young and aged mice, and we determined the effect of age on the SHH function in the osteoblasts and osteoclasts during fracture healing.

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Materials and Methods

Experimental animals. The right eighth rib of 5-week-old (young) and 60-week-old (aged) male ICR mice was fractured (12). Briefly, each mouse was anesthetized, and the eighth rib on the right side was exposed and cut vertically to the axis with surgical scissors. For a sham operation as a control, the right eighth rib of another mouse was similarly exposed but not fractured. This study was carried out in accordance with the guide for the care and use of laboratory animals of Okayama University. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (2013102).

Histochemical and immunohistochemical analyses of surgically resected samples. The sections were sequentially de-waxed by passage through a series of xylene, graded ethanol, and water immersion steps. After being autoclaved in 0.2% citrate buffer for 15 min, the sections were incubated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. They were then incubated with a 1:200 dilution of antibodies against SHH (mouse IgG, R&D Systems, Minneapolis, MN) overnight at 4˚C followed by thee washes with Tris-buffer saline (TBS). The slides were then treated with a streptavidin-biotin complex; Envision System Labeled Polymer, horseradish peroxidase (HRP; Dako, Carpinteria, CA, USA) for 60 min at a dilution of 1:100. The immunoreaction was visualized by using 3,3'-diaminobenzidine (DAB) substrate-chromogen solution (Dako Cytomation Liquid DAB Substrate Chromogen System, Dako), and counterstaining was performed with hematoxylin. For the detection of tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) activities, a TRAP/ALP staining kit was used according to the manufacturer's protocol (Wako, Osaka, Japan). Finally, the sections were immersed in an ethanol and xylene bath and then mounted with Vectashield (Vector Laboratories, Peterborough, UK) and viewed under a microscope (IX8l, Olympus, Tokyo).

Alkaline phosphatase (ALP) and Alizarin red staining. For the estimation of ALP activity, we collected primary osteoblasts from the tibias of young and aged ICR mice as described (14). The primary osteoblasts were cultured in 24-well multiplates with α-Minimum Essential Medium (α-MEM) complete media containing 10% fetal bovine serum (FBS), 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate (β-GP), and then ALP and Alizarin red staining was performed as described (12). Thereafter, they were viewed under an inverted microscope, and the stained area was measured using imaging software (Lumina Vision/OL, Mitani Corporation, Tokyo).

Isolation and culture of bone marrow-derived macrophages. We isolated bone marrow-derived macrophages from the tibia of ICR mice as described (15). To generate osteoclasts, we cultured bone marrow macrophages (1×10^5 cells/well) with M-CSF (30 ng/ml) and RANKL (30 ng/ml, Peprotech, Rocky Hill, NJ) in 48-well (500 μl/well) tissue culture plates. The complete medium was changed every 2 days. After 10 days of incubation, the cells were fixed and stained for TRAP activity (Sigma, St. Louis, MO, USA) and then the number of TRAP-positive multinucleated cells (nuclear number >3) in each well was counted. For osteoclast activity assay, bone marrow macrophages were seeded on Osteo assay plates (Corning, Corning, NY, USA) at a density of 5×10^4 cells/well. The cells were incubated with M-CSF (30 ng/ml) and RANKL (30 ng/ml) for 10 days. The remaining cells in the plate were lysed using 1 M NaOH with a 6% sodium hypochlorite solution. Five images per well were obtained using an inverted microscope (200%), and the resorbed area was measured using the imaging software Lumina Vision/OL.

RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR). We used an RNeasy Minikit (Qiagen, Valencia, CA, USA) to isolate the total RNA. Complementary DNA was generated from 1 μg of total RNA in a final volume of 20 μl with a first-strand cDNA synthesis kit (Takara, Tokyo, Japan) and then amplified for 30 cycles with the following oligonucleotide primer pairs: 5'-ACTCATCCCTA TGGCTCGTG-3' and 5'-GGTAGGGAGCTGGG TTAAGG-3' for osterix, 5'-AATGGGCGTCTCCACAGTAAC-3' and 5'-CTGAGTGG TGTTGCATCGC-3' for ALP, 5'-TTCTCC AACCCACGAATGCAC-3' and 5'-CAGGTACGTGTGGTAGTG AGT-3' for Runx2, 5'-CTTGGTGCACACCTAGCAGA-3' and 5'-TTCTGTTTCCTCCCTG CTGT-3' for osteocalcin (OCN), 5'-CTTCAGCTGGAGGAC ACCCC-3' and 5'-GGAAGACCAGCCTCACCCTG -3' for NFATc1, 5'-CACGATGCCAGCGACAAGAG-3' and 5'-TGACCCCGTATG TGGCTAAC-3' for TRAP, 5'-GGTCGGGCAATTCTGAATT-3' and 5'-ACATCTAGGACATC CATGC-3' for RANKL, 5'-ACCAAA GTGAATGCCGAGAG-3' and 5'-TCTGTGGTGAGGTTCGAGTG-3' for osteoprotegerin (OPG), 5'-CGCAATGTGACAAGCTGC TCAA-3' and 5'-TGAACTTGAGGCACTCGCTGTA-3' for PTH1R, and 5'-TGAACGGGAAGC TCACTGG-3' and 5'-TCCACCACCC TGTTGCTGTA-3' for glyceraldehyde-3-phosphatase dehydrogenase (GAPDH). Each PCR cycle was carried out for 30 s at 94˚C, 30 s at 55˚C, and 1 min at 68˚C. The PCR products were then separated on 2% agarose gels containing ethidium bromide and visualized under ultraviolet light.

Statistical analysis. We analyzed the data by using the unpaired Student's *t*-test for the analyses of two groups or a one-way analysis of variance (ANOVA) for the analysis of repeated multiple group comparisons. Results are expressed as the mean±standard deviation (SD). *p*-Values <0.05 were considered significant.

Results

Comparison of SHH expression in fractured ribs between young and aged mice. We first evaluated the time course of histological changes in the mouse bone fracture model, recording our observations on days 3, 5, 14, and 28 after rib fracture in young and aged mice. As shown by the hematoxylin-stained day 3 sections in Figure 1, ALP-positive cells were formed in the fracture site in young but not in aged mice. On day 5, the SHH expression was intense in many of the bone marrow cells in young mice but not in those of aged mice. The ALP-stained day-5 sections revealed that the numbers of ALP (light purple)-positive cells in the young mice were significantly higher compared to those in the aged mice at the margin of the cortical bone near the fracture site (Figure 1, young, 3.3±0.58 cells, old 0.75±0.5 cells, $p<0.05$). As illustrated by the hematoxylin-stained day-14 section shown in Figure 1, by this time point the callus was formed in the young mice, but the callus formation was delayed at the fracture site in the aged mice. SHH was also detected in

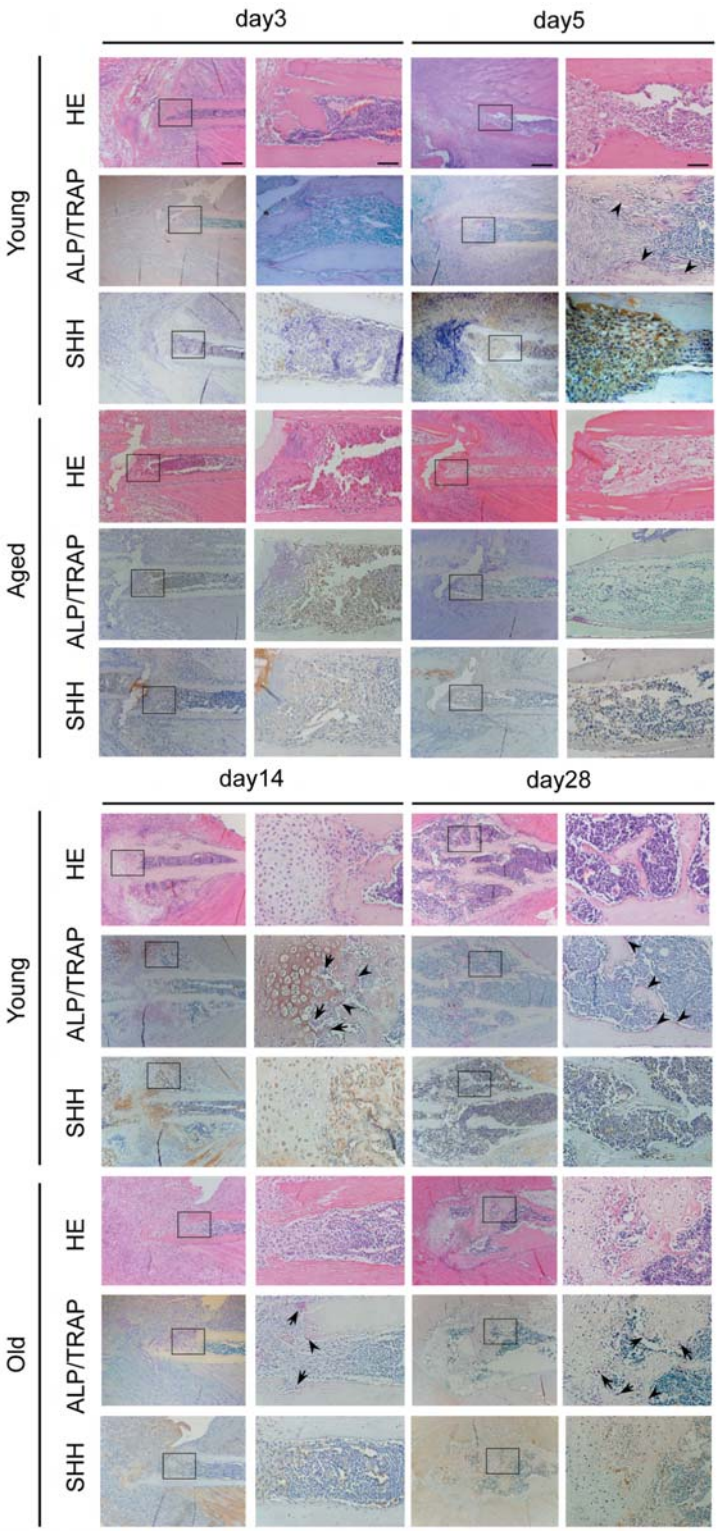

Figure 1. *The histological appearance, ALP/TRAP staining, and localization of SHH in fracture of young and aged mouse ribs at 3, 5, 14, and 28 days after fracturing. Photomicrographs show the healing process at 3 and 5 after fracturing. Sections were stained with HE or ALP and TRAP, and SHH. Each photo at the right shows a section that is a histological magnification of the rectangle-delaminated area in the corresponding left photo. Bar, 200 μm (left photo), 50 μm (right photo). Arrowheads: Osteoblasts. Arrows: Osteoclasts. Data from a typical experiment are presented; similar results were obtained in repeated experiments.*

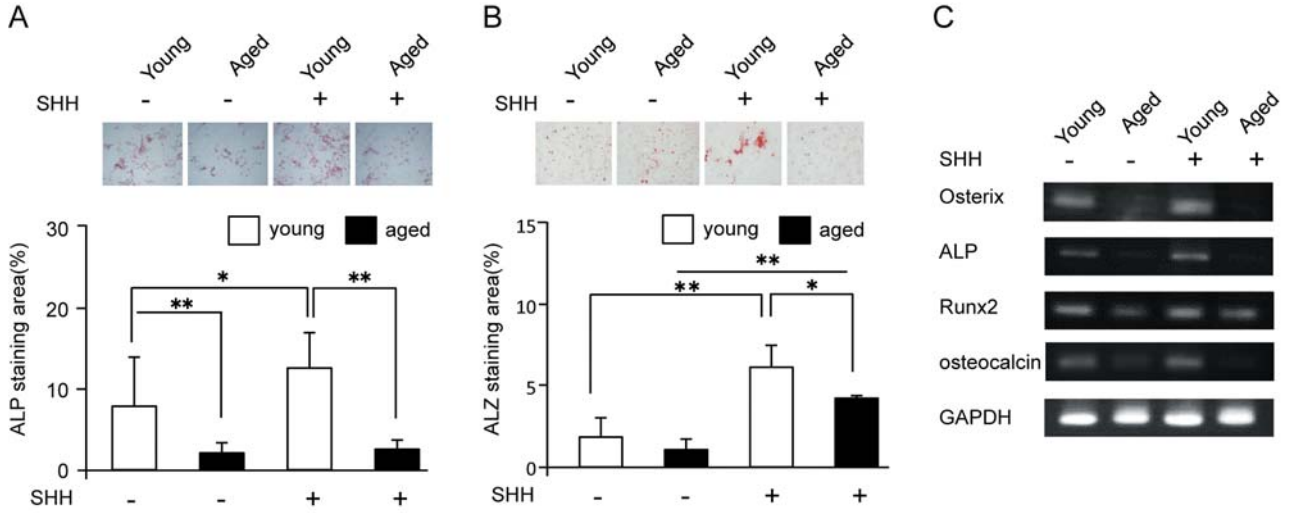

Figure 2. *Effects of SHH on young and aged mouse osteoblast differentiation. (A) SHH (500 ng/ml) stimulated the ALP staining in young-mouse osteoblasts (n=12). (B) SHH (500 ng/ml) stimulated Alizarin red staining in young osteoblasts more than aged osteoblasts (n=12). (C) RT-PCR analysis of osterix, ALP, Runx2, and osteocalcin mRNA expression in SHH-stimulated young and aged osteoblasts. Data from a typical experiment are presented; similar results were obtained in three separate experiments.*

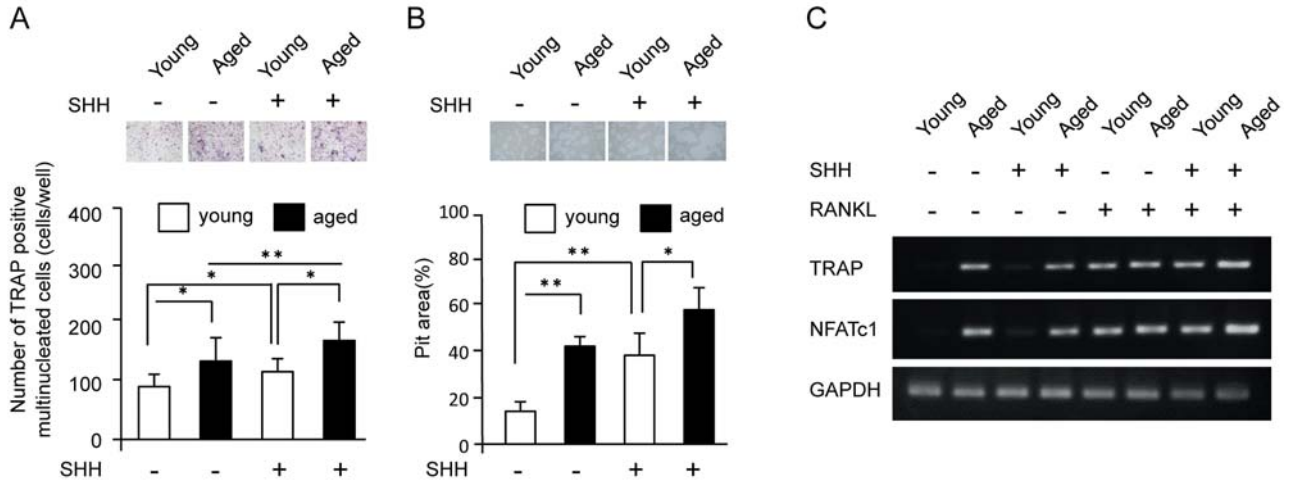

Figure 3. *Comparison of the direct SHH effect on osteoclast formation and activation between young and aged mouse bone marrow-derived macrophages. (A) SHH (500 ng/ml) stimulated an increase in the number of TRAP-positive cells among both young and aged bone marrow-derived macrophages in the presence of RANKL (30 ng/ml) (n=4). (B) SHH (500 ng/ml) stimulated osteoclast activity in both young and aged bone marrow-derived macrophages in the presence of RANKL (30 ng/ml) (n=4). *p<0.05, **p<0.01. (C) RT-PCR analysis of TRAP and NFATc1 expression levels in young and aged bone marrow-derived macrophages with or without SHH (500 ng/ml) or RANKL (30 ng/ml). The total RNA was extracted 24 h after the start of treatment. Data from a typical experiment are presented; similar results were obtained in three separate experiments.*

osteoblasts and bone marrow cells at the callus remodeling stage on days 14 and 28 in both young and aged mice.

ALP-positive osteoblasts and TRAP-positive osteoclasts were detected next to hypertrophic chondrocytes at the rib fracture site in both young and aged mice (Figure 1). The number of ALP-positive osteoblasts was significantly higher in young mice compared to aged mice (young, 9.5±0.57 cells, old 6.5±2.08 cells, $p<0.05$), whereas the number of TRAP-positive osteoclasts was significantly higher in the aged mice (young, 4.5±0.57 cells, old 7.5±1.0 cells, $p<0.05$) compared to the callus stage. The hematoxylin-stained day-28 sections showed new trabecular bone in young mice,

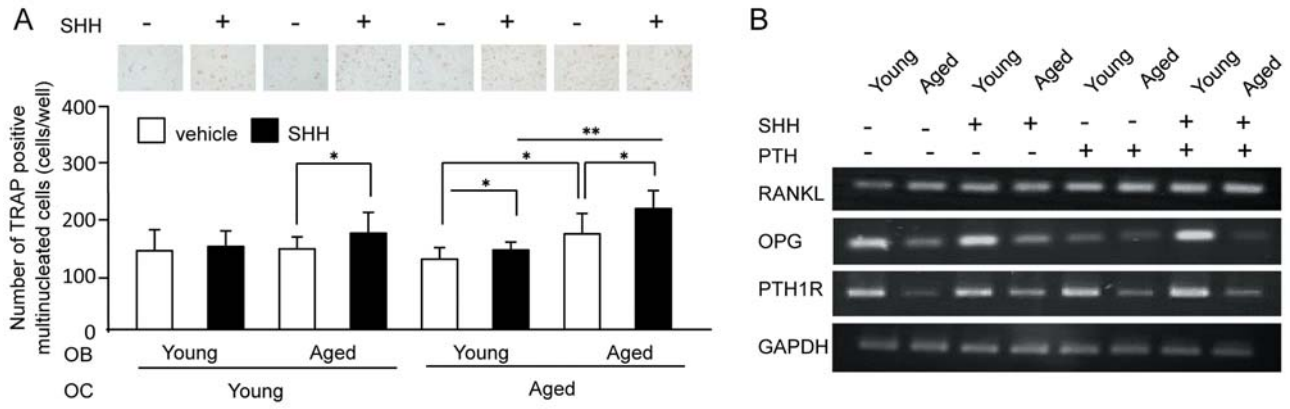

Figure 4. *Effect of SHH on the osteoblast-mediated osteoclast formation in a PTH-stimulated co-culture system. (A) SHH (500 ng/ml) stimulated an increase in the number of TRAP-positive cells in the presence of 10 nM PTH only in co-cultures of aged osteoblasts and aged bone marrow-derived macrophages (n=4). *p<0.05, **p<0.01. (B) RT-PCR analysis of RANKL, OPG, and PTH1R expression levels in young and aged osteoblasts with or without 500 ng/ml SHH or 10 nM PTH. Total RNA was extracted 24 h after the start of treatment. Data from a typical experiment are presented; similar results were obtained in three separate experiments.*

whereas hypertrophic chondrocytes had remained in aged mice. By this time ALP-positive osteoblasts and TRAP-positive osteoclasts were observed at the surface of the new trabecular bone that had been formed by endochondral ossification in the young mice.

Comparison of SHH effects on the osteoblast differentiation in young and aged mice. To determine whether there was a significant difference in the SHH-induced osteoblast differentiation between young and aged mice, we collected primary osteoblast cells from mouse long bones and stimulated the cells with 50 μg/ml ascorbic acid and 10 mM β-GP for 10 days. The number of osteoblast cells from the young mice was significantly higher in the ALP-stained areas compared to those from the aged mice (Figure 2A). SHH (500 ng/ml) significantly increased the number of ALP-positive cells in young mice osteoblasts but not in aged osteoblasts cultured for 5 days in medium containing 10% FBS, 50 ug/ml ascorbic acid and 10 mM β-GP (Figure 2A).

As shown in Figure 2B, the aged mice osteoblasts showed significantly lower Alizarin red staining areas compared to those of young mice at 10 days after the start of differentiation. SHH (500 ng/ml) treatment resulted in significantly increased areas of Alizarin red staining in the young osteoblasts compared to the aged osteoblasts for 10 days (Figure 2B). To confirm the mRNA expression changes due to the SHH stimulation of the osteoblasts, we performed an RT-PCR analysis. The mRNA expression levels for osterix, *Runx2, ALP* and osteocalcin were higher in the young osteoblasts compared to those of aged mice (Figure 2C). SHH (500 ng/ml) increased the expression levels of osterix, *ALP* and osteocalcin mRNA in the young osteoblasts but not in the aged osteoblasts (Figure 2C).

Comparison of the effect of SHH on the osteoclast differentiation and activation between young and aged mice. To further test the ability of osteoclast formation between young and aged mice, we isolated bone marrow macrophages from mice and cultured them individually with 30 ng/ml M-CSF and 30 ng/ml RANKL for 10 days. As shown in Figure 3A, the numbers of TRAP-positive multi-nucleated osteoclast cells were higher in the aged mice. SHH (500 ng/ml) significantly stimulated the osteoclast formation in the presence of 30 ng/ml RANKL for 10 days in both the young and aged macrophages (Figure 3A). The osteoclast activity was higher in aged mice than the young mice (Figure 3B). SHH stimulated the osteoclast activity in both the young and aged mice.

We next examined the expressions of *TRAP* and *NFATc1* mRNA in young and aged mouse bone marrow macrophages incubated with or without 500 ng/ml SHH and 30 ng/ml RANKL (Figure 3C). The RT-PCR analysis showed that the basal expression levels of *TRAP* and *NFATc1* mRNA in the aged mice were higher than those in the young mice. SHH stimulated greater expressions of *TRAP* and *NFATc1* mRNA in the presence of RANKL in the aged macrophages compared to the young macrophages.

SHH signaling is involved in PTH stimulated osteoblast-mediated osteoclasts formation. It has been reported that in mouse osteoblastic MC3T3-E1 cells, SHH stimulates osteoclast formation in the presence of PTHrP signaling through the upregulation of RANKL (12). Herein, in order to test the effects of SHH in osteoblasts on PTH-induced osteoclast formation in young and aged mice, we used a co-culture system composed of osteoblasts and bone marrow macrophage cells of young or aged mice with or without 500 ng/ml SHH or 10 nM PTH.

The results demonstrated that both the PTH-treated young and aged osteoblasts supported the TRAP-positive osteoclast formation with young and aged bone marrow macrophages. SHH significantly stimulated osteoclast formation in the aged osteoblasts and in both the young and aged bone marrow macrophages co-culture group (Figure 4A).

We next examined the expression of *RANKL, OPG*, and *PTH1R* mRNA in young and aged mice osteoblasts incubated with or without 500 ng/ml SHH or 10 nM PTH (Figure 4B). The results of the RT-PCR analysis showed that PTH stimulated the expressions of *RANKL* and *PTH1R* mRNA, but suppressed *OPG* mRNA expression in both young and aged mice osteoblasts. SHH stimulated the expression of *RANKL* mRNA in both young and aged mice osteoblasts. The basal expression levels of *OPG* and *PTH1R* mRNA in young mice were higher than those in the aged mice. SHH up-regulated the *OPG* mRNA expression independently and recovered the *OPG* mRNA expression suppressed by PTH.

Discussion

Although our present findings demonstrated the effects of aging on the expression of SHH during fracture healing, the extent to which decreased SHH signaling in aged mice slows down fracture healing is not known. Fracture healing is a complex event that involves a sequential series of highly linked processes including mesenchymal proliferation, callus formation and differentiation, vascularization, primary bone formation and remodeling. These processes are highly dependent on and interact with each other, and molecular alternations that occur early during the healing response may result in an alteration in the timing of subsequent events (1). Thus, the early molecular events that occur immediately after bone fracture have particular importance in the sequential series of processes.

In the present study, we observed that the phenotype of fracture repair in the aged mice is consistent with a decrease in the number of osteoblasts in the early stage of fracture, suggesting a decrease in the rates of the proliferation and differentiation of osteoblast-lineage cells. A recent study showed that the age of the host may affect the growth and mineralization ability of osteoblasts (16). However the molecular mechanisms of aging that regulate the function of osteoblasts are not well understood.

Hedgehog signaling stimulates osteoprogenitor and osteoblast differentiation *in vivo* (17) and stimulates the differentiation through *Runx2* gene expression in osteoblasts (18). Consistent with previous studies, our results clearly showed that SHH promotes osteoblast differentiation by causing up-regulation of the expression of ALP, osteocalcin and Runx2 in young osteoblasts, confirming the effects of SHH on pre-osteoblast and osteoblast differentiation. However, SHH had no effect on the early stage of aged osteoblast differentiation.

Since osterix has been reported as a Runx2 target gene that mediates osteoblastic differentiation (19), we speculated that a low level of osterix expression in aged osteoblasts is involved in this low response of SHH-induced osteoblast differentiation. The knock-down of osterix in osteogenic cells abrogated all osteogenic marker expression in both SHH-treated and non-treated cells (20). These data indicated that osterix is a master regulator during an early stage of SHH-induced osteoblast differentiation, but decreased the signaling in aged osteoblasts. In the present study, the *Runx2* mRNA in aged osteoblasts was slightly up-regulated by SHH treatment, suggesting that *the osterix* gene may not be regulated by Runx2 dependently. These data indicated that SHH induces an early-to-late stage of young and aged osteoblast differentiation *via osterix* gene that may be regulated in a Runx2-dependent or independent manner (20).

The replacement of the callus is the process of endochondral ossification, and the coupling of osteoclasts and osteoblasts during the replacement of the callus requires activation of specific pathways that promote the osteoclast differentiation (21). Based on results we obtained in an earlier study (12), we propose an aged-mouse fracture model to explain how SHH is involved in aged callus resorption. A sustained rise in PTH increased the local concentration of RANKL and thereby development of new osteoclasts (22). Despite the elevation of osteoclast differentiation by PTH in the present co-culture system, the level of PTH-responsive *RANKL* mRNA expression was low in both young and aged osteoblasts with or without SHH. This is most likely because PTH also suppressed the *OPG* mRNA expression in both the young and aged osteoblasts. Hence PTH significantly increased the osteoclast formation.

The evidence that SHH co-treatment completely recovered the *OPG* mRNA in young osteoblasts indicates that SHH might suppress the PTH-stimulated osteoblast-mediated osteoclast formation. One explanation for the PTH-stimulated aged osteoblast co-cultured cells' osteoclast formation is that the SHH did not respond to the OPG mRNA expression. On the other hand, SHH signaling directly induces osteoclast differentiation and promotes bone resorption in the presence of RANKL. The direct effect of SHH might be involved in aged osteoblasts and osteoclasts' co-culture osteoclast formation. In addition, an age-related increase in osteoblastic cells was reported to mediate osteoclast formation (23).

The expression of OPG was decreased and osteoclast formation was greater when osteoblasts from old, rather than young donors were used to induce osteoclast formation (23). In a previous study, OPG expression was significantly lower in human bone marrow cells obtained from subjects older than 65 years (24). Thus both osteoblast-dependent and -independent mechanisms of SHH in progressive bone loss with aging may operate in concert to generate and activate enough osteoclasts for callus resorption.

In summary, we showed the involvement of SHH in age-related fracture healing. SHH did not respond to age-related changes in the decrease of osteogenic potentials, and PTH suppressed the anti-osteoclastogenic OPG expression in osteoblasts. In addition to the osteoblast-mediated osteoclast formation, SHH directly stimulated the age-related osteoclast formation and resorption. These findings strongly suggest that an aged-related delay of fracture healing could contribute to the primary bone formation and remodeling, which were regulated by hedgehog signaling. Further studies are required to investigate the roles of other signaling molecules in the regulation of bone remodeling.

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