Age-dependent Wnt gene expression in bone and during the course of osteoblast differentiation

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Abstract Wnt signaling is vital for osteoblast differentiation and recently has been associated with aging. Because impaired osteoblastogenesis is a cellular characteristic of age-induced bone loss, we investigated whether this process is associated with an altered expression of Wnt signaling-related proteins in bone and osteoblasts. Bone marrow cells were isolated from male C57BL/6 mice, aged 6 weeks, 6 months, and 18 months, respectively. Osteogenic differentiation was induced for 3 weeks and assessed using alizarin red staining. Gene expression of Wnt1, 3a, 4, 5a, 5b, 7b, 9b, 10b, lipoprotein receptor-related protein (LRP)-5/6,

as well as dickkopf-1 (Dkk-1), sclerostin, and secreted frizzled related protein-1 (sFRP-1) was determined in bone tissue and osteoblasts on days 7, 14, and 21 by real-time RT-PCR. Osteoblast differentiation was significantly reduced in aged mice compared with young and adult mice. In bone tissue, expression levels of all genes assessed were decreased in adult and old mice, respectively, compared with young mice. Mature osteoblasts of aged compared with those of young mice showed enhanced expression of Wnt9b, LRP-6, and Dkk-1, and decreased expression of Wnt5a and 7b. In early osteoblasts, mRNA levels of Wnt1, 5a, 5b, and 7b were increased significantly in aged mice. The expression of Wnt3a, 4, LRP-5, and sclerostin was not altered in aged osteoblasts. In conclusion, osteoblastic expression of each Wnt-related protein is regulated individually by aging. The overall decreased expression of Wnt-related proteins in bone tissue of aged mice underlines the newly discovered association of Wnt signaling with aging.

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Introduction

Age-related osteoporosis is hallmarked by decreased bone mineral density (BMD), reduced bone strength, and compromised microarchitecture predisposing the elderly to an increased fracture risk (Parfitt 1979). Men are equally affected with age-related osteoporosis as women and its incidence increases dramatically

with advancing age (WHO Study Group 1994). Due to the increasing proportion of aged people in industrialized countries, this disabling and painful disease has emerged as a major socioeconomic health problem.

Maintenance of bone mass throughout life relies on the bone remodeling process, which continually replaces old and damaged bone with new bone. While osteoclasts are derived from hematopoietic precursor cells and degrade the bone matrix, osteoblasts originate from mesenchymal stem cells. They deposit a collagenous bone matrix and orchestrate its mineralization. Age-related bone loss represents a deficit in bone formation relative to bone resorption (Parfitt et al. 1995). Several clinical studies and investigations using rodent models of skeletal aging, including our own, have found a decrease in the number of osteoblast precursors and insufficient osteoblast activity as indicated by reduced osteocalcin serum levels and bone histomorphometric observations showing a decrease in trabecular thickness as well as a reduction of mineralization surface and mineral apposition rate (Clarke et al. 1996; Pietschmann et al. 2007). However, the underlying molecular mechanisms leading to insufficient osteoblast activity are not fully understood.

Wnt signaling has been shown to be vital for osteoblast differentiation and bone mass maintenance (Krishnan et al. 2006). Wnts transduce their signals through various pathways, of which the canonical β -catenin-dependent pathway and the non-canonical β -catenin-independent Wnt/ Ca^{2+} pathway have been found to be most critical for osteoblast differentiation (Bennett et al. 2005; Takada et al. 2007). The canonical pathway is activated upon binding of Wnt proteins to a receptor complex consisting of Frizzled (FZD) and its co-receptor, lipoprotein receptor-related protein (LRP)-5 or LRP-6. This interaction ultimately results in the stabilization of β -catenin, which then translocates into the nucleus to induce transcription of osteoblastic proteins. The Wnt/ Ca^{2+} pathway on the other hand transduces signals through the calcium-dependent calcineurin/nuclear factor of activated T cells (NFAT) pathway, and Wnt5a, an activator of this pathway, has also been identified to operate in osteoblasts (Baksh and Tuan 2007).

Wnt signaling is regulated at various levels, such as through the presence or absence of multiple Wnt proteins, co-receptors, intracellular signaling molecules, and transcription factors (Krishnan et al. 2006). Furthermore, Wnt signaling is tightly regulated by a series of inhib-

itors, including members of the Dickkopf (Dkk) and secreted frizzled-related protein (sFRP) family as well as sclerostin and Wnt inhibitory factor (WIF). In bone, Dkk-1, sclerostin (SOST), and sFRP-1 have been shown to inhibit Wnt signaling specifically, thus diminishing osteoblast differentiation and reducing bone mass (Winkler et al. 2003; Morvan et al. 2006; Wang et al. 2005).

Currently, there is accumulating evidence that Wnt signaling regulates cellular aging; however, the mechanism by which it affects aging remains controversial (Decarolis et al. 2008). There are multiple convincing studies that found activated Wnt signaling to delay aging, while at the same time emerging evidence supports the opposite concept (Brack et al. 2007; Liu et al. 2007). For bone tissue, it could be shown that the sustained expression of Wnt10b in bone marrow maintained bone mass in aged mice (Bennett et al. 2005). Another study demonstrated that sFRP-4, a protein increasing osteoblast differentiation, was negatively associated with peak bone mass in senescence-accelerated mice strain P6 (SAMP6) mice, a murine model of accelerated aging (Cho et al. 2008; Nakanishi et al. 2006). These data suggest that Wnt signaling is involved in age-related processes in bone.

In this study, we assessed the direct effects of aging on the mRNA expression of various bone-related Wnt proteins, including Wnt1, 3a, 4, 5a, 5b, 7b, 9b, and 10b, the Wnt co-receptors LRP-5 and LRP-6, as well as the Wnt inhibitors Dkk-1, sclerostin, and sFRP-1 in bone tissue of aged mice and during ex-vivo osteoblast differentiation.

Materials and methods

Animals

Six male C57BL/6J mice per age group were purchased from Janvier, Le Genest St Isle, France. The age groups were “young” (6-weeks old), “adult” (6-months old), and “old” (18-months old). The animals were held and euthanized according to Austrian laws and institutional regulations.

Primary bone marrow-derived osteoblast culture

The isolation of murine bone marrow-derived osteoblasts was conducted as described previously (Rauner et al. 2007). Briefly, mice were killed by cervical

dislocation and the hind legs were dissected. Flesh was aseptically removed and, after abscising the epiphyseal ends, bone marrow was flushed out with α -MEM (minimal essential medium) supplemented with 10% FCS (fetal calf serum), 100 U/ml penicillin G, 100 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B (all from Invitrogen, Vienna, Austria), 50 μ g/ml ascorbic acid and 5 mM β -glycerophosphate (both from Sigma, Vienna, Austria). Cells were seeded at a density of 1×10^7 cells per 10-cm Petri dish and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. To allow optimal attachment of the cells to the plastic surface medium was not changed until day 5; thereafter, medium was changed every 2–3 days.

Mineralization assay

Two-million cells were cultured per well in a 24-well plate in osteogenic medium for 21 days. At the end of the culture period, cells were fixed for 1 h in 70% ethanol and incubated for 10 min at room temperature in a 40 mM alizarin red S solution, pH 4.2 (Sigma, Munich, Germany). Excess dye was removed by washing the plates with distilled water. The amount of bound calcium was quantified by eluting with a 0.1 M HCl/0.5% sodiumdodecyl sulphate (SDS) solution for 30 min at room temperature. Aliquots were taken and measured with a spectrophotometer at 415 nm.

RNA extraction and real-time RT-PCR analysis

Total RNA was extracted from the bone tissue and bone marrow cultures using TRIzol (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's protocol. To isolate RNA from the bone tissue, long bones from the hind legs of the mice were crushed in liquid nitrogen. Trizol was directly added to the bone powder and further proceedings were performed according to the protocol. RNA was reverse transcribed into cDNA (Superscript II First-Strand, Invitrogen) and used for quantitative PCR. Real-time RT-PCR was performed for Wnt1 (NM_021279.3), Wnt3a (NM_009522.1), Wnt4 (NM_009523.1), Wnt5a (NM_009524.2), Wnt5b (NM_009525.2), Wnt7b (NM_009528.2), Wnt9b (NM_011719.2), Wnt10b (NM_011718.1), Dkk-1 (NM_145592.3), SOST (NM_024449.3), sFRP-1 (NM_011356.2), LRP-5 (NM_008513.1), LRP-6 (NM_008514.1), and type I collagen (NM_007743.2) using assay-on-demand primers and probes following

the manufacturer's instructions. The analyses were performed on the ABI Prism Sequence Detection System 7700 (Applied Biosystems, Foster City, Calif.). All experiments were performed in duplicates and were normalized to an invariant endogenous control (GAPDH, NM_001615). PCR conditions were 50°C for 2 min and 94°C for 2 min, followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. The results were calculated applying the $\Delta\Delta$ CT method and are presented as fold increase relative to GAPDH expression.

Statistical analysis

Results are presented as means $\pm$ standard deviation from the data of six mice per age group. Statistical evaluations were performed using a Student's *t*-test. *P* values < 0.05 were considered statistically significant.

Results

Gene expression of Wnt-related proteins in bone tissue of aged mice

Gene expression levels of Wnt1, 3a, 4, 5a, 5b, 7b, 9b, 10b, the Wnt co-receptors LRP-5 and LRP-6, as well as the Wnt inhibitors Dkk-1, sclerostin (SOST), and sFRP-1 were assessed in the bone tissue of young, adult, and old mice in order to directly investigate age-related changes in their expression. The expression levels of Wnt4, 5a, 5b, 10b, LRP-5, Dkk-1, and sFRP-1 were significantly reduced in adult and old animals (Fig. 1). Wnt1, 7b, and 9b expression levels were markedly reduced in old, but not adult mice. Interestingly, mRNA levels of Wnt3a, LRP-6, and sclerostin were significantly reduced in adult animals only. Although a decrease in their expression was apparent also in old mice, it did not reach statistical significance. No differences were found between the mRNA expression levels of any genes investigated in adult and old mice. The mRNA expression of type I collagen (COL) and osteocalcin (OCN), representing typical proteins found in bone, was also significantly reduced in adult and old animals.

Gene expression of Wnt-related proteins during osteoblast differentiation

After finding that all Wnt signaling-related genes assessed were down-regulated with aging, we next

Fig. 1 Gene expression of Wnt proteins, Wnt co-receptors, and Wnt inhibitors in bone tissue of aged mice. RNA was isolated from the long bones of young, adult, and old male mice aged 6 weeks, 6 months, and 18 months, respectively, and subjected to real-time RT-PCR analysis to determine the mRNA expression

levels of Wnt1, 3a, 4, 5a, 5b, 7b, 9b, 10b, LRP-5, LRP-6, Dkk-1, sFRP-1, and SOST relative to GAPDH expression. Expression levels of type I collagen and osteocalcin mRNA were assessed as a positive control. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$

analysed the mRNA expression pattern of Wnt1, 3a, 4, 5a, 5b, 7b, 9b, 10b, LRP-5, LRP-6, Dkk-1, SOST, and sFRP-1 in the course of osteoblast differentiation in adult mice, which have a fully developed skeleton and therefore their skeleton best reflects mature bone. The mRNA expression of type I collagen was assessed as a typical intermediate osteoblast marker and osteocalcin as a typical late osteoblast marker to confirm osteoblast maturation. Indeed, mRNA levels of type I collagen were highest in intermediate osteoblasts and slightly decreased again in late osteoblasts, whereas the expression of osteocalcin increased with osteoblast maturation. The mRNA expression of Wnt1 and 4 increased in intermediate osteoblasts and decreased again in late, mineralized ones. Wnt5a, 5b, 7b, and 10b expression was highest in intermediate osteoblasts. Gene expression levels of Wnt3a and 9b did not change significantly in the course of osteoblast maturation. LRP-5, LRP-6, and sFRP-1 showed a similar gene expression pattern, increasing dramatically in intermediate and late osteoblasts. Dkk-1 was expressed highest in early osteoblasts and significantly declined with maturity. SOST mRNA levels on the other hand increased with osteoblast maturity (Fig. 2).

Age-dependent gene expression of Wnt ligands during osteoblast differentiation

After investigating the gene expression patterns of the Wnt proteins in osteoblast differentiation, we next

assessed age-related changes in their expression pattern. Therefore, we first examined the differentiation potential of osteoblasts in dependence of age. Osteoblast differentiation was significantly decreased by 60% and 70%, respectively, in old animals compared with young and adult ones (Fig. 3). Next, the mRNA expression levels of runx2 and osterix, two osteoblast-specific transcription factors, were assessed as well as the expression of type I collagen and osteocalcin, to evaluate age-related changes of typical osteoblast markers (Fig. 4). While osterix, type I collagen and osteocalcin showed the highest expression in adult animals, runx2 was expressed highest in young animals in intermediate osteoblasts. Type I collagen further showed a significant decline in aged animals compared with young animals in intermediate osteoblasts, whereas osteocalcin was significantly decreased in mature osteoblasts from old animals compared with young ones.

After assessing the expression pattern of typical osteoblast markers, we next analyzed the age-related changes in the expression pattern of Wnt ligands. Individual Wnt expression levels were highly heterogeneous with respect to osteoblast differentiation state as well as to age. Expression levels of Wnt3a and 4 mRNA were not influenced by age. In early osteoblasts, the expression level of Wnt1 and 10b was significantly increased in adult animals compared with young ones, whereas the expression of Wnt5a and 9b was decreased (Fig. 5). Furthermore, Wnt5a, 5b, and

Fig. 2 Gene expression of Wnt proteins, Wnt co-receptors, and Wnt inhibitors in the course of osteoblast differentiation. Osteoblasts were isolated from the bone marrow of adult mice aged 6-months and cultured in osteogenic medium for up to 21 days. Gene expression analysis of Wnt1, 3a, 4, 5a, 5b, 7b, 9b, 10b, LRP-5, LRP-6, Dkk-1, sFRP-1, and SOST was

performed on days 7, 14 and 21 representing early, intermediate and late osteoblasts. Results are presented as fold-increase relative to GAPDH expression. Expression of type I collagen and osteocalcin served as a positive control. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$

7b mRNA levels were significantly increased in old mice. In intermediate osteoblasts, Wnt1 expression was significantly increased in adult and old mice, while Wnt5a mRNA levels were decreased with aging. In late osteoblasts, the expression of Wnt5a and 7b was markedly decreased in adult and old animals. Wnt5b expression was only reduced significantly in adult animals compared with young ones. In contrast, mRNA level of Wnt9b was significantly increased in old mice.

Age-dependent mRNA expression of Wnt co-receptors and Wnt inhibitors during osteoblast differentiation

After investigating changes in the age-dependent expression of Wnt ligands, we further examined the expression pattern of the Wnt co-receptors LRP-5 and LRP-6 as well as Wnt inhibitors in bone, namely Dkk-1, SOST, and sFRP-1 (Fig. 6). LRP-5 mRNA levels significantly increased in adult mice in intermediate and late osteoblasts. While LRP-6 mRNA levels decreased significantly in early osteoblasts of adult and old animals, its expression was significantly increased in late osteoblasts. The expression of Dkk-1 was highest in early osteoblasts in adult mice, whereas in late osteoblasts, mRNA levels of Dkk-1 were highest in old mice compared to young and adult ones. The expression level of sclerostin in early osteoblasts was lowest in adult animals but did not change

with aging in intermediate or late osteoblasts. Whereas levels of sFRP-1 mRNA were highest in early osteoblasts of young animals, they were lowest in intermediate and late osteoblasts.

Discussion

Advanced age is a high-impact risk factor for osteoporosis. Due to our increasingly aging society, senile osteoporosis has emerged as a serious health problem

Fig. 3 Influence of aging on osteoblast differentiation potential. Osteoblasts were isolated from the bone marrow of young, adult, and old male mice aged 6 weeks, 6 months, and 18 months, respectively, and cultured under osteo-inductive conditions for 21 days. Cells were stained with alizarin red S to visualize mineralization, a hallmark of mature osteoblasts. Subsequently, the dye was eluted for quantification. ** $P \leq 0.01$, *** $P \leq 0.005$

Fig. 4 Age-dependent expression of osteoblast-specific markers. Osteoblasts were isolated from the bone marrow of mice aged 6 weeks, 6 months, and 18 months, respectively, and cultured under osteo-inductive conditions for up to 21 days. Gene expression analysis of runx2, osterix, type I collagen and

osteocalcin was performed on days 7, 14 and 21. Results are presented as fold-increase relative to GAPDH expression. White bars young mice; gray bars adult mice; black bars old mice; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$

in industrialized countries. Osteoblast insufficiency has been identified as a main contributor to low bone mass in aged individuals and experimental animal models of aging. However, the underlying molecular mechanisms of impaired osteoblast differentiation are poorly characterized. Lately, Wnt signaling has been linked to age-related processes (Brack et al. 2007; Liu et al. 2007). As it is well known that Wnt signaling critically regulates osteoblast differentiation and bone mass maintenance, we have questioned whether aging decreases the expression of Wnt proteins in bone, and thereby may be associated with the insufficient osteoblast differentiation and function seen with aging.

Our observational study shows that mRNA expression levels of various bone-related Wnt proteins, including Wnt1, 4, 5a, 5b, 7b, 9b, 10b, and LRP-5, are significantly decreased with advanced age. Surprisingly, also the expression of the Wnt inhibitors Dkk1 and sFRP-1 in bone tissue is decreased in old animals. This result suggests that although expression levels of both Wnt inhibitors and Wnt ligands are decreased, the ratio of Wnt ligands to Wnt inhibitor may be altered in a way that the expression of Wnt inhibitors prevails over that of Wnt ligands, thereby blocking osteoblastogenesis. Our study extends previous investigations assessing the expression of Wnt10b in bone and muscle with aging (Krishnan et al. 2006; Vertino et

al. 2005). Those studies have demonstrated that mice overexpressing Wnt10b in bone marrow maintain bone mass throughout life, and that the decrease of Wnt10b expression in myocytes drives the cells into adipocyte differentiation, which leads to the accumulation of lipids in muscle tissue in aged animals. The observation that Wnt10b suppresses the expression of adipocyte-related genes in myocytes was also found to be true for osteoblasts and, although not yet investigated, decreased expression of Wnt10b may also contribute to the increase in fatty marrow (Kang et al. 2007). The age-dependent expression of other Wnt and Wnt-related proteins has not yet been assessed in bone. Hence, our study is the first to show that the expression of additional Wnt ligands, including Wnt1, 4, 5a, 5b, 7b, and 9b, is reduced in bones of aged mice. Interestingly, the expression of Wnt inhibitors such as Dkk1 and sFRP-1 is also reduced with aging, thus suggesting that the decrease in expression of Wnt genes may be the result of the insufficiently working gene expression machinery in aged osteoblasts displaying impaired differentiation and function.

Various Wnt proteins have been shown to positively or negatively regulate osteoblast differentiation. While some reports have demonstrated that Wnt1 and 3a inhibit osteoblast differentiation, other studies indicate that Wnt3a induces the expression of alkaline

Fig. 5 Age-dependent expression of Wnt ligands during osteoblast differentiation. Osteoblasts were isolated from the bone marrow of young, adult, and old male mice aged 6 weeks, 6 months, and 18 months, respectively, and cultured under osteo-inductive conditions for up to 21 days. Gene expression analysis of Wnt1, 3a, 4, 5a, 5b, 7b, 9b and 10b was performed on days 7, 14 and 21. Results are presented as fold-increase relative to GAPDH expression. *White bars* young mice; *gray bars* adult mice; *black bars* old mice; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$

phosphatase and supports osteoblastogenesis (Boland et al. 2004; de Boer et al. 2004; Si et al. 2006). Although our study does not investigate the effects of Wnt3a on osteoblast differentiation, we found that its gene expression was constantly very low. Moreover, expression of Wnt1, 5b, 7b, 10b and the non-canonical Wnts 4 and 5a was highest in intermediate osteoblasts independent of age. In the case of Wnt10b, it is conceivable that the decrease thereafter may lead to a fragile osteoblast phenotype, allowing the cell to trans-differentiate into adipocytes under appropriate conditions, such as aging. Along with the increase of most Wnt proteins in intermediate osteoblasts, also gene expression of the co-receptors LRP-5 and LRP-6 were enhanced, suggesting optimal conditions for the induction of Wnt signaling. Interestingly, Wnt9b was the

only gene investigated that decreased during osteoblast differentiation. As reported previously, our study confirmed the predominant expression of Dkk1 in early proliferating and of sclerostin in late, mature osteoblasts (van der Horst et al. 2005; Poole et al. 2005). Further, our data indicate that sFRP-1 is also expressed highest in late osteoblasts, suggesting that this protein may also have a role in mechanotransduction similar to sclerostin by suppressing osteoblast activity and enabling the transition into an osteocyte. Notably, another group observed sFRP-1 peak expression during the transition from preosteoblast to osteoblast (Bodine et al. 2005). Despite the early expression of this protein in the osteoblast ontogenesis, the authors observed a differentiation-inhibitory and proapoptotic role for sFRP-1.

Fig. 6 Age-dependent mRNA expression of Wnt co-receptors and Wnt inhibitors during osteoblast differentiation. Osteoblasts were isolated from the bone marrow of young (6 weeks), adult (6 months) and old (18 months) male mice and cultured under osteo-inductive conditions for up to 21 days. Gene expression analysis of LRP-5, LRP-6, Dkk-1, sFRP-1 and SOST was performed on days 7, 14 and 21. Results are presented as fold-increase relative to GAPDH expression. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$

Next to investigating direct effects of aging on Wnt gene expression in bone tissue and the endogenous expression of Wnt proteins during osteoblast differentiation, we further explored the influence of age on ex-vivo osteoblast generation and Wnt gene expression. Our results demonstrate a significant age-dependent defect in ex-vivo osteoblast differentiation similar to the study of Zhou et al. (2008). While it is difficult to hypothesize on the consequences of the altered Wnt signaling in osteoblasts, it has become clear that age influences the gene expression only of certain Wnts and related proteins in osteoblasts. Our study shows that Wnt3a, 4, LRP-5, and sclerostin are not affected significantly with aging, while Wnt5a, 5b, 7b, and 10b are significantly down-regulated and Wnt1, 9b, LRP-6, Dkk-1, and sFRP-1 are up-regulated with aging at different stages of osteoblast differentiation. Hence, our study provides novel information about the age-related regulation of Wnt and Wnt-related gene expression during osteoblast differentiation and points out certain Wnt proteins that may be interesting targets for further investigations aimed at elucidating mechanisms of osteoblast aging.

Although Wnt ligands may be considered as a potential therapeutic option to maintain bone mass in osteoporosis (Sipos et al. 2008), this and other studies highlight the complexity of Wnt signaling and underline the importance of investigating this pathway in a tissue- and cell-specific manner as well as taking into account the developmental stage of the organisms investigated to fully comprehend the consequences of interfering with this pathway. Although protein levels of the Wnt-related proteins have not been assessed, this study highlights that even the gene expression pattern of certain molecules in tissues may differ from that in specific cells within the tissue, such as exemplified by different Wnt inhibitor expression patterns in whole bone tissue and cultured osteoblasts. This, once more, gives evidence for the necessity for investigating expression profiles in whole tissues as well as cell cultures in order to better understand the complexity of pathophysiological processes.

In summary, we show that the gene expression of various Wnt proteins as well as Wnt co-receptors and, surprisingly, also Wnt inhibitors is down-regulated in

the bone tissue of aged mice. Furthermore, our results demonstrate that each Wnt protein has an individual gene expression pattern during osteoblast differentiation and is differentially regulated by aging. Hence, our study underlines the newly discovered association of Wnt signaling with age-related processes.

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