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Aging activates adipogenic and suppresses osteogenic programs in mesenchymal marrow stroma/stem cells: the role of PPAR-γ2 transcription factor and TGF-β/BMP signaling pathways

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Summary

Osteoblasts and adipocytes originate from a common progenitor, which arises from bone marrow mesenchymal stroma/stem cells (mMSC). Aging causes a decrease in the number of bone-forming osteoblasts and an increase in the number of marrow adipocytes. Here, we demonstrate that, during aging, the status of mMSC changes with respect to both their intrinsic differentiation potential and production of signaling molecules, which contributes to the formation of a specific marrow microenvironment necessary for maintenance of bone homeostasis. Aging causes a decrease in the commitment of mMSC to the osteoblast lineage and an increase in the commitment to the adipocyte lineage. This is reflected by changes in the expression of phenotype-specific gene markers. The expression of osteoblast-specific transcription factors, Runx2 and Dlx5, and osteoblast markers, collagen and osteocalcin, is decreased in aged mMSC. Conversely, the expression of adipocyte-specific transcription factor PPAR-γ2, shown previously to regulate osteoblast development and bone formation negatively and to regulate marrow adipocyte differentiation positively, is increased, as is a gene marker of adipocyte phenotype, fatty acid binding protein aP2. Furthermore, production of an endogeneous PPAR-γ activator(s) that stimulates adipocyte differentiation and production of autocrine/paracrine factor(s) that suppresses the osteoblastic phenotype are also increased. In addition, expression of different components of TGF-β and BMP2/4 signaling pathways is altered, suggesting that activities of these two cytokines essential for bone homeostasis change with aging.

Keywords

adipocyte; aging; marrow stem cells; osteoblast; PPAR-γ; TGF-β/BMP

Introduction

Age-related bone loss or type II osteoporosis occurs universally in animals and humans and, in contrast to post-menopausal bone loss or type I osteoporosis, affects individuals regardless of their sex steroid status (Frost, 1973;Manolagas, 1998). Maintenance of bone homeostasis throughout life relies on the bone remodelling process, which continually replaces old and damaged bone with new bone in order to maintain bone strength and elasticity (Parfitt, 1994). Two types of cells are involved in bone remodelling: osteoclasts, originating from haematopoietic cells, are responsible for bone resorption; and osteoblasts, originating from mesenchymal cells, are responsible for formation of new bone. Age-related bone loss results from attenuated and unbalanced bone turnover and occurs only on the surface in contact with

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bone marrow. Thus, an oversupply of osteoclasts, relative to the need for bone resorption, and/or an undersupply of osteoblasts, relative to the need for cavity repair, are critical pathogenic factors in type I and type II osteoporosis, respectively (Manolagas, 1998).

Although aging has a negative effect on osteoblast production, it has a positive effect on the proportion of fatty marrow (Tavassoli, 1984;Moore & Dawson, 1990;Gimble *et al*., 1996a;Robey & Bianco, 1999). In neonatal mammals, adipocytes are all but absent in the bone marrow, which is primarily haematopoietic at this stage. However, with advancing age, the number of adipocytes in the bone marrow increases, resulting in the appearance of fatty marrow. In humans, most of the femoral cavity is occupied by fat by the third decade of life.

Osteoblasts and adipocytes are derived from mesenchymal marrow stroma/stem cells (mMSC). mMSC are also progenitors for marrow fibroblasts and cartilage cells, and function as haematopoiesis-supporting stroma (Bianco *et al*., 2001;Jiang *et al*., 2002). The milieu of intracellular and extracellular signals controls mMSC differentiation into osteoblast or adipocyte. Activation of phenotype-specific transcription factors, such as osteoblast-specific Runx2/Cbfa1 and adipocyte-specific PPAR-γ2, determines lineage commitment (Tontonoz *et al*., 1994b;Ducy *et al*., 1997;Komori *et al*., 1997;Karsenty, 2001;Rosen & Spiegelman, 2001). We previously demonstrated that the adipocyte-restricted PPAR-γ2 transcription factor is a key regulator of osteoblast and adipocyte differentiation (Lecka-Czernik *et al*., 1999). In a cellular *in vitro* model of murine mMSC differentiation, PPAR-γ2 acts as a positive regulator of adipocyte differentiation and a dominant-negative regulator of osteoblast differentiation. Ectopic expression of recombinant PPAR-γ2 in osteoblastic UAMS-33 cells irreversibly suppressed Runx2/Cbfa1 expression and the osteoblast phenotype and simultaneously converted these cells to terminally differentiated adipocytes. *In vivo*, an essential role of PPAR-γ in maintaining bone homeostasis was demonstrated in two opposing, but complementary, models (Akune *et al*., 2004;Rzonca *et al*., 2004). First, in a model of bone loss, a high-affinity ligand and activator for PPAR-γ, rosiglitazone (Werner & Travaglini, 2001), was administered to mice for 7 weeks and resulted in a significant decrease in bone mineral density (BMD), bone volume and changes in bone microarchitecture. Moreover, rosiglitazone treatment decreased the number of osteoblasts while simultaneously increasing the number of marrow adipocytes (Rzonca *et al*., 2004). Second, in a model of increased bone formation due to PPAR-γ insufficiency, heterozygous PPAR-γ-deficient mice exhibited high bone mass and increased osteoblastogenesis (Akune *et al*., 2004). In humans, PPAR-γ polymorphism, resulting from a silent C to T transition in exon 6, is associated with reduced BMD (Ogawa *et al*., 1999).

signaling through TGF-β/BMP cytokines exemplifies extracellular mechanisms that modulate intracellular processes (Miyazono *et al*., 2001;Derynck & Zhang, 2003). TGF-β regulates osteoblast differentiation in a biphasic manner. It stimulates development and proliferation of early osteoblasts, but it inhibits their maturation and expression of phenotype-specific genes, such as osteocalcin and alkaline phosphatase (Alliston *et al*., 2001;Nishimura *et al*., 1999;Lee *et al*., 2000;Banerjee *et al*., 2001). In contrast, BMP2 and BMP4 cytokines are essential for osteoblasts to achieve their mature phenotype, which is characterized by the ability to form collagen-based extracellular matrix and mineral deposits (Abe *et al*., 2000;Canalis *et al*., 2003;Devlin *et al*., 2003). BMP2/4 cytokines positively regulate expression of osteoblast-specific genes, such as Runx2/Cbfa1, Dlx5, collagen and alkaline phosphatase. Inhibition of this pathway by noggin, a natural antagonist, leads to suppression of the osteoblast phenotype and lack of mineralization *in vitro* and *in vivo* (Abe *et al*., 2000;Devlin *et al*., 2003).

TGF-β/BMP cytokines also control adipocyte formation. TGF-β inhibits adipocyte differentiation by inactivating C/EBP transcription factors via physical interaction with Smad3 or an unclear mechanism involving Smad6 and Smad7 proteins (Choy *et al*., 2000;Choy & Derynck, 2003). In contrast, BMP2/4 cytokines stimulate adipocyte differentiation by either

committing multipotential cells to adipocyte lineage and/or augmenting their adipocyte differentiation, through both Smad-dependent and p38 kinase-dependent mechanisms (Sottile & Seuwen, 2000;Hata *et al*., 2003;Tang *et al*., 2004).

Both TGF-β and BMP2/4 cytokines communicate with cells through Smad proteins, which serve as signal mediators between cell surface receptors and the nuclear transcriptional apparatus (Derynck & Zhang, 2003;Shi & Massague, 2003). In general, extracellular binding of TGF-β and BMP2/4 to their receptors recruits pathway-restricted Smads (R-Smads), which are phosphorylated by the serine/threonine kinase activity of the receptors. Smad2 and -3 are activated in response to TGF-β signals, whereas Smad1, -5 and -8 are activated in response to BMP2/4 signals. These receptor-activated R-Smads are released from the receptor complex to form a new complex with Smad4 and translocate into the nucleus, where they serve as transcriptional regulators. The Smad signaling pathway also includes two inhibitory Smads (I-Smads), Smad6 and Smad7, which help regulate the cellular response to TGF-β/BMP cytokines. I-Smads operate through a negative feedback loop mechanism, and their expression is under the positive control of activated R-Smads (Stopa *et al*., 2000;von Gersdorff *et al*., 2000;Ishida *et al*., 2003).

Here, we have examined the effects of aging on the differentiation potential of mMSC and the role of PPAR-γ2 transcription factor in this process. Moreover, we have examined the effects of aging on expression of different components of TGF-β/BMP2/4 signaling pathways. Our results indicate that changes in the differentiation potential of mMSC are accompanied by alterations in the intracellular mechanisms and extracellular signaling that control their fate.

Results

Age-related changes in bone and bone marrow structure

Age-related changes in the rate of bone formation are accompanied by an increase in the proportion of marrow occupied by adipocytes (Moore & Dawson, 1990). Histological examination of the bone marrow in proximal tibiae of old (26-month-old) C57BL/6 mice indicates the presence of significantly more fat cells than in the marrow of adult (8-month-old) mice (Fig. 1). The increase in the number of adipocytes in the marrow of old mice is accompanied by a decrease in the number of bone-forming osteoblasts at bone remodelling sites and reduced BMD, which indicates lower bone mass.

Aging alters the differentiation potential of mMSC

Bone marrow consists of a variety of cell types, including cells of haematopoietic and mesenchymal lineage. The conditions of primary bone marrow cultures select for cells that adhere to a plastic surface. This population of cells, historically referred to as marrow stroma cells (MSC), is primarily composed of cells of mesenchymal lineage but also includes a substantial number of macrophages and a relatively small number of myeloid and endothelial cells (Owen *et al*., 1987;Phinney *et al*., 1999). A population of adult mesenchymal MSC is heterogeneous and consists of both multipotential primitive stem cells and cells at various stages of differentiation toward specific lineages (Nuttall *et al*., 1998;Pittenger *et al*., 1999;Jiang *et al*., 2002). For clarity, we will refer to these cells as mesenchymal marrow stroma/stem cells or mMSC.

Prompted by the evidence that osteoblasts and adipocytes of the bone marrow are derived from a common set of mesenchymal progenitor cells, we used bone marrow aspirates of adult and old mice to assess the number of mMSC that were able to differentiate into osteoblasts, as judged by the ability to form mineralized colonies, and adipocytes, as judged by the ability to form colonies of fat-laden cells. mMSC were cultured at conditions that allowed a single

mesenchymal cell to proliferate and form a separate colony, referred to as a colony forming unit (CFU). For formation of mineralized colonies, mMSC were cultured in the presence of pro-osteoblastic stimuli, whereas the adipocytic phenotype was stimulated by treatment with a pro-adipocytic cocktail, as described in the Experimental procedures. Bone marrow derived from old animals developed relatively fewer osteoblastic colonies (CFU-OB) and more adipocytic colonies (CFU-AD) than marrow derived from adult animals (Fig. 2A,B). A calculated ratio of CFU-OB to CFU-AD formation indicates changes in the differentiation potential of mMSC. In adult animals the prevalence of CFU-OB formation was fourfold greater than CFU-AD, whereas in old animals the frequency of CFU-OB and CFU-AD formation was equal (Fig. 2C). Thus, aging changes the differentiation potential of mMSC for more adipogenic and less osteoblastogenic.

A detailed examination of the adipogenic potential of mMSC revealed that marrow derived from old C57BL/6 mice possessed a greater number of cells able to differentiate spontaneously into adipocytes in the absence of pro-adipocytic stimuli. Bone marrow cultures derived from old mice spontaneously developed approximately three times more CFU-AD than cultures derived from adult animals (Table 1). This, together with our former observation, suggests not only that aging increases the commitment of mMSC to adipocytic lineage but also that a higher proportion of these committed cells are present at the late stages of differentiation.

We used quantitative real-time RT-PCR to examine whether changes in the differentiation potential of mMSC were accompanied by changes in the expression of phenotype-specific gene markers (Fig. 3). In basal, non-differentiating conditions, mMSC derived from old animals express more mRNA encoding fatty acid binding protein aP2, a marker for the adipocyte phenotype, and less mRNAs for osteoblast-specific transcription factors, Runx2/Cbfa1 and Dlx5, and phenotype-specific markers, collagen and osteocalcin, than mMSC isolated from adult animals. These data support our previous observation that aging increases mMSC commitment to adipocyte and decreases commitment to osteoblast lineage.

PPAR-γ 2 transcription factor accounts for increased adipogenic potential of mMSC with aging

PPAR-γ is a key regulator of adipocyte differentiation and is essential for maintenance of adipocyte phenotype and function (Rosen & Spiegelman, 2001). The thiazolidinedione, rosiglitazone, is an artificial, highly specific PPAR-γ agonist, causing induction of pro-adipocytic and anti-diabetic activities of this transcription factor (Lehmann *et al*., 1995). Treating bone marrow cultures with rosiglitazone significantly increased the pro-adipocytic response of old marrow as compared with adult marrow (Table 1). In addition to increasing in number, adipocytes developed in cultures derived from old animals accumulated more fat than adipocytes in the cultures derived from adult animals (Fig. 4A). These results indicate that sensitivity to PPAR-γ is increased in old marrow. This increased sensitivity was evidenced by both the large number of cells responding to rosiglitazone and the robust adipocytic response in individual cells.

This finding suggests that expression of PPAR-γ is increased in old marrow as compared with adult marrow. PPAR-γ nuclear receptor is expressed in two isoforms, PPAR-γ1 and PPAR-γ2, which result from alternative splicing and alternative promoter usage (Zhu *et al*., 1995). Although both isoforms play important roles in fat tissue homeostasis, recent evidence indicates that PPAR-γ2, but not PPAR-γ1, is essential for adipocyte formation (Ren *et al*., 2002). Moreover, whereas PPAR-γ2 expression is restricted to adipocytes, PPAR-γ1 is expressed in many cell types, including osteoblasts (Lecka-Czernik *et al*., 1999). Therefore, we focused our attention on the PPAR-γ2 isoform.

Quantitative real-time RT-PCR analysis revealed that the expression of PPAR-γ2 mRNA is 10-fold higher in old than in adult marrow (Fig. 4B). These data, together with the observations on the essential role of PPAR-γ2 in adipocyte development (Ren *et al*., 2002) (Tontonoz *et al*., 1994a;Mukherjee *et al*., 1997), the requirement of this isoform for terminal differentiation of marrow adipocytes and irreversible suppression of the osteoblast phenotype (Gimble *et al*., 1996b;Lecka-Czernik *et al*., 1999), indicate that PPAR-γ2 plays a critical role in the decision of mMSC to differentiate into either osteoblasts or adipocytes.

Aging increases production of adipocytic activator(s) and osteoblastic inhibitor(s) in the bone marrow

In vivo, two factors are necessary to achieve increased adipocyte differentiation: increased expression of PPAR-γ2 and increased availability of natural PPAR-γ ligand and/or activator. Therefore, we examined whether bone marrow produces an endogeneous PPAR-γ activator, and whether its level changes with aging. We used conditioned media derived from the cultures of adult and old marrow to assess their pro-adipocytic activities in U-33/γ2 and U-33/c cells (Lecka-Czernik *et al*., 1999). U-33/γ2 cells represent an *in vitro* model of mMSC differentiation that is under the control of the PPAR-γ2 transcription factor. U-33/c cells, which lack PPAR-γ2 but naturally express PPAR-γ1, served as a negative control for the processes mediated through the PPAR-γ2 isoform. As a positive control for adipocyte differentiation, U-33/γ2 cells were treated with rosiglitazone. Conditioned media collected from old bone marrow cultures induced fat accumulation in a significantly greater number of U-33/γ2 cells than conditioned medium collected from adult bone marrow cultures (Table 2). No effect on fat accumulation was seen in U-33/c cells (data not shown), indicating that the pro-adipocytic effects of tested conditioned media were mediated through PPAR-γ2.

The same conditioned media were also evaluated for effects on the osteoblastic phenotype of U-33/γ2 and U-33/c cells. Conditioned media from cultures of old marrow cells effectively suppressed the osteoblast phenotype in both U-33/γ2 and U-33/c cells, as measured by formation of mineralized extracellular matrix (Fig. 5). When cells were cultured in the presence of conditioned medium derived from cultures of adult bone marrow, neither cell line demonstrated a significant change in mineralization. These results indicate that old bone marrow not only produces PPAR-γ2 activator(s), but also possesses a PPAR-γ2-independent activity that inhibits osteoblast function. Whether this activity is mediated by the same or different factor(s) remains unclear.

Effects of aging on mRNA expression of components of TGF-β/BMP signaling pathways

In bone, both TGF-β and BMP cytokines are produced mainly by cells of the mesenchymal lineage; they control osteoblast and adipocyte differentiation and are essential for bone formation and bone homeostasis. Therefore, changes in the balance between osteoblast and adipocyte differentiation during aging may result from changes in the activities of the TGF-β/BMP signaling pathways. To investigate this possibility, we analysed whether aging changes the expression of different components of these signaling pathways. mRNA expression of two genes encoding I-Smads, Smad6 and Smad7, was decreased in mMSC from old mice (Fig. 6). Because expression of Smad7 is under positive control of TGF-β cytokines, whereas expression of Smad6 is positively regulated by BMP2/4 cytokines, the decrease in expression of these two genes suggests decreased activities of the TGF-β/BMP signaling pathways. These decreased activities may result from changes in the levels of gene expression and activity of components that are limiting factors for TGF-β/BMP signaling. Indeed, whereas mMSC from old mice demonstrated an increase in the mRNA expression of TGF-β1 cytokine, they showed a significant decrease in the mRNA expression of two other cytokines, TGF-β2 and TGF-β3, and the receptor Tβ-R1 (Fig. 6A). Among signaling components of the BMP2/4 pathway, we

observed an increase in the mRNA expression of BMP4 cytokine and a 10-fold decrease in the mRNA expression of the receptor BMPR-1B in mMSC derived from old animals (Fig. 6B).

Discussion

We have presented evidence that, during aging, the status of mMSC changes with respect to both their intrinsic differentiation potential and production of signaling molecules that contribute to the formation of a specific marrow microenvironment necessary for maintenance of bone homeostasis. With aging, the number of mMSC committed to the adipocytic lineage increases, whereas the number of mMSC committed to the osteoblastic lineage decreases. Increased expression of the adipocyte-specific transcription factor PPAR-γ2 and increased production of its activator might be a driving force for pro-adipocytic and anti-osteoblastic changes in the differentiation potential of mMSC.

As osteoblasts and adipocytes originate from a common progenitor and PPAR-γ2 plays an important, although opposite, role in their differentiation, it is reasonable to hypothesize that with aging, differentiation toward adipocytes occurs at the expense of osteoblast differentiation. Similar age-related changes occur in another type of mesenchymal stem cell, muscle satellite cells (Taylor-Jones *et al*., 2002). With aging, satellite cells acquired adipocyte-like characteristics in part due to activation of PPAR-γ protein. Thus, it is possible that the common feature of mesenchymal stem cell aging is attaining an adipocytic phenotype. Whether this alteration has physiological reasons or it is just a default and/or by-product of the aging process remains unclear.

Production of an endogeneous PPAR-γ activator by adipocytes was reported previously, but the identity and nature of this activator has not yet been characterized (Kim *et al*., 1998). Natural ligands for PPAR-γ include polyunsaturated fatty acids and their oxidized forms, certain alkyl phospholipids, and derivatives of prostaglandin J2 (Kliewer *et al*., 1995,1997;Davies *et al*., 2001). We previously reported that oxidized derivatives of linoleic acid, which are found in oxidized LDL and whose levels increase with aging, effectively activate pro-adipocytic and anti-osteoblastic properties of PPAR-γ2 in U-33/γ2 cells (Lecka-Czernik *et al*., 2002). *In vivo*, a high-fat atherogenic diet, which increases levels of oxidized LDL, elicited significant bone loss in mice (Parhami *et al*., 2001). By contrast, mice deficient in 12/15-lipoxygenase production, an enzyme responsible for fatty acid oxidization, exhibit increased bone mass (Klein *et al*., 2004). These observations suggest that fatty acids and their oxidized derivatives may be good candidates for endogenously produced PPAR-γ2 activators.

Another interesting feature of aging marrow is the production of anti-osteoblastic activity that inhibits the mineralization process *in vitro*, a hallmark of osteoblast phenotype and function *in vivo*. This activity is different from the pro-adipocytic activity discussed above because it affects the osteoblastic phenotype regardless of the presence of PPAR-γ2. It is a matter of speculation whether such activity is produced *in vivo* and whether it affects the function of osteoblasts involved in bone formation at the bone remodelling sites.

Finally, we have demonstrated that aging changes the expression of components of the TGF-β/BMP signaling pathways in mMSC, suggesting changes in the cellular responses to these cytokines. Indeed, the negative effect on Smad7 gene expression, which is positively regulated by TGF-β signaling, suggests that the activity of the TGF-β pathway is decreased with aging. Changes in the expression of TGF-β cytokines and a decrease in the expression of a common type I receptor may contribute to the decrease in the activity of this signaling pathway. It has been demonstrated that TGF-β activity and availability is decreased in murine bone during aging (Gazit *et al*., 1998). Consistent with TGF-β stimulating osteoblast proliferation and inhibiting adipocyte differentiation, a decrease in TGF-β activity with aging would lead to a

decrease in the formation of new osteoblasts and an increase in the formation of new adipocytes. These are the features of mMSC aging that we have reported here.

Similarly, decreased expression of Smad6, which is positively regulated by BMP2/4 cytokines, indicates decreased activity of BMP2/4 signaling. Although BMP2/4 cytokines are necessary for bone formation and osteoblast maturation, they also synergize with signals promoting adipocyte differentiation (Sottile & Seuwen, 2000;Hata *et al*., 2003;Tang *et al*., 2004). BMP2/4 cytokines communicate with cells through two type I receptors, BMPR-1A and -1B, that form a complex with a common type II receptor. Studies in pre-osteoblastic 2T3 cells suggest that BMPR-1A controls adipocytic differentiation, whereas BMPR-1B controls osteoblastic differentiation (Chen *et al*., 1998). According to this model, our findings that aging increases the expression of BMP4 cytokine that commits stem cells to adipocyte lineage (Tang *et al*., 2004), and decreases the expression of BMPR-1B, a receptor which conveys osteoblast-specific signaling (Chen *et al*., 1998), are particularly interesting. These results suggest a switch with aging in the activity of the BMP signal transduction pathway from pro-osteoblastic to pro-adipocytic. This possibility needs further investigation.

At the dawn of development of new medical therapies that employ adult stem cell transplants to cure, repair or even grow a new organ, it is necessary to gain a better understanding of their biology and changes that occur in these cells with aging. We have shown that mesenchymal marrow stroma/stem cells undergo age-related changes. Therefore, the therapeutic potential of adult and aged mMSC differs and should be taken into account whenever these cells are considered for therapies against osteoporosis.

Experimental procedures

Animals

Adult (6–8-month-old) and old (20–26-month-old) C57BL/6 mice were obtained from the colony maintained by the National Institute of Aging under contractual agreement with Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). Animals were housed with free access to water and were maintained at a constant temperature on a 12-h light–dark cycle. The animal treatment and care protocols conformed to National Institute of Health guidelines and were performed using protocols approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee.

BMD measurements and bone histomorphometry

BMD was measured on anaesthetized animals using the small animal dual energy X-ray absorptiometry (DXA) instrument and software V1.46 (GE Lunar, Madison, WI, USA) (Rzonca *et al*., 2004). Internal variations in repeated measures of total murine body BMD have been determined to be 1.7–2.0%.

For bone histomorphometry measurements, undecalcified tibiae were embedded in methyl methacrylate and sectioned on an automatic, retractable Microtom 355 with a D-profile, tungsten carbide steel knife at 4 μm. Adjacent sections were stained with Masson Trichrome and Von Kossa (Jilka *et al*., 1996). The histomorphometric examination was performed using an OsteoMeasure system, which includes a Nikon microscope with motorized stage, interfaced with a computer and digitizer tablet (OsteoMetrics Inc., Atlanta, GA, USA). All cancellous measurements were two-dimensional, confined to the secondary spongiosa, and made using a 40× objective lens (numerical aperture 0.75). The terminology and units used were those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (Parfitt *et al*., 1987). Measurements were performed on six representative fields per bone sample.

Murine primary bone marrow cultures

Bone marrow cultures were established from femur marrow aspirates as previously described (Kajkenova *et al*., 1997) and maintained in basal medium consisting of α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 0.25 μg mL^{-1} amphotericin at 37 °C in a humidified atmosphere containing 5% CO_2.

Differentiation and assessment of adipocyte and osteoblast cultures

For differentiation assays, bone marrow isolates from individual mice ($n = 8$) were seeded separately in triplicate at a density of 2.5×10^5 cells cm^{-2} on six-well plates in basal medium. One-half of the medium was changed every 6 days.

To stimulate adipogenesis, after 10 days of growth, cultures were exposed for the next 3 days to IHI medium [0.5 mM iso-butylmethylxanthine (IBMX), 60 μM indomethacin, and 0.5 μM hydrocortisone (Sigma Chemical Co., St. Louis, MO, USA)]. The medium was then changed to basal medium and cells were maintained in culture for a further 3 days (Dorheim *et al*., 1993). Alternatively, after 10 days of growth in basal medium, cultures were maintained for 3 days in medium supplemented with 1 μM rosiglitazone (Tularik Inc., South San Francisco, CA, USA). Fat-containing cells were visualized with Oil Red O staining and adipogenesis was quantified by enumerating colonies containing at least 10% Oil Red O-positive cells (Lecka-Czernik *et al*., 2002).

To stimulate osteoblastogenesis, cells were maintained in osteoblastic medium (basal medium supplemented with 50 μg mL^{-1} ascorbic acid and 10 mM β-glycerophosphate) for 28 days. Mineralization was determined by Von Kossa staining.

Collection and testing activities of conditioned media

Primary bone marrow cultures (three independent bone marrow isolates per age group) were established at a density of 0.5×10^6 cells cm^{-2} and allowed to grow for 6 days with no media change, and then one-half of the media was replaced with fresh media every 2 days for the next 10 days. At these 2-day intervals, conditioned media were collected and frozen at −70 °C.

Murine marrow-derived UAMS-33 cells stably transfected with PPAR-γ2, referred to as U-33/γ2 cells, and UAMS-33 transfected with an empty vector, referred to as U-33/c cells, have been previously described (Lecka-Czernik *et al*., 1999). Cultures of U-33/γ2 and U-33/c cells were established in α-MEM supplemented with 10% heat-inactivated FBS and 30 mg mL^{-1} G418. When cultures were 70% confluent, cells were fed every 2 days for 6 days with conditioned media from 16-day bone marrow cultures that were preselected for their pro-adipocytic activity. At the end of the experiment, U-33/γ2 and U-33/c cells were stained for fat with Oil Red O, and fat cells were counted. As a positive control for adipocyte formation, cells were grown in the presence of 5 μM rosiglitazone for the same period of time as above cultures.

To assess the effects of bone marrow-conditioned media on the osteoblastic phenotype, U-33/γ2 and U-33/c cells were grown in the conditioned media collected from 16th-day primary bone marrow cultures; these conditioned media were freshly supplemented with pro-osteoblastic components (50 μg mL^{-1} ascorbic acid and 10 mM β-glycerophosphate). As a control for mineralization, U-33/γ2 and U-33/c cells were cultured in non-conditioned media, referred to as naïve, supplemented with 15% FBS, ascorbic acid and β-glycerophosphate. After 6 days of treatment, calcium content was measured using a calcium binding assay (Sigma Chemical Co.) as previously described (Lecka-Czernik *et al*., 2002).

Gene expression analysis using quantitative real-time RT-PCR

For RNA isolation, primary bone marrow cultures were established from pooled groups of marrow isolates in basal medium. Cells were plated at a density of 2×10^5 cells cm^{-2} on 100-mm plates. After 10 days of growth, RNA was isolated using RNAeasy (Qiagen, Valencia, CA, USA) and was subjected to DNase I digestion. Gene-specific primer sequences were selected using the Taqman Probe and Primer Design function of the Primer Express v1.5 software (Applied Biosystems, Foster City, CA, USA) and are listed in Table 3. Reverse transcription reactions were carried out using 2 μg RNA and TaqMan Reverse Transcription Reagents (Applied Biosystems), followed by real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems) and ABI Prism 7700 Sequence Detection System (Applied Biosystems). Reactions were performed in the following cycling conditions: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min, with the exception for PPAR-γ2, which was performed in the following conditions: 95 °C for 10 min, then 40 cycles of 94 °C for 15 s, 53 °C for 15 s, and 72 °C for 20 s. Concentrations of primers and templates used in each reaction were optimized based on the standard curve created prior to the reaction and corresponding to nearly 100% efficiency of the reaction. Results were then normalized to expression of 18S rRNA in the same sample.

Statistical analysis

Statistically significant differences between groups were detected using one-way ANOVA followed by *post-hoc* analysis by Student–Neuman–Keuls within the SigmaStat software (SPSS, Inc., Chicago, IL, USA) after establishing the homogeneity of variances and normal distribution of data. In all cases, $P < 0.05$ was considered significant.

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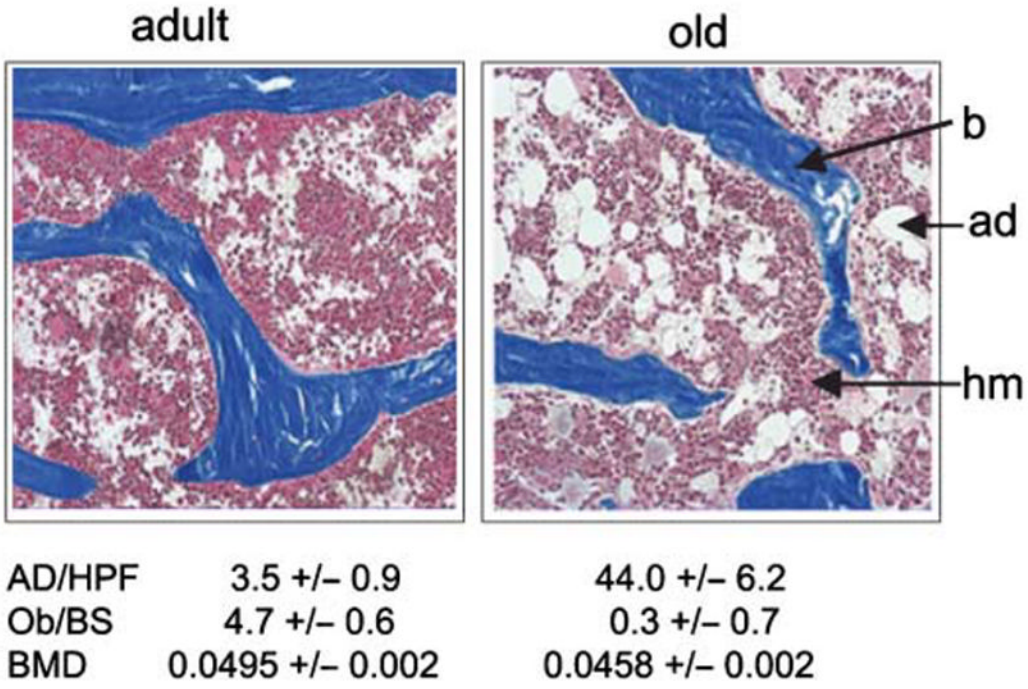

Fig 1.

Histological examination of proximal tibiae sections of 8-month- and 26-month-old C57BL/6 mice. Vertical sections of undecalcified tibiae specimens were stained with Masson Trichrome, and images were taken at 4× magnification. The number of osteoblasts at the osteoid sites and the number of adipocytes were determined in six randomly chosen microscopic fields per specimen and calculated as the average of eight animals per group (± SD). b, bone: ad, adipocytes; hm, haematopoietic marrow; AD/HPF, number of adipocytes per field at 20× magnification; Ob/BS, number of osteoblasts per osteoid; BMD, bone mineral density.

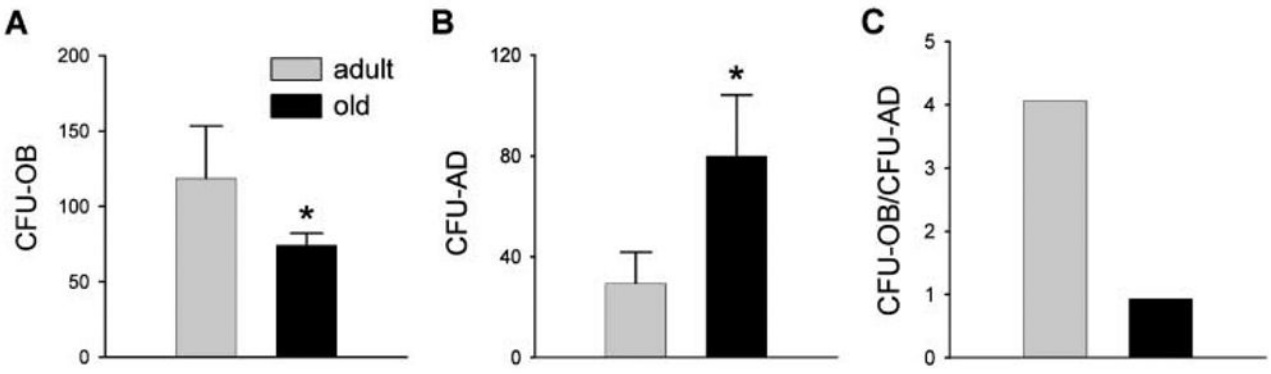

Fig 2.

Formation of osteoblastic (CFU-OB) and adipocytic (CFU-AD) colonies in primary bone marrow cultures. Primary bone marrow cell cultures were prepared from 8-month- and 26-month-old C57BL/6 mice. Cells from each animal were cultured separately and examined for the formation of: (A) colonies containing mineralized bone nodules (CFU-OB), which developed in osteoblastic medium; (B) colonies containing fat laden cells (CFU-AD), which developed in adipogenic medium; and (C) ratio of CFU-OB to CFU-AD formation. Each bar represents the average from eight animals; the number of colonies was calculated per 2.5×10^6 plated cells. Error bars indicate SD. Shaded bars, 8-month-old; black bars, 26-month-old animals. $*P < 0.05$.

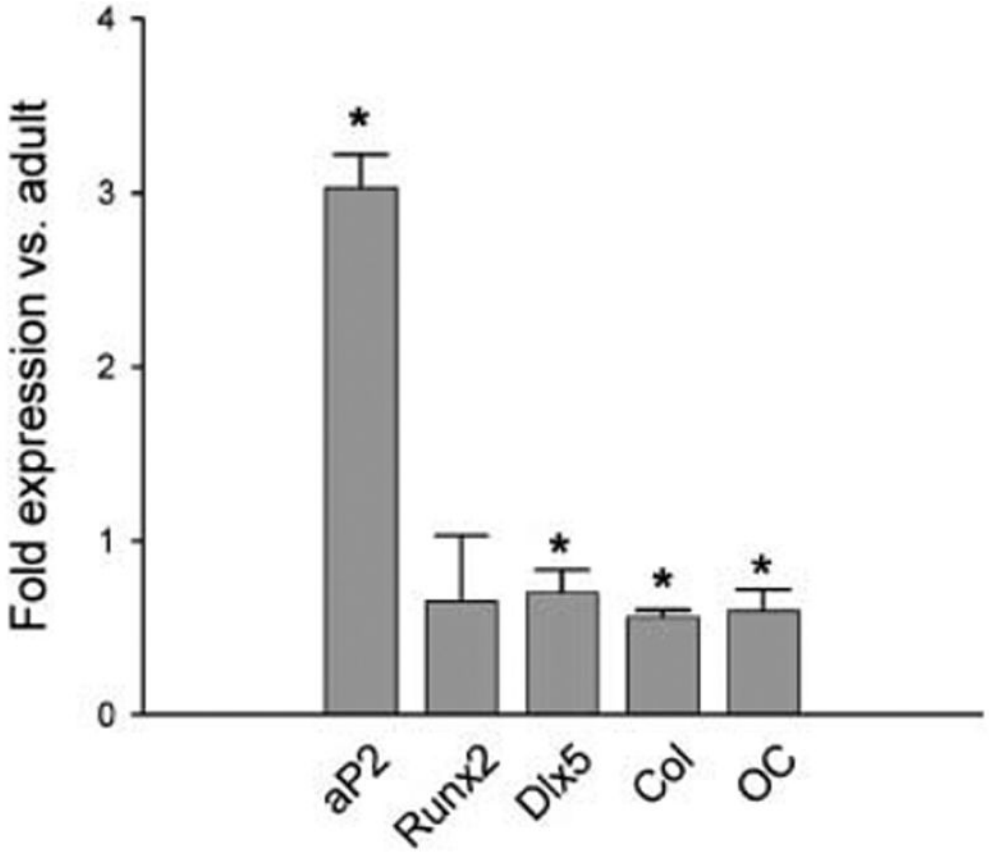

Fig 3.

Gene expression of adipocyte and osteoblast markers in mMSC derived from 6-month- and 20-month-old mice. Gene expression was determined using quantitative real-time RT-PCR as described in Experimental procedures. Values were normalized to the expression of GAPDH, and expression in old marrow (20-month-old) is presented as the fold increase or decrease of that in adult marrow (6-month-old). Error bars represent SD. Col, collagen; OC, osteocalcin. *$P < 0.05$.

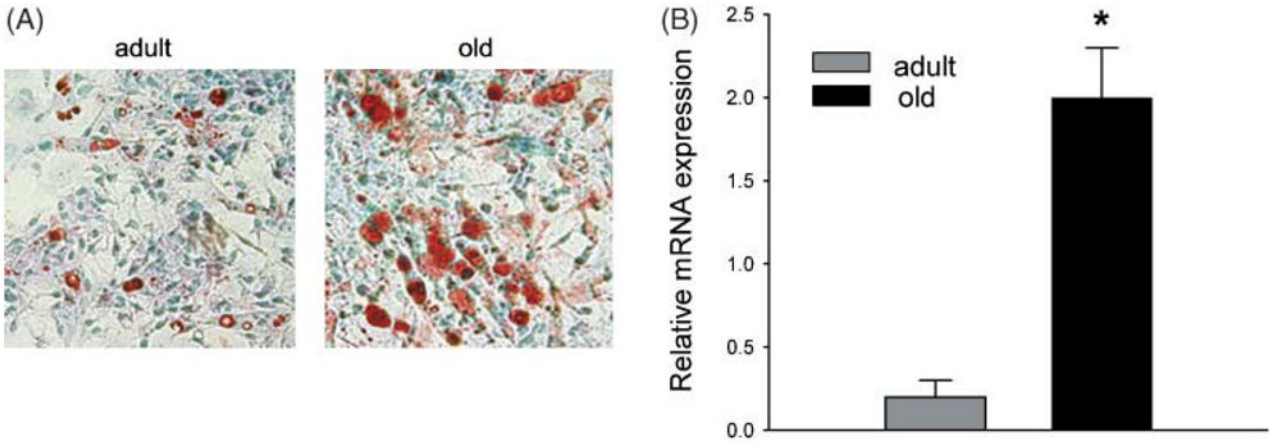

Fig 4.

Effects of aging on mMSC sensitivity to rosiglitazone and expression of PPAR-γ2 transcription factor. (A) Photomicrographs (40× magnification) show a response to 5 μM rosiglitazone treatment of mMSC derived from adult (6-month-old) and old (20-month-old) mice. Cells stained with Oil Red O (red) for fat and counterstained with methyl green (blue). (B) Expression of PPAR-γ2 in mMSC derived from 6-month- and 20-month-old mice. mMSC were grown for 10 days in basal medium followed by RNA isolation and analysis of PPAR-γ2 expression using quantitative real-time RT-PCR. Values were normalized to the expression of 18S rRNA, and bars represent average expression from three independent experiments; error bars indicate SD. Shaded bars, 6-month-old; black bars, 20-month-old. *$P < 0.001$.

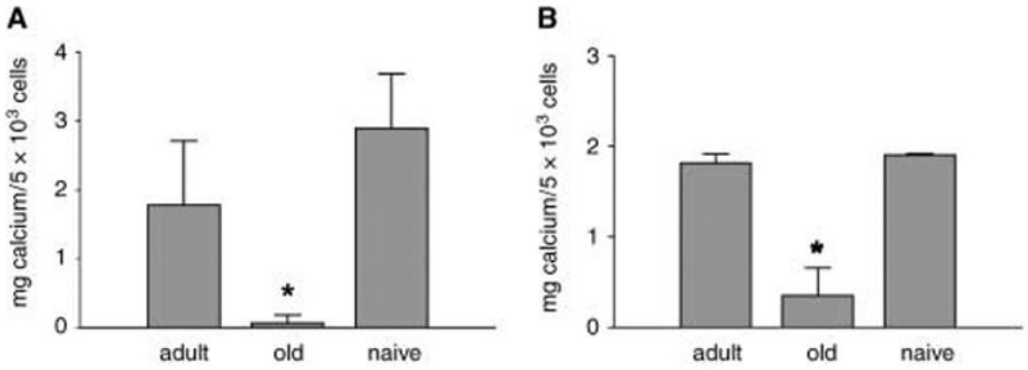

Fig 5.

Effects of bone marrow-conditioned media on osteoblast differentiation of U-33/γ2 (A) and U-33/c cells (B). Cell cultures of U-33/γ2 and U-33/c were fed with conditioned media collected from 16th-day primary bone marrow cultures from adult (6-month) and old (20-month) animals, or naïve (non-conditioned medium), freshly supplemented with ascorbic acid and β-glycerophosphate as described in Experimental procedures. After 6 days of treatment extracellular calcium content was measured. *$P < 0.05$.

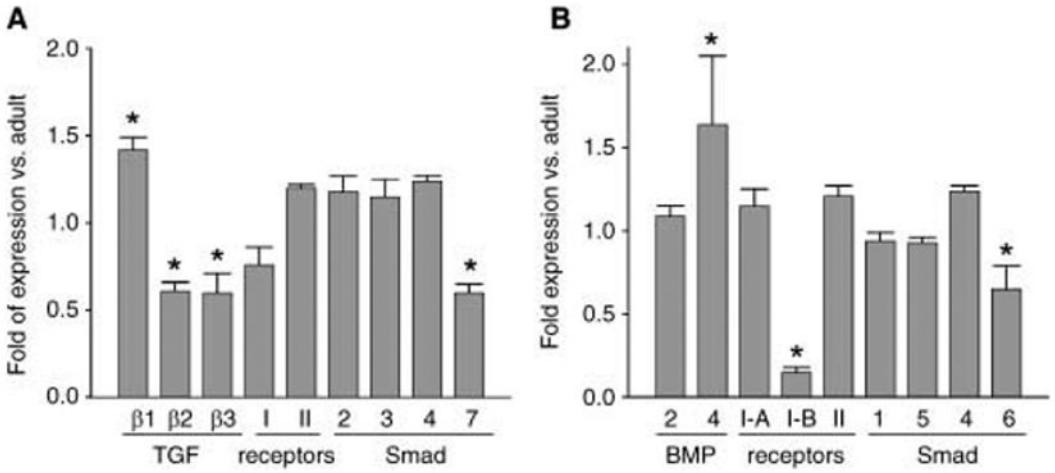

Fig 6.

Expression analysis of components of TGF-β (A) and BMP2/4 (B) signaling pathways in mMSCs derived from adult (6-month) and old (20-month) mice. Quantitative real-time RT-PCR was performed as described in Experimental procedures. Values were normalized to the expression of GAPDH, and expression in mMSC from old mice is presented as fold increase or decrease of that in adult animals. Error bars indicate SD. *$P < 0.001$.

Table 1

Potential of adult and old mMSC for spontaneous and rosiglitazone-stimulated CFU-AD development

Mice age (months)	Basal conditions (CFU-AD)‡	Rosiglitazone (CFU-AD)†‡
8	19.3 ± 13.1	485.7 ± 216.0
26	57.5 ± 28.6*	1131.7 ± 268.8*

† Culture medium supplemented with 1 μM rosiglitazone.

‡ Mean values of six individual cultures are presented per two femora bone marrow aspirates from a single mouse.

* $P < 0.05$ vs. 8-month-old animals.

Table 2

Effects of conditioned medium derived from bone marrow cultures of adult and old mice on fat accumulation in U-33/γ2 cells

Medium	ORO/total[†]	ORO (%)
Conditioned		
6-month	40.7 (10.3)/475.0 (44.6)	8.6
20-month	107.3 (35.5)/412.7 (30.9)[*]	26.0
Rosiglitazone (5 μM)	528.5 (136.5)/689.5 (207.2)	76.6

[†] Average number of cells calculated from five microscopic fields (20× magnification) ± SD (in parentheses).

ORO, cells positively stained for fat with Oil Red O.

[*] $P < 0.05$ vs. 8-month-old animals.

Table 3

Sequences of primers used for quantitative real-time RT-PCR

Gene	Accession no.	cDNA sequence (5′–3′) F, forward; R, reverse	Amplicon length (bases)	Corresponding cDNA sequence position
18S rRNA	X56974	F: TTCGAACGTCTGCCCTATCA	49	1535–1555
		R: ATGGTAGGCACGGCGACTA		1584–1566
aP2	NM_024406	F: GCGTGGAATTCGATGAAATCA	67	246–266
		R: CCCGCCATCTAGGGTTATGA		313–294
Osteocalcin	L24430	F: CGGCCCTGAGTCTGACAAA	208	1023–1041
		R: GCCGGAGTCTGTTCACTACCTT		1231–1210
Runx2/ Cbfal	NM_009820	F: GGGCACAAGTTCTATCTGGAAAA	54	169–191
		R: CGGTGTCACTGCGCTGAA		240–223
Dlx5	AF072453	F: TGACAGGAGTGTTTGACAGAAGAGT	64	184–208
		R: CGGGAACGGAGCTTGGA		248–232
α1(I) Collagen	NM_007742	F: ACTGTCCCAACCCCCAAAG	59	311–329
		R: CGTATTCTTCCGGGCAGAAA		370–351
PPAR-γ2	U09138	F: GCTGTTATGGGTGAAACTCTG	351	34–54
		R: ATAAGGTGGAGATGCAGGTTC		384–364
TGF-β1	NM_011577	F: TACAGCAAGGTCCTTGCCCT	62	1873−1892
		R: GCAGCACGGTGACGCC		1935−1920
TGF-β2	BC011055	F: CAACACCATAAATCCCGAAGC	66	682–702
		R: GGTCAGTGGTTCCAGATCCTG		748–728
TGF-β3	BC014690	F: GCAACTAGCTATCTCAGGTCCCTT	79	2221–2244
		R: CCAGGGAATACATGAGAGAACCA		2300–2278
TβR-1	NM_009370	F: AGCAGTGACTGCCATGCG	67	2337–2354
		R: CAGGCTAAACGTCTCAACTGCA		2404–2383
TβR-2	D32072	F: CATGTGAGAAGAATAAAATACGAGAACA	93	3615–3642
		R: AATGTGTAAGGGAAGTTGCCTATGT		3708–3684
BMP2	AY050249	F: AACTGGCTAGAATATTAAGCACTGCA	71	6082–6107
		R: AGTGATTTCCTAACTGCCCAGG		6153–6132
BMP4	BC052846	F: TCAAGGGAGTGGAGATTGGG	60	916–935
		R: GCCATCATGGCCAAAAGTG		976–958
BMPR-1 A	BC042611	F: TGCATCAAGACTCCAATCCTGA	83	2086–2107
		R: ACAGAAAGCACCACTTTATGGACA		2169–2146
BMPR-1B	BC065106	F: GCTGGGCGCAGAATCCT	73	1560–1576
		R: GGACTCTGACATTTTGGCAAGG		1633–1612
BMPRII	U78048	F: TCCACCTGGGTCATCTCCA	63	2975–2993
		R: CCCTGTCACTGCCATTGTTG		3038–3019
Smad1	BC058693	F: TCCGTCTCTTGCAAACTATCGA	75	1809−1830
		R: TTCGTCAGGTCTCCATCCTGT		1884−1864
Smad2	BC021342	F: CCCTTCAGTGCGATGCTCA	74	1504–1522
		R: GAATACTACGACGGAGGAGCTGTT		1578–1555
Smad3	NM_016769	F: CACGCAGAACGTGAACACC	100	485–503
		R: GGCAGTAGATAACGTGAGGGA		585–565
Smad4	BC046584	F: ACAGAGAACATTGGATGGACGA	69	662–683
		R: ACGGGCATAGATCACATGAGG		731–711
Smad5	BC050001	F: CAAGGGCCTTGCCTGCT	76	3469–3483
		R: GTCCGAGACCTATGACATGAAGACT		3545–3521
Smad6	BC047280	F: TGGCTGGAGATCCTACTCAACA	62	1753–1774
		R: GGACGCTGCGGCACAG		1815−1800
Smad7	NM_008543	F: GCCCCCCCTTCCTGCT	63	3103–3119
		R: CCAGCCAAGGGATGGTACC		3166–3148