Characterization of skeletal alterations in a model of prematurely aging mice

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Abstract An age-related bone loss occurs, apparently associated with the concomitant increase in an oxidative stress situation. However, the underlying mechanisms of age-related osteopenia are ill defined since these studies are time consuming and require the use of many animals (mainly rodents). Here, we aimed to characterize for the first time the bone status of prematurely aging mice (PAM), which exhibit an increased oxidative stress. Tibiae from adult (6 months) PAM show an increase in bone mineral density (BMD) and bone mineral content (assessed by bone densitometry) versus those in their normal counterparts (non-prematurely aging mice, NPAM) and similarly decreased in both kinds of mouse

with age. However, at this bone site, trabecular BMD (determined by μ -computerized tomography) was similar in both adult PAM and old (18 months) NPAM. Femurs from these groups of mice present an increase in oxidative stress, inflammation, osteoclastogenic, and adipogenic markers, but a decrease in the gene expression of osteoblastic differentiation markers and of the Wnt/ β -catenin pathway. Our findings show that adult PAM recapitulate various age-related bone features, and thus are a suitable model for premature bone senescence studies.

Keywords Aging · Osteoporosis · Oxidative stress · Mouse

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Introduction

Osteoporosis, defined as the loss of bone mass and deteriorated bone quality, is one of the most prevalent diseases in developed countries which leads to an increase in skeletal fragility (Reginster and Burlet 2006). It has been widely demonstrated that patients with osteoporosis have an increase in fracture risk (NIH Consensus Panel 1993; NIH Consensus Development Panel 2001). In fact, a high incidence of vertebral and femoral fractures related to osteoporosis occurs in humans over 35 years old (Cooper and Melton 1992). As a consequence of bone fracture, a reduction in motor skills ensues with concomitant progressive health deterioration. Aging itself is a factor that favors the development of osteopenia and accelerates osteoporosis onset. Accordingly, osteoporosis might be considered as one of the factors contributing to the age-associated development of frailty, defined as “a transient period before the onset of disability, and perhaps the most appropriate life period for prevention of such disability” (Runge and Hunter 2006).

There is compelling evidence that aging is associated with an increase in cellular oxidative stress (Harman 1956; Miquel et al. 1980) and consequently with higher levels of reactive oxygen species (ROS), which interfere with cell metabolism (De la Fuente and Miquel 2009). Moreover, oxidative stress interferes with osteoblastic, osteoclastic, and vascular differentiation, and thus, it has been proposed as a good candidate to explain bone loss occurring from the third decade of life, before sex hormone reduction (Garrett et al. 1990; Mody et al. 2001; Bai et al. 2004; Manolagas 2010). Among the defensive mechanisms against oxidative stress, forkhead box O (FoxO) transcription factor family is involved in the response to ROS by driving the transcription of several anti-oxidant genes like catalase and growth arrest and DNA damage 45 (Gadd45) (Kops et al. 2002; Tran et al. 2002). In bone cells, one important effect of FoxO activation is that this transcription factor binds β -catenin to promote the expression of anti-oxidant gene program but avoiding that β -catenin might drive the transcription of several genes implicated in osteoblastic function (Almeida et al. 2007a; Hooeboom et al. 2008). Other consequences of ROS increase include lipid peroxidation and phosphorylation of p66^{Shc} leading to an increase in osteoblast and osteocyte apoptosis (Trinei et al. 2002; Almeida et al. 2007a).

Taken together, all these effects contribute to the inability of osteoblasts to appropriately exert their function.

Animal models for studying the effects of aging on frailty, and the underlying cellular and molecular mechanisms, are scarce. A model of prematurely aging mice (PAM) in adult age has been proposed to study several molecular and physiological changes characteristic of premature senescence (Perez-Alvarez et al. 2005; Viveros et al. 2007). PAM are obtained from a regular colony of female ICR (CD-1) mice after submitting animals to a T-shaped maze. Mice that take longer to explore this maze, which represent a poor stress response to a new environment, also show higher anxiety levels than those performed faster in this behavior test, which are considered non-prematurely aging mice (NPAM). PAM also show a neurochemistry similar to that of aged mice, a premature immunosenescence and oxidative stress situation as well as a lower life span than NPAM (Viveros et al. 2001; Guayerbas et al. 2002). Nevertheless, currently, no data are available about the bone status of these mice. In the present study, we aimed to evaluate the gene and protein profiles as well as the structural parameters in the long bones of both PAM and their normal counterparts (NPAM) in a comparative manner and its response to age.

Materials and methods

Animals and sample extraction

Adult female mice (28 $\pm$ 2 week old) ICR/CD-1 were purchased from Harlan (Barcelona, Spain). The mice were pathogen free, as tested by Harlan according to the Federation of European Laboratory Science Associations' recommendations, and maintained (five animals/cage) in a temperature-controlled room (22 $\pm$ 2°C), with a 12-h light/dark reversed cycle. All mice were fed standard Sander Mus pellets (A04 diet, Panlab, Barcelona, Spain) and tap water ad libitum. Mice were treated according to guidelines of European Union directives (86/6091 ECC).

Animals were sorted out as PAM or NPAM as previously described (Guayerbas et al. 2002; Guayerbas and De La Fuente 2003). Briefly, mice at 29 $\pm$ 2 weeks of age were tested once a week for four consecutive weeks in a T-shaped maze. PAM were defined as those that complete the exploration in more than 10 s in all tests. These tests were performed between 9:00 a.m. and

11:00 a.m. to minimize circadian variations, under red light. Adult PAM (33 ± 2 weeks old), old PAM (72 ± 2 weeks old), and their NPAM counterparts ($n=6$ per age/mouse type) were weighed and then sacrificed, and femurs and tibiae were removed and cleaned of soft tissue. One femur and one tibia were snap-frozen and kept in liquid N_2 until total RNA and protein extraction, respectively. The remaining tibia and femur were maintained in neutral formaldehyde for posterior micro-computed tomography (μ CT) and histology evaluation, respectively. Non-fasting glucose was tested in blood drawn from the mouse tail using a glucometer (Glucocard G meter, A. Menarini Diagnostics, Firenze, Italy) (Lozano et al. 2009).

Bone densitometry

Bone mineral density (BMD), bone mineral content (BMC), and percent fat in whole body, vertebrae (L1–L4), femur, and tibia were determined in anesthetized mice by dual-energy X-ray absorptiometry using PIXImus (GE Lunar Corp., Madison, WI, USA). The PIXImus software calculates the aforementioned parameters with a coefficient of variation $\pm 2\%$, and data are recorded in Microsoft Excell files (Microsoft Corp., Redmond, WA, USA) (Lozano et al. 2009).

Bone histology

The femoral specimens were dehydrated in graded ethanols and embedded in methylmethacrylate. Seven micron-thick sagittal longitudinal sections of the femur were obtained with a rotation microtome for hard materials (Leica RM2255, Leica Microsystems, Nussloch, Germany) and were stained with Goldner's trichrome. The number of osteoclast and osteoblasts was evaluated in trabecular and endosteal bone compartments in a large area (2.8 mm^2) at the femoral metaphysis below the growth plate (Lozano et al. 2009, 2011; Nuche-Berenguer et al. 2011). The perimeter of endosteal and trabecular compartment in each femur was measured and used to normalize the number of osteoblasts and osteoclasts at each localization: number of trabecular osteoclasts/bone perimeter, number of endosteal osteoblasts/bone perimeter, and number of trabecular osteoblasts/bone perimeter. Histological evaluations were performed by at least two independent observers in a blinded fashion, using a light microscope with reticule-mounted eyepiece grid,

and were calculated from the corresponding mean score value obtained for each.

μ CT analysis

Mouse tibiae were scanned using the GE eXplore Locus μ CT scanner (GE Healthcare, London, Canada). X-ray tube settings were 80 kV of energy and 450 μ A of current. The μ CT image acquisition consisted of 400 projections collected in one full rotation of the gantry in 20 min. The resulting raw data were reconstructed using a filtered back-projection algorithm to a final image with a resolution of 93 μm in all three spatial dimensions. The reconstructed images were viewed and analyzed using MicroView software, version 2.2 with Advanced Bone Analysis + (GE Healthcare). Parameters given for trabecular compartment were: volumetric BMD (vBMD) and BMC, percent bone volume (BV) per total tissue volume (TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp), whereas those for cortical compartment were vBMD, BMC, and cortical area.

Real-time PCR

Total RNA was extracted from the bone samples with Trizol (Invitrogen, Groningen, The Netherlands). Synthesis of cDNA was performed using the high-capacity cDNA reverse transcription kit following manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed in an ABI PRISM 7500 system (Applied Biosystems) as described (Lozano et al. 2009). TaqMan MGB probes were obtained from Applied Biosystems (Assay-by-DesignSM) for gene amplification of runt-related transcription factor 2 (Runx2), osteocalcin (OC), parathyroid hormone-related peptide (PTHrP), dickkopf-related protein 1 (DKK1), cyclin 1, peroxisome-proliferator-activated receptor gamma (PPAR- γ) 2, adipocyte fatty acid-binding protein 4 (FABP-4), monocyte chemotactic protein-1 (MCP-1), tartrate-resistant acid phosphatase (TRAP), FoxO1, and catalase, using Premix ex Taq (Takara, Otsu, Japan). The mRNA copy numbers were calculated for each sample using the cycle threshold (Ct) value, and normalized against 18S rRNA, as reported previously (Livak and Schmittgen 2001), and results were expressed as n-fold mRNA values versus corresponding values in adult NPAM.

Western blot analysis

After crushing the tibia with a mortar in liquid nitrogen, total protein was extracted with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP-40, 1 mM dithiothreitol, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) (Bellido et al. 2011). Protein concentration was measured by BCA protein assay (Pierce, Rockford, IL, USA). Proteins (60 µg per lane) were loaded in 10–12% polyacrylamide-SDS gels under reducing conditions. Detection of β -catenin and sclerostin was performed with a rabbit polyclonal anti- β -catenin antibody (Abcam, Cambridge, UK), at 1:200 dilution, and a goat polyclonal anti-sclerostin antibody (R&D systems, Minneapolis, MN, USA), at 0.4 µg/ml, followed by incubation with an anti-rabbit or anti-goat IgG (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), at 1:2,000 dilution, as secondary antibody, respectively. Protein normalization was carried out with a mouse monoclonal α -tubulin antibody (Sigma-Aldrich, St. Louis, MO, USA), at 1:1,000 dilution, followed by incubation with goat anti-mouse IgG (Santa Cruz Biotechnology), at 1:5,000 dilution. ECL™ western blotting detection reagents (GE Healthcare) or Super Signal® West Dura reagents (Pierce) were used for developing, as necessary. Films were scanned and quantified with Image Jv1.41o (NIH, Bethesda, MD, USA).

Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical evaluation was carried out with nonparametric analysis of variance (Kruskal–Wallis) and a post hoc test (Dunn's) or Mann–Whitney test when appropriate. A value of $p<0.05$ was considered significant.

Results

Changes in body weight in PAM and NPAM with age

Body weight decreased only in old NPAM. Of note, this parameter was similar in both types of adult mice, but was significantly higher in aged PAM than in their NPAM counterpart (Table 1).

Age-related alterations of bone mass and structure in PAM and NPAM

We first assessed putative changes in BMD, BMC, and percent fat (in bone surrounding tissue) at different skeletal locations. In NPAM, we found an age-related decrease in BMD and BMC in total body and in the tibia as well as in percent fat at all of the bone sites evaluated (Table 1). A similarly consistent trend was observed in PAM, but the differences with age in these mice did not reach statistical significance (Table 1). Moreover, values for these parameters in the tibia were higher in adult PAM than in NPAM.

We next evaluated bone microarchitecture in the tibia of these mice by μ CT analysis. Regarding the trabecular compartment, as expected (Halloran et al. 2002), BV/TV, vBMD, BMC, and Tb.N significantly decreased, whereas Tb.Sp increased, without significant changes in Tb.Th, in NPAM with age. PAM showed a similar but non-significant pattern of changes with aging (Table 2). In fact, trabecular BV/TV and vBMD were lower in adult PMA than in NPAM. In addition, an increase of vBMD and BMC was observed in the cortical compartment of the tibia in old NPAM (but not in old PAM) compared to these adult mice, with no changes in Ct.Ar (Table 2).

Aging and oxidative stress have been shown to influence the number of osteoclasts and osteoblasts in bone (Almeida et al. 2007b; Jilka et al. 2010). Therefore, in the present study, we examined the number of these cells in the femur of all of the experimental groups of mice. In adult PAM, we found a significant increase in the number of osteoclasts lining trabeculae at the femoral metaphysis, compared to that in adult NPAM, but similar to that in old NPAM. On the other hand, a significant and similar decrease of the number of endosteal osteoblasts occurred in both adult PAM and old NPAM, compared to that in adult NPAM; adult PAM also showed a significant decrease of trabecular osteoblasts at the femoral metaphysis, compared to those in adult NPAM (Table 3).

Bone gene expression profiles in PAM and NPAM

We also examined the putative age-associated changes in the expression of genes related to bone remodeling and oxidative stress in the femur of both types of mice. We observed that mRNA levels of Runx2—an early

Table 1 Body weight and bone densitometry values of NPAM and PAM (BMD, BMC and percent fat)

Localization	Weight (g) Parameter	Adult NPAM 45.2±3.6 Adult NPAM	Adult PAM 47.4±1.7 Adult PAM	Old NPAM 38.6±2.1* Old NPAM	Old PAM 45.3±3.0*** Old PAM
Total body	BMD (g/cm ²)	0.064±0.001	0.067±0.001	0.059±0.0002**	0.063±0.001***
	BMC (g)	0.794±0.017	0.819±0.034	0.711±0.037**	0.731±0.01
	%Fat	28.00±0.82	27.7±1.30	20.53±0.97**	22.76±1.27
Vertebrae	BMD (g/cm ²)	0.082±0.005	0.087±0.006	0.088±0.004	0.089±0.0008
	BMC (g)	0.085±0.011	0.081±0.007	0.087±0.004	0.089±0.002
	%Fat	19.42±1.49	20.46±0.96	12.00±0.76**	13.17±0.75
Femur	BMD (g/cm ²)	0.091±0.003	0.098±0.002	0.10±0.003	0.0950±0.001
	BMC (g)	0.041±0.001	0.0436±0.001	0.042±0.0019	0.042±0.002
	%Fat	28.8±2.22	29.32±1.11	16.83±0.63**	21.15±1.71
Tibia	BMD (g/cm ²)	0.069±0.00007	0.077±0.0004*	0.061±0.001**	0.067±0.001
	BMC (g)	0.038±0.0006	0.042±0.0008*	0.033±0.0008**	0.03±0.001
	%Fat	24±2.29	25.8±0.75*	20.27±0.45**	22.00±1.53

Each value is the mean ± SEM ($n=6$ mice)

NPAM non-prematurely aging mice, PAM prematurely aging mice, BMD bone mineral density, BMC bone mineral content

* $p<0.05$

** $p<0.01$ with respect to the corresponding value in adult NPAM

*** $p<0.05$ with respect to the corresponding value in old NPAM

osteoblast differentiation marker (Banerjee et al. 1997; Ducy et al. 1997)—similarly decreased in aged PAM and NPAM (Fig. 1a). Furthermore, this age-related diminution also occurred for the gene expression of

OC, a late osteoblast differentiation marker (Pockwinse et al. 1992; Zhang et al. 2010). It has been demonstrated that PTHrP production—an important modulator of bone formation and remodeling in adult bone (Bisello

Table 2 Analysis of several bone structural parameters by μ CT in the tibia of adult and old NPAM and PAM

Compartment	Parameter	Adult NPAM	Adult PAM	Old NPAM	Old PAM
Trabecular	BV/TV (%)	25.1±0.8	21.7±0.5*	20.5±0.8**	19.7±1.8*
	vBMD (mg/cm ³)	68.0±4.7	36.6±6.6*	26.6±6.8*	23.2±5.8**
	BMC (mg)	2.45±0.15	2.19±0.34	0.9±0.21**	1.10±0.28**
	Tb. N (mm ⁻¹)	0.69±0.03	0.66±0.02	0.58±0.03*	0.57±0.02*
	Tb.Sp (mm)	1.07±0.06	1.14±0.05	1.35±0.08*	1.40±0.05*
	Tb.Th (mm)	0.37±0.02	0.36±0.01	0.35±0.01	0.36±0.01
Cortical	vBMD (mg/cm ³)	705±25	755±10	1116±137*	709±28
	BMC (mg)	0.116±0.004	0.12±0.007	0.17±0.02*	0.10±0.01
	Ct.Ar (mm ²)	1.93±0.06	2.14±0.07	2.14±0.09	1.986±0.19

Each value is the mean ± SEM ($n=6$ mice)

PAM prematurely aging mice, BV/TV bone volume/tissue volume (in percent), vBMD volumetric bone mineral density, BMC bone mineral content, Tb.N trabecular number, Tb.Sp trabecular separation, Tb.Th trabecular thickness, Ct. Ar cortical area, NPAM non-prematurely aging mice

* $p<0.05$

** $p<0.01$ with respect to the corresponding value in adult NPAM

Table 3 Femoral bone histology in the experimental groups of mice

	Adult NPAM	Adult PAM	Old NPAM	Old PAM
$n_{\text{T}}\text{OC/BPm}$ (cm^{-1})	0.8 ± 0.1	$7 \pm 2^*$	$6 \pm 1^*$	3 ± 3
$n_{\text{E}}\text{OB/BPm}$ (cm^{-1})	7.5 ± 0.9	$3.0 \pm 0.5^*$	$2.7 \pm 0.7^*$	3.4 ± 1.6
$n_{\text{T}}\text{OB/BPm}$ (cm^{-1})	9.5 ± 0.1	$6.7 \pm 0.7^*$	8.0 ± 0.8	7.5 ± 2.0

Each value is the mean $\pm$ SEM ($n=6$ mice)

$n_{\text{T}}\text{OC/BPm}$ number of trabecular osteoclasts/bone perimeter, $n_{\text{E}}\text{OB/BPm}$ number of endosteal osteoblasts/bone perimeter, $n_{\text{T}}\text{OB/BPm}$ number of trabecular osteoblasts/bone perimeter, *PAM* prematurely aging mice, *NPAM* non-prematurely aging mice

* $p < 0.05$ versus adult NPAM

et al. 2004)—decreases in human osteoblastic cells with the age of the donor (Martinez et al. 2002). Here, we found that PTHrP gene expression was significantly downregulated in both NPAM and PAM with aging. Of interest, this downregulation of PTHrP and OC was recapitulated in adult PAM (Fig. 1a).

DKK-1, an endogenous inhibitor of canonical Wnt/ β -catenin pathway (Fedi et al. 1999), and that of a final target gene of this pathway, cyclin D1 (Shtutman et al. 1999; Tetsu and McCormick 1999), also decreased with age in both types of old mice. Meanwhile, the decreased expression of the latter gene was already observed in adult PAM (Fig. 1b).

Both adipogenic genes, PPAR γ 2 and FABP-4, were upregulated in adult PAM, but only FABP-4 remained so in both kinds of aged mice (Fig. 1c). The pro-inflammatory gene MCP-1 and the osteoclast-related gene TRAP were both upregulated in adult PAM and further increased with age in both PAM and NPAM (Fig. 1d). A similar pattern of increase with age was evidenced for oxidative stress-related genes, FoxO1 and catalase, which were both yet upregulated in adult PAM (Fig. 1e).

Age-related changes in bone β -catenin protein and sclerostin levels in PAM and NPAM

Given that the activation of canonical Wnt pathway is a major mechanism to increase bone accrual (Glass et al. 2005; Khosla et al. 2008; Deschaseaux et al. 2009), we measured the protein levels of β -catenin, the main effector of this pathway, in the tibia of both types of aging mice. We found that these levels were significantly diminished in adult PAM, and further decreased to almost undetectable values in both types of aged mice (Fig. 2a). We also analyzed by western blotting

the protein expression of sclerostin, the product of Sost gene, which is another important inhibitor of canonical Wnt pathway (Winkler et al. 2003; Baron and Rawadi 2007). Sclerostin levels were found to be similar in adult PAM and NPAM, and showed an increase with age in both types of mice (Fig. 2b).

Discussion

Nowadays, there is an increasing demand of animal models that would allow us to characterize the molecular mechanisms of age-related osteoporosis. Mice present a continuous growth at the metaphysis; they do not exhibit estrogen depletion, and they lack Haversian system. Nevertheless, the mouse is a well-recognized model that generally mimics the human bone changes with aging (Bar-Shira-Maymon et al. 1989; Halloran et al. 2002). Bone structure deterioration has been described in the trabecular compartment of long bones and vertebra in aging rodents (Wang et al. 2001; Banu et al. 2002; Almeida et al. 2007b; Glatt et al. 2007). We focused here on the study of the long bone status in a mouse model of spontaneous premature aging. PAM are characterized by an impairment in the immune and neurological functions, and by an increased oxidative stress (Guayerbas et al. 2002; De la Fuente et al. 2003; Guayerbas and De La Fuente 2003; De la Fuente and Gimenez-Llort 2010), but no data about the status of bone in these mice were available. Although an age-related increase in body weight has been observed in females of the CD-1 mouse strain until 11 weeks (Harlan Laboratories, Inc, Indianapolis, IN, USA), and in female C57BL/6L mice (Halloran et al. 2002), as well as in several other mice strains as reflected in the Mouse

Fig. 1 Gene expression analysis in the femur of both adult and old NPAM and PAM. Total RNA was isolated from femoral homogenates and gene expression was determined by real-time PCR as described in the “Materials and methods” section. Expression of genes related to osteoblastic function, Runx2 (solid bars), OC (open bars), and PTHrP (gray bars) (a). Expression of genes related to Wnt-pathway, DKK-1 (solid bars) and cyclinD1 (open bars) (b). Expression of genes related to

adipogenic function, PPAR-γ2 (solid bars) and FABP-4 (open bars) (c). Expression of osteoclastic gene TRAP (solid bars) and inflammation marker MCP-1 (open bars) (d). Expression of genes related to oxidative stress status, FoxO1 (solid bars) and catalase (open bars) (e). Results are mean $\pm$ SEM of six measurements in duplicate. * $p < 0.05$, ** $p < 0.01$ versus the corresponding value in adult NPAM

Phenome Database (<http://phenome.jax.org>), a decrease in body weight was here observed in NPAM with age. This decrease was associated with a decrease in BMD and BMC in the total body and a reduction of percent fat in all of the skeletal sites examined. In this regard, we speculate that a lower physical activity in NPAM than in PAM with aging might explain at least in part these differences. In fact, PAM show a “freezing” behavior in

response to an unavoidable novel stimuli such as a T maze, generating a high level of anxiety which might subsequently increase the physical activity once back into the cage. Consistent with this hypothesis, in the tibia—that together with ulnae can support a high mechanical loading in mice—adult PAM showed higher bone mass than their NPAM controls. In this line, PAM might be better protected from age-related bone loss

Fig. 2 Analysis of Wnt pathway-related proteins in the tibia of NPAM and PAM of different ages. Total protein was isolated from femoral samples and analyzed by Western blot as described in the “Materials and methods” section. Total protein from four different mice in each experimental group was pooled (15 µg/mouse) for Western analysis of β-catenin (a) and sclerostin (b), detecting specific bands corresponding to an apparent molecular weight of 85 and 26 kD, respectively. Protein loading in each well was assessed by using α-tubulin. Representative autoradiograms are shown. Relative intensities of corresponding β-catenin and sclerostin signals to α-tubulin are indicated

than NPAM. Further studies evaluating and comparing the physical activity of these mice during aging are needed to confirm this hypothesis.

In spite of the fact that some mouse strains exhibit a continuous increase in BMD, bone microarchitecture has been shown to consistently deteriorate with age in mice (Halloran et al. 2002; Glatt et al. 2007). In the present study, old NPAM showed a decrease in vBMD, BMC, and Tb. N. and an increase in Tb. Sp. in the tibial trabecular compartment with respect to that in the adult animals. Moreover, a decrease in vBMD was already observed in adult PAM compared to their NPAM counterparts. This was not reflected in the corresponding densitometric BMD values. However, bone densitometry cannot sort out the trabecular and the cortical compartments, and we observed opposing BMD changes in these compartments in both types of adult mice by µCT, which might account for the different results using both techniques.

The observed changes in the gene expression of several bone remodeling markers also indicate a decreased osteoblastic function and an increased osteoclastic function in the mouse femur with age. Furthermore, the onset of these changes occurred earlier in PAM than in NPAM. An age-related decrease in PTHrP mRNA levels in the

mouse long bones is consistent with previous findings in human osteoblastic cells derived from hip and knee of elderly donors (Martinez et al. 2002). It has previously been reported that a decrease in Wnt pathway activation occurs with aging (Almeida et al. 2007a). Our findings here agree with this concept and demonstrate that age-related changes in some components of this pathway, namely, β-catenin and cyclin D1 expression, in the long bones of NPAM are recapitulated in adult PAM. Of interest, the upregulation of DKK1 or sclerostin, two major Wnt pathway inhibitors, are unlikely to account for β-catenin reduction in the long bones of adult PAM, since their gene or protein expression levels, respectively, were similar to those in age-matched NPAM controls. However, the observed decrease in β-catenin in both groups of old mice might be related, at least in part, to the increased sclerostin, an important modulator of the canonical Wnt pathway (Winkler et al. 2003; Baron and Rawadi 2007). Nevertheless, a destabilization of β-catenin has been shown to occur related to an increased adipogenesis (Ross et al. 2000). In this regard, the femur of adult PAM showed an upregulated gene expression of PPAR-γ2 and FABP-4, two markers of adipogenic differentiation (Wu et al. 1999; Urs et al. 2006).

PAM have previously been shown to have an increase of serum corticosterone (Perez-Alvarez et al. 2005), that might contribute to the bone alterations as observed here in these mice (Clowes et al. 2001; de Castro et al. 2010). In addition, PAM was earlier characterized as a model of high oxidative stress in leukocytes (Alvarado et al. 2006) and the neurological system (Viveros et al. 2007). In the present study, we demonstrate that the gene expression levels of both FoxO1 and catalase, a FoxO1 target gene (Kops et al. 2002), were increased in the femur of adult PAM. Therefore, FoxO1 may divert β-catenin from inducing transcription of genes implicated in osteoblast differentiation program to anti-oxidative stress gene transcription (Hoogeboom et al. 2008). Oxidative stress is also linked to an increase of inflammation because ROS produces various inflammation-related factors (Kulinsky 2007). In fact, adult PAM showed upregulation of both MCP-1, a well known pro-inflammatory chemokine which likely recruits osteoclast precursors to bone remodeling areas (Deshmane et al. 2009), and TRAP, an osteoclast differentiation maker. Consistent with these findings, adult PAM showed an increment in the number of trabecular osteoclasts in the femoral metaphysis. This was accompanied by a concomitant

decrease in the number of trabecular osteoblasts in the femur of these mice, suggesting a predominance of bone resorption in these animals. In any event, this pro-inflammation status in long bones of adult PAM recapitulates that previously observed in aging bone (Garrett et al. 1990; Cao et al. 2005).

Taken together, these data demonstrate that adult PAM display bony features mimicking those observed in aged mice of the same strain, making them a suitable model for bone senescence studies. We found that adult PAM exhibit an increase in oxidative stress possibly related to an altered bone remodeling. Moreover, the use of different kinds of antioxidants has been suggested to be a good alternative for preventing the age-related oxidative stress (De la Fuente and Miquel 2009; De la Fuente 2010) and the deleterious effects of oxidation in musculoskeletal system (Runge and Hunter 2006; Manolagas 2010). Thus, the present results indicate that adult PAM model can be a good candidate to proof the efficacy of antioxidant strategies to prevent age-related bone deterioration.

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