Increased Bone Resorption and Osteopenia in *Dlx5* Heterozygous Mice

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ABSTRACT

Distal-less (Dlx) homeobox transcription factors play a central role in the control of osteogenesis. In particular, Dlx5 regulates osteoblasts/osteoclasts coupling during perinatal bone formation. We analyze here the effect of *Dlx5* allelic reduction in the control of bone remodeling. We first show that *Dlx5* expression persists during postnatal bone development. We then compare the skeletal phenotype of 10- and 20-week-old $Dlx5^{+/-}$ mice to that of wild-type (WT) littermates. $Dlx5^{+/-}$ male mice exhibit lower bone mineral density (BMD) at both ages while only 20-week-old females are affected. μCT analyses reveal a reduction in cortical thickness of femoral midshafts in $Dlx5^{+/-}$ mice. Histomorphometry on distal femora shows no changes in trabecular structure and confirms a reduction in $Dlx5^{+/-}$ cortical thickness. The cortical decrease of 10-week-old mice does not derive from a reduction in periosteal bone apposition, but results from increased bone resorption with a significantly higher number of endosteal osteoclasts per bone surface and a larger marrow diameter. Urinary level of deoxypyridinoline is also higher in heterozygous mice confirming an increase in bone resorption activity. Our findings might be relevant for understanding complex, multifactorial diseases such as osteoporosis in which quantitative deregulation of gene expression leads to disruption of bone homeostasis. J. Cell. Biochem. 107: 865–872, 2009. © 2009 Wiley-Liss, Inc.

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Bone development and remodeling are highly integrated processes essential to assure the morphogenesis of skeletal elements, as well as their harmonious growth, maintenance, and repair during the whole lifespan of an individual. These processes depend on the concerted action of different cell types, which assure bone apposition (osteoblasts) and resorption (osteoclasts). The communication between these different cellular compartments is assured by soluble molecular signals, which activate transcriptional regulatory networks.

Gene inactivation studies have shown that Distal-less (Dlx) homeobox transcription factors play an important role in regulating the proliferation and differentiation of the major cell types which constitute bone: chondrocytes, osteoblasts, and osteoclasts. Vertebrate *Dlx* genes are transcription factors which share a highly conserved homeodomain, homologous to that of *Drosophila Distal-less* (*Dll*). The mammalian *Dlx* gene family is formed by three bigenic clusters: *Dlx1* and *Dlx2*; *Dlx5* and *Dlx6*; and *Dlx3* and *Dlx4* [Cohen et al., 1989; O'Hara et al., 1993; Panganiban and Rubenstein, 2002]. All *Dlx* genes might play a role in chondrogenesis and/or osteogenesis [Samee et al., 2007]. In particular, Dlx5 is a homeodomain transcription factor expressed in osteoblasts from very early stages of bone development [Zhao et al., 1994]. Long bones of $Dlx5^{-/-}$ mutant mice present a narrower hypertrophic zone [Bendall et al., 2003] and a defective trabecular component [Acampora et al., 1999; Samee et al., 2008]. The calvaria of the *Dlx5* mutants display delayed ossification resulting in open fontanellae [Acampora et al., 1999]. In vitro studies have shown that *Runx2*, a key regulator of osteogenesis [Komori et al., 1997; Hassan et al., 2004], contains multiple *Dlx5* responsive elements in the Runx2 P1 promoter [Lee et al., 2005; Hassan et al., 2006] and many early (Osterix) and late markers (Bone

Sialoprotein (BSP) and osteocalcin) of osteoblastic differentiation are potential indirect/direct targets of *Dlx5* [Benson et al., 2000; Lee et al., 2003; Samee et al., 2008; Ulsamer et al., 2008].

Most functional studies on skeletal regulatory factors have been performed during embryonic development as early lethality of null mutant mice has often hampered the elucidation of their postnatal role. However, emerging findings from studies using bone-specific conditional strategies or heterozygous mice indicate that several of the factors involved in early osteogenesis play also important roles during postnatal modeling and remodeling of the skeleton [Ducy et al., 1999; Orestes-Cardoso et al., 2001; Nissen-Meyer et al., 2007]. Our previous studies have shown that *Dlx5* deficiency in vivo affects bone formation and leads to bone defects in mouse embryo. $Dlx5^{-/-}$ mice at birth have a significant decrease in bone volume and their primary osteoblasts display reduced proliferation and differentiation capacity with a higher potential to induce osteoclastogenesis [Samee et al., 2008]. However, as *Dlx5*-null mice exhibit perinatal lethality, the function of Dlx5 in bone modeling/remodeling remains still unexplored. Indeed, several indirect results have supported the notion that Dlx5 may be also implicated in the regulation of adult bone homeostasis: (1) *Dlx5* is expressed by mature osteoblasts and osteocytes in vitro [Li et al., 2008]. (2) During fracture repair, *Dlx5* is strongly expressed in the regenerating blastema [Miyama et al., 1999]. (3) Five-day-old mice infected with retroviral vectors expressing wild-type (WT) *Dlx5* exhibit an enhanced mineralization due to an increase in the expression level of BSP and osteopontin [Zhang et al., 2008]. (4) The overexpression of Dlx5 in mesenchymal stem cells leads to reduced bone differentiation [Muraglia et al., 2008]. However, despite these, often contradictory, results no direct evidence has so far demonstrated a direct role for Dlx5 in the maintenance of adult bone integrity.

Here, we analyze the effects of allelic reduction of *Dlx5* on adult bone phenotype. Our results demonstrate that $Dlx5^{+/-}$ mice have a significant decrease in femoral bone mineral density (BMD) exclusively due to a decrease in cortical thickness. We bring direct evidence showing that *Dlx5* allelic reduction results in an increase in bone resorption. These findings suggest that Dlx5 has a role in maintaining osteoblast/osteoclast coupling throughout lifespan and makes this gene a candidate target for tackling human bone disease.

MATERIALS AND METHODS

ANIMALS

Mice with targeted disruption of *Dlx5* have been previously described [Acampora et al., 1999]. In these mice, the first and second exons of *Dlx5* are replaced by the *lacZ* reporter. PCR genotyping and β-galactosidase staining were performed as described [Acampora et al., 1999], and the gender of newborn mice was determined by PCR detection of the chromosome Y *sry gene.* Ten- and 20-week-old heterozygous mice and their WT littermates were used for the experiment and they were allowed free access to food and water in full compliance with the French government animal welfare policy. The animals were sacrificed by cervical elongation. To evaluate the dynamic bone formation parameters by histomorphometry, the mice were given two fluorochrome markers by intraperitoneal injection; 20 mg/kg oxytetracycline (Pfizer, Amboise, France) and 20 mg/kg calcein (Sigma–Aldrich, St. Louis, MO), respectively, at an interval of 2 days for the 10-week-old mice and 4 days for the 20-week-old mice.

MEASUREMENT OF BONE MINERAL DENSITY BY DUAL–ENERGY X–RAY ABSORPTIOMETRY (DXA)

Dual-energy X-ray absorptiometry (DXA) analysis was carried out under anesthesia. Total body, whole femur, and caudal vertebral bone mineral content (BMC, mg), bone area (area, cm^2), and BMD (mg/cm^2) were measured using a PIXImus Instrument (Lunar, France; Software version 1.44) in ultrahigh resolution mode (resolution 0.18 mm × 0.18 mm). The precision and reproducibility of the instruments had previously been evaluated by calculating the coefficient of variation of repeated DXA measurements. The coefficient of variation was <2% for all the parameters evaluated. A phantom was scanned daily to monitor the stability of the measurements.

HISTOMORPHOMETRY AND MICROCOMPUTED TOMOGRAPHY ANALYSIS

The left femur from each animal was excised at death, and the surrounding soft tissue was cleaned off. After storage in 70% ethanol at 4°C, femurs were trimmed and the distal halves of bones were postfixed in 70% ethanol, dehydrated in xylene at 4°C, and embedded without demineralization in methyl methacrylate. Histomorphometric parameters were measured in accordance to the ASBMR nomenclature [Parfitt et al., 1987; Parfitt, 1988] on 5 μm sections using a Nikon microscope interfaced with the software package Microvision Instruments (Evry, France). Sections were stained with aniline blue. For tartrate-resistant acid phosphatase (TRAP) detection, sections were stained with 50 mM sodium tartrate and naphthol ASTR phosphate (Sigma, St Louis, France). Two 10-μm thick sections were taken for measuring the dynamic parameters. The measurements of the trabecular bone were performed in a region of the secondary spongiosa. In particular, we measured the trabecular bone volume (BV/TV), the trabecular bone thickness (Tb.Th), and the trabecular separation (Tb.Sp). For cortical bone, we measured the average bone and marrow diameters at the femoral metaphysis and calculated the cortical thickness (Cort.Th). Dynamic parameters were determined on unstained sections examined under UV light.

The other femurs from 10- and 20-week-old male heterozygous mice and gender- and age-matched control littermates were used for three-dimensional (3D) microcomputed tomographic (μCT) analyses of the cortical thickness at the femoral midshaft.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed in Tris-buffered saline (Tris 50 mM, pH 7.6, NaCl 150 mM), using standard protocols. Deparaffinized sections were incubated overnight at 4°C with the primary antibody (rabbit polyclonal anti-β-galactosidase) and revealed with Anti-Rabbit Ig (peroxidase) and DAB (ImmPRESS™; Vector Laboratories). Negative controls included sections without the primary antibody or with irrelevant antibodies.

RNA EXTRACTION AND REAL-TIME QUANTITATIVE PCR (qPCR)

For the preparation of RNA from long bones, the soft tissues surrounding the bones were stripped off. The epiphyses were cut off and the bone marrow flushed out with PBS. Total RNAs were isolated using Trizol reagents (Invitrogen, Carlsbad, CA) and cleaned using an RNeasy cleanup kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and then reverse transcribed into cDNA using the Reverse-iT Max Blend (ABgene, Surrey, UK). Quantitative real-time PCR expression analysis was performed on Lightcycler1.5 (Roche Diagnostics) using Absolute® SYBR Green capillary mix (ABgene) at 56°C for 40 cycles. Primers product was designed from the online mouse library probes of Roche Diagnostics. mRNA levels were normalized by using either *Aldolase A* or *18S* as housekeeping genes.

STATISTICAL SIGNIFICANCE

Results are expressed as mean ± standard error (SEM). Statistical analysis was carried out by Statview analysis program using two-way ANOVA to compare differences between genotypes. P-values less than 0.05 were considered to be significant.

RESULTS

DYNAMICS OF *Dlx5* EXPRESSION IN POSTNATAL BONE

We determined, by qPCR, the expression levels of *Dlx5* in femurs of WT mice at different (1, 4, 6, 10, and 20 weeks) postnatal time points. *Dlx5* expression levels were normalized (*Aldolase A* and *18S*) and the arbitrary values so obtained were reported to that of 1-week-old male mice. As indicated in Figure 1, *Dlx5* transcripts were present at all time points examined, but their level of expression decreased significantly during bone maturation; this reduction was observed both in males and females. Comparing 1-week-old to 20-week-old mice, a fourfold and a sevenfold reduction was noted in males and in females, respectively. In addition, the transcript level of *Dlx5* in
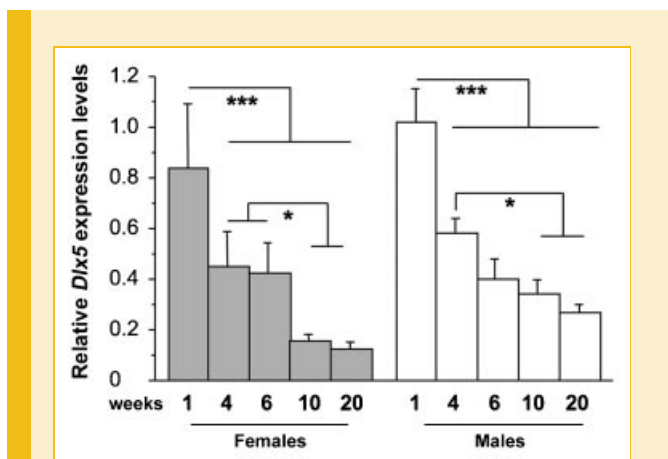

Fig. 1. Dynamics of Dlx5 expression in postnatal bone. Relative expression levels of *Dlx5* in femurs of 1-, 4-, 6-, 10-, and 20-week-old female (gray bars) and male (open bars) wild-type mice. Results are presented as mean ± SEM normalized to the expression level of 1-week-old male mice. *$P < 0.05$ and ***$P < 0.001$; n = 4–5 mice per gender at each time point.

10- and 20-week-old males was significantly higher when compared to females at the same time points (10-week-old male: 0.337 ± 0.06 vs. 10-week-old female: 0.151 ± 0.02; $P < 0.05$; n = 4/gender and 20-week-old male: 0.265 ± 0.03 vs. 20-week-old female: 0.120 ± 0.02; $P < 0.01$; n = 5/gender). A similar diminution of the levels of expression of *Dlx5* during bone maturation was observed in *Dlx5*$^{+/-}$ mutant mice (data not shown).

During embryogenesis, *Dlx5* is expressed by all developing cartilages and bones [see, for example, Zhao et al., 1994; Acampora et al., 1999; Robledo et al., 2002; Samee et al., 2008] prevalently in pre-osteoblasts and osteoblasts. So far, however, *Dlx5* expression has not been examined in adult bones. We have shown that the pattern of β-galactosidase expression in heterozygous *Dlx5*^{lacZ} mice recapitulates faithfully that of *Dlx5* in WT mice in all analyzed tissues. Immunohistochemical staining against β-galactosidase on whole femurs sagittal sections of 10-week-old mice *Dlx5*^{lacZ} (Fig. 2) showed that *Dlx5* is expressed mainly by cells on the endosteal and periosteal surfaces of the cortical bone, most likely pre-osteoblasts and osteoblasts, in cells surrounding the trabeculae, in few osteocytes within the mineralized bone and in some bone marrow cells (blue arrowhead in Fig. 2).

REDUCED FEMORAL BONE MINERAL DENSITY IN *Dlx5*$^{+/-}$ MICE

BMD was measured by DXA (DXA) on groups of 10- and 20-week-old *Dlx5*$^{+/-}$ and WT mice. *Dlx5*$^{+/-}$ mice were normal in appearance and their body size and weight were not different from those of WT littermates (data not shown). We detected a significant decrease of femoral BMD (Fig. 3) in *Dlx5*$^{+/-}$ mice. These animals did not present, however, any variation in whole body, tibia, and caudal vertebrae BMD. Femurs of male *Dlx5*$^{+/-}$ mice presented a significantly lower BMD already at 10 weeks of age (-7.8%; $P < 0.05$; n = 14 per genotype), and the difference increased at 20 weeks of age (-13%; $P < 0.001$; n = 10 per genotype); in females this reduction was only significant in the 20-week-old group (-6%; $P < 0.05$; n = 10 per genotype). The impact of *Dlx5* haploinsufficiency on bone mass appeared, therefore, to be site-specific and more pronounced in males than in females.

Dlx5 HAPLOINSUFFICIENCY AFFECTS CORTICAL THICKNESS

3D μCT analyses of femoral bones showed alterations of the cortical thickness of *Dlx5*$^{+/-}$ mice. At 20 weeks of age, the cortical thickness at the femoral midshafts was significantly lower in both male (-17%; $P < 0.001$, n = 12) and female (-10%; $P < 0.05$, n = 10) *Dlx5*$^{+/-}$ mice compared with WT (Fig. 4). Ten-week-old male *Dlx5*$^{+/-}$ mice also exhibited a significant decrease in cortical thickness (-12%; $P < 0.01$, n = 13) associated with a significant increase of the marrow diameter; this finding suggests that the endosteal resorption activity was affected in these mice. On the contrary, in 20-week-old heterozygous males we observed a reduction in the external femoral diameter while the marrow diameter did not change. These findings suggest that Dlx5 controls femoral bone maturation through different mechanisms at different postnatal ages, first regulating endosteal resorption activity and then acting predominantly on periosteal bone formation.
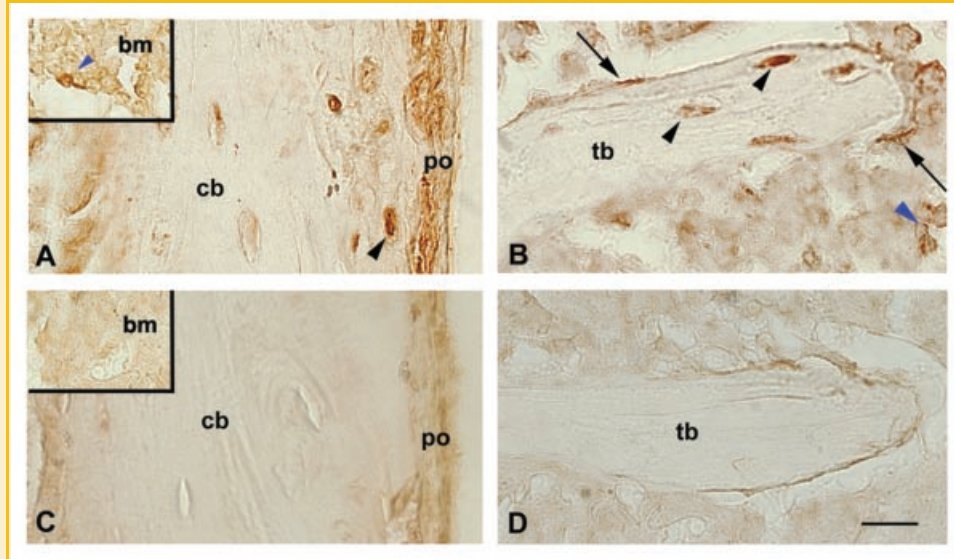
Fig. 2. Immunolocalization of β–galactosidase protein on femoral sections of wild–type and $Dlx5^{LacZ+/-}$ bone. Longitudinal femoral sections from 10–week–old $Dlx5^{LacZ+/-}$ and wild–type male mice were immunostained with anti–β–galactosidase antibodies and peroxidase–coupled secondary antibody. A positive signal was detected only in sections from $Dlx5^{LacZ+/-}$ mice (A,B) and not in those from control littermates (C,D). Insert in A,C details bone marrow immunostaining. Staining was seen in the periosteum (po), in trabecular bone lining cells (arrows), in certain osteocytes (arrowheads), on endosteal–lining cells, and in few bone marrow cells (blue arrowheads). bm, bone marrow; cb, cortical bone; po, periosteum; tb, trabecular bone. Bar: 20 μm.

MAINTAINED TRABECULAR BONE FORMATION AND INCREASED ENDOSTEAL BONE RESORPTION IN 10-WEEK-OLD $Dlx5^{+/-}$ MICE

To better clarify the mechanism underlying the decrease in cortical thickness, we focused our analysis on male $Dlx5^{+/-}$ mice as their cortical phenotype is more pronounced. Data obtained by histomorphometry on distal femora from 10- and 20-week-old mice confirmed to the μCT data, showing no difference in the structure of the trabecular bone (Table I), but a significant reduction of the cortical thickness in $Dlx5^{+/-}$ mice of both ages (Table II). Double fluorochrome labeling did not reveal any significant change

Fig. 3. Effects of Dlx5 haploinsufficiency on femoral BMD. BMD of 10- and 20-week-old wild–type female (gray bars) and male (open bars) and $Dlx5^{+/-}$ mice (hatched bars) were evaluated on the femurs by DXA. Results are presented as mean ± SEM. n = 7–14 per genotype and gender. $^*P < 0.05$ and $^{***}P < 0.001$.

of the mineral apposition rate (MAR) and of the mineralized surfaces (DM/BS) of trabecular and endosteal bone. Double-labeled mineralized surfaces on the periosteum were quite rare making it impossible to assess the cortical bone apposition rate. Interestingly, while trabecular or periosteal osteoclast surfaces were unmodified, a significantly ($P < 0.05$; n = 10/genotype) higher number of endosteal osteoclasts per bone surface (Oc.S/BS, %) were observed only in 10-week-old $Dlx5^{+/-}$ compared to WT mice (Fig. 5). Taken together, these data reinforce the notion that reduced cortical thickness in 10-week-old $Dlx5^{+/-}$ mice results from a defect in endosteal bone resorption rather than in bone formation.
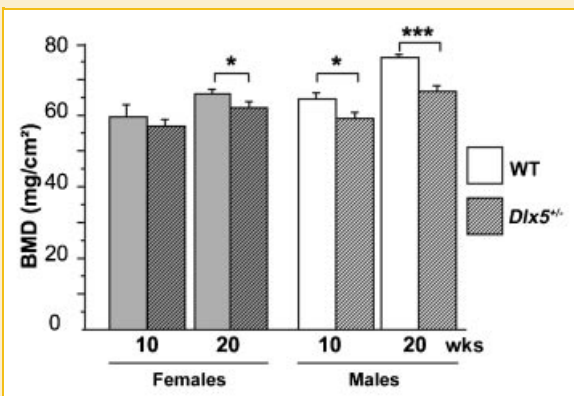
INCREASE IN URINARY DEOXYPYRIDINOLINE EXCRETION IN 10-WEEK-OLD $Dlx5^{+/-}$ MICE

To confirm the enhanced resorption activity observed in 10-week-old $Dlx5^{+/-}$ bones, we evaluated the levels of urinary deoxypyridinoline. $Dlx5^{+/-}$ mice did indeed display a significant increase in the level of this marker when compared to WT littermates ($Dlx5^{+/-}$: 29.7 ± 3.12 nmol; $P < 0.05$ vs. WT: 22.1 ± 1.46 nmol) (Fig. 6) supporting the notion that allelic reduction of $Dlx5$ leads to a higher rate of bone resorption. In 20-week-old $Dlx5^{+/-}$ mice, urinary deoxypyridinoline levels were also increased, but the difference did not reach significance.

EXPRESSION OF OSTEOBLASTIC AND OSTEOCLASTIC MARKERS IN $Dlx5^{+/-}$ ADULT BONE

We have previously shown that $Dlx5$ is not expressed by spleen-derived osteoclast precursors cultured in the presence of M-CSF and RANK-L [Samee et al., 2008], it is therefore highly unlikely that $Dlx5$
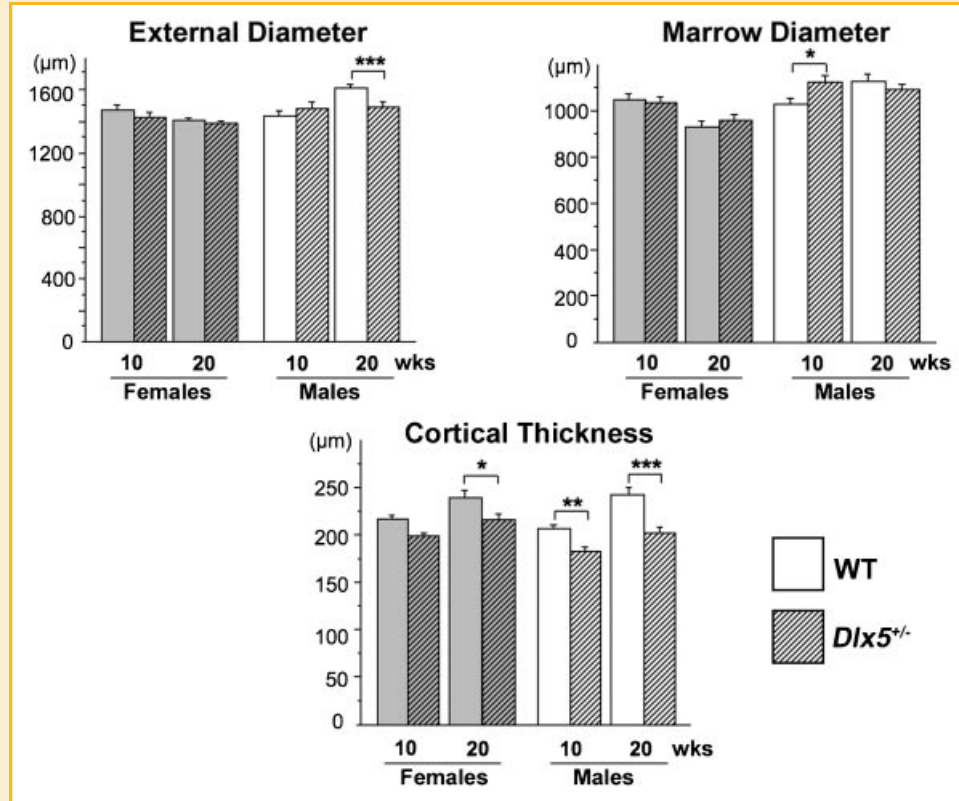
Fig. 4. μCT analysis of cortical bone thickness of $Dlx5^{+/-}$ mice. We compared, by μCT analysis, the femoral thickness at midshafts of 10- and 20-week-old wild-type (open bars) and $Dlx5^{+/-}$ mice (hatched bars). The analysis was performed both on female (gray bars) and male (white bars) mice. Values of external diameter, marrow diameter, and cortical thickness are presented as mean ± SEM (n = 9–13 per group). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

has a direct effect on osteoclast differentiation. We therefore considered the possibility that haploinsufficiency of $Dlx5$ could affect osteoblast/osteoclast coupling resulting in an increased osteoclast differentiation. We first monitored the levels of expression of $Dlx5$, OPG, RANKL, the osteoclastic markers TRAP and cathepsin K as well as genes involved in the acquisition of the osteoblastic phenotype in femurs of 10- and 20-week-old mice. The inactivation of one allele of $Dlx5$ resulted in a strong reduction of $Dlx5$ bone expression at both ages without affecting

that of $Dlx6$ (data not shown). In only 10-week-old $Dlx5^{+/-}$ male, this reduction was accompanied by an increase of RANKL, OPG, and osteoclastic markers (Fig. 7). We did not notice, however, any significant variation of the expression levels of the osteoblastic markers $Runx2$, Osx, $osteocalcin$, BSP, and of the RANKL/OPG ratio at both studied time points. The expression of other coupling factors such as members of the ephrine family or IFN-γ were also unmodified in $Dlx5^{+/-}$ femurs when compared to controls (data not shown).

TABLE I. Trabecular Bone Histomorphometry of $Dlx5^{+/-}$ and Wild-Type Mice

	10-week-old male[a]		20-week-old male[b]	
Trabecular bone parameters	WT	$Dlx5^{+/-}$	WT	$Dlx5^{+/-}$
Bone structure				
Bone volume/tissue volume (BV/TV; %)	11.42 ± 0.84	9.28 ± 0.70	9.40 ± 1.40	8.30 ± 1.20
Trabecular number (Tr.Nb; 1/mm)	2.5 ± 0.21	2.5 ± 0.20	3.3 ± 0.29	3.3 ± 0.42
Trabecular separation (Tr.Sp; μm)	210 ± 12.1	240 ± 12.3	293 ± 32	396 ± 68
Trabecular thickness (Tr.Th; μm)	26.0 ± 0.99	23.8 ± 0.99	29.1 ± 2.3	31.0 ± 2.7
Bone resorption				
Osteoclast surface/bone surface (Oc.S/BS; %)	4.51 ± 1.42	7.59 ± 1.83	1.79 ± 1.42	3.18 ± 1.83
Osteoclast number/bone surface (N.Oc/BS; 1/mm^2)	15.0 ± 4.68	18.8 ± 4.54	6.27 ± 2.77	9.38 ± 2.60
Bone dynamics				
Mineralizing apposition rate (MAR; μm/day)	3.21 ± 0.19	3.32 ± 0.19	1.55 ± 0.15	1.53 ± 0.16
Bone formation rate (BFR/BS; μm^3/μm^2/day)	1.18 ± 0.03	1.29 ± 0.25	0.15 ± 0.09	0.16 ± 0.08

[a]Values are shown as mean ± SEM (n = 10 per group).
[b]Values are shown as mean ± SEM (n = 6 per group).

TABLE II. Cortical Bone Histomorphometry of $Dlx5^{+/-}$ and Wild-Type Mice

Cortical bone parameters	10-week-old male[a]		20-week-old male[b]	
	WT	$Dlx5^{+/-}$	WT	$Dlx5^{+/-}$
External diameter (mm)	2.36 ± 0.09	2.40 ± 0.06	2.63 ± 0.09	2.35 ± 0.15
Endocortical diameter (mm)	1.99 ± 0.28	2.10 ± 0.17	2.25 ± 0.10	2.05 ± 0.15
Cortical thickness (mm)	0.18 ± 0.02	$0.15 \pm 0.03^*$	0.19 ± 0.01	$0.15 \pm 0.01^*$

[a]Values are shown as mean ± SEM (n = 10 per group).
[b]Values are shown as mean ± SEM (n = 6 per group).
*Significantly different from WT, $P < 0.05$.

DISCUSSION

The tight mutual control of bone formation and resorption, mediated, respectively, by osteoblasts and osteoclasts, constitutes the core regulation of bone development, growth, and remodeling [Olsen et al., 2000]. Alteration of this balance in favor of osteoclasts leads to excessive bone resorption and to deterioration of bone architecture. Bone homeostasis largely depends on the exchange of signals between osteoblasts and osteoclasts leading to the coupling of the transcriptional regulatory cascades that govern their proliferation and differentiation [Theoleyre et al., 2004]. Several homeodomain proteins, including members of the Dlx family are involved in the control of osteoblast/osteoclast coupling [Hassan et al., 2006; Samee et al., 2007]. Dlx5 is expressed by periosteal and trabecular osteoblasts since very early stages of embryonic bone formation [Zhao et al., 1994; Acampora et al., 1999; Samee et al., 2008] and Dlx5-null mice die at birth rendering it impossible to perform functional studies on bone maturation. Their role in postnatal bone growth and remodeling remains, therefore, largely unexplored. Here, by analyzing the effect of Dlx5 allelic reduction on postnatal skeletal morphogenesis, we show that Dlx5 plays a late
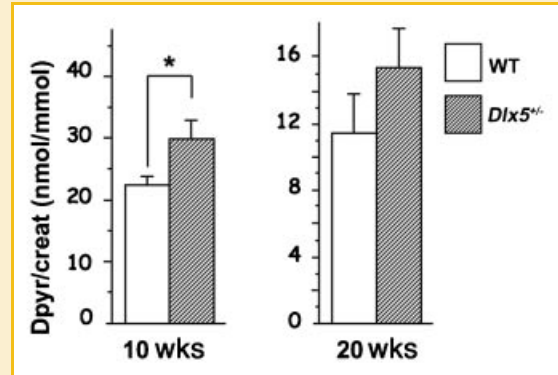

Fig. 6. Higher bone resorption activity in 10- and 20-week-old $Dlx5^{+/-}$ male mice. Urinary deoxypyridinoline cross-links normalized by the amount of creatinin (Dpyr/creat) was measured in 10- and 20-week-old wild-type (open bar) and $Dlx5^{+/-}$ mice (hatched bar). Results are presented as the mean ± SEM; n = 9 per genotype and *$P < 0.05$.

role in the control of bone remodeling and maintenance of adult bone mass.

In this study, we show that the expression pattern of Dlx5 in adult long bone is reminiscent, but not identical, to that found at the end of embryogenesis. Indeed, Dlx5 expression is present, in both cases, in trabecular, cortical, and periosteal osteoblast, however, in adult bone we also observed expression in some osteocytes. The relative level of expression of Dlx5 in postnatal bone is, however, lower than that observed at birth and diminishes progressively in aging animals. After allelic reduction of Dlx5, we did not observe any significant change in the expression of osteoblastic markers nor any modification in the number and/or the activity of mineralizing trabecular osteoblasts. The only indication of defective osteoblast function in $Dlx5^{+/-}$ mice was the decrease in the external femoral diameter of 20-week-old males which suggests a reduction in
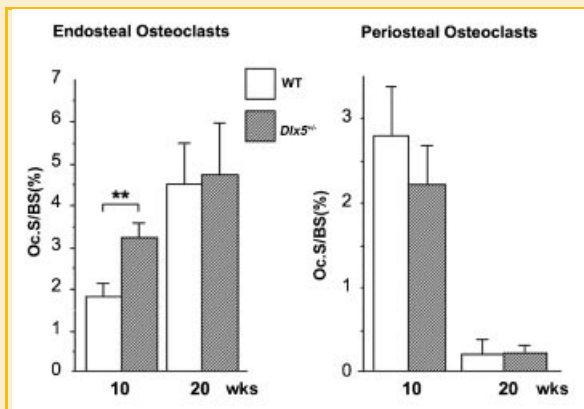

Fig. 5. Higher endosteal osteoclastic surface in 10-week-old $Dlx5^{+/-}$ mice. TRAP-positive cells were counted on periosteal and endosteal surfaces of 10- and 20-week-old male wild-type and $Dlx5^{+/-}$ mice femurs. Results are presented as number of active osteoclasts per bone surface (Oc.S/BS) mean ± SEM; 10-week-old mice, n = 10 per genotype; 20-week-old, n = 5–7 per genotype. **$P < 0.01$.
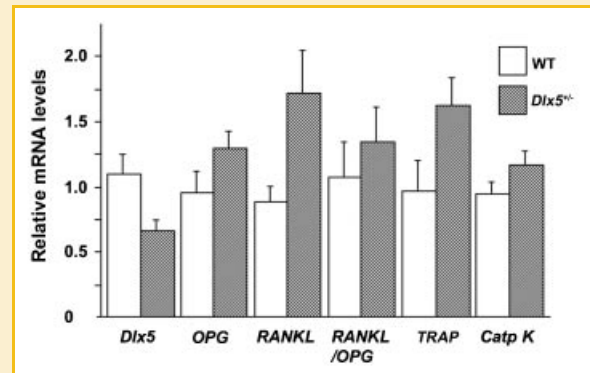

Fig. 7. Relative expression levels of Dlx5 and osteoclastogenesis-related markers in wild-type and $Dlx5^{+/-}$ mice femurs. The relative expression levels of Dlx5, OPG, RANKL, TRAP, and cathepsin K were measured by qPCR in wild-type (open bars) and $Dlx5^{+/-}$ (hatched bars) littermates. While the level of Dlx5 expression was reduced by about 42% in $Dlx5^{+/-}$ mice, OPG, RANKL, TRAP, and cathepsin K were all increased in $Dlx5^{+/-}$ mice. The RANKL/OPG ratio did not change significantly.

periosteal apposition. As we used whole femurs for quantitative PCR assays, the modest change in osteoblast and osteoclast markers and in RANKL/OPG ratio could result from dilution of more pronounced effects taking place only on cortical surfaces. Thus, the phenotype observed during embryogenesis, which is characterized by a decrease in the total and cancellous bone volume of $Dlx5^{+/-}$ and $Dlx5^{-/-}$ mice, differs substantially from that of adult heterozygotes that exhibit a phenotype only in cortical bone.

$Dlx5$ and $Dlx6$ are co-expressed at multiple sites and share redundant functions [Merlo et al., 2000]. One could speculate that, the mild osteoblastic phenotype of $Dlx5^{+/-}$ mice might derive from partial compensation by Dlx6 which is also expressed in adult bone (data not shown). However, our results do not evidence a deregulation of $Dlx6$ following $Dlx5$ allelic reduction supporting the notion that $Dlx6$ up-regulation does not compensate for the loss of one allele of $Dlx5$.

The loss of one $Dlx5$ allele was associated to a significant change in cortical, but not in trabecular, bone parameters in adult mice. The increase in urinary deoxypyridinoline and endosteal osteoclast surfaces resulting in higher marrow diameter suggests that, in 10-week-old $Dlx5^{+/-}$ mice, bone resorption activity along endosteal surfaces is higher. This increased resorption in adult male $Dlx5^{+/-}$ is consistent with our findings in homozygous mutants at the end of gestation where we have shown that Dlx5 affects directly osteoblast/osteoclast coupling [Samee et al., 2008]. The increased osteoclast activity associated to $Dlx5$ haploinsufficiency is, however, observed only in prepubertal mice and is lost in older (20-week-old) individuals. By contrast, in older mice we observe a reduced external diameter, secondary to decreased cross-sectional growth. This reduction of cortical thickness is more pronounced in males than in females where it was significant only at 20 weeks of age. This difference might possibly be related to the lower levels of $Dlx5$ expression in 10- and 20-week-old female when compared to male at the same time point (Fig. 1).

Cortical bone is a major determinant of bone strength not only for long bones but also for vertebrae. An increase in both periosteal apposition and endosteal bone erosion might be at the origin of the changes in bone cortical structure occurring during human aging [Bousson et al., 2001; Szulc et al., 2005]. Furthermore, knowledge about the magnitude and extent of periosteal bone formation and endosteal resorption with age has important implication for fracture etiology because both the amount and location of cortical bone contribute to the biomechanical strength [Chavassieux et al., 2007]. Loss of cortical bone with aging is primarily caused by endosteal surface negative balance, and in mice model this surface remains sensitive to resorption. In our model, we observed a similar mechanism, an increase in endosteal bone resorption in 10-week-old $Dlx5^{+/-}$ male mice. In addition, we suggested a decrease in periosteal apposition in 20-week-old mice describing a similar pattern as aging. The two different mechanisms indicate that Dlx5 has distinct function at different time points during age-related bone loss.

Gonadal steroids (testosterone and estrogen) have been shown to affect BMD and to have markedly different action on cortical bone [Vanderschueren et al., 2004]. The decreased femoral BMD observed in $Dlx5^{+/-}$ mice was not likely to be the effect of these hormones as both sexes were affected and no change in testosterone level was observed when 10-week-old $Dlx5^{+/-}$ males were compared to WT mice (data not shown). However, Dlx5 could be part of the hormonal sex effect on cortical bone. Indeed, the cortical defect observed in $Dlx5^{+/-}$ mice appeared later in females than in males; this difference might possibly be related to the role of Dlx genes on the activation of steroidogenic acute regulatory protein expression [Nishida et al., 2008].

In summary, our study showed that $Dlx5$ haploinsufficiency in adult mice resulted in reduced femoral BMD affecting preferentially cortical bone. The decrease in cortical thickness was associated with an increase in endosteal bone resorption before $Dlx5^{+/-}$ male mice attained peak bone mass and, at later stages, with a reduction of periosteal bone apposition. We conclude that Dlx5 is a determinant of cortical bone remodeling which has distinct functions in the control of bone homeostasis during embryogenesis and in postnatal development. The analysis of mice carrying a conditional inactivation of $Dlx5$ in bone will be decisive to evaluate the potential implication of $Dlx5$ in osteoporosis.

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