The immune regulatory protein B7-H3 promotes osteoblast differentiation and bone mineralization

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B7-H3, a member of the B7 family of the Ig superfamily proteins, is expressed on the surface of the antigen-presenting cells and down-regulates T cell functions by engaging an unknown counterreceptor on T cells. Although B7-H3 is ubiquitously expressed, its potential nonimmune functions have not been addressed. We found that B7-H3 is highly expressed in developing bones during embryogenesis and that its expression increases as osteoblast precursor cells differentiate into mature osteoblasts. *In vitro* bone formation by osteoblastic cells was inhibited when B7-H3 function was interrupted by the soluble recombinant protein B7-H3-Fc. Analysis of calvarial cells derived from neonatal B7-H3 knockout (KO) mice revealed normal numbers of osteoblast precursor cells possessing a normal proliferative capacity. However, the B7-H3-deficient calvarial cells exhibited impaired osteogenic differentiation, resulting in decreased mineralized bone formation *in vitro*. These results suggest that B7-H3 is required for the later phase of osteoblast differentiation. Although B7-H3 KO mice had no gross skeletal abnormalities, they displayed a lower bone mineral density in cortical (but not trabecular) bones compared with WT controls. Consistent with the reduced bone mineral density, the femurs of B7-H3 KO mice were more susceptible to bone fracture compared with those of WT mice. Taken together, these results indicate that B7-H3 and its unknown counterreceptor play a positive regulatory role in bone formation. In addition, our findings identified B7-H3 as another molecule that has a dual role in the bone-immune interface.

Bone homeostasis is maintained through a highly coordinated balance between bone formation and bone resorption, which are mediated by osteoblasts and osteoclasts, respectively (1, 2). Dysregulated bone metabolism caused by hormonal changes or chronic inflammation often leads to a loss of bone mass. Intensive research has focused on elucidating the regulatory mechanisms governing osteoclast differentiation and activation with the aim of preventing hyperactive bone resorption. This effort led to the discovery of the receptor activator of nuclear factor κB (RANK)–RANK ligand–osteoprotegerin system that regulates the final stage of osteoclast differentiation and activation (2–4). Drugs targeting this pathway may prove effective in reducing bone resorption rate. However, treatment of osteoporosis by preventing bone resorption alone is of limited value, especially in elderly humans because of the age-associated reduction of osteogenic potential (5, 6). Thus, it is important to understand the mechanisms controlling osteoblast differentiation and function. Although key transcription factors, such as Runx2/Cbfa1 and osterix, that drive osteoblast lineage determination have been identified (7–9), cell surface proteins that regulate osteoblast differentiation and activity that are more amenable to clinical manipulation have not been well defined.

The immune system and bone have an intimate relationship manifested in multiple ways. First, bone provides bone cavities in which hematopoietic stem cells differentiate into specialized blood cells including immune cells. In the bone cavity, osteoblasts lining the trabecular bones provide the "niche" for hematopoietic stem cells (10, 11). Secondly, cells of macrophage and possibly B cell lineage serve as precursors to osteoclasts (12, 13). Thirdly, as shown in rheumatoid arthritis, autoimmune inflammation can cause bone loss. RANK ligand (RANKL) and other inflammatory cytokines produced by activated T cells play an important role in osteoclastogenesis (14, 15). RANKL [also known as TNF-related activation-induced cytokine (TRANCE)] was originally identified as a molecule that enhances dendritic cell survival (16). Fourthly, osteopontin [also known as early T lymphocyte activation 1 (Eta-1)], a bone protein that regulates osteoblast and osteoclast function (17), is induced in activated T cells and regulates T cell-mediated immune responses (18, 19). Lastly, the gene encoding the osteoclast associated receptor (OSCAR), a regulator of osteoclastogenesis, is located in a genetic locus termed leukocyte receptor complex (LRC). The LRC harbors multiple genes encoding stimulatory or inhibitory immunoreceptors (20). Collectively, the extensive sharing of cellular and molecular components between bone and the immune system suggests that these two biological systems have established a close relationship during evolution.

B7-H3 was originally identified from a human dendritic cell cDNA library by virtue of its sequence identity to members of the B7 family (21). The B7 family of proteins are type I transmembrane glycoproteins containing two extracellular Ig domains. By engaging their cognate receptors on T cells, B7 family proteins provide accessory signals to T cells during T cell activation, which could be either stimulatory or inhibitory (22). Initial *in vitro* experiments have shown that, through binding to an unknown receptor, B7-H3 might enhance T cell activation (21). However, we and others recently reported that B7-H3 in fact inhibits T cell function both *in vitro* and *in vivo* (23–25). Like many other B7 family members, B7-H3 is expressed in multiple organs and tissues, implying that it may also play roles outside the immune system. Here, we show that B7-H3 is highly expressed in osteoblasts during bone formation and that disruption of B7-H3 function decreases bone mineralization both *in vitro* and *in vivo*.

Materials and Methods

Animals. The generation of B7-H3 knockout (KO) mice and their immune phenotypes has been described (23). All animal experiments were conducted with the approval of the University Heath Network Animal Care Committee, Toronto.

Cell Culture. MC3T3-E1 (murine osteoblastic cells) and RSBMC-D8 [clonal cells derived from rat stromal bone marrow cell line RSBMC (26)] cells were cultured in αMEM (Sigma) supplemented with 10% FCS and antibiotics (100 μg/ml peni-

Abbreviations: BMD, bone mineral density; KO, knockout; DEXA, dual energy x-ray absorptiometry; E*n*, embryonic day *n*.

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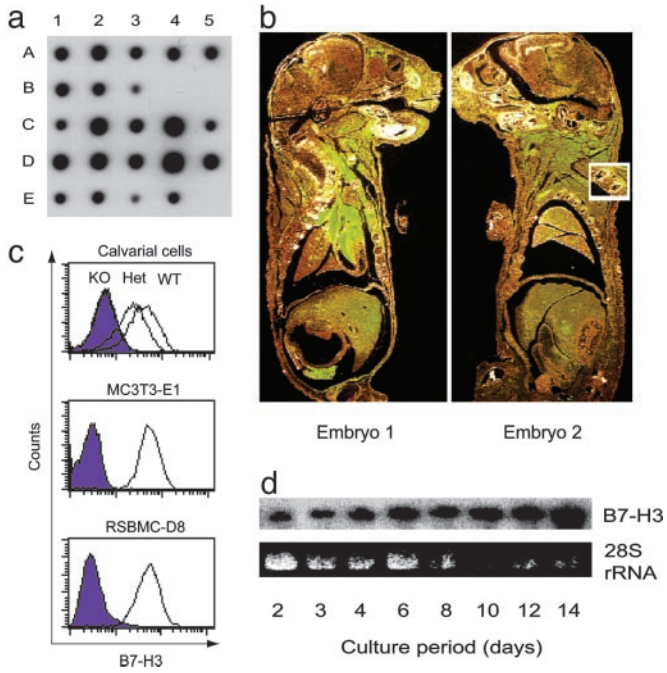

Fig. 1. B7-H3 is highly expressed in osteoblasts. (*a*) Tissue expression pattern of B7-H3. Shown is Northern analysis of a mouse multiple tissue Northern blot (Clontech) probed with a mouse B7-H3 probe. A1–A5: brain, eye, liver, lung, kidney. B1–B3: heart, skeletal muscle, smooth muscle. C1–C5: pancreas, thyroid, thymus, submaximal gland, spleen. D1–D5: testis, ovary, prostate, epididymus, uterus. E1–E4: embryo day 7, embryo day 11, embryo day 15, embryo day 17. (*b*) Embryonic B7-H3 expression. Saggital sections of mouse embryos (E18.5) were hybridized with an antisense B7-H3 probe and then processed as described in *Materials and Methods*. The two panels represent sections obtained from two embryos embedded in the same block. The *Inset* in the right panel shows a magnified view of the ribs. White spots represent B7-H3 mRNA. No signal was observed when a sense B7-H3 probe was used (not shown). (*c*) B7-H3 expression by osteoblasts. Mouse calvarial cells of the indicated genotypes, mouse osteoblastic cells (MC3T3-E1), and rat osteoblastic cells (RSBMC-D8) were stained with the anti-B7-H3 antibody followed by FITC-conjugated secondary antibody and then analyzed by flow cytometry. Filled histograms represent staining with rat IgG (control). (*d*) Increased B7-H3 expression during the late phase of osteogenesis. Total RNAs were prepared from RSBMC-D8 cells that had been cultured under osteogenic differentiation conditions (*Materials and Methods*) for the indicated periods of time. Radiolabeled B7-H3 probe was used to detect B7-H3 mRNA. 28S rRNA, loading control.

cillin G, 50 μg/ml gentamycin, and 0.3 μg/ml fungizone). To induce *in vitro* osteogenic differentiation, RSBMC-D8 cells were grown until confluency in αMEM plus 10% FCS. Ascorbic acid (50 μg/ml) was added at confluency, and β-glycerophosphate (10 mM) was added after nodules had formed ($\approx$day 10 after confluency). Murine calvarial cells from day 3 neonatal mice were prepared by enzymatic digestion as described (27) and cultured in αMEM supplemented with 10% FCS and antibiotics. For *in vitro* osteogenic differentiation, mouse calvarial cells were cultured until confluency, followed by addition of dexamethasone (10 nM) and ascorbic acid (50 μg/ml). β-glycerophosphate (10 mM) was added after nodules had formed ($\approx$day 21 after confluency). The medium was changed every 2–3 days.

Proteins and Antibodies. The rabbit anti-rat B7-H3 IgG used in this study has been described (23). This antibody recognizes mouse, rat, and human B7-H3. Recombinant protein B7-H3-Fc, in which the two extracellular Ig domains of murine B7-H3 are fused in-frame to the Fc portion of human IgG1, has been described (23). Human IgG purchased from Jackson ImmunoResearch was used as the control for B7-H3-Fc protein.

Northern Blot Hybridization. A 1.1-kb cDNA fragment comprising the ORF of murine B7-H3 was used as a template to generate a radioactive probe using Ready-To-Go DNA labeling beads (Amersham Pharmacia) and [α-^{32}P]dCTP (NEN). A mouse multiple tissue Northern blot was purchased from Clontech and hybridized according to the manufacturer's instructions. Total RNAs were prepared from RSBMC-D8 cells by using TRIzol (GIBCO) as described (28).

***In Situ* Hybridization.** ^{33}P-labeled antisense and sense cRNA probes corresponding to the ORF of B7-H3 were generated by *in vitro* transcription using linearized plasmid DNAs as templates. Saggital paraffin sections of 18.5-day CD1 mouse embryos were hybridized with the probes as described (29). After hybridization, the slides were exposed to NTB2 emulsion (Kodak), developed, and then counterstained with hematoxylin/eosin. Images were acquired by using dark-field microscopy.

Flow Cytometric Analysis. Cultured osteoblastic cells were suspended by trypsinization and then incubated with anti-B7-H3 antibody. After washing, the bound antibodies were detected by using FITC-conjugated goat anti-rabbit IgG (Pharmingen). Stained cells were analyzed by flow cytometry using FACSCalibur instrument equipped with CELLQUEST software (Becton Dickinson). Rabbit IgG (Pharmingen) was used as the isotype control.

Alkaline Phosphatase Assays. Osteoblastic cells were identified based on positive staining for alkaline phosphatase activity by using FAST RED (Sigma) and Naphthol-AS-MX-phosphate (Sigma) as previously described (28). Alkaline phosphatase activities in cell lysates were quantified by using a colorimetric method (28) and normalized according to the total protein concentration in the lysate as determined by the Bio-Rad protein assay reagent.

von Kossa Staining. Mineralized nodules were stained with silver nitrate solution according to the von Kossa method as described (30).

Dual Energy X-Ray Absorptiometry (DEXA) Analysis. The bone mineral content (BMC) of whole mice or excised bones was determined by DEXA by using a PIXImus scanner (GE Medical System). Bone mineral density (BMD) was calculated by dividing the BMC by the bone area.

Mechanical Testing of Excised Bones. Excised femurs were tested in torsion on a home-made instrument at 35°/min with a 0.35 N·m torque cell as described (31). Excised vertebral bodies were subjected to compression at 0.5 mm/min with a 100-N load cell by using a material testing system (Model 1011, Instron, Canton, MA) as described (32).

Statistical Analysis. The DEXA and mechanical data were analyzed by using the SPSS 11.0 statistical program (SPSS, Chicago). Statistical significance of the data was judged by the Student's *t* test.

Results

B7-H3 Expression in Osteoblasts. Initially, we found that B7-H3 was expressed throughout mouse embryogenesis and in multiple adult tissues as shown by a Northern blot analysis (Fig. 1*a*). *In situ* hybridization analysis of embryonic day (E) 18.5 mouse embryos revealed that murine B7-H3 was particularly highly expressed in bone tissues such as the mandible, vertebrae, and ribs (Fig. 1*b*). The prominent periosteal staining pattern of the ribs (Fig. 1*b* *Right Inset*) suggested that osteoblasts might be the main cells that express B7-H3. We therefore examined B7-H3 expression

on the surface of osteoblastic cells of mouse, rat, or human origin by using flow cytometry. First, we stained calvarial cells derived from B7-H3 WT, Het, and KO mice with the anti-B7-H3 antibody. As shown in Fig. 1*c Top*, B7-H3 was detected on the murine calvarial cell surface in a gene dosage-dependent manner. Osteoblastic cell lines of mouse (MC3T3-E1), rat (UMR 106-01, ROS 17/2.8, and RSBMC-D8), and human (SaOS-2) origin also expressed high levels of B7-H3 on the cell surface (Fig. 1*c Middle* and *Bottom* and data not shown). We further analyzed the expression pattern of B7-H3 in RSBMC-D8 osteoblastic cells while they were undergoing osteogenic differentiation *in vitro*. As shown in Fig. 1*d*, B7-H3 mRNA level increased over the course of osteoblast differentiation. These findings indicated that B7-H3 might be involved in osteoblast differentiation and/or function.

Inhibition of *in Vitro* Osteogenesis by B7-H3 Disruption. We reasoned that B7-H3 may function by binding to a putative counterreceptor on the osteoblastic cell surface, as it does when it regulates T cell activation (21, 23). To test this hypothesis, we performed *in vitro* osteogenic differentiation assays by using RSBMC-D8 cells in the presence of a soluble recombinant B7-H3 protein B7-H3-Fc or human IgG as a control. Under osteogenic conditions, RSBMC-D8 cells undergo an osteogenic differentiation program *in vitro* that closely resembles the bone formation process *in vivo* (A.H.J. and J.S., unpublished results). Within 2 weeks in culture, cells aggregate and produce extracellular matrix (termed "nodules"), which is readily mineralized in the presence of β-glycerophosphate. The abundance of mineralized nodules after 12 days in culture was assessed by von Kossa staining. B7-H3-Fc treatment greatly reduced the number of nodules in RSBMC-D8 cultures compared with treatment with human IgG (Fig. 2*a*). These results raised two possibilities. First, B7-H3 and its counterreceptor could provide a positive regulatory role in osteoblast differentiation and/or function. In this model, B7-H3-Fc would behave as an inhibitor. Alternatively, B7-H3-Fc could bind to and activate the B7-H3 counterreceptor such that B7-H3 function was augmented. This model postulates that B7-H3 and its counterreceptor play an inhibitory role in osteoblast differentiation and/or function. To resolve this issue, we prepared calvarial cells from neonatal WT and B7-H3 KO mice and cultured them under osteogenic conditions. As shown in Fig. 2*b*, the number of mineralized nodules was substantially lower in the B7-H3-deficient calvarial cell culture compared with that in the WT control. This difference in nodule formation was not due to a defective expansion of osteoblastic precursor cells in the B7-H3 KO calvaria because B7-H3 WT and KO calvarial cells did not show any major differences in alkaline phosphatase activity during the proliferation phase (Fig. 2*c*). Nor was there any difference in the number of alkaline phosphatase-positive cells between the WT and B7-H3 KO calvarial cell cultures (Fig. 2*d*). Collectively, these results indicate that, when B7-H3 engages its putative counterreceptor expressed on osteoblasts, a positive regulatory signal is delivered that enhances osteoblast differentiation and bone mineralization at the later phase of the osteogenic program.

Reduced BMD in B7-H3 KO Mice. To determine the *in vivo* consequences of B7-H3-deficiency on bone, we analyzed bones from WT and B7-H3 KO mice. B7-H3 KO mice were born healthy and grew to normal size without obvious skeletal abnormalities. Anatomical analysis of calvaria, teeth, and long bones revealed no developmental defects in B7-H3 KO mice (data not shown). We then determined the whole-body BMD of age- and sex-matched WT and B7-H3 KO mice by using DEXA. At 6–7 months of age, both male and female B7-H3 KO mice tended to show reduced whole-body BMD compared with WT mice, with a more pronounced difference in the females (Fig. 3*a*). By 10

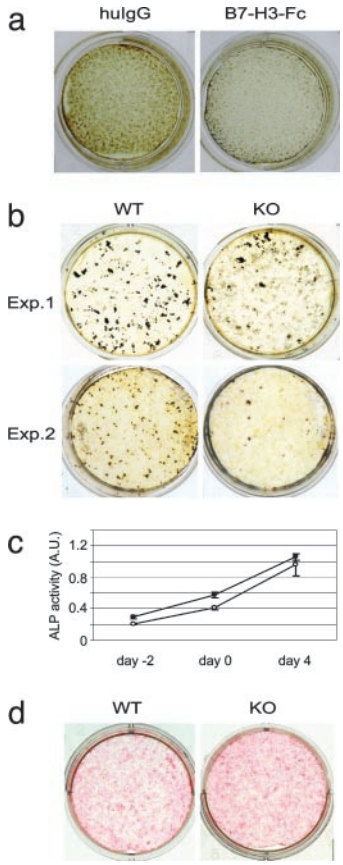

Fig. 2. Inhibition of *in vitro* osteogenesis by B7-H3 disruption. (*a*) Reduced number of mineralized nodules in response to B7-H3-Fc. RSBMC-D8 cells were cultured under osteogenic conditions (*Materials and Methods*) for 12 days in the presence of B7-H3-Fc (10 μg/ml) or human IgG (10 μg/ml). Mineralized nodules were visualized by von Kossa staining. Results shown are representative of two independent experiments. (*b*) Reduced number of mineralized nodules in the absence of B7-H3. Calvarial cells prepared from WT or B7-H3 KO neonatal mice were cultured for 21–24 days under osteogenic conditions (*Materials and Methods*) and subjected to von Kossa staining. Results shown are representatives from two independent experiments. (*c*) Normal osteoblastic expansion in the absence of B7-H3. Protein extracts of WT (filled circle) and B7-H3 KO (open circle) calvarial cells were prepared at the indicated times relative to the initiation of osteogenic differentiation (day 0). Alkaline phosphatase (ALP) activity (OD_{405}) was normalized to the total protein concentration (μg/ml). Data shown are mean $\pm$ SD of triplicate samples. (*d*) Normal number of ALP-positive cells in the absence of B7-H3. Calvarial cells from WT or B7-H3 KO mice were cultured for 4 days under osteogenic conditions followed by fixation and staining to detect ALP^+ cells (*Materials and Methods*). Results shown are representative of three independent experiments.

months of age, the whole-body BMD in the female B7-H3 KO mice was 7.65% lower compared with that of WT female mice (mean BMD $\pm$ SEM: WT, 58.79 $\pm$ 1.08; KO, 54.29 $\pm$ 0.73 mg/cm^2; $n = 12$, $P = 0.0022$). We then analyzed the BMD of excised bones from the 10-month-old female mice. Consistent with the whole mouse analysis, the excised femurs from the B7-H3 KO mice showed reduced BMD compared with those from the WT mice [Fig. 3*b* (Femur, left); mean BMD $\pm$ SEM: WT, 51.34 $\pm$ 1.88; KO, 45.02 $\pm$ 3.16 mg/cm^2; $n = 12$, $P = 0.099$]. However, no significant difference was detected in the vertebrae [Fig. 3*b* (Vertebrae, right); mean BMD $\pm$ SEM: WT, 33.58 $\pm$ 2.37; KO, 32.75 $\pm$ 1.89 mg/cm^2, $n = 12$, $P = 0.786$]. Thus, a lack of B7-H3 causes a substantial deficit in BMD in the cortical bones but not in the trabecular bones.

Mechanical Properties of B7-H3-Deficient Bones. To test whether the reduced BMD of the cortical bone observed in the absence of

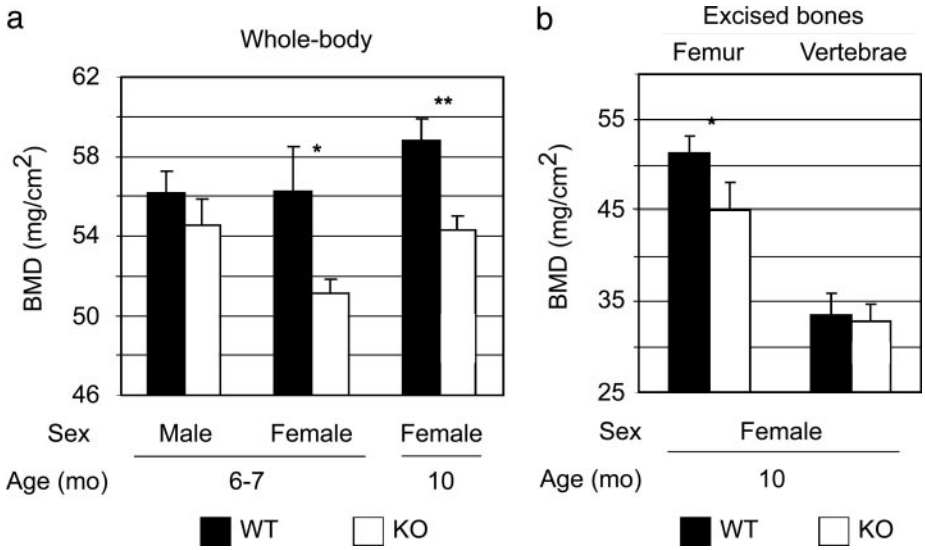

Fig. 3. Reduced BMD in B7-H3 KO mice. (*a*) Reduced whole-body BMD. Age- and sex-matched WT (filled bars) and B7-H3 KO (open bars) mice were analyzed by DEXA. Twelve mice of each genotype were used for analyses of 6- to 7-month-old males and 10-month-old females. Six WT and five KO mice were used for analysis of 6- to 7-month-old females. *, $P < 0.1$, **, $P < 0.01$. (*b*) Reduced BMD of cortical bone. Right femurs and vertebral bodies (L6) were excised from 10-month-old female WT (filled bars) and B7-H3 KO (open bars) mice and analyzed by DEXA. The twelve mice of each genotype that had been used for the whole-body BMD measurements in *a* were used. *, $P < 0.1$.

B7-H3 translated into altered mechanical properties, we subjected the excised femurs of WT and B7-H3 KO mice to a mechanical testing. We examined bone deformation and fracture mediated by torsion that is predominantly dependent on the quality of the cortical bone. The degree of bone deformation (strain) as a function of the force applied to the unit area of the cortical bone (stress) is schematically depicted as a stress–strain plot in Fig. 4. Compared with WT femurs, femurs from B7-H3 KO mice failed at a lower level of stress (reduced strength), and required less energy to fracture (reduced toughness). Thus, femurs from the B7-H3 KO mice displayed significantly reduced mechanical strength and toughness in torsion as quantitatively described in Table 1. The observed differences in bone mechanical properties were not likely due to altered bone geometry because the cortical bone area measured in the cross sections of the femurs was not different between the genotypes (Table 1). Rather, the reduced mechanical strength and toughness of the B7-H3 KO femur is consistent with the reduced BMD in the cortical bones and the impaired capacity of the B7-H3-deficient osteoblastic cells to form nodules *in vitro*. We also measured the mechanical properties of the vertebral bodies in compression. Consistent with the normal BMD, we did not observe any differences in mechanical properties between WT and B7-H3 KO vertebral bodies (data not shown).

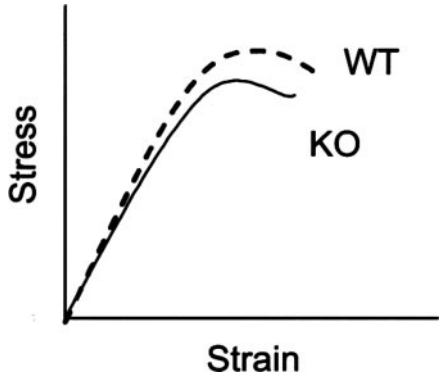

Fig. 4. Altered mechanical properties of B7-H3 KO femurs. WT and B7-H3 KO femurs excised from 10-month-old female mice ($n = 12$ per genotype) were subjected to torsion as described in *Materials and Methods*. The stress–strain curve shows that the WT femurs are stronger and tougher than the B7-H3 KO femurs.

Discussion

The first role identified for B7-H3 was that of an immunomodulatory molecule that down-regulates T cell-mediated immune responses. Here, we showed that B7-H3 is also required for the later stages of normal bone formation by osteoblasts. B7-H3 KO mice display reduced BMD and weakened bone mechanical properties in the cortical bones but not in the trabecular bones. These defects can be attributed to compromised osteoblast differentiation and mineralization capacity in the absence of B7-H3.

The putative counterreceptor for B7-H3 is not known. Because B7-H3-Fc does not bind to cells expressing a high level of B7-H3 (data not shown), a homophilic interaction of B7-H3 molecules does not seem likely. We have tried to clone the gene that encodes the B7-H3 counterreceptor by expression cloning using B7-H3-Fc as a probe. An extensive screen of the cDNA library generated from an osteoblastic cell culture undergoing *in vitro* osteogenic differentiation failed to uncover any candidates. It may be that the counterreceptor is present in very low abundance, and/or that it binds to B7-H3 with only very low affinity. Identification of the B7-H3 counterreceptor and characterization of its downstream signaling pathways would help us understand how B7-H3 exerts its osteogenic effects. It might then also be possible to design tools to

Table 1. Mechanical properties of WT and B7-H3 KO femurs subjected to torsion

	WT (mean ± SEM)	KO (mean ± SEM)
Strength, N·mm	23.73 ± 6.16	19.16 ± 2.31**
Stiffness, N·mm/rad	192.66 ± 23.88	152.42 ± 15.40
Toughness, N·mm·rad	1.64 ± 0.68	1.38 ± 0.28*
Cross section, mm^2	1.05 ± 0.08	0.99 ± 0.03

$n = 12$ female 10-month-old mice per genotype; *, $P < 0.05$; **, $P < 0.005$.

enhance the ostogenic potential in patients suffering from osteoporosis or bone fracture.

We found that B7-H3 deficiency affects the BMD of cortical bone but not that of trabecular bone. This result suggests that the mechanisms controlling cortical bone BMD could be different from those controlling trabecular bone BMD. Consistent with this notion, it is known that the genetic loci that regulate the microstructure and mechanical strength of cortical bone are not identical from those that govern trabecular bone structure and strength (32, 33).

Both RANK ligand and osteopontin have dual roles in bone metabolism and immunity. Our results show that B7-H3 is another component in the growing interface between bone and the immune system. Many newly identified B7 family proteins have been known to be expressed in multiple organs of nonimmune function (22). It would be interesting to investigate whether these proteins also have important functions outside the immune system.

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