Control of bone resorption in mice by Schnurri-3

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Mice lacking the large zinc finger protein Schnurri-3 (Shn3) display increased bone mass, in part, attributable to augmented osteoclastic bone formation. Here, we show that in addition to regulating bone formation, Shn3 indirectly controls bone resorption by osteoclasts in vivo. Although Shn3 plays no cell-intrinsic role in osteoclasts, Shn3-deficient animals show decreased serum markers of bone turnover. Mesenchymal cells lacking Shn3 are defective in promoting osteoclastogenesis in response to selective stimuli, likely attributable to reduced expression of the key osteoclastogenic factor receptor activator of nuclear factor-xB ligand. The bone phenotype of Shn3-deficient mice becomes more pronounced with age, and mice lacking Shn3 are completely resistant to disuse osteopenia, a process that requires functional osteoclasts. Finally, selective deletion of Shn3 in the mesenchymal lineage recapitulates the high bone mass phenotype of global Shn3 KO mice, including reduced osteoclast-bone catabolism in vivo, indicating that Shn3 expression in mesenchymal cells directly controls osteoclastic bone formation and indirectly regulates osteoclastic bone resorption.

Results

We previously demonstrated that the adult-onset high bone mass phenotype of mice lacking Shn3 persists following WT bone marrow (BM) transplantation, and that Shn3-deficient BM cells display normal osteoclast differentiation and resorptive function in vitro (5). To rule out a role for Shn3 in regulating bone resorption in an osteoclast-intrinsic manner further, we performed reciprocal BM transplantation experiments. Good hematopoietic chimerism was achieved (Fig. S14), and, as expected, the Shn3-deficient high bone mass phenotype mapped to the genotype of the host animal (Fig. S1B). We conclude that Shn3 deficiency exerts its effect on bone mass through its expression in a radiosensitive (i.e., nonosteoclast) cell type in vivo.

Reduced Osteoclast Activity in Shn3-Deficient Mice in Vivo. We sought to characterize osteoclast activity in Shn3−/− mice further in vivo. Because these animals show dramatic elevations in osteoclast behavior as assayed by dynamic histomorphometry (5), we predicted that these mice might show a compensatory increase in serum markers of bone resorption. This was not the case. As shown in Fig. 1A and B, serum markers of collagen type 1 cross-linked C-telopeptide (CTX) and Pyd were significantly reduced in young (6-wk-old) Shn3−/− animals compared with WT littermates.

Previously, we reported comparable numbers of osteoclasts in WT and Shn3−/− skeletal tissue as assessed by histomorphometry just below the growth plate in the proximal tibia (5). Given the unexpected decrease in markers of bone resorption, we performed a more extensive histochemical investigation and observed qualitative reductions in osteoclast numbers in whole-mount skull preparations (Fig. 1C) and along the surfaces of increased diaphyseal trabecular bone (Fig. 1D) in Shn3−/− animals.

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Interestingly, this analysis confirmed normal numbers of osteoclasts just below the growth plate in the tibiae and femurs of Shn3−/− animals, suggesting that Shn3 may control osteoclast numbers and/or activity in a skeletal region-selective manner. In Fig. 1D, WT control sections were not analyzed because of the drastically different qualitative nature of bone mass and quality at the diaphyseal level. Taken together, these data suggest that Shn3 expression in nonosteoclastic cells may regulate osteoclast development and/or activity.

Mesenchymal Cells Lacking Shn3 Are Defective in Driving Osteoclastogenesis. Radioresistant cells of stromal/osteoblastic lineage are known to support osteoclast development in vitro in response to calcitropic stimuli by expression of RANKL (18–21). To interrogate the ability of Shn3-deficient cells to support osteoclastogenesis, we performed coculture experiments. In these assays, we observed that osteoblastic/stromal cells lacking Shn3 were defective in driving osteoclastogenesis in response to prostaglandin E2 (PGE2) and the β2-adrenergic receptor agonist isoproterenol but not PTH (Fig. 2A). We did not specifically address the resorptive capacity of the osteoclasts generated during these in vitro coculture assays. However, we do note that mice lacking Shn3 (Fig. 1A and B; see Fig. 5J) display reduced serum markers of bone turnover, indicating reduced osteoclast activity in vivo.

Morphological analysis of osteoclasts from these coculture assays revealed a consistent lack of giant multinucleated cells in the presence of Shn3−/− stromal cells (Fig. 2B). Consistent with this, RNA obtained from these cocultures showed reduced expression of terminal markers of osteoclast differentiation (cathepsin K and calcitonin receptor) comparing WT with Shn3−/− stromal cells/osteoblasts (Fig. 2C).

Reduced Levels of RANKL in the Absence of Shn3 in Vivo. To explore the expression pattern of RANKL in bone tissue lacking Shn3 further, we performed immunohistochemistry for RANKL and histochemical labeling for the osteoclast marker tartrate resistant acid phosphatase (TRAP). These studies demonstrated comparable levels of RANKL in growth plate hypertrophic chondrocytes (Fig. S2A) and p10 proximal metaphyseal bone lining cells (Fig. S2B) but qualitatively reduced levels of RANKL and TRAP in bone lining cells more distant from the growth plate (Fig. 1C). Shn3-deficient osteoblastic/mesenchymal cells are defective in driving osteoclastogenesis. (A) WT or Shn3−/− cells were cocultured with WT BM osteoclast precursors in the presence of the indicated calcitropic agents. After 5 d, tissue culture supernatants were assayed for TRAP activity via colorimetric readout (A405). Error bars represent SD of absorbance from three independent wells. *P < 0.05. This experiment was repeated four independent times with similar results. (B) Representative photomicrographs of cocultures. (Magnification: 40×) C  RNA was harvested from cocultures, and expression of cathepsin receptor and cathepsin K was determined by quantitative real-time PCR assay. Levels of the indicated genes were normalized to actin and expressed relative to levels obtained with WT osteoblastic cells. Error bars represent SD of values obtained from PCR triplicate assays. This experiment was repeated three independent times with similar results.
growth plate [Fig. 3 C (metaphyseal region) and D (diaphyseal region)]. Another cell type known to express RANKL is the CD4+ T helper 17 (Th17) cell (22). Shn3 is dispensable for Th17 cell differentiation and RANKL expression (Fig. S2 C). Taken together, these data indicate that Shn3 controls RANKL expression by osteoblastic/stromal cells in vivo but not in hypertrophic chondrocytes and Th17 cells.

**Reduced Expression of RANKL by Osteoblastic/Stromal Cells Lacking Shn3.** Primary calvarial osteoblasts lacking Shn3 show reduced levels of RANKL mRNA after a 7-d in vitro culture period. These cells also display increased levels of the antiosteoclastogenic factor OPG compared with WT cells (Fig. 3 E). However, RANKL levels are known to decrease, and OPG levels to increase, during the course of osteoblast differentiation using this in vitro system (23). To circumvent the possibility that the differences observed reflect disparate differentiation states, we acutely altered Shn3 levels in transformed osteoblast cell lines using lentivirus-based shRNA-mediated gene silencing and overexpression. As shown in Fig. 3 F, these manipulations led to the previously observed alterations in RANKL levels but not in OPG levels.

As shown in Fig. 24, Shn3-deficient osteoblastic cells fully support osteoclastogenesis in response to PTH but not in response to isoproterenol and PGE2. Accordingly, Shn3−/− osteoblastic cells are defective in up-regulating RANKL in response to isoproterenol and PGE2 but not in response to PTH (Fig. 3 G and H). Acute reductions in Shn3 levels by shRNA-mediated gene silencing reduced responsiveness to PGE2 as expected (Fig. S3 A). When coculture experiments were performed in the presence of a neutralizing anti-OPG antibody, the defect in the ability of Shn3−/− osteoblasts to drive osteoclastogenesis was reversed (Fig. S3 B), suggesting that reduced RANKL expression by these cells contributes to their inability to support osteoclast differentiation.
Shn3 Controls Expression of RANKL through cAMP Response Element Binding Protein and an Upstream Regulatory Element. We next sought to determine the mechanism whereby Shn3 controls RANKL expression. Tnfsf11 gene expression is controlled by a variety of distal and proximal regulatory regions (21, 24, 25). We focused on a conserved regulatory region located 76 kb upstream of the transcriptional start site that had been described by two independent groups as important for calcitropic agent responsiveness in vitro and in vivo (26). Shn3 overexpression can enhance activity of this upstream promoter element but not that of the proximal RANKL and OPGL gene regulatory regions (Fig. S4A).

Transcription factors, such as vitamin D receptor (VDR), Runx2, cAMP response element binding protein (CREB), and STAT3, are known to associate with this RANKL gene regulatory region (24, 27). We found that Shn3 does not activate transcription from this reporter when the CREB binding sites are deleted (Fig. S4B). In overexpression studies, Shn3 can bind CREB (Fig. S4C) and regulates its transcriptional activity (Fig. S4 D and E). Taken together, these data suggest that Shn3 controls RANKL expression in osteoblastic cells in vivo and in vitro, at least in part, through a mechanism that involves binding to CREB in the context of a conserved upstream regulatory region.

Shn3-Deficient Animals Are Protected from Bone Loss Attributable to Aging and Disuse but Not Secondary Hyperparathyroidism. To explore the functional impact of RANKL regulation by Shn3 further, three models of stimulated bone resorption were used: aging, dietary-induced hypocalcemia, and disuse osteopenia. Young (8-wk-old) Shn3−/− mice show high bone mass associated with an increased rate of bone formation and decreased resorptive markers (5) (Fig. 1A). Interestingly, aged (>3-mo-old) Shn3−/− animals continue to accrue bone, ultimately leading to obliteration of the marrow cavity and extramedullary hematopoiesis (not shown) but display reduced histomorphometric indices of bone formation (Fig. 4A). This phenotype of increasing bone mass with reduced bone formation rates in aged Shn3−/− mice is associated with reduced serum levels of the resorptive markers Pyd and CTX and RANKL (Fig. S5) (A). These findings suggest that the continued accrual of bone mass in aged Shn3−/− mice is driven by reduced bone resorption. Therefore, it appears that the cause of the high bone mass phenotype in Shn3-deficient mice varies with age. Young mice lacking Shn3 predominantly display an osteoanabolic phenotype, although as the mice age, bone formation slows down and the major phenotype is that of reduced bone resorption. Further studies are required to identify and dissect the age-related switch whereby this transition occurs.

Due to PTH being able to increase RANKL expression normally in Shn3-deficient osteoblastic/mesenchymal cells, we wondered whether secondary hyperparathyroidism in vivo would lead to bone loss in Shn3-deficient mice. To test this notion, we placed 11-wk-old WT and Shn3−/− animals on a control diet or a low-calcium diet for 2 wk (28). Shn3−/− mice showed reductions in trabecular bone volume/total volume (BV/TV) (Fig. 4B) and increases in serum markers of bone resorption (Fig. S5B), and were able to maintain normocalcemia (Fig. S5C) in this model. Percent reductions in bone loss comparing WT with Shn3−/− mice were comparable. These data are consistent with our observations that Shn3 is dispensable for PTH-mediated induction of osteoclastogenesis in coculture models (Fig. 2A) and RANKL up-regulation in osteoclastic cells (Fig. 3H). Moreover, these data suggest that the Shn3−/− bone matrix is not “unresorbable,” thereby discounting the notion that biochemical properties alone cause the high bone mass phenotype observed in these mice.

We next used an osteoblast-driven model of disuse osteopenia (29, 30) to test the physiological relevance of our findings further. In this model, botulinum toxin is injected into the hind-limb muscles (quadriceps and/or calf muscle groups), which leads to transient muscle paralysis and subsequent bone loss in the ipsilateral but not contralateral tibia. Both genotypes showed similar muscle atrophy (Fig. SSD). The subset of imaged Shn3−/− animals demonstrated no changes in the contralateral limb attributable to muscle paralysis (−4.2 ± 3.9%) during the 21-d study period. Although WT animals showed expected ipsilateral bone loss following this manipulation, Shn3−/− animals were completely protected at multiple time points (Fig. 4 C and D). This observation further supports a model in which Shn3 plays an important role in regulating bone resorption in response to a variety of physiological stimuli.

Deletion of Osteoclasts Does Not Alter the Diaphyseal High Bone Mass Phenotype in Shn3-Deficient Mice. Because we had observed reduced diaphyseal osteoclast populations in Shn3−/− mice (Fig. 1D), we wondered whether elimination of osteoclasts in the setting of Shn3 deficiency would alter this phenotype in any way. To this end, we intercrossed mice lacking Shn3 with animals harboring a conditional Nfatc1 allele and bearing an Mx1-Cre
Recently, it has been suggested that Shn3 (also known as ZAS3) may play a cell-intrinsic role in regulating osteoclastogenesis by regulating receptor activator of nuclear factor-κB signaling (33). Consistent with our findings, these authors observed increased bone mass with increased fracture resistance in an independent model of germline Shn3 deletion. Similar to our findings, Liu et al. (33) observed decreased numbers of osteoclasts in vivo. However, we believe that our current findings of persistent high bone mass phenotype in WT animals receiving Shn3-deficient BM transplantation (Fig. S1B) and, more importantly, a high bone mass phenotype in mice lacking Shn3 selectively in mesenchymal cells (Fig. 5) highlight that the predominant effect of Shn3 deficiency in vivo maps to the mesenchymal, rather than hematopoietic, compartment. Moreover, in our previous experiments (5), we found no defect whatsoever in osteoclast differentiation and function comparing WT and Shn3-deficient BM cells.

It is interesting that Shn3 deficiency leads to qualitative reductions in osteoclasts in calvariae and diaphyseal bone but not in metaphyseal regions. This observation suggests that different cell populations are responsible for RANKL expression and osteoclastogenesis at these different anatomical sites. Indeed, it has previously been suggested that hypertrophic chondrocytes play a critical role in driving the differentiation of metaphyseal osteoclasts/chondroclasts during development, a finding that is consistent with preserved expression of RANKL in these cells in situ in Shn3−/− mice (34, 35). More recently, cell type-specific RANKL deletion has shown that hypertrophic chondrocytes play a major role in regulating metaphyseal osteoclasts (19), whereas late-stage [dentin matrix protein 1 (DMP1)-expressing] osteoblasts/osteocytes are more important sources of RANKL for osteoclasts involved in adult skeletal remodeling (18, 19). Future studies are required to interrogate the activity of Shn3 in DMP1-expressing bone cells.

We found that Shn3 controls RANKL expression, at least in part, through a regulatory region 76 kb upstream of the transcriptional start site. Just as Shn3 interacts with Jun to coordinate AP-1 complexes in the context of the IL-2 gene in T cells (6), Shn3 seems to function as a context-dependent transcriptional coactivator in the setting of the RANKL gene in mesenchymal cells. Little is known about its structure/function relationship, and the mechanism whereby it functions as a transcriptional coactivator in the setting of the RANKL gene is likely attributable to both increased bone formation and reduced bone resorption. Shn3-deficiency seems to uncouple these processes.

Discussion
We had previously observed that Shn3 functions as an important inhibitor of osteoclastic bone formation (5). Here, we show that the dramatic high bone mass phenotype seen in Shn3-deficient animals is likely attributable to both increased bone formation and reduced bone resorption. Shn3-deficiency seems to uncouple these processes.

Reduced Bone Resorption in Mice Lacking Shn3 Only in Mesenchymal Cells
To interrogate further the mechanism whereby Shn3 controls the balance between bone formation and resorption in vivo, we generated mice harboring a Shn3 allele in which exon 4 is flanked by loxP sites (Fig. S7A), hereafter called Shn3f/f mice. To determine definitively whether Shn3 expression in mesenchymal cells plays a role in controlling bone resorption, we intercrossed Shn3f/f mice with mice expressing Prx1-Cre, which expresses in cells in situ in Shn3f/f mice with mice expressing Prx1-Cre, which expresses in cells in situ in Shn3f/f mice (data not shown), confirming that Prx1-Cre deletion causes high bone mass through a local (i.e., not humoral) mechanism. Furthermore, femurs from Prx1-Cre Shn3f/f mice showed improved performance in biomechanical testing compared with transgene-negative controls (Fig. S7 C–E).

Histomorphometric analysis was then performed to investigate further the effects of selective Shn3 deletion in mesenchymal cells. As was the case in global Shn3 deletion, removing Shn3 only in Prx1-expressing cells led to a robust increase in bone formation rate (Fig. 5F) and serum procollagen type 1 amino-terminal propeptide (P1NP) levels (Fig. 5G). In contrast to our previous histomorphometry data from global Shn3-deficient animals, we observed a significant increase in osteoblast numbers when Shn3 was deleted with Prx1-Cre (Fig. 5H).

Based on our proposed model that Shn3 deficiency in the mesenchymal lineage leads to a cell-extrinsic reduction in bone catabolism, in part, via reduced RANKL expression, we expected that Prx1-Cre Shn3f/f mice would show reduced osteoclast numbers and activity in addition to increased bone anabolism. Indeed, Prx1-Cre transgene-positive mice showed reduced osteoclast populations via histomorphometry (Fig. 5I) and reduced serum CTX levels (Fig. 5J). Taken together, these findings further solidify our model that Shn3 expression in mesenchymal cells directly controls osteoblastic bone formation and indirectly regulates osteoclastic bone resorption.
coactivator in certain situations. It is interesting that Shn3, like ATF4, is required for RANKL up-regulation in osteoblastic cells in response to isoproterenol but not PTH (36). Future work is required to determine if there is a relationship between Shn3 and ATF4.

Mice lacking Shn3 are completely protected from bone loss induced by muscle paralysis achieved via Botox-induced transient paralysis of calf muscles. Bone loss in this model is osteoclast-driven, as evidenced by complete protection observed in the absence of NFATc1 (30). Although it is clear that osteoclastic bone resorption via RANKL is critical for bone loss in the model, the cellular source of RANKL remains unclear. Recent work suggests that an intercellular communication pathway between osteocytes, RANKL-expressing cells, and osteoclasts involving the secreted protein sclerostin may be involved (37, 38).

Mice lacking Shn3 in DMP1-expressing cells are protected from bone loss in a similar model (19). Future studies will be required to determine what, if any, role there is for Shn3 in mechanotransduction in osteocytes. In summary, we have shown that the high bone mass phenotype of Shn3-deficient mice is likely attributable to a combination of increased bone formation and reduced bone resorption. We do note that it is difficult to conclude from our current study which aspect of the Shn3-deficient phenotype (increased bone formation or reduced bone resorption) is more important for the overall high bone mass phenotype. Additional studies are required to address this important outstanding question definitively. That being said, it is provocative that the aged Shn3-deficient mice maintain a dramatic high bone mass phenotype yet show reduced bone formation rates. Based on our findings, one would predict that small-molecule Shn3 inhibitors would increase bone mass through both of these mechanisms.

Materials and Methods

Mice. Shn3-deficient animals were as previously described (5). Conditional Shn3 KO mice were generated at Taconic and on a C57/BL6 background. Prx-1-Cre mice were purchased from the Jackson Laboratory. Conditional NFATc1-deficient mice were as described (31) and intercrossed with Shn3<sup>−/−</sup> mice.

Serum Measurements. Levels of Pyd (Quidel), CTX (IDS), RANKL (R&B Systems), and calcium (BioAssay) were determined per the instructions of the manufacturers.

Histology and Immunohistochemistry. Decalcified femur sections were stained with RANKL antibodies (sc-7628, Santa Cruz Biotechnology) and TRAP (Sigma).

Osteoblast/Osteoclast Cocultures. Calvarial osteoblasts were grown in medium supplemented only with 10% (vol/vol) FCS and antibiotics. Osteoclast precursors were isolated from adult male BM cells precultured in macrophage-colony stimulating factor (M-CSF) and isolated by Histopaque 1083 (Sigma) gradient centrifugation.

Luciferase Assays and Coimmunoprecipitations. Experiments were performed using previously described methods (5).

In Vivo Muscle Paralysis. Experiments were performed as previously described (29) using 6-mo-old WT and Shn3<sup>−/−</sup> mice on a BALB/c genetic background. Additional details are provided in SI Materials and Methods.

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