Id1 Represses Osteoclast-Dependent Transcription and Affects Bone Formation and Hematopoiesis

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Introduction

Hematopoietic stem and progenitor cell (HSPC) self-renewal, proliferation, and differentiation are tightly regulated processes that depend on the microenvironment in which they reside [1,2]. This microenvironment is located in the bone marrow (BM) where hematopoietic and bone remodeling cells, osteoblasts and osteoclasts, are anatomically juxtaposed. Due to the close proximity of these cells, it is reasonable to hypothesize that these hematopoietic and bone cells communicate with and/or regulate one another. However, the complex microenvironmental interactions and the molecular pathways that govern these connections occurring at this bone-bone marrow interface are still not well understood.

At the bone surface, the dynamic process of bone remodeling involves the constant interaction between bone-forming osteoblasts and bone-resorbing osteoclasts [3–7]. While the roles of osteoblasts and osteoclasts in bone remodeling have been well established, recent studies have demonstrated that these cells are also crucial components of the hematopoietic stem cell (HSC) niche. Accumulating evidence suggests that osteoblasts enforce the quiescence of HSCs, while osteoclasts induce hematopoietic...
activity. In particular, osteoblasts have been shown to physically anchor HSCs to the endosteal surface of the trabecular regions of the bone, thereby keeping them in an undifferentiated and quiescent state [9,10]. Conversely, osteoclasts secrete bone-resorbing enzymes, such as cathepsin K (CTSK), which promote mobilization of HSCs from their quiescent state. This process involves the cleavage of cytokines, such as stem cell factor (SCF), that are responsible for HSC anchorage to the niche [11,12]. Thus, the balance between osteoblastic and osteoclastic activity not only dictates healthy bone volume but also contributes to maintaining hematopoietic homeostasis.

Although a great deal of progress has been made in defining the BM microenvironment, the molecular regulators involved, their interactions, and how they affect bone remodeling, hematopoiesis, and cell fate decisions are largely unknown. Here, we report a new role for the inhibitor of differentiation (Id1) gene in bone remodeling and maintenance of the BM microenvironment. Id1 is one of four proteins in a family of transcriptional regulators that all function by inhibiting basic helix-loop-helix (bHLH) transcription factors from binding DNA [13,14]. Id1 proteins have a helix-loop-helix domain that allows them to dimerize with bHLH transcription factors, but lack a DNA-binding domain. As a result, Id-bHLH transcription factor heterodimers cannot bind to DNA, leading to the disruption of the events mediating the cellular differentiation and proliferation of a variety of different cell types.

In this study, we report several lines of evidence that Id1 is a critical regulator of bone and BM homeostasis. In particular, Id1−/− mice exhibited an osteoporotic phenotype, with significantly reduced bone mineral content and density. This is a novel finding, as Id1 has not been previously linked to osteoporosis. We also show that the expression of genes necessary for osteoclast maturation, such as CTSK, TRAP, and Oscn, are upregulated in osteoclasts derived from Id1−/− mice and repressed in osteoclasts overexpressing Id1, suggesting that Id1 is a master regulator of a set of genes required for osteoclast function and bone resorption. Examination of the hematopoietic compartment of Id1−/− mice revealed an increase in myeloid differentiation and HSPC proliferation. Thus, in the absence of Id1, the HSPC pool is likely depleted by the increased flux towards myeloid differentiation, resulting in osteoclast-driven changes to the BM microenvironment. Results from this study will shed light on the molecular cues at the bone-bone marrow interface that regulate both bone and BM homeostasis. Moreover, Id1 may potentially be a novel therapeutic target for the treatment of skeletal disorders.

Results

Id1−/− Mice Exhibit an Osteoporotic Phenotype

To assess the effect of loss of Id1 on skeletal structure and function, we analyzed the bones of Id1−/− mice. Id1−/− mice were born at the expected mendelian frequency and showed no obvious abnormalities. We observed that Id1−/− mice were slightly, but significantly smaller than wild-type littermates (Figure 1A, Figure S1A). Morphological analysis of the skeleton revealed a phenotype that previously has not been reported in these mice. Microradiographs showed a marked decrease in the radiodensity of Id1−/− long bones, a feature that was most pronounced in regions of rapid longitudinal growth, such as the distal femur and the proximal tibia (Figure 1B). Three-dimensional microstructural analysis using micro-computed tomography (micro-CT) confirmed that loss of Id1 resulted in a significant reduction in bone mass (Figure 1C). Furthermore, this analysis revealed significant decreases in bone volume fraction, bone mineral content, bone mineral density (BMD), trabecular thickness, and marrow area (Table 1, Figure S1B). To determine whether the differences in the microstructural properties influenced the mechanical strength of the bone, we performed a three-point bending test on the femurs of wild-type and Id1−/− mice. The bending moment and bending rigidity were significantly decreased in Id1−/− mice (Figure 1D), indicating that the bones of Id1−/− mice are weaker and more prone to fractures as compared to wild-type bones. Together, these results demonstrate an osteoporotic phenotype in Id1−/− mice, characterized by low bone mass and micro-architectural deterioration of bone tissue, with a resultant increase in bone fragility and susceptibility to fracture.

Osteoblast Development and Function Appear Normal in Id1−/− Mice

Since proper bone remodeling is determined by a delicate balance between the bone-forming osteoblasts and the bone-resorbing osteoclasts, the observed osteoporosis in Id1−/− mice could be a consequence of decreased osteoblast activity, increased osteoclast activity, or both. We first examined whether the loss of Id1 affects osteoblast development and function. Staining for osteoblast marker, pro-collagen I, on femur sections showed normal ratios of osteoblast numbers to bone surface in Id1−/− mice as compared to wild-type littermates (Figure 2A). Double tetracycline labeling also showed normal mineral apposition rates (Figure 2B), indicating normal osteoblast activity in vivo. In addition, the expression of osteoblast-associated cytokines receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), and stromal cell-derived factor 1 (SDF-1) was not altered in Id1−/− and wild-type bones (Figure S2). Thus, the decrease in bone mass in Id1−/− mice did not appear to be due to defects in osteoblast number or function.

Id1 Negatively Regulates Osteoclastogenesis In Vivo and In Vitro

Next, we examined whether Id1 affects osteoclast differentiation and function. Enzyme histochemistry for osteoclast marker, tartrate-resistant acid phosphatase (TRAP), on femur sections showed significantly increased osteoclast numbers in Id1−/− long bones (Figure 2C). To determine whether increased osteoclast differentiation could be caused by a cell-autonomous role for Id1 in the osteoclast lineage, BM mononuclear cells were isolated and differentiated in vitro. In support of our in vivo findings, differentiation of Id1−/− BM cells gave rise to 1.6 fold more TRAP-positive osteoclasts as compared to wild-type cells (Figure 2D). Moreover, in vitro resorption assays showed that Id1−/− osteoclasts were functionally active (Figure 2E). Thus, our in vivo and in vitro data suggest that Id1 has a cell-intrinsic role as a negative regulator of osteoclast differentiation.

Myeloid Differentiation Is Enhanced in Id1−/− Mice

Whereas osteoblasts arise from mesenchymal stem cells, osteoclasts differentiate from hematopoietic monocyte/macrophage precursors of the myeloid lineage. Consequently, the observed osteoporotic bone phenotype and increased osteoclastogenesis in Id1−/− mice could be due to altered homeostasis of myeloid precursor cells. To explore this possibility, we analyzed various subsets of committed myeloid progenitor cells in the BM, represented by the lineage negative, c-kit+ and sca-1+, (LKS−) progenitor population. The common myeloid progenitor (CMP) population, the most immature of the myeloid progenitor cells, was decreased in Id1−/− mice, while the more mature megakaryocyte-erythrocyte progenitor (MEP) subset was significantly expanded.
Additionally, analysis of differentiated myeloid cell subpopulations showed a higher fraction of CD11b⁺ monocytes in the BM (Figure 3B) and a higher fraction of monocytes and neutrophils in the peripheral blood of Id1⁻/⁻ mice compared to wild-type littermates (Figure 3C, Table S1). Thus, in Id1-deficient mice, myeloid differentiation is increased, indicating that Id1

Figure 1. Skeletal phenotype of Id1⁻/⁻ and wild-type mice at 6 weeks of age. (A) Photograph of a representative Id1⁻/⁻ and wild-type mouse. (B) X-ray microradiographs of hindlimbs. White arrows indicate trabecular bone regions. (C) Representative micro-CT images of the femoral trabecular and cortical areas in wild-type and Id1⁻/⁻ mice. (D) Three-point bending tests for evaluating the mechanical strength of femurs from wild-type (n = 8) and Id1⁻/⁻ (n = 8) mice. The parameters measured include the bending moment and the bending rigidity (*P<0.05, **P<0.01). Error bars represent ±S.E.M.

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Table 1. Characteristics of femurs in 6-week old wild-type and Id1<sup>−/−</sup> mice.

|                      | WT (n = 8) | Id1<sup>−/−</sup> (n = 8) | p-value |
|----------------------|------------|---------------------------|---------|
| Bone Volume Fraction | 0.199 ± 0.024 | 0.135 ± 0.021            | <0.001  |
| Bone Mineral Content (mg) | 1.514 ± 0.176 | 1.015 ± 0.173         | <0.001  |
| Bone Mineral Density (mg/cc) | 665.8 ± 16.25 | 578.1 ± 19.37          | <0.01   |
| Trabecular Thickness (mm) | 0.035 ± 0.003 | 0.031 ± 0.003       | <0.05   |
| Marrow Area (mm<sup>2</sup>) | 1.022 ± 0.065 | 0.552 ± 0.019         | <0.001  |

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normally acts to maintain myeloid progenitors in an undifferentiated state. These findings are consistent with the previously suggested role of Id1 in restraining both myeloid [15] and erythroid [16] differentiation.

To determine whether the loss of Id1 alters the kinetics of myeloid differentiation, we challenged Id1<sup>−/−</sup> and wild-type mice with a single, low dose of 5-fluorouracil (5-FU), a myeloblastic drug that mobilizes stem cells from their quiescent state to reconstitute the hematopoietic system (Figure 3D). We observed an increase in total numbers of white blood cells at day 15 after 5-FU administration due to an increase in both neutrophils and monocytes (Figure 3E), demonstrating that Id1 also regulates myeloid differentiation during hematopoietic recovery after myeloablation.

Proliferation of Id1<sup>−/−</sup> LSK Cells Is Enhanced In Vivo, but Not In Vitro

After a single, low dose injection of 5-FU, hematopoietic recovery in Id1<sup>−/−</sup> mice was delayed by 3 days, as compared to wild-type littermate controls (Figure 3D). To test whether this delay was due to increased cell cycling of Id1<sup>−/−</sup> progenitor cells, we performed BrdU labeling at steady state and during reconstitution following myeloablation. We observed a significant increase in the number of BrdU-labeled, Lin<sup>−</sup> sca-1<sup>+</sup> c-kit<sup>+</sup> (LSK) cells both at steady state (Figure 4A) and during hematopoietic recovery (Figure 4B), suggesting that a higher fraction of Id1 deficient LSK cells enter the cell cycle as compared to wild-type cells. In contrast, when we isolated and cultured the LSK cells in vitro, Id1<sup>−/−</sup> and wild-type cells proliferated at the same rate (Figure 4C), signifying that Id1 might indirectly affect the proliferation of LSK cells via factors secreted in the BM microenvironment.

To investigate whether the enhanced proliferation status of LSK cells in Id1<sup>−/−</sup> mice would lead to premature exhaustion of HSPCs after continuous myeloblastic stress, we treated wild-type and Id1<sup>−/−</sup> mice weekly with 5-FU. We found that the survival rate was much lower in Id1<sup>−/−</sup> mice than in wild-type controls (Figure 4D). Analysis of blood and BM from both Id1<sup>−/−</sup> and wild-type mice severely affected by the 5-FU treatment showed a complete absence of myeloid cells, low hemoglobin/RBC counts, and low platelet counts (data not shown). These observations suggested that both Id1<sup>−/−</sup> and wild-type mice succumbed to BM failure, but that Id1<sup>−/−</sup> mice did so at a faster rate. Thus, Id1 deficient LSK cells showed altered proliferation kinetics with more progenitor cells entering cell cycle both at steady state and under conditions that lead to hematopoietic stress.

Intrinsic and Extrinsic Mechanisms Operate in the Bone Marrow Microenvironment

Recently, a number of studies have highlighted the importance of both intrinsic and extrinsic cues within the BM to maintain effective control over HSPCs [17,18]. To explore the relative contributions of these intrinsic and extrinsic signals in the BM, we performed a series of BM transplant experiments in which we subjected the mice to weekly injections of 5-FU. Wild-type BM was transplanted into Id1<sup>−/−</sup> mice and Id1<sup>−/−</sup> BM was transplanted into wild-type mice. As a control, wild-type BM was transplanted into wild-type mice and Id1<sup>−/−</sup> BM was transplanted into Id1<sup>−/−</sup> mice. For the purpose of following engraftment by Y chromosome specific PCR, all recipient mice were female and all donor mice were male. Six weeks after transplantation, these mice were treated weekly with 5-FU and their survival was monitored (Figure 5A). If Id1 is exclusively an intrinsic regulator, we would expect a complete rescue of the hematopoietic phenotype, such that Id1<sup>−/−</sup> mice transplanted with wild-type BM would have the same survival rate as wild-type mice transplanted with wild-type BM. However, the hematopoietic rescue that we observed was partial. Transplantation of wild-type BM into Id1<sup>−/−</sup> mice significantly increased their survival, but not to the level of wild-type mice transplanted with wild-type BM. Similarly, transplantation of Id1<sup>−/−</sup> BM into wild-type mice significantly reduced their survival, but not to the same level as Id1<sup>−/−</sup> mice transplanted with Id1<sup>−/−</sup> BM.

To investigate whether BM transplantation could have a similar effect in rescuing the bone phenotype seen in Id1<sup>−/−</sup> mice, we performed the same BM transplants as described above and analyzed the bone properties by micro-CT. To allow sufficient time for bone turnover to occur, we waited 3.5 months post-transplantation before sacrifice. Micro-CT analysis showed that transplantation of wild-type BM into wild-type mice and Id1<sup>−/−</sup> BM into Id1<sup>−/−</sup> mice recapitulated the same bone phenotype seen in wild-type and Id1<sup>−/−</sup> mice, respectively. Id1<sup>−/−</sup> mice transplanted with Id1<sup>−/−</sup> BM had significantly reduced BMD and trabecular bone compared with wild-type mice transplanted with wild-type BM (Figure 5B). Following the same reasoning as above, we would expect that if Id1 is exclusively an intrinsic regulator, we would see a complete rescue of the bone phenotype, such that Id1<sup>−/−</sup> mice transplanted with wild-type BM would have the same bone microstructural properties as wild-type mice transplanted with wild-type BM. However, once again, the rescue that we observed was partial. Transplantation of wild-type BM into Id1<sup>−/−</sup> mice significantly increased their BMD and trabecular bone, but not to the level of wild-type mice transplanted with wild-type BM. Correspondingly, transplantation of Id1<sup>−/−</sup> BM into wild-type mice significantly reduced their BMD and trabecular bone, but not to the same level as Id1<sup>−/−</sup> mice transplanted with Id1<sup>−/−</sup> BM. Together, these results show that the loss of Id1 impacts both intrinsic and extrinsic signals in the BM microenvironment.

To rule out the possibility that the partial rescue of hematopoietic and bone phenotypes in these mice was not due to post transplant donor/host chimerism, we performed qPCR for the Y chromosome specific, sex determining region (SRY) gene. Complete donor chimerism is often defined as the detection of greater than 95% donor DNA [19]. By this definition, all of the mice we transplanted achieved complete donor hematopoietic chimerism (Figure S3), indicating the sustained engraftment of donor-derived cells. Therefore, it is unlikely that autologous hematopoiesis had any impact on the partial rescue of phenotypes in these mice.

Next, we examined the microenvironment of Id1<sup>−/−</sup> and wild-type mice to explore differences that might be responsible for the observed phenotypes. Recently, it was shown that CTSK, a proteolytic enzyme secreted by osteoclasts, can cleave cytokines that regulate stem cell proliferation, survival, and mobilization
We found that CTSK was upregulated at the mRNA and protein level in the BM of Id1<sup>−/−</sup> mice as compared to wild-type littermates (Figure 5C,D). Expression levels of other cathepsins remained unchanged (Figure S4). One of the cytokines that can be cleaved by CTSK is SCF, which is involved in anchoring stem cells to the niche. Accordingly, we found elevated levels of SCF (Figure 2).
Figure 3. Enhanced myeloid differentiation in Id1<sup>−/−</sup> mice. (A) Representative flow-cytometric profiles of myeloid progenitor populations (CMP, GMP, MEP) in wild-type and Id1<sup>−/−</sup> BM (n = 6). (B) Monocytes in the BM were measured by flow cytometry using antibodies against CD11b and CD45 (n = 8). (C) Monocytes in the blood were measured by a complete blood count test (n = 40). (D) White blood cell (WBC) counts in wild-type and Id1<sup>−/−</sup> mice during hematopoietic challenge. Mice were given a single injection of 5-FU and bled every 3 days (n = 5). (E) Monocyte and neutrophil counts measured 15 days after 5-FU injection (n = 5). (A–E) *P < 0.05, **P < 0.01, ***P < 0.001. Error bars represent ± S.E.M. doi:10.1371/journal.pone.0007955.g003
weekly injections of 5-FU (P = 0.013; n = 10). Error bars represent proliferation of sorted LSK cells in response to cytokine stimulation evaluate the percentage of BrdU injection of 5-FU. Flow cytometry was performed 15 days post 5-FU to proliferation of LSK cells from mice challenged with a single in vivo proliferation of LSK cells, at steady state, assessed by flow cytometry for the percentage of BrdU. In vivo proliferation of LSK cells, at steady state, assessed by flow cytometry for the percentage of BrdU.

A Mechanism for Id1 Function in Bone and the Bone Marrow Microenvironment

Our findings suggested that Id1 is critical for proper function of the BM microenvironment and that loss of Id1 impacts HSPC homeostasis via osteoclast-induced modifications in the bone. Thus, we hypothesized that Id1 acts to inhibit differentiation of both myeloid cells and osteoclasts, thereby keeping HSPCs quiescent and in the BM (Figure S5). In support of this model, we observed that osteoclast differentiation was increased in the absence of Id1, leading to increased secretion of CTSK. As a result, SCF cleavage by CTSK released soluble SCF into the circulation and mobilized HSPCs out of the BM.

Since Id proteins are known to negatively regulate bHLH transcription factor function, we hypothesized that Id1 interacts with specific bHLH transcription factors to inhibit osteoclast differentiation. Association between Id proteins and Mitf, a bHLH transcription factor that targets genes such as CTSK, TRAP, and osteoclast associated receptor (Oscar), has been demonstrated in vitro [20]. During osteoclast differentiation, Mitf has been shown to cooperate with other transcription factors, PU.1 and nuclear factor of activated T cells 1 (NFATc1), to synergistically induce expression of CTSK, TRAP, and Oscar [21–24]. In addition, a positive feedback loop has been described between Oscar and NFATc1, such that increased Oscar expression leads to increased NFATc1 expression [25]. To investigate the role of Id1 in transcriptional regulation of these osteoclast-associated genes, we performed qPCR for CTSK, TRAP, Oscar, and NFATc1 in BM isolated from Id1−/− and wild-type mice. In the absence of Id1, these genes were significantly upregulated (Figure 6A), suggesting that the loss of Id1 may allow increased binding of Mitf to PU.1 and NFATc1, subsequently resulting in increased expression of target genes.

To explore this further, we transplanted lethally irradiated Id1−/− mice with Id1−/− BM that was transduced with a lentivirus vector overexpressing Id1 (Figure S6A, B). In these transplanted mice, the genetic expression profile was reversed. CTSK, TRAP, Oscar, and NEAT-1 expression were significantly downregulated in mice overexpressing Id1, as compared to mice receiving the empty vector control (Figure 6B). Furthermore, overexpression of Id1 in the BM of transplanted mice led to decreased plasma levels of SCF (Figure 6C) and the restoration of BM myeloid progenitor cell populations (Figure 6D).

Finally, we assessed whether phenotypic effects due to the loss of Id1 could be reversed by abrogating CTSK activity. We transplanted lethally irradiated Id1−/− mice with Id1−/− BM that was transduced with a lentivirus vector containing a shRNA targeted against CTSK (shCTSK) (Figure S7A). A shRNA targeted against GFP (shGFP) was used as a control. To allow sufficient time for bone turnover to occur, we performed micro-CT on the bones of these mice 3.5 months after transplantation. Mice transplanted with BM containing shCTSK had increased bone volume fraction, trabecular number and thickness (Figure 6E, Table S2, Figure S7B). In comparison, control mice exhibited a severely reduced and more separated trabecular bone network, similar to that of Id1−/− mice. Hence, inhibition of CTSK activity was able to restore trabecular bone in Id1−/− mice. Overall, our results support a model wherein Id1 interacts with bHLH transcription factor Mitf to inhibit transcriptional activation of osteoclast-associated genes, subsequently leading to regulation of bone remodeling and the BM microenvironment.

Discussion

The roles for Id1 in differentiation, cell growth, senescence, angiogenesis, and tumorigenesis have been extensively studied [14,26–29]. Yet, how Id1 deficiency contributes to aging-associated phenotypes, such as osteoporosis, has not been well characterized. Previous studies have indicated that Id proteins likely inhibit osteoclast differentiation from BM monocyte cells grown in vitro [20]. However, the present study is the first to demonstrate the direct correlation between Id1 and bone growth in an in vivo animal model. Several novel findings have been generated from this study. First, Id1−/− mice exhibited osteoporosis due to increased osteoclast differentiation, a phenotype that has not been previously described in these mice. Second, the osteoporotic phenotype was associated with a reduction in the
Figure 5. Loss of Id1 alters extrinsic niche factors. (A) Survival over time of BM transplanted mice treated with weekly injections of 5-FU. Lethally irradiated wild-type and Id1−/− mice were transplanted with either wild-type or Id1−/− BM. After 6 weeks to allow for hematopoietic reconstitution, the mice were injected weekly with 5-FU and their survival was monitored (n = 10). (B) Representative micro-CT images of the femoral distal trabecular region and bone mineral density measurements in BM transplanted mice (**P < 0.01, ***P < 0.001; n = 5). (C) CTSK staining of femoral sections (400X). Arrowheads indicate CTSK+ osteoclasts stained in brown (**P < 0.01; n = 5). (D) Level of expression of CTSK in the BM of wild-type and Id1−/− mice (**P < 0.01; n = 8). (E) SCF levels in plasma assayed by ELISA (***P < 0.001; n = 8). Error bars represent ± S.E.M.

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Figure 6. A proposed mechanism for Id1 function in the BM microenvironment.

(A) Expression of osteoclast-associated genes, TRAP, Oscar, and NFATc1 in the BM of wild-type and id1−/− mice (n = 6). (B) Expression of osteoclast-associated genes from the BM of transplanted mice (n = 6). Lin− BM cells from id1−/− mice were transduced with lentivirus containing PGEW-Id1 or PGEW-empty vector overnight and transplanted into id1−/− mice. After 8 weeks, the mice were sacrificed and BM from the femur was collected for qPCR analysis. (C) SCF levels in plasma assayed by ELISA. (D) Representative flow-cytometric profiles of myeloid progenitor populations (CMP, GMP, MEP) in mice transplanted with PGEW-Id1 or PGEW-empty BM (n = 6). (E) Representative micro-CT images of the femoral distal trabecular region in id1−/− mice transplanted with BM containing a shRNA targeted against CTSK or GFP. (A-D) *P<0.05, **P<0.01, ***P<0.001. Error bars represent ±S.E.M.

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number of reserve HSPCs and an increased tendency toward myeloid differentiation. Id1 impact on myeloid and osteoclast cell numbers and their shared microenvironment can help explain the functional significance of the drive toward the myeloid compartment, as seen in our results, as well as others [15,30]. Thus, the Id1−/− mouse represents a model that simulates the bone and BM features seen in an aged host. Disruption of normal osteoclast function via the loss of Id1 had profound effects on the behavior and activity of cells in the BM microenvironment. These observations demonstrate that bone cells provide critical regulatory cues to the hematopoietic system, confirming that crosstalk between these different cell types at the bone-marrow interface does indeed occur.

The BM microenvironment is a specialized region of the bone responsible for a host of homeostatic functions, including maintenance of stem cell number, protecting stem and progenitor cells from exhaustion over the lifetime of an organism, and providing cues that dictate the self-renewal or differentiation of HSPCs to other cell types [1,31]. Within this niche, the endosteme lines the inner surface of the bone and serves as the interface between the bone and BM. Even though traditionally, bone and BM functions have been regarded as distinct, unrelated processes, it is becoming increasingly clear that the bone remodeling cells, osteoblasts and osteoclasts, are crucial components of the BM microenvironment. Osteoblasts and osteoclasts interact with each other through cytokines secreted by either cell type, as well as by membrane-bound ligands and receptors to initiate intracellular signaling [3,6]. Osteoblasts and BM stromal cells secrete two cytokines, macrophage colony stimulating factor (M-CSF) and RANKL, which are both necessary and sufficient to induce osteoclast differentiation. M-CSF induces the expression of RANK in osteoclast precursor cells, priming them to differentiate in response to RANKL [6]. In addition, osteoclasts stimulated with M-CSF undergo cytoskeletal reorganization, cell spreading and migration [32,33]. The downstream effects of RANKL-induced signaling include the expression of CTSK and TRAP, genes which encode crucial bone-resorbing enzymes that are secreted into the BM microenvironment and stimulate mobilization of HSPCs [6,11,12,34]. RANKL also induces expression of integrin αvβ3, which plays an important role in osteoclast adhesion, migration, and cell signaling [33,34]. Therefore, intrinsic and extrinsic regulatory pathways converge to maintain bone and BM homeostasis.

Despite recent studies that have recognized the importance of osteoblasts in regulating hematopoiesis [8,9,35], we did not find any differences in osteoblast number or function between Id1−/− mice and wild-type controls. However, the present study does show the importance of Id1 during osteoclast differentiation. Osteoclast numbers were increased in the bones of Id1−/− mice and differentiation of BM monocytes to osteoclasts was increased by Id1 deficiency, suggesting a cell-autonomous role for Id1 in osteoclastogenesis. In addition, the absence of Id1 resulted in increased expression of CTSK, TRAP, Oscar, and NFATc1, while overexpression of Id1 decreased expression of these genes. Therefore, interaction between Id1 and Mif may normally act to inhibit a cascade of gene expression events required for osteoclast differentiation.

As a consequence of the increased osteoclast differentiation and activity, we observed increased secretion of extrinsic factors, such as CTSK, into the BM microenvironment. In keeping with a previous report showing the potential of CTSK to cleave stem cell regulating cytokine SCF [11], we found increased plasma levels of SCF in Id1−/− mice. We propose that this alteration in the level of secreted factors in the microenvironment directly contributed to the increased tendency of Id1−/−-LSK cells to enter the cell cycle, leading to increased turnover and premature exhaustion of HSPCs. Id1−/− mice reconstituted with BM overexpressing Id1 were able to reestablish the levels of HSPCs and myeloid cells comparable to those observed in wild-type mice. Furthermore, knockdown of CTSK in the BM of Id1−/− mice decreased osteoclast function and enhanced trabecular bone formation. Finally, our finding that transplantation of wild-type BM into Id1−/− mice only partially restored the bone and hematopoietic phenotype in these animals provides more evidence that the intrinsic program of cells in the microenvironment integrate with multiple extrinsic molecules and pathways needed to regulate bone and BM homeostasis. Although the present data are highly suggestive that Id1 can play a regulatory role in both intrinsic and extrinsic cues necessary for maintaining a balanced microenvironment, the challenge for future investigations will be to sort out cell intrinsic versus cell extrinsic activities in vivo. Undoubtedly this will be a difficult task, as they are most certainly interconnected, but by doing so it will elucidate the precise molecular mechanisms responsible for the role of Id1 in the bone and BM.

Finally, it has been shown that Id proteins often have overlapping functions and can compensate for each other. Specifically, Id1 and Id3 were shown to have very similar expression patterns and targeting of both genes is often required to produce a phenotype [36]. Previously, we reported that tumor growth was inhibited more effectively in Id1−/−, Id3−/− than in Id1+/−, Id3+/− mice [29]. Similarly, other studies have shown that simultaneous targeting of Id1 and Id3, compared to targeting of each gene alone, is more effective at inhibiting tumor growth and metastatic potential of breast, colorectal, gastric, and pancreatic cancers [37–40]. In the current study, the absence of Id1 alone produced a significant bone and hematopoietic phenotype, suggesting that Id3 does not compensate for Id1 in this instance. However, we found that Id3 expression is slightly elevated in the BM of Id1−/− mice compared to wild-type litters (data not shown). The significance of this finding is so far unclear, but it will be of interest to observe if Id1−/−, Id3+/− mice have a more severe bone and hematopoietic phenotype compared to Id1−/−, Id3−/− mice. Clearly, more work will be necessary to elucidate the precise role of Id3 in the bone and hematopoietic phenotypes described here.

It is clear that our understanding of the fundamental mechanisms underlying the connections between the bone and BM is still in its infancy. In the future, many fruitful discoveries may come from investigating the commonalities between these two systems. This study offers insight into the cellular and molecular interactions of Id1 in regulation of bone and BM physiology. Although the clinical relevance of our findings has yet to be determined, the knowledge acquired from our results has the possibility of translating into potential therapeutic treatments in the areas of osteoporotic and malignant diseases. Moreover, microenvironments have garnered increased attention in recent years, due to findings that highlight the importance of BM cells, stem cells, and niches in areas of diseases such as cancer. We previously showed the role of Id proteins in supporting the growth of primary tumors and metastatic lesions [29,41]. We also demonstrated the importance of niches in metastatic spread of tumor cells [42]. From the results of the present study, it is intriguing to speculate that long-standing osteoporosis may result in a reduced HSC pool, thus impairing the mobilization of HSCs to future peripheral sites of metastasis. It would also be of interest to investigate whether cooperation between osteoclasts and HSPCs can create a favorable environment for tumor cells to engraft and proliferate in the bones. A better understanding of the
function of Id genes in osteoclasts, hematopoietic cells, tumorigenesis, and metastasis is clearly necessary in order to intelligently design tailored therapies. As the molecular and cellular events that regulate the function of the BM microenvironment are unraveled, novel methods for its specific manipulation for tailored therapies should also be revealed.

Materials and Methods

Mice

Generation of Id1−/− mice has been previously reported [13]. Animals used in all experiments were matched for sex, age (6–8 weeks old) and genetic background (C57B6/Sv129). All animal procedures were approved and performed under the guidelines of the Institutional Animal Care and Use Committee (IACUC).

Micro-Computed Tomography (Micro-CT)

To assess bone microarchitecture, the right femurs of the mice were removed and cleaned of all soft tissue. The bones were scanned in saline, using the Enhanced Vision Systems Model MS-8 In Vitro Micro-CT Scanner (GE Healthcare). 2-D projections of 4 femurs per scan, 4 hours per scan, were collected by Evolver software (GE Healthcare). Microwiew (GE Healthcare) was used to calculate 2-D images, 3-D volume generation, and threshold analysis.

Mechanical Testing

To assess bone strength, the left femurs of the mice were removed and cleaned of all soft tissue. A 3-point bending test was performed by placing the bones, anterior face up, on two supports equidistant from the ends and 7 mm apart. The load was applied to the center of the femoral shaft with a velocity of 0.05 mm/second until fracture. Load displacement curves for each individual femur were recorded and used to calculate the bending moment (PL/4) and bending rigidity (mL3/48), where P is the applied load, L is the span of the support points (7 mm), and m is the slope of the linear portion of the load-displacement curve.

Osteoclast Formation and Resorption Assays

BM cells from long bones were cultured in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS), penicillin, streptomycin, and 5 ng/mL M-CSF (PeproTech) for 16 hours. Nonadherent cells were harvested and cultured for 3 more days in the presence of 30 ng/mL M-CSF. Floating cells were removed and adherent cells were used as osteoclast precursors. The cells were further cultured in medium supplemented with 30 ng/mL M-CSF and 50 ng/mL RANKL (PeproTech) for 3 days. The culture plate was stained for TRAP-positive multinuclear cells (TRAP⁺ MNCs) using the leukocyte acid phosphatase (TRAP) kit (Sigma-Aldrich). TRAP⁺ MNCs containing more than 3 nuclei were counted. For resorption assays, cells were plated on BD BioCoat Osteologic slides (BD Bioscience). After 10 days in culture in medium supplemented with 30 ng/mL M-CSF and 50 ng/mL RANKL, the cells were removed with bleach and resorption pits were visualized by von Kossa staining.

Histology, Immunohistochemistry, and TRAP Staining

The femurs were cleaned of muscle and fixed in 4% paraformaldehyde for 48 hours. Following an overnight running water rinse, the bones were decalcified in 10% EDTA until the bones were soft and flexible, and processed in a VIP tissue processor to paraffin. Embedded sections were sampled to a thickness of 5 μm and stained for H&E for standard histology. The following antibodies were used for immunohistochemical analysis: Procollagen type I clone SP1.D8 (Developmental Studies Hybridoma Bank, University of Iowa) and Caspase 9 clone C-16 (Santa Cruz Biotechnology). For TRAP staining, the use of hexazonium pararosaniline for localization of acid phosphatase activity was previously described [44]. When this acid phosphatase method is used histochemically, in the presence of tartrate, the resulting stain is due to TRAP activity [45]. To prevent bias, all slides were coded and a total of 10 randomly chosen fields were assessed for each slide. For determination of common bone parameters, such as quantitation of cell types, determination of bone surfaces, and mineral apposition rate, the slides were evaluated with use of the Bioquant Morphometric System (Bioquant Image Analysis Corp., Nashville, TN).

Bone Histomorphometry

At 5 weeks of age, each animal was labeled by intraperitoneal injection with Tetracycline (5 mg/kg body weight), followed by Xenol Orange (90 mg/kg body weight) with a 4 day interval between labels. One day after the second label, the animals were sacrificed and the femurs were removed. The bones were subsequently fixed in 10% neutral buffered formalin for forty-eight hours, washed overnight in running water, dehydrated through a graded ethanol treatment, cleared in xylene, embedded in methyl methacrylate, and sectioned to a thickness of 5 μm, as previously described [46]. The distance between the two labels was measured in 10 randomly chosen fields per slide, and the mineral apposition rate (μm/day) was determined by dividing the mean distance between the double labels by the interlabel time (4 days).

Cell Preparation and Flow Cytometry

BM cell numbers were determined with a FACSCalibur (BD). Red blood cells were lysed using ACK buffer (Gibco, Invitrogen) according to the manufacturer’s instructions. For the preparation of cells for flow cytometry, before cells were stained with specific antibodies, nonspecific binding sites were blocked, when needed, with purified anti-FcγRII/III (93; eBioscience). All cells were stained at 4°C in PBS with 5% (vol/vol) FCS. The following antibodies were used for staining: anti-CD3 (2C11), anti-CD11c (HL-3), anti-CD45R (S7; all from Pharmingen); anti-CD34 (Ram34), anti-CD19 (1D3), anti-NK.1.1 (PK136), anti-TER119 (Ter119), anti-Grl (RB6-8C5), anti-CD11b (M1/70), anti-CD16/32 (93), anti-CD117 (2B8), anti-Sca-1 (D7), anti-CD45R (RA3-6B2), anti-IgM (11/41) and anti-CD25 (PC61.5; all from eBioscience). The following reagents were used for secondary steps: DAPI (4,6-diamino-2-phenylindole; Molecular Probes) and streptavidin-phycoerythrin-carbocyanine 5.5 (Caltag).

Differential Blood Cell Counts

Blood samples were obtained by retro-orbital bleeding and diluted 1:4 in PBS with 2 mM EDTA, 5% BSA. Complete blood cell counts were analyzed on an ADVIA 120 (Bayer).

Cell Cycle Analysis of Progenitor Cells

For BrdU incorporation, mice were first injected intraperitoneally with BrdU (1 mg per 6 g of mouse weight), followed by administration of BrdU in the drinking water (0.8 mg/ml) for 5 days. BM cells were harvested and stained for surface markers. Intracellular staining with anti-BrdU (PE) was carried out using the BrdU Flow Kit (BD Pharmingen) following the manufacturer’s instructions. Sca-1 and c-kit antibodies were used as stem cell surface markers. For assessing in vitro proliferation, Lin⁺, Sca-1⁺, c-kit⁺ (LSK) stem cells were sorted from the BM into a 48 well plate
Isolation and Transduction of Hematopoietic Cells

BM of donor mice was harvested by flushing femurs and tibias with PBS+2% FBS. Lin− cells were purified by lineage-marker negative selection using the Mouse Hematopoietic Progenitor Enrichment kit (StemCell Technologies), plated at a density of 1x10^6 cells/ml in StemSpan Serum Free Expansion Medium (STEMCELL Technologies). Lin− cells were transduced with concentrated virus for 12 h (MOI of 50–60), washed, and resuspended in PBS for transplantation.

Statistical Analysis

Statistical and graphical analyses were performed using GraphPad Prism software (version 3.0). The data was analyzed using Student’s unpaired t-test and results were considered significant at the 95% significance level (P<0.05). Results were representative of two or more independent experiments, and data was expressed as mean ± SEM of at least 3 replicates.

Supporting Information

Figure S1  
Id1−/− mice weigh less and have less trabecular bone. (A) Weights of 6-week old wild-type and Id1−/− mice (**P<0.001; n = 12). Error bars represent ± S.E.M. (B) Representative H&E staining of femoral sections from wild-type and Id1−/− mice. Arrowheads indicate areas of trabecular bone; M, marrow; GP, growth plate.  
Found at: doi:10.1371/journal.pone.0007955.s001 (2.17 MB PPT)

Figure S2  
Absence of Id1 does not alter the expression of osteoblast-associated genes. Results of qPCR for the expression of RANKL, OPG, and SDF-1 in the BM of wild-type and Id1−/− mice (n = 6). Error bars represent ± S.E.M.  
Found at: doi:10.1371/journal.pone.0007955.s002 (0.04 MB PPT)

Figure S3  
Detection of Y chromosome DNA sequences in BM transplanted mice. DNA samples were isolated from 200 μL of peripheral blood from lethally irradiated wild-type and Id1−/− mice that were transplanted with either wild-type or Id1−/− BM. Control DNA was isolated from wild-type male and female mice, and admixed to generate standards with known ratios of male and female DNA. Thus, XY male DNA was serially diluted in XX female DNA. Standards and samples were assayed by using TaqMan Gene Expression Assays (Applied Biosystems) for the sex determining region (SRY) gene. The cycle threshold (Ct) readings of the standards were used to generate a standard curve by plotting the mean of triplicate Ct values versus the log of the percentage of Y DNA in the background of XX DNA and calculating a regression line. The amount of Y DNA in unknown samples was determined by applying the mean Ct value of triplicates to the regression line. The amount of Y DNA in unknown samples was determined by applying the mean Ct value of triplicates to the standard curve and correcting for the total amount of DNA in the sample to determine the percentage of male sequence within a female background. Error bars represent ± S.E.M.  
Found at: doi:10.1371/journal.pone.0007955.s003 (0.04 MB PPT)

Figure S4  
Absence of Id1 specifically upregulates the expression of CTSK and not other cathepsins. Results of qPCR for the expression of other cathepsin family genes, CTSL and CTSB in the BM of wild-type and Id1−/− mice (n = 6). Error bars represent ± S.E.M.  
Found at: doi:10.1371/journal.pone.0007955.s004 (0.04 MB PPT)

Figure S5  
A model for the role of Id1 in regulating myeloid and osteoclast differentiation. Id1 inhibition of myeloid and osteoclast differentiation regulates HSC niche factors and limits HSC mobilization (left). In the absence of Id1, osteoclast differentiation increases and results in increased CTSK secretion (right).
Representative H&E staining of femoral sections from Id1 was collected for qPCR analysis. Error bars represent portion of the gag gene (GA); Rev-response element (RRE); splice fluorescent protein (EGFP) driven by the promoter of the human overnight and transplanted into lethally irradiated sacrificed and BM from the femur was collected for qPCR

Table S1 Steady state peripheral blood cell counts in wild-type and Id1−/− mice.

Table S2 Characteristics of femurs in CTSK-shRNA and GFP-shRNA BM transplanted mice.

Acknowledgments

Author Contributions

References

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Figure S6 Use of lentiviral vectors for the overexpression of Id1. (A) Schematic drawings of the lentiviral vector containing Id1 (PGEW-Id1) and the empty vector control (PGEW-empty). Both vectors contain the promoter of the elongation factor 1 alpha (EF1α) gene and carry an internal cassette for the enhanced green fluorescent protein (EGFP) driven by the promoter of the human phosphoglycerate kinase (PGK) gene. The following viral cis-acting sequences are labeled: long terminal regions (LTR); major splice donor sites (SD), encapsidation signal (ψ) including the 5′ portion of the gag gene (GA); Rev-response element (RRE); splice acceptor sites (SA); and post-transcriptional regulatory element of woodchuck hepatitis virus (Wpre). (B) Expression of Id1 in the BM of transplanted mice (***P<0.001; n = 6). Lin- BM cells from Id1−/− mice were transduced with lentivirus containing PGEW-Id1 or PGEW-empty vector overnight and transplanted into lethally irradiated Id1−/− mice. After 8 weeks, the mice were sacrificed and BM from the femur was collected for qPCR analysis. Error bars represent ±S.E.M.

Figure S7 Use of lentiviral vectors to knockdown expression of CTSK. (A) Expression of CTSK in the BM of transplanted mice (***P<0.001; n = 6). Lin- BM cells from Id1−/− mice were transduced with lentivirus containing shCTSK or shGFP vector overnight and transplanted into lethally irradiated Id1−/− mice. After 3.5 months, the mice were sacrificed and BM from the femur was collected for qPCR analysis. Error bars represent ±S.E.M. (B) Representative H&E staining of femoral sections from Id1−/− mice transplanted with BM containing a shRNA targeted against CTSK or GFP. Arrowheads indicate areas of trabecular bone; M, marrow; GP, growth plate.
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