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Quiescent Bone Lining Cells Are a Major Source of Osteoblasts During Adulthood

Igor Matic^a, Brya G. Matthews^a, Xi Wang^a, Nathaniel A. Dymant^a, Daniel L. Worthley^b, David W. Rowe^a, Danka Grcevic^c, and Ivo Kalajzic^a

^aDepartment of Reconstructive Sciences, University of Connecticut Health Center, Farmington, Connecticut, USA

^bDepartment of Medicine and Cancer Theme, University of Adelaide & SAHMRI, Adelaide, South Australia, Australia

^cDepartment of Physiology and Immunology, School of Medicine, University of Zagreb, Zagreb, Croatia

Abstract

The in vivo origin of bone-producing osteoblasts is not fully defined. Skeletal stem cells, a population of mesenchymal stem cells resident in the bone marrow compartment, are thought to act as osteoprogenitors during growth and adulthood. Quiescent bone lining cells (BLCs) have been suggested as a population capable of activation into mature osteoblasts. These cells were defined by location and their morphology and studies addressing their significance have been hampered by their inaccessibility, and lack of markers that would allow for their identification and tracing. Using lineage tracing models, we have observed labeled osteoblasts at time points extending beyond the reported lifespan for this cell type, suggesting continuous reactivation of BLCs. BLCs also make a major contribution to bone formation after osteoblast ablation, which includes the ability to proliferate. In contrast, mesenchymal progenitors labeled by Gremlin1 or alpha smooth muscle actin do not contribute to bone formation in this setting. BLC activation is inhibited by glucocorticoids, which represent a well-established cause of osteoporosis. BLCs express cell surface markers characteristic of mesenchymal stem/progenitors that are largely absent in osteoblasts including Sca1 and Leptin Receptor. BLCs also show different gene expression profiles to osteoblasts, including elevated expression of *Mmp13*, and osteoclast regulators RANKL and macrophage colony stimulating factor, and retain osteogenic potential

Correspondence: Ivo Kalajzic, M.D, Ph.D, Department of Reconstructive Sciences, MC 3705, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, Connecticut 06032, USA, Telephone: 860-679-6051; Fax: 860-679-2910; ikalaj@uchc.edu.

I.M. and B.G.M. contributed equally to this work.

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Author contributions

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upon transplantation. Our findings provide evidence that bone lining cells represent a major source of osteoblasts during adulthood.

Keywords

Bone lining cell; Mesenchymal stem cell; Osteoblasts; Osteogenesis

Introduction

Skeletal tissue undergoes constant remodeling, a process critical for maintenance of bone strength and mineral homeostasis. Remodeling involves numerous growth factors and cytokines, and participation of various cell lineages. The in vivo origin of bone-producing osteoblasts is not fully defined. Part of the complexity of identifying osteoprogenitor cells arises from the use of different models of bone formation. During bone development osteoblasts are derived from mesenchymal stem cells (MSC) within condensing mesenchyme that differentiate along the edge of the cartilage anlagen. Following blood vessel invasion into the cartilaginous matrix, bone marrow forms, and osteoblasts expressing cells have been reported to move along with the vasculature into the marrow space, making significant contributions to the formation of new trabecular bone [1]. Hypertrophic chondrocytes may also contribute to the osteoblast pool during development [2, 3]. MSC activation has also been implicated during fracture or bone defect healing. The periosteum is a critical contributor to fracture callus formation and periosteal progenitor cells differentiate into chondrocytes and osteoblasts following injury [4]. These cells have been identified in lineage tracing studies using Prx1-CreERT2, Osx-CreERT2, α SMACreERT2, and Gremlin1CreERT2 [1, 5–7].

Osteoblasts in adult animals are thought to originate from multipotent MSCs resident in the bone marrow. In recent years, a number of markers have been postulated to identify this cell population. The best-characterized marker in human bone marrow is CD146, which labels a perisinusoidal cell population [8]. In mouse, markers used to identify MSCs include Sca1, CD51, PDGFR α , CD105, CD90, Nestin-GFP, α SMA, and combinations thereof [9–13]. The presence of multipotent MSCs, mainly established by in vitro studies, has recently been challenged by experiments using lineage tracing in murine models. Gremlin1CreERT2 labeled cells give rise to osteochondro and reticular progenitors (OCR), while Mx1Cre labels lineage-restricted osteoprogenitors [7, 14]. Other markers that label mesenchymal progenitor cells include α -smooth muscle actin (α SMACreERT2) [5], and Leptin receptor (Lepr-Cre) [15]. Notably, all of these markers label cells that contribute to a proportion of the osteoblasts on the bone surface, but not the entire lineage.

These observations indicate potential for the presence of other osteoprogenitor populations responsible for the maintenance of osteoblastic lineage turnover during adulthood. Quiescent, lineage committed progenitors have an important role during tissue turnover or repair in other tissues, such as satellite cells in muscle [16]. A similar quiescent population exists within the bone lineage. Osteoblast lifespan has been calculated to be about two weeks, although may be as little as 3 days in rapidly growing animals, or over a month in

young adult mice [14, 17, 18]. Following completion of matrix formation, osteoblasts have three possible fates: become osteocytes embedded in mineralized bone matrix and lose most of their cytoplasmic organelles, die by programmed cell death or transform into inactive quiescent bone lining cells [19, 20]. Bone lining cells (BLCs) are post-mitotic flat osteoblast lineage cells lining the bone surface. To date, there are no accepted markers of BLCs or effective techniques to selectively isolate these cells. One proposed function of BLCs is removal of demineralized matrix on the bone surface prior to bone formation [21]. BLCs have been reported as a source of active osteoblasts after intermittent parathyroid hormone administration, basic fibroblast growth factor (FGF2) administration or following acute exposure to high dose γ -radiation [22–24]. This cell population therefore represents an alternative to MSCs as a source of osteoblasts in adult bone.

In this study, we evaluate the contribution of BLCs to bone formation in adult mice. Using a transgenic approach, we were able to label and trace BLCs following elimination of mature osteoblasts. Our results identify BLCs as a major contributor of osteoblasts and preosteoblasts in adult bone remodeling and as a potential therapeutic target for osteoporosis.

Materials and Methods

Mice

All animal protocols were approved by an institutional animal care committee. The following transgenic mouse lines were previously described: Dmp1CreERT2 [25], Col2.3 TK [26], α SMACreERT2 [5], Gremlin1-CreERT2 [7], Col2.3GFP [27]. Ai9 reporter mice (B6;129S6-Gt(ROSA)26Sortm9(CAG-tdToma-to)Hze/J, stock # 007905), Ai6 reporter mice (B6.Cg-Gt(ROSA)26-Sortm6(CAG-ZsGreen1)Hze/J, stock # 007906), and immunodeficient NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, stock # 005557) were obtained from Jackson Laboratories (Bar Harbor, ME, www.jax.org). Cre recombination was induced by low (7.5 μ g/g) or high dose (75 μ g/g) of tamoxifen (Sigma-Aldrich Corp., St. Louis, MO, www.sigmaaldrich.com) by intraperitoneal (i.p.) injection. Ganciclovir (GCV, Fresenius Kabi, Lake Zurich, IL, www.fresenius-kabi.us) treatment was administered twice a day for 16 days at a dose of 8 mg/kg i.p.

Flow Cytometry

For preparation of the endosteal cell fraction, hind limbs were dissected, soft tissue was removed, bone marrow flushed and periosteum removed. Bones were opened, then enzymatically digested with 0.05% trypsin (Lonza, Anaheim, CA) and 0.2% collagenase A (Roche, Basel, Switzerland, www.roche.com) in PBS for 30 minutes at 37°C with ~200 rpm shaking followed by crushing bones and digesting in fresh enzyme for a further 30 minutes. Cells from multiple mice were pooled in order to obtain enough tdTomato⁺ cells for sorting and analysis. The staining protocol and antibodies were described previously [28], with the exception of CD44 (clone IM7). Dead cells were excluded using Fixable Viability Dye eFluor 455 (UV). Analysis and cell sorting were performed using a FACS Aria II (BD Biosciences, San Jose, CA, www.bdbiosciences.com). Gates were set using appropriate cell preparations from Cre⁻ mice and unstained cell preparations from tamoxifen treated Cre⁺

mice. Analysis was performed using FACSDiva (BD Biosciences) or FlowJo (Flowjo LLC, Ashland, OR, www.flowjo.com).

Prednisolone Treatment

Aged matched (within 2 days) iDMP/TK mice were treated with GCV for 16 days beginning at 6 weeks of age, with Tam labeling on day 15 of treatment. One day after completion of GCV, mice were randomized to receive either placebo or prednisolone pellets (Innovative Research of America, Sarasota, FL, <http://www.innovrsrch.com/>), which were administered subcutaneously. Prednisolone pellets contained 1.5 mg for 21-day release, equivalent to 2.3–3.5 µg/g/day which is sufficient to cause bone loss [29]. Calcein was given 24 hours before sacrifice, and mice were sacrificed either 7 or 21 days after prednisolone was administered. Image analysis of femur sections was performed by a blinded observer.

Statistical Analysis

Changes in histomorphometric parameters and FACS markers were evaluated by one-way ANOVA. Changes were compared to an appropriate control (generally day 1 of labeling), using Dunnett's post-test, with $p < .05$ considered statistically significant. In instances where only two groups were compared, including micro CT data, Student's t tests were performed. Multiphoton measurements were compared via Mann Whitney U tests corrected for multiple comparisons ($p < .017$).

Additional methods are described in the Supporting Information Experimental Procedures section.

Results

Tracing the Fate of Mature Osteoblasts

To investigate the fate of mature osteoblasts in vivo, we utilized dentin matrix protein 1 (*Dmp1*) promoter driven inducible Cre (*Dmp1CreERT2*) mice previously shown to label osteoblasts and osteocytes [23, 25]. *Dmp1CreERT2* mice were crossed with the Ai9 reporter line to generate iDMP mice (Fig. 1A). Ai9 is a much more sensitive Cre reporter than the Rosa26R lacZ reporter used in previous studies. iDMP mice were treated with tamoxifen at 2 months of age to label Cre-expressing cells, and the fate of the iDMP⁺ osteoblasts assessed over a 3 or 6-month time-course (Fig. 1 and Supporting Information Fig. S1). Given an expected lifespan of 2–4 weeks, we expected to observe loss of iDMP⁺ osteoblasts or conversion to BLCs within 3–6 weeks. One day after labeling, over 90% of the endocortical bone surface and 60% of trabecular surfaces are covered with iDMP⁺ cells that are associated with new bone formation assessed by calcein deposition (Fig 1C, 1G, 1J, and Supporting Information Fig. S1B, S1G, S1J). The cells on the bone surface were confirmed as osteoblasts based on coexpression of the Col2.3GFP transgene (Supporting Information Fig. S2). By 21 days after labeling, the iDMP⁺ cell surface in both cortical and trabecular bone significantly decreased, however a similar proportion of the bone surface remained iDMP⁺ at later time points, up to 6 months after initial labeling (Fig. 1 and Supporting Information Fig. S1). Active osteoblasts are plump cells associated with a mineralization label while BLCs are much flatter in relation to the bone surface, so we evaluated iDMP⁺

cell thickness. There was a slight decrease in the thickness of iDMP⁺ cells at later time points, however this change was not always significant (Fig 1H, 1K and Supporting Information Fig. S1H, S1K). Some flat iDMP⁺ cells start to appear on endocortical and trabecular bone surface by day 21, however many plump cells associated with calcein label characteristic of osteoblasts remain as long as 6 months after tamoxifen labeling (Fig. 1D–1F, Supporting Information Fig. S1C–S1F). In addition, the proportion of iDMP⁺ osteocytes in cortical bone continued to increase up to 3 months after labeling, suggesting continued contribution to bone formation by iDMP⁺ cells (Fig. 1I, 1L and Supporting Information Fig. S1I, S1L).

In the absence of tamoxifen, a significant level of spontaneous activation of iDMP was observed within the osteocyte population, labeling 10%–25% osteocytes in cortical bone and 30%–40% osteocytes in trabecular bone, but with no increase in the proportion of tdTomato⁺ cells between 2 and 5 months of age (Fig. 1I, 1L and Supporting Information Figs. S1I, S1L, and S3A, data not shown). *Dmp1* expression is highest in osteocytes resulting in leakiness of Cre activity in this population [30]. In addition, a small population of bone marrow cells is labeled by iDMP after tamoxifen injection. To determine if these cells represent a population of bone marrow mesenchymal cells with osteogenic potential we initiated bone marrow stromal cell cultures (BMSC) after in vivo labeling of iDMP mice (Fig. 2A). Morphology of iDMP⁺ cells in the bone marrow was dendritic (Fig. 2B), and FACS analysis indicated that <0.2% of cells within bone marrow express the transgene and most of these cells express the hematopoietic cell marker CD45 (Fig. 2D). In culture, iDMP⁺ cells have a distinct appearance (large, non-fibroblastic cells) and do not form CFU-F colonies (Fig. 2C). FACS analysis showed the percentage of iDMP⁺ cells decreased over time in BMSC culture (Fig. 2E–2G), indicating that iDMP does not label bone marrow MSCs. Together, these results indicate that while the majority of iDMP⁺ osteoblasts disappear within 3 weeks of labeling, some osteoblasts persist on the bone surface for much longer than their expected lifespan, suggesting a continuous supply of iDMP⁺ osteoblasts cells that appear to be independent of bone marrow resident MSCs.

Identification and Lineage Tracing of Bone Lining Cells

Given that we were unable to selectively label BLCs on the bone surface, even after long term lineage tracing of osteoblasts as has been reported in other studies [23], we sought to identify a more enriched BLC population by genetic ablation of osteoblasts. For these studies we used the Col2.3 TK mouse, which allows ablation of Col2.3 expressing cells when treated with ganciclovir (GCV). Ablation of osteoblasts can be achieved by 16 days of GCV treatment, while osteocytes are retained, as GCV does not affect non-dividing cells [9, 26]. We crossed iDMP mice with Col2.3 TK to generate iDMP/TK mice (Fig. 3A). Osteoblast ablation studies were initiated in young adult animals (6–7 weeks) as indicated (Fig. 3B). GCV does not alter iDMP labeling in the absence of the Col2.3 TK transgene (Supporting Information Fig. S3B, S3C). As expected, after ablation iDMP⁺ osteoblasts were absent from the bone surface, and calcein labeling was minimal (Fig. 3C). However, iDMP⁺ osteocytes are still present, and thin cells characteristic of BLCs are evident on many bone surfaces. Electron microscopy of iDMP⁺ cells in mice following osteoblast ablation confirmed that these cells demonstrated characteristics of BLCs including a small basic

network of rough endoplasmic reticulum (rER) around the nucleus (Supporting Information Fig. S4A–S4C) compared to rER composed of branched cisternae in active osteoblasts from mice that had not undergone ablation (Supporting Information Fig. S4D–S4F). By day 7 of recovery, calcein label was evident on some surfaces and plump iDMP⁺ osteoblasts, presumably derived from iDMP⁺ BLCs are present (Fig. 3D). By day 21, rapid bone formation has occurred to create a new trabecular region, with iDMP⁺ cells evident on many surfaces (Fig. 3E). Quantification of labeled bone surfaces showed dramatic increases in iDMP⁺ trabecular surface over the course of recovery (Fig. 3I). iDMP⁺ cell thickness significantly reduced after ablation by at least 50%, consistent with loss of osteoblasts, but returned to baseline by day 21 of recovery, suggesting conversion of BLCs to active osteoblasts (Fig. 3G, 3J), and iDMP⁺ osteocyte proportion also increased in both cortical and trabecular bone after recovery indicating conversion of iDMP⁺ cells to osteocytes (Fig. 3H, 3K). Labeling of iDMP cells prior to osteoblast ablation (day-17) resulted in similar contribution to osteoblasts after recovery, and to marrow fibrosis, suggesting that a BLC population is retained throughout the osteoblast ablation (data not shown). A number of previous studies have shown evidence of activation of BLCs to osteoblasts as a mechanism for rapid increases in bone formation, particularly in response to PTH treatment, but they have generally suggested that proliferation of these cells is minimal or absent [22, 23]. Given the dramatic increases we observed in the frequency of iDMP⁺ cells, we assessed EdU incorporation after a single dose of EdU the day before sacrifice. As expected, immediately after ablation, iDMP⁺ BLCs show no evidence of proliferation (Fig. 3L). In contrast, later in recovery, iDMP⁺ EdU⁺ cells are evident on or near the bone surface (Fig. 3M, 3N). These results were confirmed by FACS analysis of EdU incorporation, which indicated that BLCs were quiescent immediately after osteoblast ablation, and a small proportion of iDMP⁺ osteoblasts were EdU⁺, but that 7 days into recovery, around 20% of BLC-derived cells incorporated EdU (Supporting Information Fig. S5).

In order to rule out contribution of iDMP⁺ osteocytes to bone recovery, or spontaneous activation of iDMP in osteoblasts during recovery, we performed similar ablation experiments without tamoxifen treatment. Consistent with previous observations (Fig. 1I, 1L and Supporting Information Fig. S1I, S1L), a proportion of osteocytes were labeled in iDMP/TK mice after ablation, but very few labeled cells were evident on the bone surface (Supporting Information Fig. S6A). After 21 days recovery, newly formed bone has iDMP⁺ osteocytes, but very few iDMP⁺ osteoblasts (Supporting Information Fig. S6B). Therefore, if osteocyte dedifferentiation is occurring as we previously demonstrated in vitro, it is making a minimal contribution to this model of bone regeneration in vivo [31]. We also initiated BMSC cultures on day 1 of recovery as described in Figure 2, and similarly failed to see formation of labeled colonies (data not shown). We, therefore, propose that during recovery following osteoblast ablation, BLCs are a major source of osteoblasts.

α SMA- and Gremlin1-Labeled Resident Cells Do Not Contribute to Bone Formation During Recovery

Bone marrow resident mesenchymal progenitors are postulated to be the major source of mature osteoblasts in adult mice. In order to determine whether we could identify bone marrow resident osteoprogenitors contributing to recovery in this model, we performed

similar osteoblast ablation studies using markers of mesenchymal progenitors; α SMA^{CreERT2}/Ai9/TK (iSMA/TK) and Gremlin1^{CreERT2}/Ai6/TK (iGremlin/TK). Both have been shown to label osteoprogenitors during growth, fracture healing, and in young adult mice [5, 7, 28]. When tamoxifen was administered to iSMA/TK mice near the end of ablation, as described for the iDMP/TK model, a small proportion of iSMA⁺ cells were detectable within the marrow compartment (Fig. 4A). Following recovery, iSMA⁺ cells contributed to marrow fibrosis in the newly formed trabecular region, but very few osteoblasts associated with bone surfaces, and almost no osteocytes within the matrix were labeled (Fig. 4B). While iSMA⁺ cells increase in abundance on bone surfaces after recovery, they occupied at least ninefold less bone surface than iDMP⁺ cells after 21 days, and there was no change in cell thickness (Fig. 4C, 4D). When tamoxifen was administered prior to osteoblast ablation there were very few iSMA⁺ cells evident in the bone marrow compartment following recovery, and no evidence of contribution to new bone formation (data not shown). However, when tamoxifen was administered during the early phases of recovery (day 0 and day 1), iSMA⁺ cells were much more abundant on bone surfaces and contributed to osteocytes in newly formed bone (Fig. 4E). Together these results indicate that α SMA does not label a resident cell population that contribute to recovery after osteoblast ablation, but that α SMA expression may be activated in cells contributing to new bone formation in the early stages of recovery. We also evaluated contribution of the recently identified OCR progenitor population labeled by Gremlin1 to recovery following osteoblast ablation. iGremlin/TK mice were administered three daily doses of tamoxifen prior to osteoblast ablation (6–7 week-old mice). Immediately following ablation, iGremlin⁺ cells were extremely rare within the bone marrow, but were evident in both articular and growth plate cartilage (Fig. 4F). After recovery, a small number of iGremlin⁺ cells were present in the trabecular area, some of which are associated with mineral label, or embedded in bone (Fig. 4G). This suggests that any contribution of iGremlin⁺ cells is very minor in comparison to iDMP⁺ BLCs.

BLCs Show Phenotypic Features Distinct from Osteoblasts

We took advantage of our osteoblast ablation model to compare BLCs to osteoblasts. Cell thickness is often used to distinguish BLCs from osteoblasts in two-dimensional sections. However, little is known regarding the three-dimensional shape differences between BLCs and osteoblasts. Therefore, using multiphoton microscopy we measured cell volume, surface area, and sphericity of iDMP⁺ cells sitting on the bone surface of ablated (BLCs, $n = 17$) and non-ablated (osteoblasts, $n = 43$) iDMP/TK mice. We also compared these shape characteristics to iDMP⁺ osteocytes ($n = 13$) from the same animals. Note that we were unable to visualize osteocyte processes, so only the shape of the main cell body was analyzed. Sphericity is a measure of the roundness of a shape with a sphere having a value of 1. BLCs displayed the lowest sphericity values followed by the non-ablated osteoblasts, then osteocytes ($p < .017$), indicating that BLCs were less spherical than the other cell populations (Supporting Information Fig. S7A, S7B). In addition, BLCs were significantly larger in volume than both osteocytes and osteoblasts ($p < .017$, Supporting Information Fig. S7A, S7C). Following K-mean clustering, close to 90% of osteocytes were in cluster 1 with no cells in cluster 3 and over 60% of BLCs were in cluster 3 with no cells in cluster 1. Additionally, over 80% of osteoblasts were in cluster 2 with the remainder equally

distributed between clusters 1 and 3, indicating that the BLCs were primarily large and less spherical while the osteocytes were small and more spherical. Finally, the osteoblasts were distributed between these two groups. Three-dimensional reconstructions of representative cells are shown in Supporting Information Figure S7D.

The BLC phenotype was further defined by cell surface marker analysis using flow cytometry. Osteoblasts were isolated from iDMP/TK mice that did not receive GCV treatment (or their iDMP/TK⁻ littermates) (Fig. 5A). While this population is enriched for osteoblasts, it is likely to also contain a small proportion of BLCs, therefore we also used Col2.3GFP mice as an alternate source of osteoblasts [27]. We confirmed that osteocytes were not represented in the endosteal preparations using mice that did not receive tamoxifen, which showed very rare occurrence of Tomato⁺ cells (Supporting Information Fig. S6C). BLCs were isolated after osteoblast ablation as indicated (Fig. 5A). iDMP⁺ cells comprised a small proportion of cells obtained by collagenase digestion of long bone endosteal surfaces (Fig. 5B). Stem cell marker Sca1 was detectable in under 3% of osteoblasts, but was present on about 40% of BLCs (Fig. 5C–5F). CD51 can be used in combination with other markers to identify either skeletal stem cells, or more committed osteogenic cells (CD51⁺ Sca1⁻) [12]. CD51 was present on over 70% of BLCs, compared to 16%–23% on the osteoblast populations (Fig. 5G), and the CD51⁺ osteoblasts were almost all Sca1⁻ (Fig. 5C). Two other potential MSC markers, CD44 and LepR, are also expressed at much higher frequency on BLCs compared to osteoblasts (Fig. 5D, 5E, 5H, 5I).

We also evaluated gene expression of BLCs compared to both iDMP⁺ and Col2.3GFP⁺ osteoblast populations. Analysis was performed on sorted single cells using a custom set of 100 genes. Clustering of samples using a several methods indicated that the osteoblast populations were very similar to each other while the BLCs mainly clustered separately (data not shown). BLCs showed significantly higher expression than both osteoblast populations of a number of genes including the collagenase *Mmp13*, Wnt inhibitor *Sfrp4* and progenitor cell markers Connective Tissue Growth Factor (*Ctgf*) and Sca1 (*Ly6a*) (Supporting Information Fig. S8). Genes encoding both major promoters of osteoclastogenesis, macrophage colony stimulating factor (M-CSF, *Csf1*) and receptor activator of nuclear factor kappa B ligand (RANKL, *Tnfrsf11*) were also significantly higher in BLCs than osteoblasts. BLCs also had elevated expression of G1/S checkpoint genes p21 (*Cdkn1a*) and p15 (*Cdkn2b*) consistent with their identity as a quiescent cell type (Supporting Information Fig. S8).

Osteogenic Potential of BLCs

In order to evaluate whether BLCs retain osteogenic potential after removal from the bone surface, we performed both in vitro cultures and transplantation experiments (Fig. 6A). When the endosteal cell fraction is cultured following osteoblast ablation, iDMP⁺ colonies that expand over time are evident (Fig. 6B), but we saw little evidence of in vitro mineralization. However, following osteoblast ablation, osteogenic differentiation of BMSCs was severely impaired, and differentiation was also reduced in the endosteal fraction (Fig. 6C, 6D), consistent with previous studies [32]. We therefore used an in vivo transplantation assay to further evaluate the osteogenic potential of BLCs. BLCs were isolated by FACS (2–

5,000 cells), mixed with nontransgenic BMSCs (as a supporting population to promote bone formation), and transplanted into a calvarial defect (Fig. 6A). Five weeks later, iDMP⁺ cells were evident within all defects, and some were associated with calcein label, or embedded as osteocytes in newly formed bone, indicating retention of osteogenic potential following transplantation (Fig. 6E). We also evaluated the osteogenic potential of iDMP⁺ cells during fracture healing (Supporting Information Fig. S9). In the absence of tamoxifen treatment, tdTomato⁺ osteocytes were present in the cortical bone and some areas of the callus (Supporting Information Fig. S9B). A small proportion of osteoblasts were also tdTomato⁺. We then traced BLC-enriched iDMP⁺ cells by performing fractures 40 days after tamoxifen treatment. The contribution of iDMP⁺ cells to the fracture callus was very similar to that observed in the untreated controls (Supporting Information Fig. S9C) indicating that iDMP⁺ BLCs do not make a major contribution to osteogenesis during fracture healing.

Glucocorticoid Treatment Inhibits Activation of BLCs

Glucocorticoid-induced osteoporosis is a significant clinical problem. Pharmacological doses of glucocorticoid increase bone resorption and reduce bone formation, and effects on the osteoblast lineage appear to be critical to the detrimental effects on bone [29, 33]. We investigated whether prednisolone treatment could alter activation of BLCs when administered after osteoblast ablation (Fig. 7A). Prednisolone significantly reduced the bone volume fraction in the distal femur after 21 days in both male and female mice (Fig. 7B, 7C). Cortical thickness was also reduced with prednisolone treatment (Fig. 7D). To evaluate whether this reduced bone formation could be explained by effects on BLCs, we examined histology seven days into recovery. Prednisolone treated bones showed less calcein label than controls (Fig. 7E, 7F), as well as dramatic reductions in the proportion of bone surfaces covered in iDMP⁺ cells (Fig. 7G, 7H). iDMP⁺ cell thickness was also significantly reduced in prednisolone-treated bones from female mice (12.4 ± 1.4 vs. $7.5 \pm 0.9 \mu\text{m}$ on cortical surfaces).

Discussion

Osteoblasts and osteocytes are generally thought to differentiate from local tissue-resident populations of multipotent mesenchymal stem or progenitor cells. A number of recent studies have identified different cell populations that contribute to osteoblasts in vitro and in vivo [5, 7, 10, 12, 15, 34]. However, there is no consensus on how to identify MSCs in vivo, and there is considerable variability in the frequency, prevalence during different stages of development, growth and adulthood, and multilineage potential using different markers. For example, $\alpha\text{SMACreERT2}$ mainly identifies osteoprogenitors in growing animals, while LepRCre identifies cells that differentiate into osteoblasts and adipocytes during adulthood, although both contribute to fracture healing [5, 15]. In contrast, Mx1Cre -labeled cells show multipotential differentiation in vitro but are mainly restricted to the osteogenic lineage in vivo, and Gremlin1CreERT2 labeled cells form osteoblasts, chondrocytes and reticular cells, but not adipocytes [7, 14]. It is noteworthy that the frequency of certain progenitor populations decline dramatically from late embryonic development/early postnatal stages to adulthood [12, 35, 36]. While it is likely that some of the populations in these studies overlap, the different characteristics of these cell populations suggest that there may be more

than one osteoprogenitor population in the bone marrow. In addition, fate mapping by a number of investigators has indicated that hypertrophic chondrocytes can be a source of osteoblasts during development and postnatal growth, particularly in the primary spongiosa region [2, 3]. The presence of hypertrophic chondrocyte-derived proliferating cells, perivascular cells, and adipocytes suggests this may occur via a progenitor intermediate, although direct transdifferentiation of some cells cannot be ruled out. In the current study, we investigated the bone lining cell as a postnatal source of osteoblasts. Based on lineage tracing data using the iDMP mouse, we propose that BLCs are continually reactivated during postnatal bone modeling, of the cortex in particular, to contribute to the osteoblast pool. They also serve as a source of osteoblasts under anabolic stimuli or conditions of marrow stress, and retain osteogenic potential after transplantation. A subpopulation (30%–40%) of BLCs express markers that have been commonly used to identify MSCs, including Sca1 and LepR, suggesting that in studies where cells are isolated from the bone using collagenase digestion, which is well established to enrich for osteoprogenitors [35], the identified progenitor populations could include BLCs.

BLCs have long been recognized as a long-lived cell type localized on the bone surface and primarily characterized by morphology [37]. BLCs were thought to constitute part of the osteoblast lineage, with the capability to activate into osteoblasts in some circumstances, however little is known about nonhistological characteristics that distinguish them from osteoblasts. Both this study and other recent reports using a similar lineage tracing model confirm that BLCs arise from mature osteoblasts [23]. Multiple factors including intermittent PTH, FGF2, sclerostin inhibition, mechanical loading, and irradiation have been shown to activate BLCs in vivo, however these studies did not show evidence of proliferation of BLCs following activation [22–24, 38, 39]. In our study we demonstrate that following osteoblast ablation stimulus, BLCs are capable of proliferating and making a major contribution to new bone formation, while the contribution of iSMA and iGremlin mesenchymal progenitors is minimal. BLCs have proved difficult to study due to a lack of selective markers. ICAM1 was proposed to selectively label BLCs, however in our hands ICAM1 (CD54), while selective for BLCs, labeled only a quarter of iDMP⁺ BLCs using FACS analysis ($3.3 \pm 0.8\%$ in osteoblasts vs. $26.3 \pm 2.2\%$ in BLCs). Recent microarray studies using laser capture derived osteoblasts, BLCs and osteocytes show subtle differences in gene expression between osteoblasts and BLCs and larger differences from osteocytes, although further probing of this dataset may still provide promising markers [40].

In addition to providing a source of osteoblasts, BLCs also appear to play a role in bone remodeling. Histological studies in human trabecular bone suggest that coupled remodeling requires sufficient cell density of osteoblast lineage cells in the canopy covering basic multicellular units (BMU), and appropriate activation of the reversal cells that lie between osteoclasts and matrix-producing osteoblasts [41, 42]. Reversal cells may constitute a type of BLC, and it is possible that BLCs also contribute to the progenitor pool in the canopy [42]. Glucocorticoid treatment inhibited sufficient recruitment/activation of reversal cells leading to uncoupling within the BMU, consistent with our data indicating that glucocorticoid treatment inhibits BLC activation. Everts and colleagues demonstrated that BLCs primed Howship's lacunae for bone formation by removing remaining demineralized collagen and depositing a thin layer of collagenous matrix and an osteopontin-rich cement

line [21]. Matrix metalloproteinases (MMP) were critical for this activity. Our gene expression results are consistent with these findings, with highly elevated expression of an important MMP with collagenase activity, *Mmp13*, in BLCs compared to osteoblasts. Everts et al. also demonstrated that BLCs reside in close proximity to osteoclasts [21]. We also observed higher expression of the two critical promoters of osteoclastogenesis, M-CSF and RANKL in BLCs compared to osteoblasts. However, it should be noted that the BLCs we analyzed are isolated from a setting in which osteoclastogenesis is almost completely suppressed [26]. Unfortunately, we were unable to compare these levels to osteocytes, which a number of recent studies indicate play a critical role in control of osteoclastogenesis in adult mice [43, 44].

Given the lack of selective markers for BLCs, the TK model used in our studies proved a valuable tool to selectively isolate BLCs. By targeting dividing cells, we can selectively ablate osteoblasts without targeting BLCs or osteocytes, both of which express most of the promoters used to target osteoblasts. The rapid trabecular bone formation during recovery is not unique to this system as it was also reported after ablation of *LepR*⁺ cells using a diphtheria toxin-mediated system [15]. Interestingly, repeated ablation of osteoblasts, despite depletion of CFU-F in the bone marrow, did not affect cortical bone density or strength [45]. There are some caveats to using an ablation model. The ablation process affects the bone marrow cellularity and composition [46], and depletes cells with osteogenic potential from the bone marrow, while also reducing in vitro osteogenic differentiation potential from the endosteal fraction. It may also affect the phenotype of the BLCs. Despite this, following ablation, the bone formation response is rapid and exacerbated, indicative of activation of resident progenitor cells, of which BLCs represent a major contributor. In addition, the rapid activation of BLCs following withdrawal of treatment in this model makes it unsuitable for studying activation of BLCs by exogenous factors like PTH, although we were able to demonstrate that BLC activation was inhibited by glucocorticoid treatment.

We selected the iDMP model for use in this study since, despite being widely considered a marker of osteocytes, it effectively labeled the majority of osteoblasts (over 90% of cortical bone surface), and BLCs in a tamoxifen-dependent manner. In addition, a subset of osteocytes are iDMP⁺, even in the absence of tamoxifen. Following tamoxifen treatment, the majority of osteocytes in trabecular bone are iDMP⁺, but labeling does not increase much in cortical bone. It is unclear whether this is related to the expression pattern of cre, or technical issues with penetration of tamoxifen. Osteoblast ablation experiments in the absence of tamoxifen were used to evaluate contribution of preosteocytes/osteocytes to the recovery process, as recent studies have suggested that osteocytes may also act as a source of osteoblasts under some circumstances [31]. The iDMP⁺ cells observed following recovery were mainly restricted to osteocytes within the newly formed bone, presumably as the result of spontaneous activation of the reporter, suggesting that if osteocyte dedifferentiation is occurring, it is making a very minor contribution to this process. iDMP also labels a small population of CD45⁺ cells in the bone marrow. Evaluation of this cell population is ongoing, but culture experiments demonstrate that they do not form CFU-Fs, making it very unlikely that they constitute an MSC-like population. In a number of experiments, we have used iDMP mice that have not undergone osteoblast ablation as a tool to label osteoblasts. It

should be noted that this population is also likely to contain a small proportion of BLCs, however given the relative prevalence of *Scal* expression, we estimate that BLCs cannot represent more than 6% of the iDMP⁺ cells in these preparations. We also evaluated Col2.3GFP⁺ osteoblasts in parallel, which in most cases showed very similar results to the iDMP⁺ osteoblast population.

Conclusion

In conclusion, we have demonstrated that in addition to MSCs, bone lining cells constitute an osteoblast precursor in adult mice both during normal modeling/remodeling, and in response to anabolic stimuli. We also show that under some circumstances, BLC-derived cells are capable of proliferation prior to bone formation. We confirmed and extended phenotypic differences between osteoblasts and BLCs, including that BLCs have a larger cell volume and lower sphericity in three dimensions, a significant subset of BLCs express cell surface markers characteristic of MSCs, and BLCs show different gene expression profiles from osteoblasts including increased *Mmp13*, M-CSF and RANKL. In addition, their activation is inhibited by glucocorticoid treatment, a common cause of osteoporosis. By understanding the mechanisms by which BLCs are activated and their population maintained, we may gain insights into the pathogenesis of osteoporosis, and identify approaches to improve bone accrual.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance statement

Our study shows that bone lining cells (BLCs) rather than bone marrow residing mesenchymal stem cells (MSC), can be a major source of osteoblasts and proliferating preosteoblasts, and contribute to osteoblasts during normal bone turnover in adults. BLCs express some markers of MSCs, as well as high levels of osteogenic markers. BLCs are potentially an underappreciated cell type involved in the response to both catabolic and anabolic bone stimuli. By understanding the mechanisms of BLC activation and maintenance, we may gain insights into the pathogenesis of osteoporosis, and identify approaches to improve bone accrual.

Figure 1.

Bone lining cells are a continuous source of osteoblasts in mature bone. (A): For lineage tracing experiments, Dmp1CreERT2 mice were crossed with Ai9 reporter mice to generate iDMP. (B): Schematic of the experimental design. Cre activity was induced by 2 doses of tamoxifen (Tam), 24 hours apart (7.5 μg/g) in 2 month old mice and the animals were sacrificed as shown. Mineralizing surfaces were labeled with calcein a day before the mice were sacrificed for histological evaluation. (C–F): Representative femur sections from males are shown at the indicated time points. (G–L): Image analysis was used to evaluate changes in labeled surface (G, J), thickness of labeled cells (H, K) and proportion of labeled osteocytes (I, L) in cortical and trabecular bone regions ($n = 3–4/\text{group}$). Data are presented as mean $\pm$ SEM. *, $p < .05$ compared to day 1 using ANOVA with Dunnett's post-test. Scale bars indicate 500 μm (C–F scans) or 50 μm (enlarged areas). Abbreviations: DMP1, dentin matrix protein 1.

Figure 2.

iDMP⁺ cells within bone marrow do not expand. (A): iDMP mice were treated as indicated before BMSC cultures were established. (B): iDMP⁺ cells in the bone marrow show a dendritic morphology (x200 magnification). (C): iDMP⁺ cells in BMSC culture at day 6 do not have a stromal morphology (x100 magnification). (D): FACS analysis of the whole bone marrow indicates that the majority of iDMP⁺ cells are CD45⁺. (E–F): FACS analysis of primary BMSC cultures at day 6 and day 8 indicated a small proportion of iDMP⁺ CD45⁺ cells. (G): Quantification of CD45⁺ iDMP⁺ cells indicated a decrease over time in culture. Data is from 5 cultures. *, $p < .05$ determined by Student's t test. Abbreviations: BMSC, bone marrow stromal cell cultures; Tam, tamoxifen.

Figure 3.

Osteoblast ablation provides a model to study bone lining cells. (A): Dmp1CreERT2 mice were crossed with Col2.3 TK mice. Double transgenic offspring were crossed with Ai9 reporter mice to produce iDMP/TK. (B): Following osteoblast ablation with GCV treatment for 16 days, iDMP was activated by Tam and histological analysis was performed at the time points indicated. Mineralizing surfaces were labeled using calcein. Animals were 6–7 weeks old at the start of GCV treatment. (C–E): Representative histological sections of female mice are shown at the indicated time points following osteoblast ablation. Boxed areas show selected cortical and trabecular regions at higher magnification. iDMP⁺ cells are red, calcein is green. (F–K): Image analysis was used to evaluate changes in labeled surface (F, I), thickness of labeled cells (G, J) and proportion of labeled osteocytes (H, K) in cortical and trabecular bone regions ($n = 3–4$ / group). Quantification from controls without osteoblast ablation (no GCV, equivalent to day 1 on Supporting Information Fig. S1), or osteoblast ablation without tamoxifen labeling (No Tam) are also shown. (L–N): EdU staining (green) was performed to identify cells that were undergoing cell division within the day before sacrifice. Arrowheads indicate iDMP⁺ EdU⁺ cells. *, $p < .05$ compared to day 1 using ANOVA with Dunnett's post-test. Scale bars indicate 500 μm (C–E scans) or 50 μm (enlarged areas, L–N). Abbreviations: BM, bone marrow; CB, cortical bone; GCV, ganciclovir; Tam, tamoxifen.

Figure 4.

αSMA and Gremlin1 osteoprogenitor cells make minimal contribution to recovery following osteoblast ablation. (A–B): iSMA/TK mice underwent ganciclovir (GCV) and tamoxifen treatment as indicated in Figure 3B. At day 1 of recovery (A), few cells were iSMA⁺. By day 21 (B) iSMA⁺ cells contributed to marrow fibrosis, and a small proportion of cells on the bone surface, but iSMA⁺ osteocytes were absent. (C–D): Image analysis ($n = 3$) indicated increased iSMA⁺ labeled surface after recovery (C), but no increase in cell thickness (D). (E): When tamoxifen was administered on day 0 and day 1 of recovery, more iSMA⁺ cells were evident on the bone surface, and some osteocytes were iSMA⁺ (arrowheads). (F–G): iGremlin/TK mice underwent tamoxifen treatment (3 daily doses of 75 μg/g starting at 6–7 weeks of age) followed by GCV. At day 1 of recovery (F) almost no iGremlin⁺ cells were evident within the bone marrow. By day 21 (G) very few iGremlin⁺ cells were present on bone surfaces. Green indicates actively mineralizing surfaces (calcein in A–B, alizarin complexone in F–G). *, $p < .05$ by student's t test. Scale bars indicate 500 μm (A, B, E–G scans) or 50 μm (enlarged areas). Abbreviation: αSMA, α-smooth muscle actin

Figure 5.

Bone lining cells express mesenchymal stem cell markers. **(A)**: iDMP⁺ osteoblast enriched population was isolated from iDMP mice treated only with tamoxifen by endosteal digest; iDMP⁺ bone lining cells were isolated from iDMP/TK mice after osteoblast ablation. Analysis was also performed using Col2.3GFP⁺ osteoblasts. **(B)**: Analysis of cell surface markers was performed in the indicated cell populations. The *x*-axis represents tdTomato or GFP expression. **(C–E)**: Representative FACS plots indicating the presence of Sca1 and CD51 (C), CD44 (D), and LepR, (E) on the different cell populations. Percentages are averages from at least three replicates. **(F–I)**: Percentages of cells in each population positive for (F) Sca1, *n* = 4–10; (G) CD51, *n* = 4–9; (H) CD44, *n* = 3–4; and (I) LepR, *n* = 4–9; are indicated. *, *p* < .05 compared to iDMP osteoblasts using ANOVA with Dunnett's post-test. Abbreviations: GCV, ganciclovir; LepR, leptin receptor; Tam, tamoxifen.

Figure 6.

BLC form colonies in vitro and bone in vivo. **(A)**: Experimental design for culture and transplantation experiments. For transplantation, freshly sorted cells (2–5,000) isolated from iDMP/TK mice were combined with wild-type bone marrow stromal cells and transplanted into a critical sized calvarial defect. **(B)**: iDMP+ BLCs in the unpurified endosteal fraction form colonies that expand in vitro over time (scans performed at x50 magnification). **(C)**: Following osteoblast ablation (GCV-treated), osteogenic differentiation is dramatically impaired in BMSC cultures, indicated by alkaline phosphatase (pink) and von kossa (brown) staining at day 21. Differentiation is also impaired in the endosteal fraction, shown at day 15. **(D)**: Effect of osteoblast ablation on in vitro differentiation of BMSC and endosteal cells indicated by expression of osteogenic markers bone sialoprotein and osteocalcin. Data representative of 3–4 cultures is shown. **(E)**: Histology of a calvarial defect transplanted with sorted BLCs indicating cells engrafted in the defect and contributing to new bone formation indicated by iDMP+ surface cells associated with calcein label, and labeled osteocytes embedded in newly formed bone (arrows). A representative image of three independent transplant experiments is shown. Scale bars indicate 100 μ m. Abbreviations: BLC, bone lining cells; BMSC, bone marrow stromal cell; GCV, ganciclovir; ND, not detectable; Tam, tamoxifen.

Figure 7.

Prednisolone inhibits activation of bone lining cells. **(A)**: iDMP/TK mice underwent osteoblast ablation, followed by administration of prednisolone (Pred.) or placebo pellets according to the indicated experimental design. $n = 3-5$ /group at each timepoint. **(B)**: MicroCT reconstructions of representative bones after 21 days recovery indicate smaller trabecular regions in the prednisolone treated animals. Quantification of microCT is shown for the trabecular region **(C)** and cortical bone **(D)**. Histology at day 7 in bones from placebo **(E)** or prednisolone **(F)** treated mice indicated reduced activation of bone formation and bone lining cell activation. Labeled cells are indicated in red, calcein in green, and DAPI in blue. Quantification of the proportion of bone surface covered in labeled cells in both trabecular **(G)** and cortical **(H)** bone is shown. *, $p < .05$ by student's t test. Scale bars indicate 1 mm (B), 500 μm (E, F scans) or 50 μm (E, F, enlarged areas). Abbreviations: GCV, ganciclovir; Tam, tamoxifen.