Osteoblastic regulation of B lymphopoiesis is mediated by $G_s\alpha$-dependent signaling pathways

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Osteoblasts play an increasingly recognized role in supporting hematopoietic development and recently have been implicated in the regulation of B lymphopoiesis. Here we demonstrate that the heterotrimeric G protein α subunit $G_s\alpha$ is required in cells of the osteoblast lineage for normal postnatal B lymphocyte production. Deletion of $G_s\alpha$ early in the osteoblast lineage results in a 59% decrease in the percentage of B cell precursors in the bone marrow. Analysis of peripheral blood from mutant mice revealed a 67% decrease in the number of circulating B lymphocytes by 10 days of age. Strikingly, other mature hematopoietic lineages are not decreased significantly. Mice lacking $G_s\alpha$ in cells of the osteoblast lineage exhibit a reduction in pro-B and pre-B cells. Furthermore, interleukin (IL)-7 expression is attenuated in $G_s\alpha$-deficient osteoblasts, and exogenous IL-7 is able to restore B cell precursor populations in the bone marrow of mutant mice. Finally, the defect in B lymphopoiesis can be rescued by transplantation into a WT microenvironment. These findings confirm that osteoblasts are an important component of the B lymphocyte niche and demonstrate *in vivo* that $G_s\alpha$-dependent signaling pathways in cells of the osteoblast lineage extrinsically regulate bone marrow B lymphopoiesis, at least partially in an IL-7-dependent manner.

B lymphocyte | G protein | osteoblast

Beginning in late embryogenesis, mammalian hematopoietic development occurs primarily in the bone marrow, where the presence of a supportive microenvironment, or niche, has long been postulated (1). The contribution of marrow stromal cells to this niche has been well documented and is supported by the identification of stromal cell lines that have the ability to support hematopoietic cell differentiation *in vitro*. The stroma of the bone marrow is composed of cells of various lineages, including osteoblasts, endothelial cells, fibroblasts, and adipocytes, but the relative contributions of each lineage have remained elusive. The importance of osteoblastic cells to the hematopoietic stem cell (HSC) niche has been demonstrated by several groups (2–5). In addition, a vascular component of the HSC niche has been proposed based on histological localization of stem cells (6). Whether or not osteoblastic and vascular cells represent distinct niches remains to be clarified.

Beyond its support for HSCs, the stromal microenvironment within the bone marrow appears to provide specific niches for more differentiated hematopoietic lineages, including B lymphocytes (7) and megakaryocytes (8). The relevance of extrinsic control of hematopoiesis to disease pathogenesis has been underscored by the finding that the microenvironment plays an integral role in the development of myeloproliferative syndromes (9, 10). Thus, the bone marrow may harbor distinct niches for differentiating cells of hematopoietic origin. The cellular constituents and relevant signaling molecules at each stage remain largely undefined, however.

A specific niche within the bone marrow for B lymphocyte differentiation was first proposed by Tokoyoda *et al.* (7), who demonstrated that within the bone marrow, B cell precursors are in direct contact with stromal cells that express CXCL12 or interleukin (IL)-7, two factors that play crucial roles in B lymphopoiesis. These stromal cells are located within the marrow space and do not co-localize with the osteoblasts lining the bone surface. Because the HSC niche has been proposed to lie along the endosteal surface (11, 12), these findings suggest that a B lymphocyte niche may reside in a distinct anatomic localization within the bone marrow. Moreover, Tokoyoda *et al.* (7) found that although prepro-B cells (the most immature identifiable population of B lymphocyte precursors) are in direct contact with cells expressing CXCL12, more differentiated pro-B cells instead contact stromal cells expressing IL-7. Thus, distinct subsets of stromal cells may be involved in regulating the transition of B lymphocytes from one stage of differentiation to the next.

Osteoblasts cultured from neonatal calvariae are capable of supporting all stages of B lymphocyte development from HSCs *in vitro* (13). Moreover, ablation of osteoblasts *in vivo* results in a rapid reduction in the number of B lymphocytes preceding the loss of HSCs (4, 13). These findings point to an integral role for osteoblastic cells in supporting B lymphopoiesis. Although mature osteoblasts line the bone surface, osteoblastic progenitors are present within the stromal cells of the bone marrow. In addition, cells of the osteoblast lineage can produce both CXCL12 and IL-7 (2, 13, 14). Therefore, immature osteoblast precursors within the marrow may play an important role in regulating B lymphocyte differentiation, perhaps in part through production of regulatory growth factors, such as CXCL12 and/or IL-7.

In recent years, the contribution of signaling through the parathyroid hormone (PTH)/PTH-related peptide receptor (PPR) in osteoblastic cells to the regulation of HSC numbers was demonstrated in mice expressing a constitutively active form of PPR targeted to osteoblasts. These mice displayed a dramatic increase in trabecular bone, accompanied by an increase in HSC numbers (2). Intriguingly, the addition of PTH to calvarial osteoblast cultures was found to stimulate the support of B lymphopoiesis *in vitro* (13). Moreover, PTH is known to upregulate production of both CXCL12 and IL-7 by osteoblastic cells *in vitro* (2, 13, 14), suggesting that signaling downstream of the PPR may be relevant to regulation of B lymphopoiesis in addition to HSCs.

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The PPR is a G protein-coupled receptor (GPCR) that signals through multiple G proteins. One major downstream mediator of PPR signaling is $G_s\alpha$, which activates adenylyl cyclase, increasing levels of adenosine 3′,5′-cyclic monophosphate (cAMP) (15). cAMP activates such effectors as protein kinase A (PKA), which in turn regulates gene transcription through phosphorylation of cAMP response element-binding proteins and other targets (16). Besides PPR, other GPCRs also activate $G_s\alpha$ in cells of the osteoblast lineage, including type 2 β adrenergic receptor (17), prostaglandin E2 receptors EP2R and EP4R (18), and thyroid-stimulating hormone receptor (19). Thus, the actions of $G_s\alpha$ on osteoblastic cells may reflect multiple inputs and may exceed the actions of any single ligand/receptor system. Indeed, deletion of $G_s\alpha$ in differentiated osteoblasts has demonstrated the importance of $G_s\alpha$ in trabecular bone (20). Because the aforementioned *in vitro* studies (13) suggest that signals downstream of the PPR might be able to regulate B lymphopoiesis, we hypothesized that $G_s\alpha$ may play an important role in regulating B lymphocyte development *in vivo*.

To test this hypothesis, we ablated $G_s\alpha$ in early osteoprogenitors using Cre recombinase driven by the promoter for osterix, a transcription factor expressed early in cells of the osteoblast lineage (21). The resulting mutant mice displayed a profound decrease in trabecular bone. Analysis of the bone marrow revealed a specific reduction in B cell precursors, with preservation of other hematopoietic lineages. The defect in B lymphopoiesis led to a lower number of precursors after the prepro-B cell stage. Our findings confirm that osteoblast lineage cells participate in a specific B lymphocyte niche within the bone marrow. Furthermore, we found that $G_s\alpha$-dependent signaling pathways are crucial to the regulation of B lymphopoiesis by cells of the osteoblast lineage, and that IL-7 may be an important mediator of this process.

Results

Trabecular Bone Is Reduced in Mice with Conditional Knockout of $G_s\alpha$ in Osteoblast Precursors. $G_s\alpha$ was ablated in osteoblast precursors using transgenic mice in which Cre recombinase is fused to green fluorescent protein (GFP), under the control of the promoter for osterix, a transcription factor expressed early in osteoblastogenesis (Osx1-GFP::Cre) (22). These mice were mated to mice carrying loxP sites flanking exon 1 of the gene encoding $G_s\alpha$ (23). The resulting mutant mice (Osx1-GFP::Cre^+;$G_s\alpha^{fl/fl}$, designated $G_s\alpha^{OsxKO}$ mice) are born at the expected Mendelian ratio and are indistinguishable from control ($G_s\alpha^{fl/fl}$) littermates at birth; however, $G_s\alpha^{OsxKO}$ mice develop postnatal growth retardation [supporting information (SI) Fig. S1*A* and *B*] and early mortality, with most $G_s\alpha^{OsxKO}$ mice dying by postnatal day 14 and none surviving past the first month of life. $G_s\alpha^{OsxKO}$ mice display a dramatic reduction in the amount of trabecular bone (Fig. 1*A* and *B*). GFP driven by osterix is expressed throughout the stages of osteoblast differentiation (22); thus, in Osx1-GFP::Cre^+ mice, the GFP^+ cell population will include most of the osteoblast lineage cells (Fig. S1*C*). To confirm efficient deletion of $G_s\alpha$ in the osteoblasts of $G_s\alpha^{OsxKO}$ mice, we isolated GFP^+ osteoblastic cells by fluorescence-activated cell sorting (FACS) from mice carrying the Osx1-GFP::Cre^+ transgene and their $G_s\alpha^{OsxKO}$ littermates. We found that $G_s\alpha$ mRNA levels were reduced by almost 90% in these cells in $G_s\alpha^{OsxKO}$ mice (Fig. 1*C*).

Ablation of $G_s\alpha$ in Osteoblast Precursors Leads to a Failure of B Lymphopoiesis. To determine whether the loss of $G_s\alpha$ alters the ability of osteoblast precursors to support hematopoietic development within the bone marrow, we analyzed mature hematopoietic lineages in the marrow using flow cytometry. Because the $G_s\alpha^{OsxKO}$ mice were significantly smaller than their control littermates, skeletal size and thus bone marrow cellularity also were decreased in $G_s\alpha^{OsxKO}$ mice (Fig. S1*D*); therefore, the

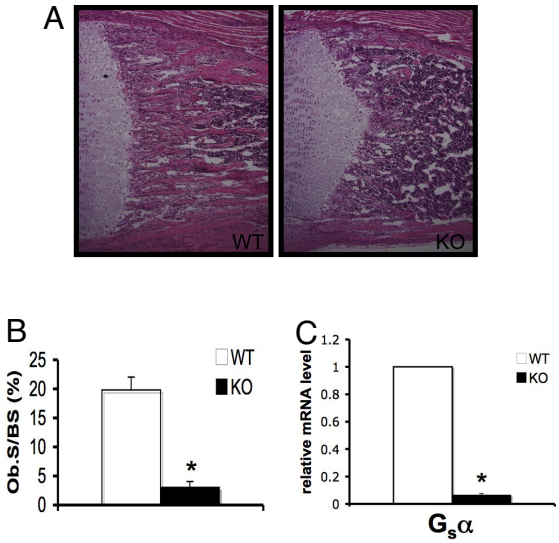

Fig. 1. Trabecular bone is decreased in $G_s\alpha^{OsxKO}$ mice. (*A*) Hematoxylin and eosin–stained sections of proximal tibia at postnatal day 9 from WT and $G_s\alpha^{OsxKO}$ (KO) mice. (*B*) Osteoblast surface (ObS/BS) as a percentage of bone surface (n = 7 [WT] or 5 [KO]). (*C*) Quantitative real-time PCR for $G_s\alpha$ mRNA levels from GFP^+ osteoblastic cells isolated by FACS. *$P < .005$.

distribution of hematopoietic lineages is reported as a percentage of bone marrow cells. In the bone marrow of the $G_s\alpha^{OsxKO}$ mice, we found a specific reduction in the $B220^+$ population, which includes cells of the B lymphocyte lineage. A statistically significant increase in the percentage of $CD11b^+$/$GR\text{-}1^+$ granulocytes was found, whereas $F480^+$/$CD11b^+$ monocytes, $Ter119^+$ erythrocytes, and $CD4^+$ and $CD8^+$ mature T lymphocytes were unaffected (Fig. 2*A*). This defect in bone marrow B lineage cell content was accompanied by a reduction of circulating $B220^+$ cells. By postnatal day 10, there were 68% fewer $B220^+$ cells in the peripheral blood of the $G_s\alpha^{OsxKO}$ mice; in contrast, the circulating $Gr\text{-}1^+$ myeloid content remained unchanged (Fig. 2*B*). Furthermore, the spleens of $G_s\alpha^{OsxKO}$ mice were markedly smaller than those of their littermate controls. Even when corrected for body weight, a 50% reduction in the ratio of spleen to body weight was seen in the $G_s\alpha^{OsxKO}$ mice; other organs (e.g., kidney, heart, lung) remained unaffected (Fig. S2*A* and data not shown). Histological analysis demonstrated that white pulp, consisting of lymphocytes in a follicular arrangement, was dramatically reduced in the spleens of the $G_s\alpha^{OsxKO}$ mice (Fig. S2*B*). Consistent with this finding, the absolute number of $B220^+$ cells within the spleen of $G_s\alpha^{OsxKO}$ mice was <20% of that in their WT littermates (Fig. 2*C*). This finding indicates that the ablation of osteoblast-specific $G_s\alpha$ led to a generalized decrease in $B220^+$ cells, suggesting impaired bone marrow B lymphopoiesis.

B Lymphopoiesis Is Impaired Along the Pro-B to Pre-B Cell Transition. B lymphocyte development shifts to the bone marrow in late embryogenesis, with commitment of the common lymphoid progenitor to the B lymphocyte lineage marked by expression of B220, followed by sequential differentiation to prepro-B, pro-B, and pre-B cells (24). Immature, newly formed B lymphocytes, which express IgM, then migrate to the spleen, where further maturation occurs and expression of IgD is acquired. Therefore, a defect in the ability of the bone marrow to support B lymphopoiesis would be expected to affect B cell precursors. Because B220 expression is not exclusive to B lineage cells, we analyzed bone marrow cells for expression of the additional B lineage markers CD93 and IgM. Indeed, the reduction in B

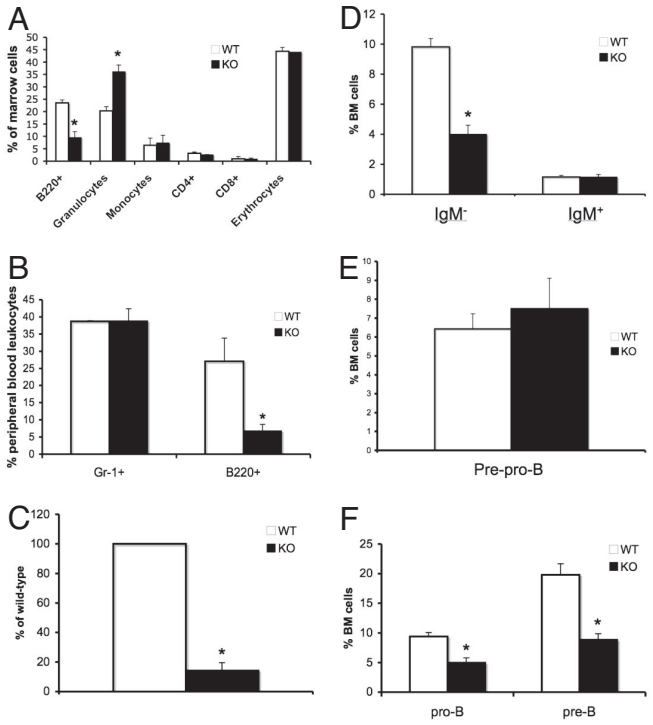

Fig. 2. B lymphopoiesis is impaired in $G_s\alpha^{OsxKO}$ mice. (*A*) Frequency of each lineage, shown as percentage of total marrow cells in postnatal day 10 bone marrow ($n = 4$). *, $P < .005$. (*B*) Gr-1$^+$ myeloid cells and B220$^+$ cells as a percentage of peripheral blood leukocytes at postnatal day 10 ($n = 4$). *, $P < .005$. (*C*) Number of splenic B220$^+$ cells, as a percentage of the number of B220$^+$ cells in WT littermates, is reduced in KO spleens at postnatal day 10 ($n = 4$). *, $P < .001$. (*D*) Percentage of bone marrow cells that are B220$^+$/CD93$^+$/IgM$^-$ (*Left*) or B220$^+$/CD93$^+$/IgM$^+$ (*Right*) at postnatal day 8 ($n = 10$ WT or 7 KO). *, $P < .05$. (*E*) Percentage of B220$^+$/CD19$^-$/CD43$^+$ prepro-B cells in bone marrow on postnatal day 8 ($n = 6$). (*F*) Percentages of pro-B (B220$^+$/IgM$^-$/CD43$^+$) and pre-B (B220$^+$/IgM$^-$/CD43$^-$) cells in bone marrow on postnatal day 8 ($n = 3$). *, $P < .05$.

lineage cells in $G_s\alpha^{OsxKO}$ bone marrow was entirely due to loss of the IgM$^-$ precursor population (Fig. 2*D*). To confirm that osterix-driven Cre recombinase was not expressed in B lineage cells, we analyzed bone marrow cells for GFP expression by flow cytometry. We found that the $G_s\alpha^{OsxKO}$ bone marrow B220$^+$ cells, as well as the other hematopoietic cell types, did not express high levels of GFP (Fig. S3*A*). We next isolated B220$^+$/IgM$^-$ B cell precursors from bone marrow of WT and $G_s\alpha^{OsxKO}$ mice, and, using quantitative real-time PCR, demonstrated that $G_s\alpha$ mRNA levels remained unchanged in B lineage precursors in the $G_s\alpha^{OsxKO}$ mice (Fig. S3*B*). These results indicate that impaired B lymphopoiesis in the $G_s\alpha^{OsxKO}$ mice is an indirect effect of osteoblast-specific $G_s\alpha$ ablation, and not a consequence of ectopic $G_s\alpha$ ablation in the B lymphocyte lineage.

We next sought to determine at which stage B lymphocyte development is affected in the absence of osteoblast-specific $G_s\alpha$. We found no decrease in B220$^+$CD19$^-$CD43$^+$ prepro-B cells in the $G_s\alpha^{OsxKO}$ mice (Fig. 2*E*), suggesting that the blocking of B lymphopoiesis occurred at a later stage. Indeed, the percentages of both B220$^+$IgM$^-$CD43$^+$ pro-B and B220$^+$IgM$^-$CD43$^-$ pre-B cells were significantly reduced in $G_s\alpha^{OsxKO}$ bone marrow (Fig. 2*F*). In contrast to the bone marrow, the fetal liver at e18.5 exhibited no difference in pro-B cells between the WT and $G_s\alpha^{OsxKO}$ mice (Fig. S3*C*). Thus, in the absence of $G_s\alpha$ in osteoblastic lineage cells, B lymphopoiesis was impaired along the pro-B cell to pre-B cell transition, specifically in the bone marrow.

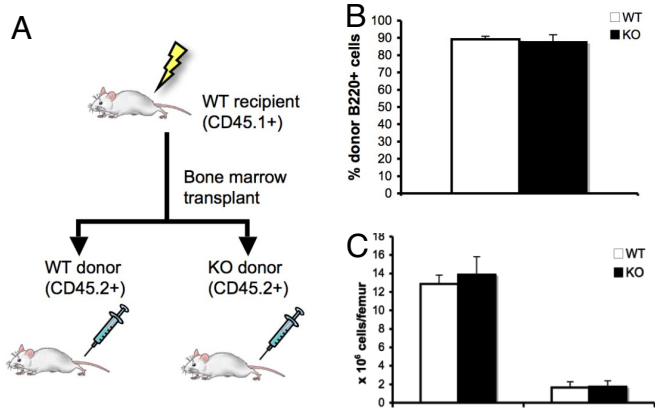

Fig. 3. Transplantation into WT microenvironment rescues B lymphopoiesis of $G_s\alpha^{OsxKO}$ bone marrow. (*A*) Adult WT lethally irradiated CD45.1$^+$ mice received bone marrow transplants from WT or KO CD45.2$^+$ donors. (*B*) Percentage of donor (CD45.1$^+$) B220$^+$ cells in recipients of WT and KO bone marrow transplantation. (*C*) Number of total bone marrow mononuclear cells (*Left*) and B220$^+$/IgM$^-$ immature B cell precursors (*Right*) in recipients of WT ($n = 5$) and KO ($n = 4$) bone marrow.

Impaired B Lymphopoiesis Is Rescued by Transplantation into a WT Microenvironment. To confirm that the abnormal B lymphopoiesis in $G_s\alpha^{OsxKO}$ mice was due to a stromal defect resulting from $G_s\alpha$ deficiency in osteoblasts, we transplanted bone marrow from WT or $G_s\alpha^{OsxKO}$ mice into WT recipients. (Transplantation of WT or $G_s\alpha^{OsxKO}$ bone marrow into $G_s\alpha^{OsxKO}$ recipients is not feasible, because $G_s\alpha^{OsxKO}$ mice die before weaning.) WT CD45.1$^+$ mice were irradiated and then underwent bone marrow transplantation, receiving either WT or $G_s\alpha^{OsxKO}$ bone marrow. Donor marrow from both WT and $G_s\alpha^{OsxKO}$ mice expressed CD45.2, allowing us to distinguish host-derived (CD45.1$^+$) from donor-derived (CD45.2$^+$) hematopoietic cells (Fig. 3*A*). We analyzed bone marrow 10 weeks posttransplantation and found successful engraftment; transplantation with either WT or $G_s\alpha^{OsxKO}$ bone marrow resulted in 90% leukocyte reconstitution by transplanted cells (Fig. 3*B*). Flow cytometric analysis of the bone marrow revealed that transplantation into a WT environment completely rescued bone marrow cellularity and B lymphopoiesis (Fig. 3*C*). Thus, the defective B lymphopoiesis in $G_s\alpha^{OsxKO}$ mice can be attributed to the loss of $G_s\alpha$ from the stromal microenvironment.

IL-7 Expression, but Not CXCL12 Expression, Is Decreased in $G_s\alpha$-Deficient Osteoblasts. To identify potential mechanisms by which $G_s\alpha$ signaling in osteoblasts might regulate B lymphopoiesis, we investigated the expression of CXCL12 and IL-7 in WT and $G_s\alpha^{OsxKO}$ osteoblasts. Both CXCL12 and IL-7 production by osteoblastic cells can be stimulated by PTH (2, 13, 14); however, these cytokines regulate distinct stages of B cell development. CXCL12 is crucial for the production of prepro-B cells (25). In contrast, cells expressing IL-7 are in close contact with pro-B cells (7), and mice lacking IL-7 or the IL-7 receptor α subunit demonstrate impaired formation of pro-B and pre-B cells (26, 27). We isolated GFP$^+$ osterix-expressing osteoblastic cells from WT and $G_s\alpha^{OsxKO}$ mice by FACS, purified total RNA, and performed quantitative real-time PCR for CXCL12 and IL-7 mRNA levels. Consistent with the finding that prepro-B cells were unaffected in the $G_s\alpha^{OsxKO}$ mice, no decrease in CXCL12 expression was seen in the $G_s\alpha^{OsxKO}$ osteoblasts. In contrast, IL-7 mRNA levels dropped to 19% of WT levels (Fig. 4*A*). This reduction is not simply a reflection of decreased osteoblast numbers in GFP$^+$ cells from which RNA was isolated, because levels of alkaline phosphatase and type I collagen mRNA were

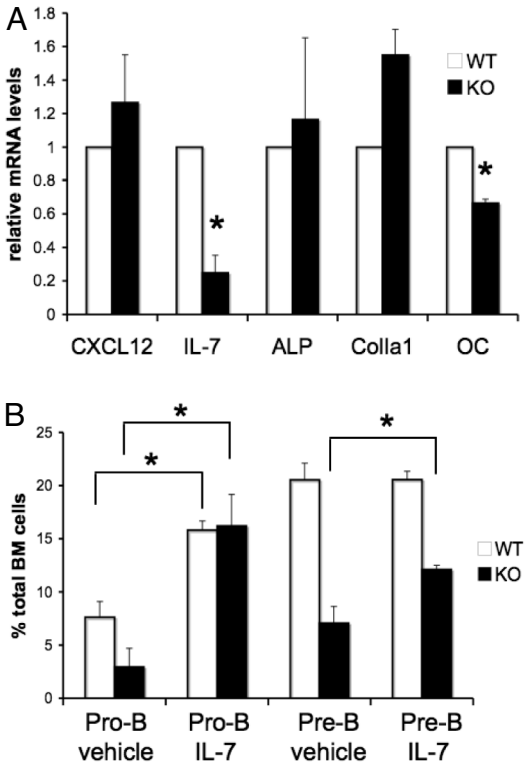

Fig. 4. IL-7 expression is reduced in $G_s\alpha^{OsxKO}$ osteoblasts. (*A*) CXCL12, IL-7, alkaline phosphatase (ALP), type I collagen (Colla1), and osteocalcin (OC) mRNA levels in Osx-GFP:Cre$^+$ osteoblasts from WT and KO littermates. *, $P < .02$. (*B*) Pro-B and pre-B cells as percentages of bone marrow cells in WT ($n = 7$) and KO ($n = 3$) mice injected with IL-7 or vehicle.

not reduced in mutant mice. As expected, expression of osteocalcin, a known target of PKA-dependent signaling (28), also was specifically reduced in the $G_s\alpha^{OsxKO}$ mice (Fig. 4*A*).

To determine whether IL-7 deficiency could explain the dramatic reduction in pro-B and pre-B cell content in $G_s\alpha^{OsxKO}$ mice, we administered recombinant murine IL-7 to WT and $G_s\alpha^{OsxKO}$ littermates on postnatal days 3–6. On postnatal day 7, a significant increase in pro-B cells in the WT mice receiving IL-7 was seen compared with the vehicle-injected mice (Fig. 4*B*). Importantly, in the $G_s\alpha^{OsxKO}$ mice, administration of IL-7 rescued pro-B cell production to WT levels. There was also a significant increase in pre-B cells in the $G_s\alpha^{OsxKO}$ mice given IL-7 compared with those given vehicle. The percentage of more differentiated pre-B cells in the IL-7-injected $G_s\alpha^{OsxKO}$ mice did not reach WT levels, likely due to the limited time course of treatment. These findings indicate that IL-7 can overcome, at least in part, the B cell precursor defect in $G_s\alpha^{OsxKO}$ mice, suggesting that IL-7 is an important mediator of $G_s\alpha$-mediated regulation of B lymphopoiesis by osteoblasts.

Besides its critical role in supporting B lymphopoiesis, IL-7 also is necessary for thymic development of T lymphocytes (29). We assessed thymic and peripheral CD4 and CD8 T lymphocyte production and found no alterations in the $G_s\alpha^{OsxKO}$ mice (Fig. S4*A* and data not shown). Consistent with this finding, IL-7 levels were not decreased in thymus tissue from the $G_s\alpha^{OsxKO}$ mice (Fig. S4*B*). Thus, as a consequence of osteoblast-specific deletion of $G_s\alpha$ in the $G_s\alpha^{OsxKO}$ mice (which would be expected to affect the bone marrow microenvironment but not the thymic stroma), B lymphopoiesis was impaired, whereas T lymphopoiesis remained unaffected.

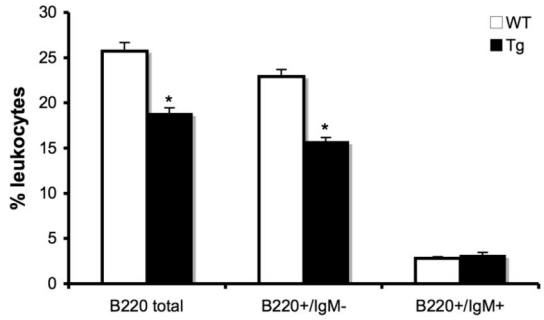

Fig. 5. Transgenic mice expressing the constitutively active PPR in osteoblasts have defective production of B220$^+$IgM$^-$ lymphocytes. Shown are the frequencies of bone marrow leukocytes that are B220$^+$, B220$^+$/IgM$^-$, or B220$^+$/IgM$^+$ in 2 week-old WT ($n = 2$) or transgenic (Tg, $n = 4$) mice. *, $P < .005$.

B Lymphopoiesis Is Attenuated in Mice with Constitutively Active PPR in Osteoblasts. Because loss of $G_s\alpha$ signaling and reduced trabecular bone leads to a dramatic reduction in the numbers of bone marrow B lymphocytes, we investigated whether augmented $G_s\alpha$ signaling in relatively mature osteoblasts might result in increased B cell numbers. Transgenic mice expressing the constitutively active PTH/PTHrP receptor driven by a collagen I(α1) promoter fragment in differentiated osteoblasts are known to exhibit a significant increase in trabecular bone (30). The mutant receptor expressed in these mice leads to amplified cAMP accumulation downstream of $G_s\alpha$ activation. Surprisingly, 14-day-old constitutively active PPR transgenic mice also had a significantly reduced number of bone marrow B lymphocytes, due entirely to a decrease in the immature IgM$^-$ fraction (Fig. 5). These findings indicate that B cell numbers are not determined solely by bone mass or osteoblast number, and that tight regulation of signaling downstream of $G_s\alpha$ at different stages of osteoblastogenesis may be needed to allow optimal B lymphopoiesis.

Discussion

We have found that ablation of $G_s\alpha$ early in the osteoblast lineage *in vivo* leads to a profound reduction in bone marrow B lineage cells. Other hematopoietic lineages are preserved, suggesting a specific impairment of B lymphopoiesis. Consistent with a defect stemming from an alteration in the bone marrow microenvironment, the production of B cell precursors is affected, resulting specifically in reduced numbers of pro-B and pre-B cells. We have further demonstrated that the abnormal B lymphopoiesis is due to the loss of $G_s\alpha$ from the stromal microenvironment. Osterix-Cre is not expressed in B lymphocytes, and $G_s\alpha$ mRNA levels are unchanged in B cell precursors isolated from $G_s\alpha^{OsxKO}$ mice. Moreover, transplantation of $G_s\alpha^{OsxKO}$-derived bone marrow into a WT host completely restores B lymphopoiesis. Thus, the defect in B lymphopoiesis is extrinsic to the hematopoietic system.

These findings confirm that cells of the osteoblast lineage are indeed an important component of the B lymphocyte niche. Here we extend our current understanding of the interactions between cells of the osteoblast and hematopoietic lineages and present the first *in vivo* evidence for a relevant signaling pathway in osteoblastic cells that regulates B lymphopoiesis. The importance of $G_s\alpha$-dependent signaling pathways to the HSC niche has been demonstrated by the finding that constitutively active PPR increases the numbers of HSCs in transgenic mice (2). In cultured cells, the constitutively active receptor mutation used in these transgenic mice signals predominantly through $G_s\alpha$ (31). Our findings demonstrate that signals downstream of $G_s\alpha$ in cells of the osteoblast lineage also are critical for the normal production of B lymphocytes by the bone marrow in early postnatal life.

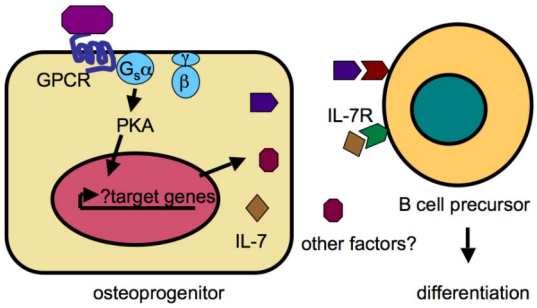

Fig. 6. Model of osteoblastic regulation of B lymphopoiesis. Within the bone marrow of early postnatal mice, B cell precursors are located in close proximity to cells of the osteoblast lineage. Stimulation of $G_s\alpha$-coupled GPCRs on the osteoblast surface leads to up-regulation of PKA target genes. These may include genes encoding for factors that stimulate B lymphopoiesis, such as IL-7.

Previous studies have found that a correlation between decreased osteoblasts and decreased B lymphocytes (4, 13). Because $G_s\alpha^{OsxKO}$ mice have fewer osteoblasts than their WT littermates, this may contribute to the loss of immature B cell precursors in mutant mice. B cell lymphopoiesis is not simply proportional to the number of osteoblasts, however. Mice lacking the proteoglycan biglycan have fewer osteoblasts than control mice, but no impairment of B lymphopoiesis (32). Conversely, the increased bone mass and numbers of osteoblasts found in constitutively active PPR transgenic mice do not lead to a greater number of B lymphocytes in bone marrow; rather, these mice also exhibit mildly impaired B lymphopoiesis. This somewhat surprising result may reflect changes in the subpopulations of cells of the osteoblast lineage needed to support B lymphopoiesis; alternatively, precise regulation of pathways downstream of $G_s\alpha$-coupled receptors may be needed for adequate production of B lymphocytes in the bone marrow.

Of the downstream target(s) of $G_s\alpha$-mediated signaling pathways critical for B lymphopoiesis, IL-7 appears to be a key mediator. IL-7 mRNA levels are decreased in cells of the osteoblast lineage in $G_s\alpha^{OsxKO}$ mice, and exogenous IL-7 completely restores pro-B cell production and significantly boosts pre-B cell numbers in these mice after only 3 days of treatment. Our results support the finding that PTH stimulates the production of IL-7 by stromal cells (13), demonstrating that PKA-dependent pathways play a significant role in regulating IL-7 expression. The upstream receptors coupling to $G_s\alpha$ to regulate the osteoblastic B lymphocyte niche remain undefined. Although PPR is an attractive candidate, other GPCRs that signal through $G_s\alpha$ have been identified in osteoblasts. In particular, the prostaglandin E2 receptors EP2R and EP4R are expressed in cells of the osteoblast lineage (18), and PGE2 has been reported to regulate the HSC niche (33).

As a working model (Fig. 6), we hypothesize that during early postnatal B cell development, B lymphocyte precursors are in close proximity to osteoprogenitors in the marrow space. Stimulation of GPCRs leads to activation of the PKA-dependent pathway through $G_s\alpha$, with up-regulation of target genes. One or more of these products, which likely include IL-7, may play important roles in B lymphopoiesis. Nagasawa (25) has proposed that whereas HSCs are initially in contact with terminally differentiated osteoblasts along the bone surface, as these cells mature, they migrate toward the central region of the marrow cavity, allowing more differentiated hematopoietic precursors to come in contact with immature stromal cells within the marrow. Whether the osteoblastic cells implicated in our findings are the same as the IL-7-expressing cells identified by Tokoyoda *et al.* (7) remains to be determined. Recently, Sapoznikov *et al.* (34) reported that perivascular clusters of dendritic cells within the bone marrow provide crucial survival signals to mature recirculating B lymphocytes. Thus, it appears increasingly plausible that each stage of B lymphocyte differentiation may occur in a specific niche, each with a potentially distinct anatomic localization.

An increased mechanistic understanding of the interactions between the hematopoietic and skeletal systems in providing regulatory niches has potential therapeutic utility. The association of both osteoporosis and declining B cell numbers with age is well known (35, 36). The finding that bone mass may be related to B cell number, and that this may be regulated by signals downstream of $G_s\alpha$, raises the possibility that treatments such as PTH, already approved for osteoporosis, may have beneficial effects for the immune system as well. In addition, it is now clear that the stromal microenvironment plays a key role in pathophysiologic processes. Given the propensity for malignant disorders of the B lymphocyte lineage (e.g., multiple myeloma) to involve the skeleton, clarifying the relevant signaling pathways may offer novel approaches with clinical benefits.

Methods

Experimental Animals. Osx1-GFP::Cre (22) and $G_s\alpha$(fl/fl) (23) mice have been described previously. Because these mice are of a mixed genetic background (C57BL/6 and CD1), WT littermates were used as controls for all experiments described. Genotyping was performed on genomic DNA isolated from tails, using previously published protocols. All animals were housed in the Center for Comparative Medicine at the Massachusetts General Hospital, and all experiments were approved by the hospital's Subcommittee on Research Animal Care.

Flow Cytometry Analysis. Bone marrow, spleen, thymus, and hemolyzed peripheral blood cells were stained for antibodies to B lymphocytes (B220, IgM, and CD93), T lymphocytes (CD4 and CD8a), granulocytes (CD11b and Gr-1), and erythrocytes (Ter119), as described previously (9). B cell precursors were analyzed with antibodies to CD2, CD19, and CD43. Isotype-matched antibodies were used for controls. Antibodies were purchased from eBioscience. Flow cytometry was performed on a FACSCalibur cytometer (BD Biosciences).

Bone Marrow Transplantation. The 8-week-old B6.SJL ($CD45.1^+$) mice were irradiated, then transplanted with 3×10^6 BM mononuclear cells from WT or $G_s\alpha^{OsxKO}$ mice ($CD45.2^+$) ($n = 5$ for each group). At 10 weeks posttransplantation, peripheral blood, bone marrow, and spleen cells were obtained for analysis of $CD45.2^+$ cells within the B lymphocyte lineage.

Isolation of Osteoblastic Cells by FACS. Osteoblastic cells were harvested from neonatal calvariae of $G_s\alpha^{OsxKO}$ and control (Osx1-GFP::Cre^+; $G_s\alpha^{+/+}$) mice by serial collagenase digestion (37). Fractions 3–6 were pooled and resuspended in phosphate-buffered saline (PBS) with 2% fetal bovine serum. Then ≈30,000 Osx1-GFP^+CD45^- cells were isolated per genotype by FACS using a FACS Aria sorter (BD Biosciences).

Quantitative Real-Time PCR. Total RNA was isolated from cells using the RNeasy kit (Qiagen) and cDNA was synthesized with the SuperScript III First Strand synthesis system for real-time PCR (Invitrogen). Quantitative real-time PCR was performed using primers for $G_s\alpha$ (38), CXCL12 (39), IL-7 (40), ALP (41), Colla1 (42), and OSC (39) according to previously published protocols, with mRNA levels normalized relative to β-actin expression. Total RNA samples subjected to cDNA synthesis reactions in the absence of reverse transcriptase were included as negative controls.

IL-7 Administration. Recombinant murine IL-7 (R&D Systems) or vehicle (PBS + 0.1% bovine serum albumin) was injected at a dose of 100 ng twice daily on postnatal days 3–6. The mice were euthanized on postnatal day 7, and their bone marrow was analyzed for B cell precursors.

Statistics. Statistical analyses were performed using a two-tailed Student's *t* test. All values are expressed as mean ± standard error of the mean.

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