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Enhancing T Lineage Production in Aged Mice: A Novel Function of Foxn1 in the Bone Marrow Niche

Erin C. Zook,†‡ Shubin Zhang,§ Rachel M. Gerstein,§ Pamela L. Witte,§†‡· and Phong T. Le§†‡

Foxn1 is essential for thymic organogenesis and T lymphopoiesis. Whereas reduced Foxn1 expression results in a decline in T lymphopoiesis, overexpression of Foxn1 in the thymus of a transgenic mouse model (Foxn1Tg) attenuates the age-associated decline in T lymphopoiesis. T lymphopoiesis begins with early T cell progenitors (ETP), derived from multipotent progenitors (MPP) in the bone marrow (BM). A decline in MPP and ETP numbers with age is thought to contribute to reduced T lymphopoiesis. Previously, we showed that reduced ETP number with age is attenuated in Foxn1 transgenic (Tg); whether the effect is initiated in the BM with MPP is not known. In this study, we report that Foxn1 is expressed in wild-type BM and overexpressed in Foxn1Tg. With age, the number of MPP in Foxn1Tg was not reduced, and Foxn1Tg also have a larger pool of hematopoietic stem cells. Furthermore, the Foxn1Tg BM is more efficient in generating MPP. In contrast to MPP, common lymphoid progenitors and B lineage cell numbers were significantly lower in both young and aged Foxn1Tg compared with wild type. We identified a novel population of lineage-CD45+EpCAM-Sca1+CD117-CD138-MHCII- cells as Foxn1-expressing BM cells that also express Delta-like 4. Thus, Foxn1 affects both T lymphopoiesis and hematopoiesis, and the Foxn1 BM niche may function in skewing MPP development toward T lineage progenitors. The Journal of Immunology, 2013, 191: 000–000.

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Materials and Methods

Mice

Generation of Foxn1Tg mice (C57BL/6) was previously described (14). Mouse Foxn1 is expressed under the human keratin 14 promoter. Two Foxn1Tg colonies, line 5 with 10−12 copies and line 60 with 4−5 copies of the transgene, were maintained at Loyola University Medical Center vi-varium. Because we have shown in the previous study that both lines...
display identical phenotypes, both Foxn1Tg lines were used in the current study and the data were pooled (14). Young and aged Wt C57BL/6 mice were from Harlan through the National Institute on Aging. BM cells from Foxn1cre-Rosa26-lacZ mice, which were previously described (15), were generously provided by V. Deep Dixit (Pennington Biomedical Research Center, Baton Rouge, LA). The H2-SVEX BM were on C57BL/6, CD45.1 background and were used to track cells with RAG activities (16).

**Flow cytometry**

Table I lists mAbs used to identify hematopoietic stem cells (HSC), MPP, CLP, committed T cell progenitors (CTP), committed intermediate T cell progenitors (CIP), and B lineage cells. Samples were analyzed on FACS-Canto II or sorted using a FACSaria (BD Biosciences, San Jose, CA). Analysis was performed using FlowJo 7.6.1 (Tree Star, Ashland, OR).

**Cell cycle**

Eight to 25,000 (8–25 × 10³) FACS-sorted progenitors (HSC, MPP, CTP, and CIP) from an individual mouse or pooled from two to six mice were washed in PBS and fixed overnight in PBS with 70% ethanol and 15% FBS. Fixed cells were washed twice in PBS and resuspended in 250 μl 0.05 mg/ml propidium iodide, 0.1 mM EDTA, plus 0.05 mg/ml RNase A at 20˚C for 1 h.

**BM adoptive transfers**

FACS-sorted Lineage⁺⁺ Scal⁻⁺⁺ c-kit⁺⁺ (LSK) cells (8–16 × 10³) from CD45.1⁺⁻⁺ H2-SVEX BM were i.v. injected into nonirradiated 17- to 21-mo CD45.2 Wt and Foxn1Tg hosts. After 10 wk, the frequency of donor HSC, MPP, CTP, and CIP was determined using flow cytometry.

**Methylcellulose colony assay**

BM cells (10⁴) from Wt and Foxn1Tg (1–4 and 19–25 mo) were cultured in MethoCult methylcellulose (Stem Cell Technologies, Vancouver, Canada) for 72 h. For staining, 5-g/ml and developed, as described above. Purified rabbit IgG was used as control.

**Immunohistochemistry**

Sternal sections were fixed for 48 h in Zamboni solution (4% paraformaldehyde with picric acid) and decalcified in 15% sucrose containing 2% acetic acid for 72 h. For staining, 5-μm sections were deparaffinized and Ag retrieval was performed using Dako’s Target Retrieval Citrate Buffer pH 6 (Carpinteria, CA) with steam at 89˚C for 1 h. Sections were treated with 3% hydrogen peroxide and blocked sequentially with human serum (Invi- 

**RT-PCR**

Total RNA from BM cells was isolated using Qiagen’s RNeasy, and cDNA was synthesized, as previously described (14). Expression of endogenous Foxn1 and transgene Foxn1 was determined by quantitative RT-PCR and calculated, as previously published (14). RT-PCR was used to determine expression of D1H and D4 in sorted cells; primers are listed in Table II. Expression of Hprt was used as control.

**Statistical analysis**

Student t test and Mann–Whitney U test were used for comparisons. For multiple comparisons, a two-way ANOVA was used. All statistical tests were performed using SigmaStat 2.03. The p values ≥ 0.05 are statistically significant.

**Results**

**MPP number does not decline with age in Foxn1Tg**

The MPP population is thought to be BM precursors to ETP (8, 11, 12). With age, both ETP and MPP populations decline (8, 13). We demonstrated that the number of thymic ETP in aged Foxn1Tg was higher than aged Wt mice (14). Thus, we determined whether the number of MPP in the BM is also higher in aged Foxn1Tg. The total number of MPP in Wt BM decreased 3.7-fold by 20–21 mo (p = 0.126) and 9.7-fold by 24–25 mo (p < 0.001) of age compared with Wt 1–2 mo (Fig. 1A). In young Foxn1Tg, the numbers of MPP were not different from young Wt. The total MPP number in the Foxn1Tg 20- to 21- and 24- to 25-mo groups was higher than age-matched Wt (p = 0.002, 20–21 mo; p = 0.03, 24–25 mo). Furthermore, the sizes of the MPP pool in Foxn1Tg were maintained because their numbers were not different among the three age groups or compared with Foxn1Tg that were 26–35 mo old (Fig. 1A). Thus, overexpression of Foxn1 in the BM prevented the decline and maintained MPP number with age.

**Foxn1Tg have a larger HSC pool**

In Wt BM, it was suggested that the decline in MPP with age is due to a developmental block in differentiation of HSC to MPP, as HSC are the immediate precursors to MPP and HSC number increases with age (13). We determined HSC number in young and aged Wt and Foxn1Tg to assess whether overexpression of Foxn1 affects age-associated changes in HSC. HSC number in Wt increased 2.1-fold by 20–21 mo compared with 1–4 mo (Fig. 1B, *p = 0.004). However, Wt that were 24–25 mo showed a 5.4-fold reduction compared with 20–21 mo (p < 0.001) and a 2.6-fold reduction compared with 1–4 mo (p = 0.036) (Fig. 1B). Compared with Wt, HSC number in 1- to 4-mo Foxn1Tg was significantly higher (1.3-fold) (Fig. 1B, *p = 0.036). As in Wt, there was an increase in HSC number in the 20- to 21-mo Foxn1Tg that was higher than age-matched Wt (Fig. 1B, †p < 0.001 Foxn1Tg 20–21 mo versus 1–4 mo; ‡p = 0.01 Foxn1Tg 20–21 versus Wt 20–21). More importantly, HSC number in the 24- to 25-mo Foxn1Tg was only reduced to the level found in 1–4 mo, whereas Wt HSC levels in the 24- to 25-mo group were approximately one-third of those in the 1- to 4-mo groups (Fig. 1B). Overall, HSC number in the 24- to 25-mo Foxn1Tg was 3.9-fold higher than age-matched Wt (Fig. 1B, *p = 0.01). Furthermore, HSC number in Foxn1Tg BM was not statistically different from Foxn1Tg mice 24– 25 mo (p = 0.800). Although the age-associated changes in the fluctuation of HSC were present in Foxn1Tg as in Wt mice, Foxn1Tg at each age group had a higher number of HSC compared with age-matched Wt, suggesting that overexpression of Foxn1 resulted in the maintenance of a larger HSC pool with age. Fig. 1C depicts the flow cytometric profiles for LSK, HSC, and MPP in Wt and Foxn1Tg mice. Table I lists Abs used to identify the above populations.

To rule out the possible contribution of a Foxn1-mediated increase in BM cellularity, we determined the total number of BM nucleated cells of Wt and Foxn1Tg mice. No significant difference was observed between total cell numbers in Wt and Foxn1Tg mice 2–4 mo (Supplemental Fig. 1A). The number of BM nucleated cells in Wt significantly increased with age, as previously reported (Supplemental Fig. 1A, *p = 0.01) (17). However, the age-associated increases were not observed in aged Foxn1Tg, although this group included mice that were up to 35 mo old (Supplemental Fig. 1A). These data demonstrated that the higher numbers of HSC and MPP in Foxn1Tg were not due to increases in BM cellularity but rather the maintenance of the HSC and MPP compartments with age.

The age-associated changes in HSC and MPP populations in Wt and Foxn1Tg were also reflected in the frequency of the LSK. In Wt 1–4 to 20–21 mo, the LSK frequency remained unchanged as the number of HSC increased and MPP decreased (Fig. 1). However, from 20–21 to 24–25 mo, the LSK frequency declined...
as did the numbers of MPP and HSC (Fig. 1). Foxn1Tg showed an increase in the frequency of LSK from 1–4 to 20–21 mo, resulting from the maintenance of MPP and the increases in HSC. At 24–25 mo, the frequencies of LSK populations were comparable to that of mice 1–4 mo relative to the changes observed in HSC populations. These data demonstrated that in mice 24–25 mo old, overexpression of Foxn1 resulted in a larger LSK population through the maintenance of the HSC and MPP populations.

HSC and MPP in Foxn1Tg are resistant to age-associated cell death

Cell cycle analysis was performed to elucidate a cellular mechanism explaining a larger HSC pool and the prevention of the decline in MPP with age. Electronically sorted HSC and MPP from young (2–5 mo) and aged (20–29 mo) Wt and Foxn1Tg were analyzed. We found no increase with age in the percentage of HSC in S,G2/M phase in Wt (Fig. 2A). In contrast, aged Foxn1Tg had a 1.7-fold increase in the percentage of HSC in S,G2/M compared with young Foxn1Tg (\( p = 0.005 \)), and the levels were significantly higher than in aged Wt (Fig. 2A, \( p = 0.001 \)). No changes were observed in the MPP frequency of S,G2/M within MPP with age in either Wt or Foxn1Tg (Fig. 2B). When cell death was determined based on the percentage of cells in sub-G0, a significant increase in the percentage of HSC in the sub-G0 fraction occurred with age in Wt mice (Fig. 2C, \( p = 0.04 \)). Whereas there was a similar trend in MPP from Wt, the increase was not significant (Fig. 2D). In contrast, the percentages of HSC and MPP in sub-G0 were not significantly different between young and aged Foxn1Tg. Strikingly, the fractions of HSC and MPP in sub-G0 in Foxn1Tg (20–29 mo) were 3.5-fold less (Fig. 2C, **\( p = 0.03 \)) and 3.7-fold less (Fig. 2D, ***\( p = 0.005 \)) compared with Wt, respectively. Thus, with age, overexpression of Foxn1 results in increased HSC proliferation and reduced cell death of both HSC and MPP.

Foxn1 is expressed in the BM of Wt and Foxn1Tg mice

It is well established that Foxn1 is expressed in epithelial cells of the thymus and skin and plays a critical role in thymic organogenesis and hair follicle development, respectively (2). However, expression of Foxn1 in the BM has not been previously interrogated. The differences in the numbers of HSC and MPP between Foxn1Tg and Wt mice provide a functional basis to investigate whether Foxn1 is indeed expressed in BM. Fig. 3A revealed that Foxn1 was expressed in the BM of Foxn1Tg mice and that expression of Foxn1 did not decline significantly with age. As we have previously shown in the thymus (14), both the transgene and endogenous Foxn1 were expressed in the BM of Foxn1Tg mice. Foxn1 was also expressed in the BM of Wt mice. Young and aged Wt mice expressed Foxn1 in the BM at low levels (average 414 copies/μg total RNA) (Fig. 3B). Foxn1Tg had a 98-fold higher Foxn1 level compared with age-matched Wt (Fig. 3A, 3B). Con-
for EpCAMpos. EpCAMpos cells were present in both Wt and Foxn1Tg cells (Fig. 3A). In aged Wt and Foxn1Tg mice did not express Foxn1 (Supplemental Fig. 3B).

FACS-sorted Linneg/low EpCAMpos cells from aged Foxn1Tg and Wt mice were assessed for Foxn1 expression by immunohistochemistry. Fig. 4B depicts the morphology of the Foxn1pos cells from the sorted population (top panels); the cellular morphology was similar to Foxn1pos cells identified in situ (Fig. 3C). On average, 12.8% of these cells were Foxn1pos (data not shown). Because BM plasma cells also express EpCAM (20), we sorted Linneg/low EpCAMpos CD138pos cells (or Syndecan-1, a common marker of plasma cells) and stained for Foxn1. Between 23 and 40% of the Linneg/low EpCAMpos were negative for CD138 in Wt and Foxn1Tg, respectively (Fig. 4B, middle panels, data not shown). Notably, the Linneg/low EpCAMpos CD138pos population was greatly enriched for Foxn1pos cells; on average, 45 and 68% of this subset were positive for Foxn1 in Wt and Foxn1Tg, respectively (data not shown). The frequencies of Linneg/low EpCAMpos CD138pos Foxn1pos cells per 100,000 BM nucleated cells were calculated to be 3 ± 1 in old Wt and 95 ± 49 in old Foxn1Tg (Fig. 4C). Additionally, Linneg/low EpCAMpos CD138pos BM cells from Foxn1Tg mice also expressed keratin 14 (Supplemental Fig. 2G–J).

To confirm that Foxn1 is normally expressed in the BM of Wt mice, BM cells were isolated from Foxn1cre-Rosa26-Lac Z reporter mice in which expression of bacterial β-galactosidase is driven by the Foxn1 promoter. The sorted Linneg/low EpCAMpos CD138pos BM cells from Foxn1cre-Rosa26-LacZ mice (24 mo) stained positive for β-galactosidase and showed identical morphology as compared with Foxn1pos cells within the Linneg/low EpCAMpos CD138pos population of Foxn1Tg and Wt mice (Fig. 4B, bottom two panels). Taken together, the data demonstrated that Foxn1 is indeed normally expressed in the BM, albeit at a low level as determined by quantitative RT-PCR (Fig. 3A, 3B).

Foxn1Tg BM microenvironment is more efficient in promoting the development of MPP and T cell progenitors

To examine whether Foxn1Tg mice are more efficient in promoting MPP development, FACS-sorted LSK cells from CD45.1 H2-SVEX BM were transferred i.v. into nonirradiated aged Wt and Foxn1Tg hosts, and the frequencies of donor-derived MPP and HSC were determined 10 wk posttransplant. H2-SVEX mice were chosen to determine whether the Foxn1Tg environment is more efficient in promoting lymphoid lineage commitment through Rag expression and activity as measured by the expression of a violet-excited GFP (VEX). No significant differences in donor frequencies were observed among HSC or MPP generated in Wt and Foxn1Tg BM hosts (Fig. 5A, 5B). The donor frequency ratios of MPP/HSC were calculated as a measurement of the efficacy in the generation of MPP. The ratios of donor MPP/HSC were higher in aged Foxn1Tg compared with Wt (*p = 0.03, Fig. 5C), suggesting that the Foxn1Tg environment is more efficient in promoting the generation of MPP from HSC. Our analyses did not detect donor MPP that were VEX positive, an observation previously seen within the LSK BM population, which includes MPP (data not shown) (21).

The ability of aged HSC from Foxn1Tg and Wt to generate multipotent progenitors in vitro was also determined using BM colony assay in methylcellulose. Compared with young Wt, aged Wt cultures produced fewer colonies with granulocyte, erythrocyte, monocyte, megakaryocyte (GEMM) cells (Fig. 6, *p = 0.03); however, no significant reduction was observed in the ability of

### Table I. List of mAbs used in the study

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<tr>
<th>Ab</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Source</th>
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<tr>
<td>CD16/32</td>
<td>2.4G2</td>
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<td>eBioscience</td>
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List of flow cytometry Abs. All staining included a blocking step with CD16/32. Asterisk (*) denotes biotinylated Abs used with strepavidin-FITC to identify lineage-positive cells. Sca-1, CD117, CD127, and CD135 were used to identify HSC, MPP, and CTP. CTP and GMP were identified within the lineage-negative (linneg) subset using CD90.2 and CD2. B220FITC and CD10PE were used to determine the number of B lineage cells in the BM. EpCAM and CD138 were used to identify Foxn1-expressing cells in the BM. Abs to CD45.1 and CD45.2 were used to identify donor and host cells in adoptive transfers.

tary to the thymus, expression of the endogenous Foxn1 did not change significantly with age (3, 14). When the endogenous level of Foxn1 was compared between Foxn1Tg and Wt, expression of the transgene increased the endogenous Foxn1 levels as seen in the thymus (14). The endogenous Foxn1 levels were 18- to 24-fold higher in young and aged Foxn1Tg mice compared with age-matched Wt (Fig. 3B compared with Fig. 3A, young p = 0.036; aged p = 0.024), supporting the previous observation that expression of Foxn1 is self-regulated (14).

Foxn1pos cells are present in the BM of Wt and Foxn1Tg

We next determined whether Foxn1-expressing cells are present and detectable in BM of Wt mice, and, if that is the case, whether Foxn1-expressing cells are associated with the endothelial or the vascular niches (18). Foxn1pos cells appeared as single cells in the central marrow cavity with proximity to sinuses of young Wt mice. On the average, 1–3 cells per 5 individual 5-μm-thickness BM sections were detected (Fig. 3C), consistent with the low copy number of transcripts determined in Wt marrow (Fig. 3B). In BM of young Foxn1Tg, Foxn1pos cells were readily detectable within the vicinity of sinuses, averaging 3–6 cells per 5-μm section (Fig. 3C). Morphologically, the Foxn1-expressing cells were round with an abundant cytoplasm and a centrally located nucleus (Fig. 3C). In aged Wt and Foxn1Tg, the Foxn1pos cell number per section increased; however, there were more Foxn1-expressing cells in aged Foxn1Tg compared with aged Wt (Fig. 3C). We also detected keratin 14pos cells with an identical morphology to the Foxn1pos cells in both Wt and Foxn1Tg mice (Supplemental Fig. 2A–C, 2E).

Medullary thymic epithelial cells (TEC) are the predominant TEC expressing Foxn1 (5). The medullary TEC are identified as TEC that express the epithelial adhesion cell molecule EpCAM (19). To identify the phenotype of Foxn1pos cells in the BM of Wt and Foxn1Tg mice, BM cells were analyzed by flow cytometry for EpCAMpos. EpCAMpos cells were present in both Wt and
aged Foxn1Tg to generate GEMM colonies compared with young Foxn1Tg (Fig. 6). The age-associated effect was specific for the multipotent GEMM progenitors because no differences were observed in the number and types of myeloid colonies arising from Wt or Foxn1Tg marrow in either young or old mice (Supplemental Fig. 1B).

A novel T cell progenitor, termed CTP, has been identified as a descendent of HSC (22–24). CTP progress toward mature T cells through the identifiable intermediate stage termed CIP, which is coupled to the proliferation of CTP (25, 26); the identification of the two populations in BM cells was shown in Supplemental Fig. 4A. In mice 2–4 mo, the total number of CTP was not significantly different between Wt and Foxn1Tg; however, Foxn1Tg had a 2.2-fold higher number of CIP (Supplemental Fig. 4B, *p = 0.001). In 21– to 25-mo mice, the numbers of CTP and CIP increased in both Wt and Foxn1Tg compared with young (Supplemental Fig. 4B). Aged Wt displayed a greater increase in the number of CTP compared with Foxn1Tg, and the CTP number in 21- to 25-mo Foxn1Tg was 1.8-fold lower than Wt (Supplemental Fig. 4B, *p = 0.002). The CIP number was not significantly different between aged Wt and Foxn1Tg (Supplemental Fig. 4B). When the ratios of CIP/CTP were calculated, Foxn1Tg displayed higher ratios in both the 2- to 4- and 21- to 25-mo groups (Supplemental Fig. 4C, **p = 0.047 young; ***p < 0.001 aged), suggesting that Foxn1Tg are more efficient in generating CIP from CTP. CTP from both 2- to 4- and 21- to 25-mo Foxn1Tg mice showed a higher percentage of CTP in S,G2/M compared with Wt, supporting previous findings that the generation of CIP is coupled to CTP proliferation (Supplemental Fig. 4D, *p = 0.002, 2–4 mo; *p = 0.054, 21–25 mo) (26).

In the adoptive transfer experiments, the frequency of donor CTP was higher but not statistically different in aged Wt than in aged Foxn1Tg hosts. This was also true for the frequency of donor CIP (Supplemental Fig. 4E). However, when the ratios of donor CIP/CTP were measured to determine the efficiency in the development of CIP, aged Foxn1Tg had a higher ratio compared with aged Wt (*p = 0.06), suggesting that the aged Foxn1Tg BM microenvironment also is more efficient in promoting the generation of CIP (Supplemental Fig. 4E). Taken together, these data suggested that the BM of Foxn1Tg mice are more efficient in promoting the generation of T cell progenitor MPP and CIP.

Maintenance of MPP homeostasis with age in Foxn1Tg does not prevent the decline in B lineage progenitors

Because ETP are downstream progenies of MPP that also give rise to the CLP in the BM, we asked whether preventing the decline in MPP restores CLPs that are reduced with age in Wt. Flow cytometric gating of CLP is shown in Fig. 7A. In Wt, the CLP number decreased 1.9-fold with age, consistent with data previously reported (Fig. 7B, *p = 0.004) (27, 28). Surprisingly, we found that CLP number in Foxn1Tg was significantly reduced compared with age-matched Wt; furthermore, overexpression of Foxn1, although preventing the decline in MPP, did not prevent the age-associated decline in its progeny CLP (Fig. 7B). Consequently, the number of B lineage cells in the BM was significantly lower in young and aged Foxn1Tg compared with Wt (Fig. 7C). Thus, maintenance of MPP homeostasis with age affects only the numbers of ETP in the thymus but not CLP in the BM.
ETP and CLP both develop from MPP; MPP with the highest expression of CD135 (Flt3) display greatest T lineage potential (29). The mean fluorescent intensity (MFI) of CD135 on MPP from young and aged Wt and Foxn1 Tg was measured to begin identifying a potential signaling pathway that would promote ETP development over CLP from MPP. The MFI of CD135 on both young and aged MPP from Foxn1 Tg were significantly higher than their Wt counterparts (p = 0.002, ANOVA) (Fig. 7D). The expression of CD135 on MPP from Foxn1 Tg varied; however, about half the mice analyzed had a MFI higher than Wt. Alternatively, skewing toward the T lineage at the expense of the B lineage could be mediated by Notch signaling (30). Thus, we examined whether the Lin<sup>neg</sup> <sup>low</sup> EpCAM<sup>pos</sup> CD138<sup>neg</sup> BM cells express Notch ligands that are responsible for the commitment to T lineage progenitors. We sorted Lin<sup>neg</sup> <sup>low</sup> EpCAM<sup>pos</sup> CD138<sup>neg</sup> BM cells from aged Foxn1 Tg and found that these cells expressed the Dl4 but not Dl1 Notch ligand (Fig. 7E, Table II).

**Discussion**

In this study, we report a novel finding that Foxn1 is expressed in the BM by Lin<sup>neg</sup> <sup>low</sup> CD45<sup>pos</sup> EpCAM<sup>pos</sup> SCA1<sup>pos</sup> CD117<sup>neg</sup> CD138<sup>neg</sup> MHCIId<sup>neg</sup> cells. This cell population also expresses Notch ligand Dl4, but not Dl1. Overexpression of Foxn1 prevents the age-associated decline in MPP, partly due to a larger HSC pool, and thus maintains a progenitor pool for ETP. Furthermore, the aged Foxn1 Tg BM environment is more efficient in promoting MPP development. Interestingly, the maintenance of MPP homeostasis does not rescue the age-associated decline in CLP and B lineage cells; rather, the Foxn1 Tg BM environment alters CLP development, suggesting that the Foxn1 Tg BM environment is biased toward T lineage at the expense of B lineage.

Reduced MPP number with age indicates that decline in T lymphopoiesis is initiated in the BM (13). Thus, we would predict that attenuation of a decline in ETP number with age in Foxn1 Tg correlates with a larger number of MPP in the BM. Whereas the

**FIGURE 3.** Expression of Foxn1 in the BM. Nucleated BM cells were used to prepare total RNA and cDNA synthesis for quantitative RT-PCR analysis. Data are presented as copy number of transcripts/µg total RNA extrapolated from a standard curve (10–100,000 copies/µL). All samples were run in triplicate. (A) Expression of endogenous and transgene Foxn1 in Foxn1 Tg 2 mo and 23–28 mo. Specific primer sets that detect only transgene transcripts or both forms of transcript were used to calculate endogenous Foxn1 expression levels. (B) Expression of Foxn1 in Wt BM 2 mo and 20–24 mo. Numbers in parentheses denote the number of mice in each age group. Error bars are SD. (C) Foxn1-expressing cells in the BM as determined by immunohistochemistry assay. Sternums from Wt and Foxn1 Tg mice were fixed, embedded in paraffin blocks, and sectioned at 5 µm. Ag retrieval was performed on rehydrated tissue sections prior to staining with either rabbit anti-mouse Foxn1 or rabbit IgG at 2 µg/ml. Primary Abs were incubated overnight at 4˚C. Dako Universal LSAB Abs or Donkey anti-rabbit biotin (6 µg/ml), followed by streptavidin-HRP was used for detection of primary Ab. Sections were developed with 3- amino-9-ethylcarbazole for 1.5 min and counterstained with hematoxylin. Pictures were taken using a Leitz Diaplan microscope (50× lens) with Retiga 2000R camera. Arrows point to Foxn1-positive cells. S denotes sinusoid (dashed lines).
number of MPP significantly declines with age in Wt mice, their number was not reduced but maintained, even in Foxn1 Tg that were 26–35 mo old. Our data indicate that preventing cell death in MPP with age is a potential mechanism for the maintenance of homeostasis with age. Alternatively, maintenance of MPP with age is possible through their immediate precursor HSC, which we will address later in this section.

Besides functioning as ETP progenitors, MPP also give rise to myeloid progenitors and CLP progenitors that display potent B lineage potential in the BM. Whereas myeloid lineage development is not affected, both young and aged Foxn1 Tg show a reduced CLP number and consequently a lower number of B lineage cells compared with age-matched Wt; thus, in contrast to ETP in the thymus, the decline in CLP is not rescued and still occurs with age.

**FIGURE 4.** Phenotypic characterization of Foxn1-expressing BM cells and their frequencies in Wt and Foxn1 Tg mice. (A) Total BM cells were analyzed by flow cytometry; the gating of Lin<sup>neg</sup>EpCAM<sup>pos</sup> BM cells in 3- to 4-mo and 18- to 23-mo Wt and Foxn1 Tg mice is shown. Foxn1 Tg Lin<sup>neg</sup>EpCAM<sup>pos</sup> cells were analyzed for the expression of CD45, Sca1, CD117, and MHCII. Although not shown, expressions of these markers were identical on Wt Lin<sup>neg</sup>EpCAM<sup>pos</sup> cells. (B) Electronically sorted Lin<sup>neg</sup>EpCAM<sup>pos</sup> and Lin<sup>neg</sup>EpCAM<sup>pos</sup> CD138<sup>neg</sup> cells from Wt, Foxn1 Tg, or Foxn1cre-Rosa26-lacZ reporter mice were cytocentrifuged and stained for Foxn1 or anti-E.coli β-galactosidase, as described in Materials and Methods. Images were captured with a 50× lens. (C) Frequencies of Foxn1<sup>pos</sup> cells were calculated based on the percentages of positive cells within the Lin<sup>neg</sup>EpCAM<sup>pos</sup> CD138<sup>neg</sup> population in combination with the frequency of Lin<sup>neg</sup>EpCAM<sup>pos</sup> CD138<sup>neg</sup> cells determined by flow cytometry and expressed as number of positive cells per 100,000 BM cells. Wt are 24 mo and Foxn1 Tg are 18–23 mo of age. Numbers in parentheses denote the number of mice in each group.

**FIGURE 5.** Differences in the ability of aged BM microenvironment of Wt and Foxn1 Tg mice to promote the generation of MPP from HSC. LSK were sorted from CD45.1<sup>pos</sup> mice, and 8–16 × 10<sup>3</sup> cells were transferred into aged Wt or Foxn1 Tg hosts via the retro-orbital route. After 10 wk, the frequencies of donor CD45.1<sup>pos</sup> HSC (A) and MPP (B) were determined using flow cytometry. (C) The ratios of donor MPP to donor HSC were calculated to determine the efficiency of generating MPP from donor HSC (*p = 0.03). Each symbol represents the result obtained from one host. Data are from three separate experiments. Error bars are SD.
the number of MPP is not reduced, but the generation of CLP is reduced with age, the Foxn1Tg BM environment may limit the development of MPP to CLP, thus providing a larger MPP pool as precursors for ETP. We suggest that skewing of T lineage at the expense of B lineage is a contributing factor to the higher number of ETP observed in aged Foxn1Tg thymus compared with Wt (14). This notion is supported by the finding that Foxn1pos BM cells express DI4, the physiological ligand for Notch in T lymphopoiesis (31). Notch signals through Foxn1pos-expressing DI4 may prime MPP to differentiate toward the T lineage as well as limit B lineage commitment and development in the BM (32, 33). It is also possible that through cell–cell contact, Foxn1pos cells alter the factors required for B lineage commitment and development such as Fms-like tyrosine kinase 3 ligand and IL-7–mediated signals (34–36). Thus, one would expect to observe more donor-derived ETP in the thymus and donor-derived CD3pos in the spleen of the hosts in the adoptive transfer experiments. We found that the frequencies of donor ETP were not significantly different between aged Wt and Foxn1Tg hosts (data not shown). We interpreted this as the Foxn1Tg host thymus expresses high levels of Foxn1, and this condition correlates with the high frequency of ETP in Foxn1Tg thymus in which ETP frequency was not reduced with age, as we have previously shown (14). We observed higher frequencies of donor CD3pos T cells and lower frequencies of B220pos B cells in the spleen, but the differences were not statistically significant (data not shown).

Previous work in Foxn1-/- nude mice suggests that lack of Foxn1 alters the BM microenvironment and hematopoiesis. Zipori and Trainin (37) demonstrated that nude BM displays a reduction in cellularity and provides limited protection when transferred into a lethally irradiated host. Thus, a reduced number of HSC could be responsible for the observed defect. In this context, Foxn1-expressing BM cells could play a potential role in regulating HSC number, seen as a larger established pool of HSC in Foxn1Tg BM. It has been observed that an increase in the numbers of osteoblasts and trabecular bone correlates with a higher number of HSC (38, 39); however, we observed no such differences between Foxn1Tg and Wt (data not shown). Alternatively, the size of HSC pool is regulated during the transition from the highly proliferative fetal HSC to the slowly proliferating adult phenotype by reduced expression of sry-related high mobility group box 17 (sox17) (40). It remains to be determined whether overexpression of Foxn1 affects the duration of Sox17 expression in HSC, leading to a larger HSC pool in Foxn1Tg BM. It is estimated that a single long-term repopulating HSC can only replicate five times in the mouse life span to maintain hematopoiesis before exhaustion (41). Thus, with a larger HSC pool, hematopoiesis would be prolonged because it would take longer to induce replicative exhaustion associated with aging.

HSC numbers can also be regulated by controlling HSC self-renewal (42). In E3 ligase Itch-deficient mice, HSC display increased cell cycling, resulting in increased HSC and MPP number (43). It is possible that, with age, Foxn1Tg BM negatively affects Itch expression or activity within HSC, leading to HSC proliferation and a larger number of HSC and MPP. Whether expression of Itch E3 ligase increases with age is unknown.

In agreement with others, the number of HSC in Wt BM increases in 20- to 21-mo-old mice as compared with 1–4 mo (44, 45). It has been suggested that this increase in HSC with age is either due to a block in the differentiation of HSC or a compensatory mechanism for the inefficiency in lymphoid lineage development with age and not by an increase in cell cycling with age (13, 45–47). We found that, with advanced age in Wt (24–25 mo), the initial increase is followed by a dramatic reduction in HSC number that is coupled with increased cell death. If the increase in HSC is in fact a compensatory mechanism to increase the number of lymphoid lineage cells derived from HSC, then the drastic decline in Wt 24 mo of age and older may result from HSC replicative exhaustion. The decline in the ability of aged Wt HSC to generate CFU-GEMM corroborates the idea that compensatory responses in aged HSC lead to their depletion with age. Because the HSC number is reduced only to levels equivalent to that found in young Foxn1Tg, we suggest that the HSC pool in aged Foxn1Tg BM is rescued from replicative exhaustion.

Increase in cell cycling also may be possible through regulation of cyclin-dependent kinase inhibitor p16Ink4a, which controls the G1 checkpoint. The cyclin inhibitor p16Ink4a is expressed in aged, but not young HSC; it was found that aged p16Ink4a-/-/- mice have an increase in the number of HSC due to increased cell cycling and decreased cell death (48). It is possible that HSC within the Foxn1pos niches of the BM environment are affected such that the age-associated increase in p16Ink4a expression is abrogated, resulting in proliferation of HSC in responding to stress with advanced age. We propose that the increase in cell cycling, reduced cell death, and intact function culminates in preventing replicative exhaustion and restoring HSC homeostasis with age in Foxn1Tg BM.

Although we could not rule out that the maintenance of HSC and MPP number in the BM of Foxn1Tg mice is the result of a feedback loop from the thymus to the BM, the presence of Foxn1-expressing cells in BM could provide a direct functional basis for the observed changes in HSC and MPP and T lineage commitment within the BM. Foxn1pos cells are located within the central marrow and are either immediately adjacent to or within an estimated three-cell length distance to sinusoids, but are rarely found adjacent to trabecular bone. Thus, they appear associated with sinusoidal or vascular niches rather than the endosteal niche. The short-term, proliferating HSC reside within the vascular niche and readily differentiate into MPP (18). Based on the location of Foxn1pos cells, we speculate that the Foxn1pos cells are vascular niche cells that affect proliferation of HSC and contribute to the maintenance and prevention of the decline of MPP with age.

The finding that Foxn1 is expressed in Wt Linneg EpCAMpos CD138neg BM cells supports the notion that expression of the Foxn1Tg is not ectopic. Approximately 68% of the Linneglow EpCAMpos CD138neg cells express Foxn1 in aged Foxn1Tg BM and 45% BM cells with identical phenotype isolated from aged Wt BM are also stained positive for Foxn1 (Fig. 4B and data not shown). Because the Linneg EpCAMpos CD138neg population isolated from Foxn1cre-Rosa26-lacZ mice expresses bacterial
β-galactosidase, we conclude that these cells are the bona fide Foxn1-expressing cells in the BM. Whereas plasma cells also express EpCAM, it is unlikely that the Foxn1pos cells are plasma cells because the majority of the Foxn1pos cells are CD138neg, furthermore, these Foxn1pos cells also express keratin 14, suggesting epithelium in nature.

Table II. List of primers used in the study

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<tr>
<th>Primer Name</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tr>
<td>Dl1</td>
<td>CTTCTTTCCGCTATGCCCTCAA</td>
<td>AGGCGGCTGATGAGTCTTTCT</td>
</tr>
<tr>
<td>Dl4</td>
<td>CGCCAGGAAACTCTCTCATCA</td>
<td>GCTCATGACAGCCAGAAAGACA</td>
</tr>
<tr>
<td>Hprt</td>
<td>AGCAGTACAGCCCCAAATGG</td>
<td>TGCGCTCATCTTAGGCTTTGT</td>
</tr>
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Forward and reverse sequences of primers used to measure gene expression.
We reason that the identification of Foxn1\textsuperscript{pos} BM cells together with the maintenance of homeostasis of MPP with age provides, to our knowledge, the first evidence to suggest that Foxn1 plays a critical role in the maintenance of hematopoiesis and particularly T lineage within the BM niche with age. This notion is supported by data from the adoptive transfer experiments showing that the generation of MPP and of CIP from donor HSC is higher in aged Foxn1\textsuperscript{tg} compared with aged WT hosts. Whereas we planned to use VEX expression as a measurement of Rag expression and activity in donor cells homed to the BM, we could not detect VEX\textsuperscript{pos} cells in the LSK and MPP populations perhaps due to a low level of expression of VEX. These findings suggest that the cells within the BM that are responsible for the generation of MPP and CIP and reinforce the notion that the increase in generation of MPP and CIP in aged Foxn1\textsuperscript{tg} mice are mediated by the Foxn1-expressing cells within the BM niches, therefore providing the rationale for future studies to establish the precise causative effect of Foxn1-expressing cells within the BM microenvironment.

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Disclosures

The authors have no financial conflicts of interest.

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